

## CYTOCHROME OXIDASE SUBUNIT AND INTERNAL TRANSCRIBED SPACER MOLECULAR REVELATION OF INFECTION WITH *FASCIOLA SP.* IN FIELD- COLLECTED *LYMNAEA NATALENSIS* SNAILS

By

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### Abstract

Fasciolosis is one of the neglected tropical diseases that negatively affect the farming industry and public health. The present study aimed to evaluate a polymerase chain reaction (PCR) assay for detection of infection with *Fasciola sp.* in field-collected *Lymnaea natalensis* snails from two rural localities in Egypt; and to compare between it and the ordinary microscopic methods. A total of 1000 lymnaeid snails were collected, and then the samples were examined for the infection by crushing, cercarial shedding and molecular methods. The average sensitivity of microscopic methods compared to PCR was 56.15% and this gives the indication that the molecular techniques were highly sensitive, specific and reliable methods for detecting *Fasciola sp.* The study detected two genes; nuclear ribosomal central internal transcribed spacer 1 (ITS1856bp) and mitochondrial cytochrome oxidase subunit I (CO1 831bp). Gel electrophoresis showed strong bands for these single snails' samples and confirmed the presence of infection in both localities. Molecular techniques gave sensitive, accurate identification of infection, established prevalence in endemic areas and planning time for proper control strategy.

Keywords: Fasciolosis; *Lymnaea natalensis*; Microscopic methods; PCR; ITS1; CO1.

### Introduction

Fasciolosis is a globally distributed helminthes infection of ruminants in tropical countries (Alba *et al.*, 2015; WHO, 2017), caused by infection of trematodes belonging to the genus *Fasciola sp.* (Lotfy and Hillyer, 2003); *Fasciola hepatica* causes huge losses in sheep and cattle worldwide (Beesley *et al.*, 2017) and human can get the infection accidentally from contaminated water or plants in endemic areas, where, millions of people are infected with fascioliasis around the world and there are more than 180 million at risk (Khan *et al.*, 2017). Freshwater snails of the family Lymnaeidae serve as intermediate hosts in the transmission of these liver flukes (Yakhchali *et al.*, 2015). *L. natalensis* snails were the main host for *Fasciola hepatica* or *gigantica*, which are widely distributed in Africa (Moema *et al.*, 2008). These snails have a wide prevalence and distribution in nearly all the Egyptian Governorates (Ashour *et al.*, 2008).

In Egypt, liver flukes of *Fasciola sp.* have negatively affected the farming industry and public health (Hussein and Khalifa, 2010),

and both *Fasciola hepatica* and *F. gigantica* are present (Hussein and Khalifa, 2010; Dar *et al.*, 2012). They cause economic losses of about US\$ 29.2 million annually (Arafa *et al.*, 2018). Also, human infection is a major public health problem in several areas of the world, including the Nile Delta where 4% were *F. hepatica* patients (Soliman, 2008).

To detect *Fasciola sp.* in field-collected snails, several techniques were used (Caron *et al.*, 2014). Simplest method was snail crushing then dissection (Dreyfuss *et al.*, 2005; Caron *et al.*, 2008), but this method detected only live free rediae and free cercariae within the snail's haemocoel. Also, cercarial shedding method detected larval stages in the intermediate host (Kaplan *et al.*, 1997; Baran *et al.*, 2017). This technique has low sensitivity, specificity and was time-consuming (Caron *et al.*, 2007). Both methods did not detect infection in the pre-patent period, compromising field trials (Magalhães *et al.*, 2004). So, studies on natural infections of field-collected snails have employed molecular tools (Lotfy *et al.*, 2008; Caron *et al.*, 2014; Yakhchali *et al.*, 2015). Molecular

