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IMMUNOLOGICAL AND MOLECULAR DETECTION OF DIGENETIC INFECTIONS IN DIFFERENT SPECIES OF EGYPTIAN FRESHWATER SNAILS

By

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Abstract

Due to the possibility of utilizing different snails in the combat of *Schistosoma* in Egypt; it is important to study the role it may play in transmitting other trematodes of medical and veterinary importance. Taking this background into consideration, polymerase chain reaction (PCR) assay was designed to identify trematode species at larval stages in intermediate hosts (cercariae in snails) using a combination of standard and molecular methods.

This PCR assay was also applied to naturally infected molluscan in order to assess the use of the procedure for detection. The importance of the present study was to demonstrate the epidemiological situation and application in control.

Keywords: *Biomphalaria alexandrina*, *Bulinus truncatus*, *Lymnaea natalensis*, *Melanoides tuberculata*, Comet assay, multiplex PCR

Introduction

Many snails act as intermediate hosts of medically and veterinary important digenean trematodes that infect humans or life stock animals (Puslednik *et al*, 2009). The correct taxonomic classification of these snails solely based on morphological characteristics was not always possible and has caused a confusing situation before additional analyses as molecular markers, allowed for an improved classification. Nowadays, sequence analyses of ribosomal RNA genes greatly helped to clarify relationships among different snail species around the world (Kaset *et al*, 2010).

Both fascioliasis and schistosomiasis are economically helminthic diseases with a significant impact on growth, development and productivity in domestic animals (Mas-Coma *et al*, 1999; Cribb *et al*, 2003). Two species *Fasciola hepatica* and *F. gigantica* are responsible for the disease in ruminant's worldwide (Fried *et al*, 2004; Soliman, 2008; Rashed *et al*, 2010). Besides, *Schistosoma mansoni*, *S. haematobium* and *S. japonicum* are responsible for about 200 million people infection in the tropic and subtropics (Adema *et al*, 2010; El-Sabah *et al*, 2011). Because of the sympatric relationship found in several metacercarial species of snail host including their morphology, which

is particularly similar in the egg forms and larval stages, it is difficult to distinguish such parasites from one another by standard methods (Aldhoun *et al*, 2009). Consequently, specific and accurate detection is needed for better definition and epidemiological control program.

A number of sensitive and specific techniques have been applied in the investigations of *Fasciola* and *Schistosoma* population dynamics (Bargues *et al*, 2001; Kolářová, 2006). As the conventional techniques used for the detection of the inter-molluscan stage of some digenetic infections such as *Schistosoma* sp., *Fasciola* sp. and *Heterophyes* sp. suffer on account of sensitivity and specificity, the development of highly sensitive and specific methods for detecting infected snails would greatly aid in epizootiological studies on digenetic infections (Kaset *et al*, 2010; Adema *et al*, 2010; Kozak and Wedrychowicz, 2010). In this context, development of RNA probe assay with its high level of sensitivity and specificity for digenean trematodes infected snails has been a forward step in this direction.

The aim of the present study was to evaluate the occurrence of trematode infections in *Biomphalaria alexandrina*, *Bulinus truncatus*, *Lymnaea natalensis* and *Melanoides tuberculata* and recognizing different types of cercariae using the crushing and emerging methods. Also, the study developed an appropriate and reliable methodology for identification of four snail species, by means of a multiplex PCR methodology of ITS-1 region.

Materials and Methods

Samples collected: Four species of freshwater snails were used *Biomphalaria alexandrina*, *Bulinus truncatus*, *Lymnaea natalensis* and *Melanoides tuberculata*. All snails were collected from agriculture canals, Abo Rawash area, Giza various streams swamps, rice fields and rivers of Egypt. The snails were transferred to the laboratory at the Department of Zoology, Faculty of Science, Cairo University. They were thoroughly cleaned and maintained for several weeks in aquaria with a thin substrate of sand, dechlorinated water and provided with blue green algae for feeding. After identification of species, each 10 snails were placed in Petri-dish containing dechlorinated water and then, they were placed against light for 3-5 h overnight at room temperature. The snails were examined for the presence of cercariae by shedding where observed snails were pressed and crushed between two square pieces of 15x15 glasses and studied in order to find cercariae, sporocysts and redia. Collected cercariae were observed carefully and mixed in 90% ethanol and 10% formalin, cleared in lactophenol and stained with azocarmine and neutral red. After measuring and drawing different parts of cercariae, the genus of cercariae was identified by a systematic key reference (Frandsen and Christensen, 1984; Christensen, 2003).

