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Fasciola hepatica infections in cattle and the freshwater snail *Galba truncatula* from Dakhla Oasis, Egypt

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Abstract

Infection by *Fasciola* species was investigated in seven districts of Dakhla Oasis, Egypt, through abattoir inspection of cattle livers for adult worms and sedimentation of faecal samples from local cattle to detect *Fasciola* eggs. In addition, lymnaeid snails collected from the study area were examined microscopically for developmental stages of *Fasciola* spp. Abattoir inspection revealed that 51 out of 458 cattle livers (11.1%) contained adult flukes, which were identified morphologically as *Fasciola hepatica*. Examination of the cattle faecal samples revealed that 142 out of 503 (28.2%) contained *Fasciola* eggs. The collected snails, identified as *Galba truncatula* and *Radix natalensis*, showed larval stages of *Fasciola* in 71 out of 731 (9.7%) *G. truncatula*, while *R. natalensis* showed no infection. Specific duplex polymerase chain reaction (PCR) targeting the mitochondrial *cox1* gene of *F. hepatica* and *Fasciola gigantica* was carried out on DNA extracted from pooled infected snails and adult worms. The *F. hepatica* size amplicon (1031 bp) was obtained from both the infected *G. truncatula* and the adult worms isolated from cattle livers from different districts. The amplicon sequences were identical to the published sequences of *F. hepatica* mitochondrial *cox1* gene. In conclusion, the zoonotic importance of *Fasciola* infection and appropriate hygienic measures must be taken into consideration in Dakhla Oasis, Egypt.

Introduction

Fasciolosis is one of the most important liver diseases of herbivores and is caused by infection with *Fasciola* spp. Fasciolosis is reported to cause economic losses of about US$29.2 million annually in Egypt (El-Shazly *et al.*, 2006). Moreover, 4% of human patients admitted to hospitals suffering from a fever of unknown origin are infected with *Fasciola hepatica* (Soliman, 2008). *Fasciola hepatica* (Linnaeus, 1758) has a wider range than its tropical counterpart, *Fasciola gigantica* (Cobbold, 1856), but their geographical distribution overlaps in many African and Asian countries (Mas-Coma *et al.*, 2005). In Egypt, both species are present (Lotfy & Hillyer, 2003; WHO, 2007; Hussain & Khalifa, 2010; Dar *et al.*, 2012).

The predominant intermediate host of *F. hepatica* in Europe, Asia, Africa and North America is *Galba truncatula* (Soulsby, 1982). However, in Egypt scant records are available incriminating *G. truncatula* in the transmission of *F. hepatica* infection (Abd El-Ghani, 1976; Brown,

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1994; El-Kady *et al.*, 2000; El-Shazly *et al.*, 2012). On the other hand, an experimental infection of *Pseudosuccinea columella* with *F. hepatica* suggested its role as an important intermediate host for *Fasciola* transmission in Egypt (Dar *et al.*, 2014).

The two main *Fasciola* species, *F. hepatica* and *F. gigantica*, are differentiated by morphological features such as body length and shape (Ashrafi *et al.*, 2006); however, these characteristics are sometimes unclear. Moreover, a form of *Fasciola* intermediate between the two species has been recorded in different countries, including Egypt (Itagaki *et al.*, 2005; Periago *et al.*, 2008; Ichikawa & Itagaki, 2010). These flukes are meiotically dysfunctional, have abnormal spermatogenesis and are considered aspermic, as they have few or no sperm in the seminal vesicles (Terasaki *et al.*, 1998; Itagaki *et al.*, 2005; Ichikawa & Itagaki, 2010). Therefore these flukes may reproduce parthenogenetically. In aspermic *Fasciola*, diploid, triploid and, sometimes, mixoploid flukes are reported (Terasaki *et al.*, 1998).