techniques are highly sensitive and specific methods in detecting these liver flukes in the snails faster and more accurate species identification (Kaplan *et al*, 1995). In Egypt *F. hepatica* & *F. gigantica* intermediate snails were morphologically proved (Periago *et al*, 2008) and molecularly based on presence of ribosomal and mitochondrial genes (Amer *et al*, 2011; Dar *et al*, 2012). The nucleotide sequences of nuclear ribosomal central internal transcribed spacer1(ITS1), (ITS2) (Ichikawa and Itagaki, 2010) and DNA sequences of mitochondrial cytochrome oxidase subunit I (CO1) genes (Itagaki *et al*, 2005; Mas-Coma *et al*, 2009) identified *Fasciola* spp. (Zarowiecki *et al*, 2007).

Cytochrome oxidase enzyme fractional monetary unit considered as a factor typically used as a deoxyribonucleic acid barcode to spot animal species (Hebert *et al*, 2003). CO1 factor sequence is appropriate for this role as a result of its mutation rate which is usually quick enough to differentiate closely connected species. Internal transcribed spacers (ITS) refers to the spacer deoxyribonucleic acid set between the small-subunit ribosomal ribonucleic acid (rRNA) and large-subunit rRNA genes and contemplate because it is the most generally sequenced deoxyribonucleic acid region in molecular ecology (Ichikawa and Itagaki, 2010). Owing to its higher degree of variation than alternative sequence regions of rDNA, variation among individual rDNA repeats typical determination among ITS regions.

The present study aimed to compare between microscopy (shedding and crushing) and the conventional PCR to detect *Fasciola* infection in the field-collected *L. natalensis* snails from two rural localities in Giza governorate, Egypt.

### **Materials and Methods**

The field groups included 1000 *L. natalensis* snails collected during March to September 2017, from different irrigation canals in two rural localities in Giza governorate (Abo-Rawash and Manshaat Radwan) every single snail in a clean screw capped tube.

The collection area covering one square meter through the agricultural canals. Snails found under water *Lemna gibba* leaves were collected manually by sweeping net. Lymnaeid snails were found in areas where water was stagnant or presented only a slow current, usually in places where livestock was present. The collected snails were transported to the lab at Theodor Bilharz research institute at the same day of collection. In Malacology laboratory, the snails were maintained in 60x30x30cm plastic containers. *Lymnaea* snails were fed by lettuce 1–2 times per week and water in the containers was changed once 24-48 hrs. Eggs were collected daily on transparent plastic sheets placed into the aquaria.

Snail's infection: Two methods were carried out either, by exposure of snails to artificial light for one hr., at 25°C, for cercarial shedding (Liang *et al*, 1987) or by crushing (Chu and Dawood, 1970), where, snails were crushed between two slides, and were carefully investigated under the dissection microscope. The collected cercariae were identified as fresh or stained samples with iodine solution using light microscope. Non-shedding snails were kept in the laboratory and re-examined weekly to verify that cercariae were not shed by snails which could have been infected only shortly before being collected. When snails proved to be negative after several examinations by the shedding method, they were gently crushed in a Petri dish containing a small amount of dechlorinated water.

Molecular detection by purification of genomic DNA from snails' tissues using a spin column technique: Soft tissue of *L. natalensis* single snail sample was dissected separately, washed in 0.01M phosphate- buffered saline (PBS, pH 7.2) and stored at -20°C. Genomic DNA was extracted by Wizard SV Genomic DNA Purification System cat. A2360 Promega. 275µl were added of the digestion solution master mix to each sample tube and the sample was completely covered. Sample tubes were incubated over-