Sampling of hemolymph: Each snail was wiped and 100 µl of hemolymph was collected by puncturing the foot

with a micropipette. The blood was placed directly into 1.5 ml Eppendorf tube. Twenty four hours before sampling, snails were stimulated by gently touching the foot with the tip of a micropipette.

Comet assay: The alkaline comet assay was performed as described by Singh *et al*. (1988) with modifications. For each animal, three microscopic slides were covered with 1.5% normal melting agarose (Sigma) dissolved in PBS (Ca^{2+} and Mg^{2+} free) and maintained overnight at room temperature. A volume of 100 µl of hemolymph dissolved in 500 µl of 0.5% low melting agarose were placed vertically in a cuvette with lysis solution (2.5 M Nacl, 100 mM EDTA, 10 mM Tris, 1% Sodium sarcosinate, 1% Triton X-100 and 10% DMSO pH 10.0) overnight at 4˚C to remove proteins. After lysis, the slides were placed side by side in a horizontal electrophoresis tank and immersed in alkaline buffer, pH 13.0 (1 mM EDTA and 300 mM NaoH) for 30 min to allow DNA damage to expressed and submitted to electrophoresis (0.74 v/cm, 105mA) for 30min at 4˚C. The slides were then neutralized with 0.4 M Tris buffer, pH 7.5, fixed with absolute ethanol for 10 min, and stained with 50µl ethidium bromide (20µg/ml) (Sigma). The comets were analyzed under a fluorescence microscope (Carl Zeiss) at 400x, with an exciting filter of 515-560 nm and a barrier filter of 590 nm.

Data Analysis: The extent of DNA migration was evaluated by the visual scoring. Comets were classified and assigned to four categories (0-3) according to the extent of DNA migration. The classification was carried out on the basis of an appearance of comets (i.e., tail length, head diameter and intensity).

DNA isolation and PCR amplification of ITS -1 region: Total genomic DNA was isolated from 25 mg of raw or processed foot sections using the protocol described by Fernandez–Tajes and Mèndez (2007). Amplification of ITS-1 sequences were carried out using primer forward 5'-GTTTCCGTAGGT GAACCTG-3' designed by Heath *et al*. (1995) and reverse 5'-TGTGCGTT CAAGATGTCG-3' designed in this work based on 5.8S rRNA gene sequences of several bivalve species. Amplification reaction were performed in 25µl of reaction volume; the reaction mixture contained 15 ng of genomic DNA, 0.24 M of each dNTP, 2 mM of $Mgcl_2$, 1µM of each primer, 0.65 U of Taq polymerase and the buffer recommended by polymerase suppliers. The thermal cycler profile consisted of an initial denaturation step of 5 min at 95˚C, 35 cycles at 94˚C for 20s, 56˚C for 20s and 72˚C for 5 min. PCR products were visualized by electrophoresis on a 2 w/v agarose gel.

Purification, cloning and sequencing of PCR products: The ITS PCR product was ligated into the plasmid pCR 2,1-TOPO, using the TOPO-TA cloning kit (Invitrogen), and transformed into TOP10F cells. Plasmid DNA purification was carried out using Q1A prep Miniprep kit (Qiagen, Barcelona, Spain). Sequencing of both strands of

the insert of 4-6 clones/individual was performed with an automatic capillary DNA sequencer (CEQTM 8000 Genetic Analysis System). The nucleotide sequences have been deposited in the EMBL database under accession numbers J966667-97 and AM 933615-31.

Analysis of sequences and design of species-specific primers: Nucleotide sequences were aligned using Clustal-x software (Thompson *et al*, 1997) and alignments were edited with GeneDoC software (Nicholas *et al*, 1997). Interspecific differences found at ITS-1 region of snails were employed for designing reverse species-specific primers 3 software (Rozen and Shaletsky, 2000). The selection was done in such a way that each species–specific primer with a universal primer identical to the four species would produce a PCR amplification product of species-specific size.

Multiplex PCR: Once all primers were tested, multiplex PCR was performed in order to simplify the species identification. Multiplex PCR was carried out using 0.2 μM of each primer, and with identical condition as described above.