The intermediate fluke forms are not identified as either *F. hepatica* or *F. gigantica*. The entity of hybrid forms was established when Japanese *Fasciola* showed ribosomal DNA sequences identical to those of *F. hepatica* and mitochondrial DNA sequences identical to *F. gigantica* (Itagaki & Tsutsumi, 1998; Itagaki *et al.*, 1998). As a result, molecular methods using both nuclear ribosomal RNA internal transcribed spacers (ITS1 and ITS2) and mitochondrial cytochrome oxidase 1 (*cox1*) and NADH dehydrogenase I genes were developed for accurate differentiation (Itagaki *et al.*, 2005). Subsequently, a single-step duplex polymerase chain reaction (PCR) based on the mitochondrial *cox1* gene was developed for detection and discrimination between *F. hepatica* and *F. gigantica* (Le *et al.*, 2012).

The aim of the present study was to investigate *Fasciola* species in cattle and lymnaeid snails in Dakhla Oasis, El-Wadi El-Gadid, Egypt. Gross and microscopic findings were confirmed by duplex PCR and sequence analysis of the resulting mitochondrial *cox1* amplicons.

Materials and methods

Collection and examination of faecal samples

This work was carried out in the Dakhla Oasis, El-Wadi El-Gadid province (coordinates: 25°29′29.6″N, 28°58′45.2″ E) in the south-western part of Egypt (fig. 1). In Dakhla Oasis water is derived from 32 wells and springs. The main source of water is the wells. Faecal samples from 503 cattle (214 males <5 years of age, 39 females <5 years of age and 250 females >5 years of age) (table 1) of local breeds were collected from seven districts (El-Raschda, $n = 85$; Al Hindaw, $n = 75$; Al Masarah, $n = 80$; El-Shiekhwali, $n = 70$; Azb El-Qasr, $n = 65$; El-Qasr, $n = 68$; and El-Aweyna, $n = 60$) in the Dakhla Oasis (table 2). Faecal samples were collected directly from the rectum into plastic bottles which were labelled and brought to Dakhla Animal Health Research Laboratory (AHRL). Coproscopic examination was performed to detect the presence of *Fasciola* species eggs by the simple sedimentation technique with tap water (Urquhart *et al.*, 1996).

Liver samples

Liver samples from 458 cattle (338 males <5 years of age and 120 females (30 <5 years of age and 90 >5 years of age)) were collected during spring 2014 in the abattoir of Mout, Dakhla province (table 2). Mout is the principal slaughterhouse in the Dakhla Oasis, serving the seven districts. Specimens were transported in an ice tank to the Animal Health Research Laboratory (AHRL), El-Dakhla, for further examination. Flukes recovered from livers were collected, counted and washed with normal saline (0.9%). Approximately 20 flukes from each district were fixed and stained (Drury & Wallingten, 1980), then morphologically identified, mainly based on non-overlapping features: distance between ventral sucker and posterior end of body (VS–P), distance between the union of the vitteline glands and posterior end of body (Vit–P), and body length/body width ratio (BL/BW), according to Ashrafi *et al.* (2006). Approximately 20 *F. gigantica* adult flukes from Beni Suef governorate were included in the study to compare the morphological findings. For PCR, intact adult flukes were preserved in 70% ethanol and stored at −20°C until DNA extraction.

Snails

A total of 1196 *Lymnaeidae* snails (≥4 mm) were collected during the morning from the edges of the main wells and their small branches during spring 2014 from the seven districts in Dakhla Oasis (table 2). The snails were either *G. truncatula* found under the mud or *Radix natalensis* found attached to grasses in the water stream. The snail collection technique adopted by the Ministry of Health and Population (Snail Eradication Office) was used with the aid of a special net (Diab, 1993). Specimens were placed in bottles with perforated caps and transported to the laboratory for further investigation. Snails were examined for the presence of different stages of *Fasciola* spp. by direct crushing in saline under a dissecting microscope, where the detected larvae were recorded (Jackson, 1958). Snails were identified according the key provided by Professor Santiago Mas-Coma, WHO, Madrid, Spain (Ibrahim *et al.*, 1999).