night (16-18hrs) in a 55°C heat block or water bath and 250µl of Wizard SV Lysis Buffer mixed to each sample by vortex. Entire sample lysate were transferred from 1.5ml microcentrifuge tube to a Wizard SV mini-column assembly and spin at 13,000×g for 3 minutes to bind the genomic DNA to the Wizard mini-column. The collected liquid was discarded and 650µl ethanol has been added as column wash. Re-centrifugation at 13,000×g for 1 minute and discard the collected liquids was done. 250µl were added of room temperature nuclease-free water to the Wizard SV mini-column and incubated for 2 minutes at room temperature (Marko *et al*, 1982; Boom *et al*, 1990). Wizard SV minicolumn/elution tube assembly placed into centrifuge and spin at 13,000×g for a minute and 250µl of nuclease-free water were added for 2 minute and recentrifuge for 1 minute. Cap the elution tube containing purified genomic DNA and store at -20°C. 250µl of nuclease-free water was added to the Wizard SV mini-column and incubated for 2 minutes at room temperature. Wizard SV minicolumn/elution tube assembly placed into the centrifuge and spin at 13,000×g for a minute and repeated twice (Soliman *et al*, 2017).

Primer design for CO1 & ITS1: Primers were designed using the online server called primer3 (<http://bioinfo.ut.ee/primer3-0.4.0/>) with specific conditions to give high quality, clear, sharp band and regarding the validation of the designed primer, done by the help of another online server IDT Oligo Analyzer ([https://www.idtdna.Com/Primer Quest](https://www.idtdna.Com/PrimerQuest)) for checking the validity and the integrity of the primers and if they have any hairpin form or hetero/homo primer dimer, which revealed a clear and absolute integrity for the structure and validity. CO1 & ITS1 primers were designed and requested from Macrogen Online Oligo Order System, Korea as follows: COX\_F: GAT CCA TAT TTT AAT TTG GTT TCT CCT & COX\_R: CCA GGA AAA GAC CTT AAT ACC C ITS\_F: CTA CTC TCA CAC AAG CGA TAC & ITS\_R:

CAA AGT GAC AGT GAC GGA AC.

Cytochrome oxidase subunit and internal transcribed spacer genes from *Fasciola* sp. were amplified by conventional polymerase chain reaction (PCR) using these specific primer sets. PCR was done in 50µl reaction mixture using 2µl of the genomic DNA extracted from single snail sample (diluted 1:30), 1.5U of Taq DNA polymerase, 50mM of each dNTPs, 2mM of MgCl<sub>2</sub>, 2.5µl of PCR reaction buffer (10×) and 0.2µM of each primer (Porta and Enners, 2012). The reaction was performed in T100 gradient thermal cycler of Bio-Rad. PCR mixtures were subjected to an initial denaturation step at 94 °C for 3 min, followed by 30 cycles of 30s at 94°C, 30s at 52°C to 65°C and 60s at 72°C, and a final extension step at 72°C for 10 min. An amount of 10µl of each PCR product along with the positive (= PCR mixture including the known DNA sample of *Fasciola* sp. and negative (= PCR mixture excluding DNA), controls were analyzed by electrophoresis on 2% agarose gel (w/v) for approximately 50 minutes at 90V, and visualized by trans illumination (Jeppson *et al*, 1979). For standardization of PCR result, laboratory inbred snail soft tissue was used as negative control and infected snail tissue as positive control (in shedding state).

Statistical analysis: Data were tabulated and analyzed. Sensitivity and specificity were calculated using the following: sensitivity (%) = TP/(TP+FN)×100 and specificity (%) = TN/(TN+FP)×100 (TP: true positive, FN: false negative, TN: true negative & FP: false positive) (Parikh *et al*, 2008).

## Results

The shell of *L. natalensis* is dextral, dark brown in color, fragile, with a wide open funnel. Its whorls range from three to five in number and are spirally coiled. The height of the shell is between 12.5-16mm (Fig. 1). Thus, the microscopic methods stated the presence of two types of cercariae. The cercariae were gymnocephalus (fasciolid) cercariae, followed by xiphidio cercariae (Fig. 2). Gymnocephalus cercariae (Fasciolidae) with

an unforked tail, and length almost as long as the body. Ventral sucker located on mid-ventral surface of body without style in oral sucker and produced by species of family *Fasciolidae* (Fig. 2, A & B). Xiphidio cercariae (Plagiorchidea) with an unforked tail; oral sucker with a style, and ventral sucker has the same size of oral sucker and located in the body middle (Fig. 2, C&D).