Results

Collection of snails based on shell morphology: Lymnaeids were selected on their general morphology of shell and body. All *M. tuberculata* snails shared on shell form with a short conical spire with 4 whorls, dextral coiling without an operculum. The shell is thinand translucent with an ear-shaped aperture, 4.5 times higher than the spire.

Morphological identification of larval trematodes: Four types of cercariae were found; Xiphidiocercarie, Furcocercous, Pleurolophocercous and Gymnocephalous among the four species of snails (*Melanoides tuberculata, Bulinus truncatus*, *Biomphalaria alexandrina* and *Lymnaea natalensis*) with a prevalence of different infection pattern (Tab. 1). *M. tuberculata* snail was the highest susceptible infection host for cercariae infection. Interestingly, it was also found that the furcocercous showed a high infection capability as it was found in *Melanoides*, *Bulinus* and *Biomphalaria* snails (Tab. 1).

Comet assay: Data on DNA strand breakage, expressed as the mean tail DNA content and the mean tail length in hemocytes of *M. tuberculata*, *L. natalensis*, *B. truncatus* and *B. alexandrina* exposed to the parasite infection (Fig. 1). On considering the four different classes of DNA damage, a highly significant difference among different snail groups. A strong dose response relationship was observed for the classes 0 and 3. Classes 2 and 3 increased up to intermediate doses and then decreases to the high doses. The difference in animal response between *L. natalensis* and *B. truncatus* although higher than in other animals, was also statically significant. However damage classes 1 and 3 showed no statistical difference between *L. natalensis* and *B. truncatus* (Tab. 2; Fig. 2).

PCR-based identification of cercariae in snails: The microscopic evolution of pepsin-digested tissue revealed furcocercous cercariae in different snails of

M. tuberculata, *B. truncatus* and *B. alexandrina*. This morphological identification was confirmed by DNA sequencing analysis of 18S rRNA gene after PCR amplification using DNA extracted from the cercariae. The use of morphology for detection of furcocercous cercariae mollusks required careful visual evaluation of features that are diagnostic for these species by highly trained professionals. Moreover, the PCR primers available at the start of this study also amplified DNA originating from the snail themselves, so the morphological finding could be confirmed by molecular analysis only after we ensured that the isolated cercariae were cleared of all molluscan DNA. To make detection of infected snails easier, new primers that allowed amplification of different snails DNA without interaction with snail DNA were developed.

PCR primers were designed to amplify 1134 bp DNA fragment of the *S. mansoni* and *F. hepatica* 185 rRNA gene. The primers were initially tested with DNA isolated as well as with the DNA extracted directly from tissue pieces from the four snails mentioned above (Figs. 3-6), and identical PCR results were obtained for *M. tuberculata* and *B. truncates* (Figs. 3,5). No amplification was observed from *B. alexandrine* snails that were negative for larval as determined by microscopy (Fig. 6). To ensure that negative PCR results were not caused by PCR inhibitors in the DNA samples, generic primers were utilized to amplify a 600bp fragment from the molluscan 18S rRNA in each samples analyzed. These results allowed us to omit larval, isolates and use this method with DNA extracted directly from the snails.

Amplification products: The smallest amplification product expected by designed primers is 42 bp, and the randomly repeated arrangement dictates ladder pattern of amplification products will size increments of 121 bp. In contrast, DNA from uninfected snails at the given high-stringency conditions was not amplified, thus enabling clear differentiation between infected and normal snails (Figs. 3-6).

Differentiation of lymnaeid snails in Egypt based on their 16S mitochondrial rRNA: To allow rapid identification of the four observed snail species, three PCR primer pairs were designed based on the obtained 16S rRNA sequences and used for molecular analysis. These additional PCR analysis confirmed that the four different 16S rRNA primer set 16S T1 or 16S T3, indicating a mixed population, whereas the snails were only positive with a single primer set, indicating non-specific populations.

Molecular diagnosis of naturally infected with the parasitic infection in lymnaeids: To allow not only identification of the snail species, but also diagnosis of the infection with *F. gigantica* was applied parasite cathepsin L, primers for 16S rRNA primers. Cathepsin L proteases are encoded by a multi-gene family in *Fasciola* and they are abundant expressed and secreted antigens in the mammalian stages of *F.*

gigantica, but their expression less well researched during development in the intermediate molluscan host. A primer pair used in a previous study to isolate partial cathepsin L cDNA from adult by RT PCR was used to identify, the corresponding *F. gigantica* genomic DNA from naturally infected snails. The PCR product had a size of 1615 bp and sequence analysis confirmed that it was a cathepsin L gene fragment containing three introns of 53, 161 and 741 bp size and 660 bp coding sequence split into a particular first exon with 57 bp followed by two complete exon of 223 bp ending with the stop codon overall. Using each specific primer together with a common forward primer (ITS), a 296 band was obtained for *M. tuberculata*/*L. natalensis*, a 223 bp PCR fragment was amplified from *B. alexandrina* individuals, and a 386 bp band was yielded from *B. truncates* (Figs. 7-10).