Molecular analyses

DNA was extracted from 71 *Fasciola*-infected and 70 uninfected *G. truncatula* snails. All infected snails from each district were pooled as one sample representing a specific district, resulting in seven pooled samples of infected snails. Uninfected snails were similarly treated. Furthermore, 7 pooled samples of 10 *R. natalensis* from each district were prepared. Snails were crushed prior to DNA extraction.

Twenty adult flukes previously isolated from cattle livers were used from each district. The worms were thoroughly washed three times with normal saline. The cone-shaped projections were removed with a sterile scalpel, crushed and homogenized, and then DNA was extracted using a PureLink® Genomic DNA Kit (Invitrogen, Carlsbad, California, USA) according to the manufacturer's instructions. The ability of the duplex PCR to differentiate between *F. hepatica* and *F. gigantica*

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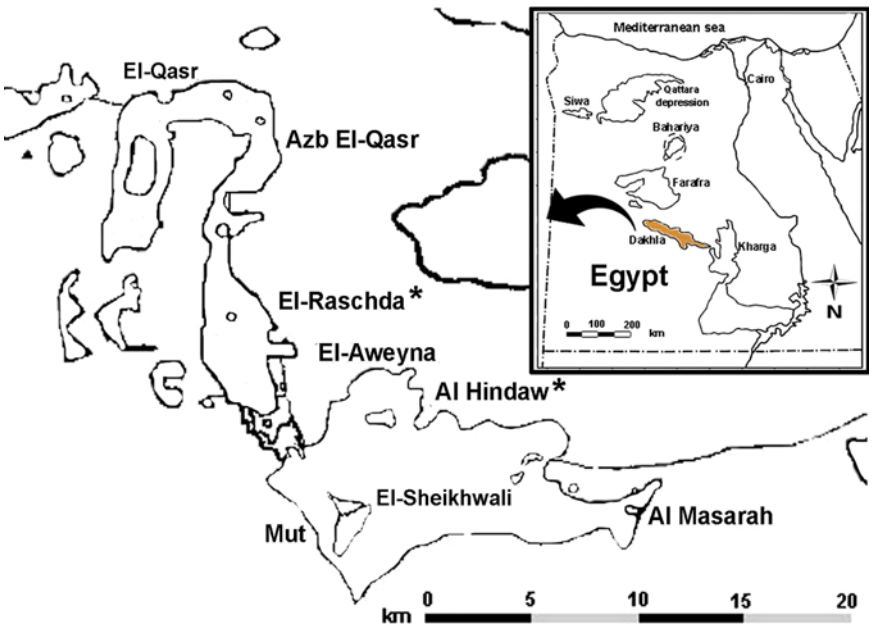

Fig. 1. A map showing different oases in Egypt. Note seven investigated districts in Dakhla Oasis: El-Qasr, Azb El-Qasr, El-Raschda, El-Aweyna, Al Hindaw, El-Sheikhwali and Al Masarah. Liver inspection (in Mut abatoir), coprological examination and examination of *Galba truncatula* snails revealed the highest percentage of fasciolosis in both El-Raschda and Al Hindaw districts (asterisks).

was checked using DNA from 20 morphologically identified *F. gigantica* adult worms obtained from Beni-Suef province in a previous study (Arafa *et al.*, 2015)

Fasciola spp. duplex PCR (Le *et al.*, 2012) was carried out on DNA from both infected and uninfected snails, and from adult worms. The PCR targets mitochondrial nucleotide sequences of the protein-coding *cox1* gene (Le *et al.*, 2012). The duplex PCR was performed in a 25-µl total reaction volume containing 1 µl (10 pmol) of both forward primers FHF (5′- GTTTTTTAGTTGTTTGGGGTTTG-3′) and FGF (5′-TGTTATGATTCATTGTTTGTAG-3′), 2 µl (20 pmol) of the reverse primer FHGR (5′-ATAAGA ACCGACCTGGCTCA-3′), 3 µl of template DNA, 12.5 µl master mix (Biomatic®; Biomatik Corporation, Ontario, Canada) and 5.5 µl nuclease-free water. Amplification was done using the following conditions: initial denaturation at 95°C for 3 min; followed by 35 cycles of denaturation at 95°C for 30 s, annealing at 52°C for 30 s and extension at 72°C for 2 min. The final extension occurred for 7 min at 72°C. The expected amplicon sizes of *F. hepatica* and *F. gigantica* were 1031 and 615 bp, respectively.