Number of gymnocephalus cercariae was higher in Abo-Rawash than xiphidio cercaria (61%), in Manshaat Radwan, xiphidio cercaria (52%) was higher than gymnocephalus cercariae (Fig. 3).

The present results showed higher infection in Abo-Rawash than in Manshaat Radwan. In Abo-Rawash 56/100(56%) snails were positive by microscopy compared to 97 (97%) positive by PCR and 95(95%) snails were positive by PCR in Manshaat Radwan (Tab. 1). By PCR, all control snails with laboratory infection were positive and control negatives were negative. This meant that PCR sensitivity and specificity were 100% (no false positive or false negative). The mi-

croscopic sensitivity to detect infected snails in Abo- Rawash was 60.8%; but negative in all uninfected ones (specificity 100%). In Manshaat Radwan, sensitivity was 51.57%, but negative in all uninfected snails with specificity 100% (Tab. 2). By comparing the sensitivity of the two methods, the average sensitivity of microscopic method compared to PCR was 56.15 % (Tab. 3).

The present molecular examination showed infection with *Fasciola* sp. larvae in field-collected *L. natalensis* snails from both sites. Notably, the present study used two genes; ITS1 fragment (856bp) and CO1 fragments (831bp) to prove infection. The optimum annealing temperature of CO1 primer was 58°C and for *Fasciola* internal transcribed spacer1 was 56°C. Agarose gel electrophoresis for both genes showed strong bands at the prospected molecular size: Numbers 4, 5 & 6 by using ITS1 primer and CO1 primer for samples 7, 8 & 9. The specimen 7 was positive but with faint band by CO1 primer but while, using ITS1 primer it confirmed by strong band (Fig. 4).

Table 1: Microscopy and PCR to detect infection in field- collected *Lymnaea natalensis*

Localities	Total No.	Microscopic examinations			PCR		
		Positive	Negative	Positive %	Positive	Negative	Positive %
Abo-Rawash	100	56	44	56	97	3	97
Manshaat Radwan	100	49	51	49	95	5	95

Table 2: Sensitivity and specificity of microscopy compared to PCR to detect infection in field- collected *L. natalensis* snails.

Localities			PCR		Sensitivity	Specificity
			Positive	Negative		
Microscopic examinations	Abo-Rawash	Positive	56	0	60.82	100
		Negative	41	3		
		Total	97	3		
Microscopic examinations	Manshaat Radwan	Positive	49	0	51.57	100
		Negative	46	5		
		Total	95	5		

Table 3: Average sensitivity of microscopy and PCR to detect infection in field- collected *Lymnaea natalensis*

Localities	Microscopic examinations	PCR
Abo-Rawash	60.8	100
Manshaat Radwan	51.5	100
Average	56.15	100

## Discussion

Morphological examination of the shell of *L. natalensis* snails revealed that they were thin, dextral, dark brown in color; the shell

whorls were ranging from three to five in number and were spirally coiled, its height ranging between 12.5 to 16mm. This agreed with El-Dafrawy and Taha (2007) they stat-

ed that the shell was thin, fragile, dextral with height ranged between 10 to 20mm and shell whorls ranged from 3 to 5 in number in *L. natalensis* snails collected from Giza Governorate, which were the most commonest Egyptian species (Ashour *et al*, 2008) especially in the northern parts of the Nile Delta (El-Dafrawy, 2002).