Identification of infected snails: control purposes, DNA from uninfected snails were individually collected, assayed by the PCR, and showed negative amplification results. Subsequently, pooled DNA from uninfected snails was used as a negative control in each amplification run in the thermocycler, when DNA extract from individual infected snails was examined different number of bands for amplification ladder bands seems to be related to the tested parasite concentration, the results suggests that different amounts of schistosomal DNA were successfully extracted by the extraction procedure.

Among 90 snails tested *M. tuberculata* with xiphidocercaria and furcocercous cercariae, (25%) were found to PCR positive, while among the 60% snails tested for pleuralphocerous and gymnocephalous cercariae (21%) and (3%) were also PCR positive. These results (Tab. 1) demonstrated that PCR sensitivity was 100%.

Identification of infected snails by PCR in DNA pools: Identification of infected snails when its DNA is pooled with DNA aliquots from several uninfected snails should increase the feasibility of using PCR assay for mass screening of infection. This was expected to be possible only when the detection sensitivity was very high.

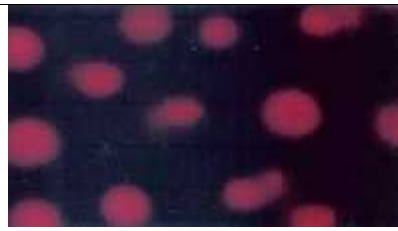
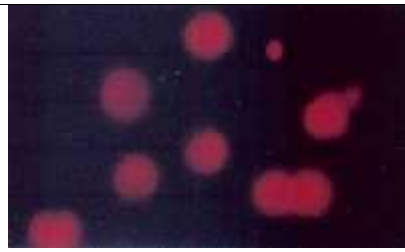
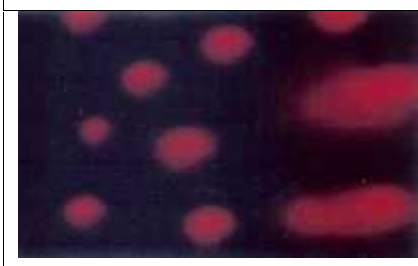
Table 1: Prevalence of cercariae infection among snail intermediate host collected from Egypt canal, Abo Rawash District, Giza Governorate.

Snail Species	Cercariae	No. of infected/examined	% Prevalence
Melanoides tuberculata	Xiphidiocercarie	32/90	37
	Furcocercous	25/90	29
	Pleurolophocercous	21/90	2
	Gymnocephalous	3/90	3
Bulinus truncatus	Xiphidiacercarie	23/90	22
	Furcocercous	17/90	4
Biomphalaria alexandrina	Furcocercous	32/90	37
Lymnaea natalensis	Gymnocephalous	8/90	6

Table 2: Comet assay: DNA damage in hemocytes of different snail species exposed to parasite infection

Species	DNA damage classes (% S.E)			
	C0	C1	C2	C3
M. tuberculata	442 (49.6)	178 (20.4)	165 (12.2)	175 (19.4)
L. natalensis	197 (22.4)	253(28.3)	173(19.3)	77(31.5)
B. truncatus	159 (16.5)	296 (30.3)	264 (26.4)	281(28.5)
B. alexandrina	58 (6.4)	49(5.2)	133 (15.2)	260 (73.5)

Fig. 1: Classes of DNA damage as detected by Comet assay in hemocytes of tested species.