For gene sequencing, PCR products of pooled samples selected for adult *F. hepatica*, *F. gigantica* and *G. truncatula* were purified with Thermo Scientific Gene JET PCR Purification Kit (#K0701; Carlsbad, California, USA) according to the manufacturer's instructions. The purified PCR products were sequenced in both forward and reverse directions on an Applied Biosystems 310 Automated DNA Sequencer using cycle sequencing ABI prism Big Dye terminator chemistry (a terminator cycle sequencing ready reaction kit) (Perkin-Elmer/Applied Biosystems, Foster City, California, USA). BLAST® analysis (Altschul *et al.*, 1990) was initially performed to establish sequence identity to GenBank accessions. In the

Table 1. The prevalence (%) of *Fasciola hepatica* infections in faecal and liver samples from cattle from Dakhla Oasis; *N* = number of samples examined.

		Faecal samples		Liver	
Host sex	Host age	*N*	%	*N*	%
<5 years	Male	214	18.7	338	5.3
	Female	39	30.8	30	10.0
>5 years	Male	0	0.0	0	0.0
	Female	250	35.3	90	33.3
Total		503	28.2	458	11.1

Table 2. The prevalence (%) of *Fasciola hepatica* infections in cattle (including mean worm numbers (*M*) in the liver) and also the freshwater snail *Galba truncatula* from seven districts in Dakhla Oasis; *N* = number of samples examined.

	Cattle						
	Liver			Faecal		*G. truncatula*	
District	*N*	%	*M*	*N*	%	*N*	%
El-Raschda	75	16.0	44	85	38.8	110	12.7
Al Hindaw	74	14.9	40	75	37.3	100	12.00
Al Masarah	70	11.4	30	80	30.0	150	11.3
El-Shiekhwali	66	7.6	20	70	28.6	120	8.3
Azb El-Qasr	58	10.3	23	65	27.7	80	78.8
El-Qasr	60	6.7	25	68	17.7	95	6.7
El-Aweyna	55	9.1	24	60	11.7	76	6.6
Total	458	11.1	206	503	28.2	731	9.7

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current study, all sequences obtained have been submitted to GenBank (accession numbers: KU058263.1, KU058264.1 and KU058265.1). Multisequence alignment of sequences was performed with selected GenBank published sequences (AB020407, AF216697, X07364, NC_024025 and KF543343).

Results

Faecal and liver samples

A prevalence of 28.2% *Fasciola* spp. eggs was observed from microscopic examination of 503 faecal samples from cattle. Based on sex and age, infection was 35.3% (102/289) in female cattle older than 5 years of age and 30.8% (12/39) in females less than 5 years old (table 1). In males, infection was 18.7% (40/214) in animals less than 5 years old (table 1). No samples of males over 5 years old could be collected as they are fattened and slaughtered before reaching this age. The El-Raschda district had the highest rate of faecal and liver infection among the seven examined districts (table 1).

Adult flukes were found in the bile ducts of 51 of 458 (11.1%) examined liver samples. The mean worm burden ranged from 20 to 44 adult flukes per liver (table 2). The adult flukes were morphologically identified as *F. hepatica*.

Snails

The snails collected were identified as *G. truncatula* (n = 731) and *R. natalensis* (n = 465). Developmental stages of *Fasciola* spp. were present in 9.7% (71/731) of the crushed *G. truncatula* (table 2). Of 465 specimens of *R. natalensis* examined, none were infected.