The present study showed that infection rate in Abo-Rawash was higher than that in Manshaat Radwan village by both methods. The infected snails contained two types of cercariae; gymnocephalus (fasciolid) cercariae and xiphidio cercariae. This agreed with Ashour *et al*. (2008) who reported that nearly all Egyptian Governorates have fasciolosis, mainly Giza, Fayoum, Damietta, Qalubia and Kafr El-Sheikh; Gharbia, Dakahlia and Sharkia, and linked the infection to the presence of aquatic plants *Lemna gibba* and *Jussiaea repens*. Also, Ahmed and Ramzy (1999); Hussein *et al*. (2005) and Soliman (2008) stated that both acute and chronic fasciolosis were found in almost all Egyptian Governorates and the infection rates of snails increased during summer months

In the present study, crushing and shedding techniques were used to facilitate microscopic detection of *Fasciola sp.* in snails compared to molecular dependent ones. The sensitivity of the two methods showed variation of microscopic method compared to PCR by 56.15 %. These results agreed with Lambert *et al*. (2012) and Arafa *et al*. (2018) who found that the microscopic methods did not give accurate or reliable results, while, PCR assays were highly specific and sensitive, providing useful and practical tools for the epidemiological investigation of *Fasciola* in the snail hosts.

Several specific molecular dependent techniques have been developed to detect *Fasciola* infections in the intermediate host snail and definitive hosts, utilizing various genetic markers, such as ITS regions, and mtDNA, including ND1 and CO1 (Ai *et al*, 2011; Reaghi *et al*, 2016). In the present study, the use of ITS1 and CO1 genes con-

firmed infection. Discrimination between *F. hepatica* & *F. gigantica* for the denovo designed primers, CO1 GenBank: AP017707.1 showed a complete homology with 100% to the *hepatica* query when using NCBI primer BLAST and ITS primers GenBank: AJ243016.1 revealed incomplete homology with *F. gigantica* while gave complete homology with *F. hepatica*. Agarose gel electrophoresis showed strong bands confirmed infection in both localities. The selected mitochondrial nucleotide sequences of the protein-coding CO1 could be used as an identification gene for the two *Fasciola* species (Arafa *et al*, 2018). These results agreed with Ai *et al*. (2010) who stated that molecular approaches based on DNA sequences of ITS1, ITS2 or the 28S ribosomal RNA gene, in combination with mitochondrial gene target were more valuable for the *Fasciola* sp. identification.

In the present study, the use soft tissue of *L. natalensis* gave reliable results. However, Arafa *et al*. (2018) reported that detection of *F. hepatica* by PCR was especially a point of strength by detecting dead and immature *Fasciola* stages giving missed data.

### Conclusion

PCR is a specific tool to detect *Fasciola* infection than the traditional methods. Using of ITS1 and mitochondrial CO1 genes to detect infection of *Fasciola sp.* gives reliable and accurate results in field-collected *L. natalensis* and help in the detection of its prevalence and in the control of this disease.

Compliance with Ethical Standards: All the applicable international, national, and/or institutional guidelines for care and use of animals were followed, but did not deal with human subjects.

*Conflict of interest:* Authors declared that they never have interest nor received funds.

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#### Explanation of figures

Fig. 1: Dorsal and ventral view of *Lymnaea natalensis* snails.

Fig. 2: Light micrographs of Gymnocephalus cercariae (A&B) and Xiphidio cercariae (C&D) Iodine stain; body (B); oral sucker (OS); stylet (ST); tail (T) and ventral sucker (VS).

Fig. 3: Percentage of gymnocephalus and xiphidio cercariae in *L. natalensis*.

Fig. 4: (2%) Agarose gel electrophoresis of PCR assay to detect *Fasciola* sp. in field-collected snails (by specific IRS & COS primers with a molecular size; 856 & 831bp, respectively). St =DNA ladder 100bp; 1 negative control; 2 & 3 negative infection; 4, 5 & 6 positive infection (ITS 1 primer); 7, 8 & 9 positive infection (by CO1 primer), 4, 5 & 7, 8 samples from Abo-Rawash; 6 & 9 samples from Manshaat Radwan.