Observed DNA Damage	DNA Migration	Damage Classes
	No migration	0
	Low	1
	Intermediate	2
	High	3

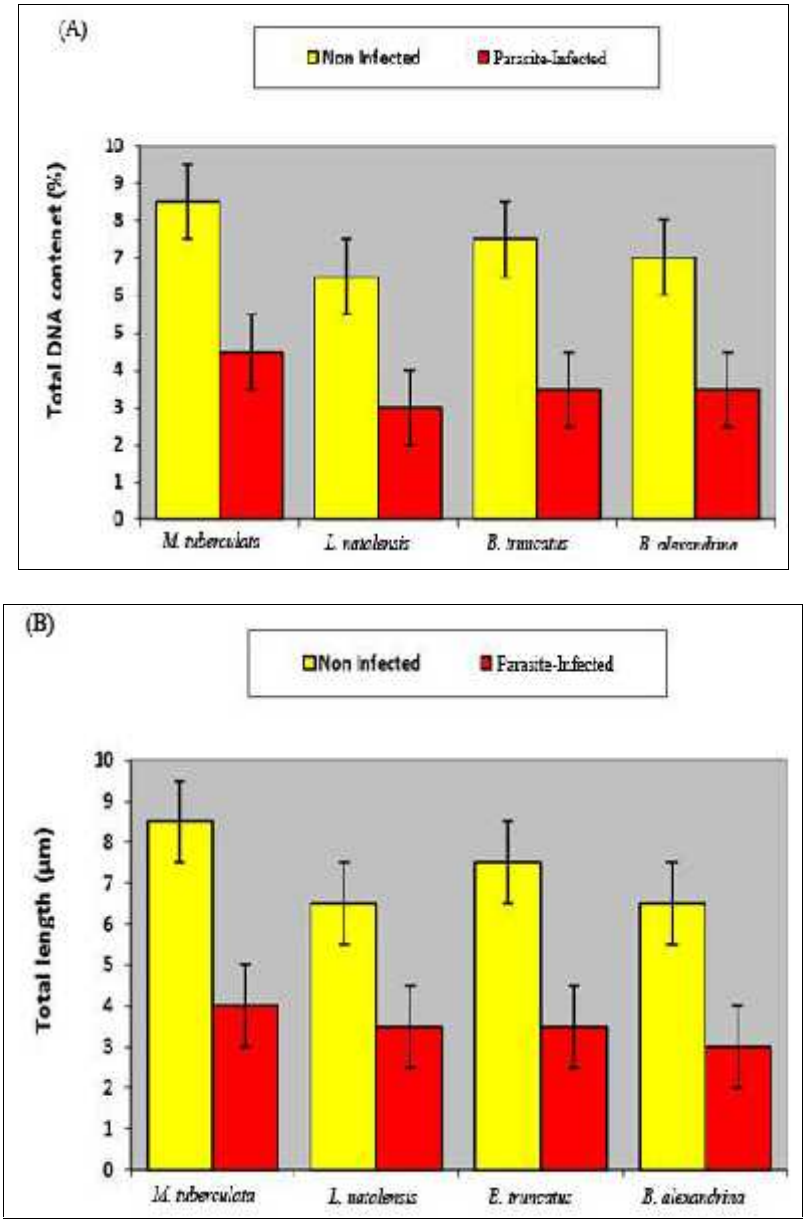


Fig. 2: Total DNA content (A) and total length (B) of non-infected and parasite infected snail of *M. tuberculata*, *L. natalensis, B. truncatus* and *B. alexandrina*, 20 specimens from each species collected and examined using comet assay. Mean ± SEM ($P<0.05$)