Molecular analyses

The pooled fluke samples from each of the seven districts revealed the specific 1031-bp amplicon of *F. hepatica*, as did infected *G. truncatula* snails of each region. However, *F. gigantica* (Beni-Suef isolates) PCR products yielded the expected amplicon size of 615 bp. Crushed samples of *G. truncatula* or *R. natalensis* with no microscopically detected *Fasciola* were negative by duplex PCR.

Sequence analysis of selected PCR products of pooled adult *Fasciola* samples from the different localities and PCR products of pooled infected *G. truncatula* were found to be closely related to *Fasciola* isolates in the GenBank database. Egyptian *F. hepatica* isolates (KU058263.1 and KU058264.1) were found to be identical to the mitochondrial *cox1* gene of *Fasciola* spp. Japanese isolate (AB020407), and shared 99% identity with *F. hepatica* (AF216697) and 97% identity with *F. hepatica* large subunit mitochondrial rRNA (X07364). The sequence of the PCR products of control *F. gigantica* obtained from Beni-Suef governorate, Egypt, (KU058265.1) showed 99% nucleotide identity to the *F. gigantica* mitochondrial genome (GenBank accessions NC_024025 and KF543342).

Discussion

Faecal examination showed that 142/503 (28.2%) samples from cattle of local breeds in Dakhla Oasis contained *Fasciola* spp. eggs. A similar finding of 28.6% was reported by Hussain & Khalifa (2010) in Qena, Egypt. Our finding is also similar to those reported in Debre Zeit, Ethiopia (28.6%) (Abdulhakim & Addis, 2012) and in Nigeria (25.8%) (Negele & Ibe, 2014). However, the prevalence obtained in the current study was higher than the 7.4% and 8.0% in Assiut, Egypt recorded by Abdo (2014) and Kuraa & Malek (2014), respectively. On the other hand, the prevalence in our study was lower than that reported in Ethiopia (35%) (Shiferaw *et al*., 2011).

The prevalence of *F. hepatica* infection was higher in cattle over 5 years of age (36.0%) than in those aged less than 5 years (30.8%) in the current study. Similar results were reported in Ethiopia by Abdulhakim & Addis (2012), who found that the prevalence of fasciolosis was 39.8% in older adult cattle and 23.3% in young cattle. Khan & Maqbool (2012) also noted a higher infection rate in older cattle than in youngsters in Pakistan. Contrary to these results, a previous report from Egypt showed that the prevalence in young animals was higher, at 13%, compared to 5.5% in older animals (Atallah, 2008). Similarly, in Nigeria, Biu *et al*. (2013) revealed that the incidence in cattle aged less than 5 years was higher, at 16.9% compared to 12.5% in animals over 5 years old. This variation may be associated with lack of veterinary care or exposure to infection.

The present study also found that the infection rate of *F. hepatica* was higher in cows (36.0%) than in bulls (18.8%). This finding is similar to that of a previous report that cows had a higher rate of infection than male cattle (6.7% versus 2.2%) in Kalyobia, Egypt (Ghoneim *et al*., 2011). This was also documented in Nigeria (18.5% vs. 12.9%) (Biu *et al*., 2013) and in northern Ethiopia (25% vs. 17.3%) (Teklu *et al*., 2015). These results were opposite to a reported higher infection rate in male cattle than females in Pakistan (Khan & Maqbool, 2012). In northwestern Ethiopia, female and male cattle had the same infection rate (Tsegaye *et al*., 2012).

It is possible that male cattle usually had a lower infection rate than females due to the anthelmintic control programme, which was applied for fattening males but not females. Additionally, males were commonly fed on dry matter during the fattening period and were slaughtered at an early age. On the contrary, females were fed green roughage, thereby increasing exposure to infection. Furthermore, the female life span is longer for breeding purposes. Variations in infection rates could also have been related to geographical distribution, grazing systems and different strategic control of helminths.