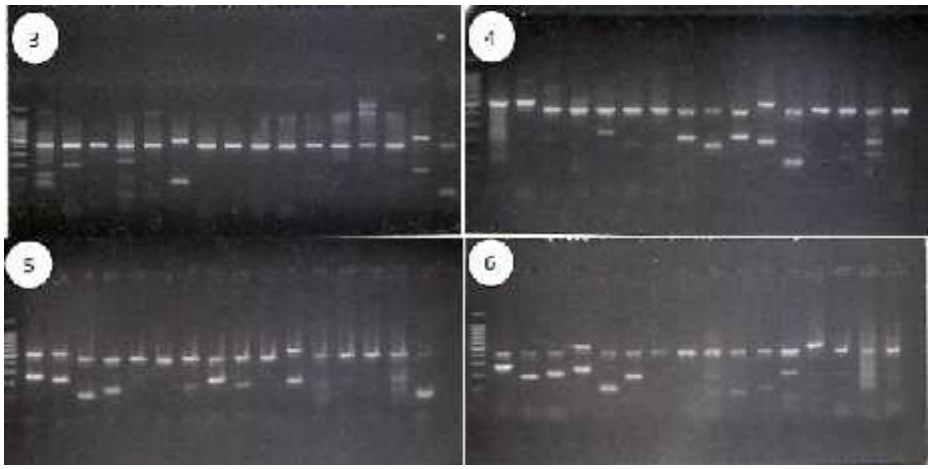


Fig. 3: Low stringency polymerase chain reaction (PCR) with uninfected and infected B. truncatus snails. Lane M size markers, lane 1–8 uninfected snails, lane 9–16 infected snails, lane 17 negative control (No DNA). Fig. 4: Low stringency PCR with uninfected and infected L. natalensis. Lane M, size markers, lane 1-16 uninfected snails, lane 17 negative control (no DNA). Fig. 5: Low stringency PCR with uninfected and infected M. tuberculuta. Lane M, size markers, lane 1-6 infected snails, lane 7-17 uninfected snails, lane 18 negative control (no DNA). Fig. 6: Low stringency PCR with uninfected and infected B. alexandrina snails. Lane M, size markers, lane 1-7 uninfected snails, lane 8-16 infected snails, lane 17 negative control (no DNA).

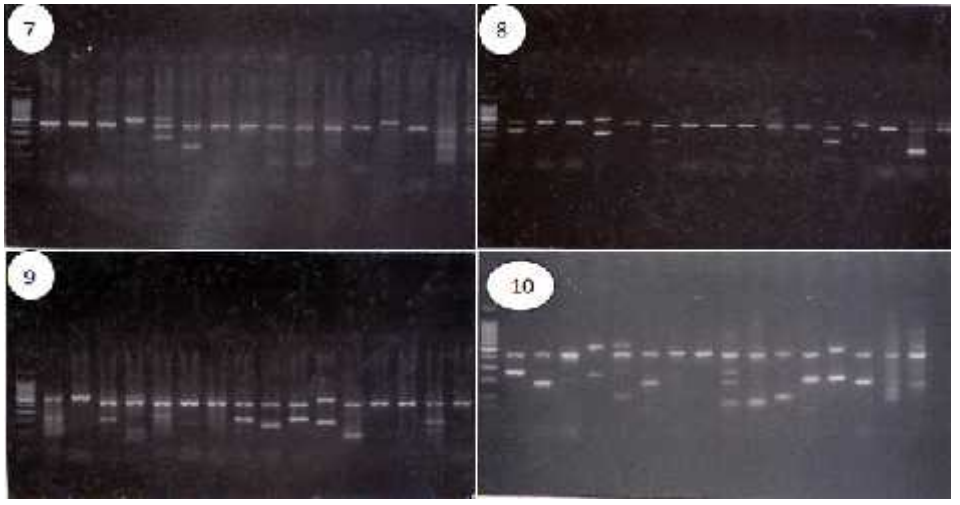


Fig. 7: Low stringency PCR with uninfected and infected *M. trberculata*. Lane M size markers, lane 1–6 uninfected snails, lane 7-15 infected snails, lane 16 negative control (No DNA). Fig. 8: Low stringency PCR with uninfected and infected *L. natalensis*. Lane M, size markers, lane 1-7 uninfected snails, lane 8-15 infected snails, lane 16 negative control (no DNA). Fig. 9: Low stringency PCR with uninfected and infected *B. truncatus*. Lane M, size markers, lane 1-15 uninfected snails, lane 16 negative control (no DNA). Fig. 10: Low stringency PCR with uninfected and infected *B. alexandrina*. Lane M, size markers, lane 1-5 uninfected snails, lane 16 negative control (no DNA).

Discussion

Many lymnaeids act as intermediate hosts of medically and veterinary important digenean trematodes that infect humans or life stock animals. The correct taxonomical classification of the snails solely based on morphological characteristics was not always possible and has caused a confusing situation where many species were at first collected into the genus *Lymnaea* before additional analyses, e.g. chromosome number and molecular markers, allowed for improved classification (Jannotti-Passos *et al*, l997; Hamburger *et al*, 1998).

In the present study, four species of Egyptian snails were analyzed for the presence of parasitic infection. The early sensitive detection of snails infected with a single miracidium, and the possibility of testing pools of DNA samples, as described in the present study, is expected to be useful for large scale screening of snail populations in nature. Furthermore, the clear positive versus negative differentiation between infected and non-infected snails should be superior to differentiation by banding pattern of the PCR products as shown by others (Hamburger *et al*, 1998). The identification was achieved by a multiplex PCR based method based on ITS-1 region. The ITS spares show more variability than their flanreing coding regions, and they can be easily amplified with universal primers (Presa *et al*, 2002; Fernande-Tajes *et al*, 2010).