It was notable that in the current study El-Raschda district recorded the highest rates of infection in cattle faecal and liver samples. This infection rate may be related to the individual ownership of three animals or fewer, a lack of available veterinary care and dependence mainly on pasture feeding.

The *F. hepatica* infection rate in cattle livers at the Mout abattoir (11.1%) in this study was lower than those reported in Kafr El-Sheikh, Egypt (18.5%), Punjab, Pakistan (22.6%), Nigeria (42.2%) or in northern Ethiopia (18.4%) (Atallah, 2008; Negele & Ibe, 2014; Teklu *et al*., 2015). On the other hand, it was higher than those detected previously in Kalyobia, Egypt (6.7%) (Ghoneim *et al*., 2011) and in Kirkuk, Iraq (1.27%) (Kadir *et al*.,

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2012). The variation in infection rates might be related to topography, the level of veterinary care, feeding and irrigation systems of animal pastures.

In the current study, faecal examination showed a 28.2% infection rate compared to 11.1% in cattle livers at the slaughterhouse. It was not surprising to record a lower infection rate at the slaughterhouse because the majority (338/458; 73.8%) of the liver samples were from male animals, which are raised under intensive management programmes that include regular deworming with different flukicides. On the contrary, faecal examination revealed a higher *Fasciola* infection rate because, under the Egyptian field conditions, female animals did not receive proper management and anthelmintics in comparison to the males.

Collected snails were identified as *G. truncatula* and *R. natalensis*. In the present study, microscopic examination detected *G. truncatula* infected with *F. hepatica*; however, no infected *R. natalensis* snails were observed. A previous study in Dakahlia, Egypt showed natural *Fasciola* spp. infection rates of 5.5% for *R. natalensis* and 3.1% for *G. truncatula* (El-Shazly *et al.*, 2002). However, the latter authors did not identify to species the *Fasciola* in *G. trancatula*. In the current study, the microscopic findings of *F. hepatica* were confirmed by PCR and sequence analysis.

In the current study, a crushing method was utilized to facilitate microscopic detection of *Fasciola* in the snails, rather than the cercaria release method. The cercaria release method for detection has been negated by several studies, which have shown that prevalence obtained from snail dissection was higher than that from cercaria release (Curtis & Hubbard, 1990; Cucher *et al.*, 2006; Martínez-Ibeas *et al.*, 2011; Lambert *et al.*, 2012). Cercaria release only detects mature infections and thus may underestimate the actual prevalence (Studer & Poulin, 2012), and sometimes snails containing mature cercariae do not shed any (Stunkard & Hinchliffe, 1952). Furthermore, the detection rate of multiple infections was higher by dissection (Curtis & Hubbard, 1990). In fact, Lloyd & Poulin (2012) found that estimation of double infections by cercaria release is more difficult than that of single infections in the same snail. Consequently, the present study utilized a crushing method for examining the snails for cercaria.

Galba truncatula is the predominant intermediate host for *F. hepatica* worldwide (Bargues *et al.*, 2012). In Egypt, *G. truncatula* was recorded in various districts, but with scarce data regarding natural infection by *F. hepatica* (Abd El-Ghani, 1976; Brown, 1994; El-Kady *et al.*, 2000; El-Shazly *et al.*, 2012). However, *G. truncatula* naturally infected with *F. gigantica* was reported (Dar *et al.*, 2005, 2010) and under experimental conditions various snail species were successfully infected with *F. hepatica* (Dar *et al.*, 2010, 2013, 2014). It is worth mentioning that in Egypt *F. hepatica* originated from imported animals (Lotfy *et al.*, 2002; Mas-Coma *et al.*, 2005; Hussain & Khalifa, 2010) and *R. natalensis* is considered to be its potential intermediate host (Dar *et al.*, 2010). In the current study, no *Fasciola*-infected *R. natalensis* were identified among the 465 snails examined.

Morphologically, adult flukes in this study were identified as *F. hepatica* and this was augmented by the presence of *F. hepatica* larval stages in its intermediate host, *G. truncatula*. Several investigations based on morphological criteria, morphometric and chemotaxonomic data showed similar findings (Lotfy & Hillyer, 2003; Periago *et al.*, 2008; Hussain & Khalifa, 2010). In this current study, mitochondrial DNA-targeting duplex PCR was used to identify *Fasciola* spp. obtained from liver samples and snail intermediate hosts. It is worth mentioning that PCR detected *F. hepatica* in pooled samples of 5–14 infected *G. truncatula* snails taken from different districts, as well as in pooled DNA samples of adult worms. Previously, *F. hepatica* DNA was detected in ten pooled *G. truncatula* snails and in up to 25 lymnaeid snails per pool (Rognlie *et al.*, 1996; Caron *et al.*, 2011). Detection of *F. hepatica* by PCR in pooled samples is especially a point of strength because it can detect dead and immature *Fasciola* stages which might be missed visually.

The sequence analysis of selected *cox1* genes amplified from *Fasciola* spp. isolates and infected *G. truncatula* from Dakhla Oasis confirmed the presence of *F. hepatica* DNA. Moreover, *cox1* amplicons from adult *F. gigantica* obtained from Beni-Suef province, Egypt, sequenced in this study, were identical to the Chinese *F. gigantica* mitochondrial genome (NC_024025 and KF543342). It is of interest to highlight that the selected mitochondrial nucleotide sequences of the protein-coding *cox1* could be used as an identification gene for the two main *Fasciola* species (Le *et al.*, 2012).

The presence of *F. hepatica* and *F. gigantica* in the same animal creates an opportunity for cross-fertilization and hybrid formation (Spithil *et al.*, 1999; Amer *et al.*, 2011). However, in the current study, the morphological features of the collected flukes were clearly identified as consistent with *F. hepatica* according to the standardized measurements of Ashrafi *et al.* (2006) and Periago *et al.* (2008). None of the worms met the morphological criteria for *F. gigantica*. Moreover, the *Fasciola cox1* gene amplified from the samples in this study was identical in sequence to that reported for *F. hepatica*. As a result, hybrid flukes between *F. hepatica* and *F. gigantica* were not taken into consideration in the current study. However, the mitochondrial DNA-targeting duplex PCR used in this study does not differentiate between diploid and triploid *Fasciola* spp., which differ only in chromosomal, not in mitochondrial, DNA. Karyotyping to determine if sperm are present in the *Fasciola* seminal vesicle, which is common in triploids and parthenogenetic diploids, or alternative molecular methods, would be needed to identify these forms (Le *et al.*, 2012). Molecular approaches based on the DNA sequences of ITS1, ITS2 or the 28S ribosomal RNA gene, in combination with mitochondrial gene markers, are recommended for precise identification (Itagaki *et al.*, 1998, 2005; Marcilla *et al.*, 2002).

The current investigation revealed that *F. hepatica* was the common liver fluke of cattle in the Dakhla Oasis subtropical area. About 2.4 million people in 61 countries are infected with *Fasciola* (Haseeb *et al.*, 2002). In Egypt, fascioliasis is endemic in certain villages, but the overall prevalence is unknown because reports show wide variations in infection rates. The existence of infected *G. trancatula* in the main water sources (wells), the low level of awareness and hygienic measures, and the distance of Dakhla Oasis from Cairo, the main capital, are contributing factors to high *Fasciola* prevalence in this area. *Fasciola*

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hepatica has been recognized by the World Health Organization as a zoonotic neglected tropical disease (WHO, 2015). Therefore, control methods must be considered in Dakhla Oasis.

In conclusion, *F. hepatica* and its intermediate host, *G. truncatula* snails, were recorded in seven districts of Dakhla Oasis, El-Wadi El-Gadid province, Egypt, both morphologically and molecularly with single-step and rapid mitochondrial DNA-targeting duplex PCR.

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Conflict of interest

None.

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