Mas-Coma (1999) and Bargues *et al*. (2004), stated that sequence analyses of ribosomal RNA genes, both mitochondrial and nuclear greatly helped to clarify relationship among the Lymnaeida of Europe, America and Australia. Comparable rDNA based studies have not been performed in Asia where the two common lymnaeid species were for some time classified as races into the super-species *L. auricularia* proposed by Hubendick (1951) as were some other Asia species. As such they were named *L. auricularia rubiginosa* and *R. auricularia swinhoei* (Burch and Upatham, 1989). Indeed, *R. rubiginosa* has been identified as the intermediate host for *S. incognitum* (Bunnag *et al*, 1983), *F. gigantica* (Srihakim and Pholpark, 1991) and various *Echinostomes* (Charoenchai *et al*, 1997). It was hard to differentiate from *R. rubiginosa* by shell morphology without expertise in canchology or by body anatomy (Brandt, 1974). Previously, molecular sequence data was not available, for *R. swinhoei* and the primary objective of the study was to determine the 16S rDNA sequences of the four tested snails for the design of species-specific PCR primers. This allows a rapid identification of the snail species by investigators who are not experts in malacology, not only at primary research institutions, but also in the provincial offices of the government agriculture department that are responsible for collecting data on infection status, implementing control strategy and advising local farmers. Used in parallel with *F. gigantica* gene-specific primers in multiplex PCR, it is possible to identify snail host and the infection status in one simple assay. With this aim, ampli-

fication and sequencing of ITS-1 space of *B. alexandrina*, *M. tuberculata*, *L. natalensis*, *B. truncatus* was done. So, PCR product of some individuals from all species were cloned and sequenced; the complete ITS-1 sequences were determined to be 566 bp in *M. tuberculata*, 535 bp in *L. natalensis* and 560 bp in *B. alexandrina* and *B. truncatus*. Although there are some small differences among species, they could not be detected by agarose electrophoresis.

The alignment of the sequence obtained with those from the databases showed 314 variable sites, 248 nucleotide substitution and 66 indels in a total of 585bp, all of them in the spacer sequence. The number of species-specific sites was 11 for *M. tuberculata*, 12 for *L. natalensis*, 89 for *B. alexandrina* and 74 for *B. truncatus*. The presence of this several species-specific sites allowed us to design reverse species-specific primers for snail characterization. In order to make an-unequivocally identification of the species, species-specific PCR can be developed because a specific sequence can be detected in a pool of sequences of a different organisms (Rodrigiuez *et al*, 2003).

In the present study, gymnocephalous, pleurolophocercous, furcocercous and Xiphidiocercariae cercariae were found by shedding and crushing methods. In this study, 37% of *M. tuberculata* and 22% *B. truncatus* were infected with species of Xiphidiocercariae. The first intermediate hosts for Xiphidiocercariae are freshwater snails; the second intermediate ones are fish, arthropods, mollusks (snails) and definitive hosts are vertebrates including human (Fried *et al*, 2004; Ghobadi and Farahnak, 2004; Yousif *et al*, 2010). 29%, 4% and 37% for snails *M. tuberculata, B. truncatus* and *B. alexandrina,* respectively; were infected with furcocercous cercariae. These cercariae may infect fish, molluscs (snails, clams) and amphibians (tadpoles, frogs) (Karamian *et al*, 2011). It was noted that cercariae of all furcocercous are fork-tailed (Cribb *et al*, 2003; Yousif *et al*, 2010). Description of furcocercous cercariae was obtained from *M. tuberculata* (El-Gindy and Hanna, 1963), *B. truncatus*, *B. alexandrina* and *Bythinia* sp. (Rysavy *et al*, 1975). However, different furcocercous cercariae were described in Thailand from *M. jugicastis*, *M. tuberculata* and *Tarebia granifera* (Ukong *et al*, 2007). 3% of *M. tuberculata* and 6% of *L. natalensis* were infected by gymnocephalous cercariae. In the life cycle of fish, snails, amphibians and reptiles are sources of metacercariae and piscivorous birds and mammals are considered as final hosts. Readev *et al*. (1999) procured a gymnocephalous cercariae from the same snail species in Israel and described it as *Philophalmus* sp. They were also procured from *L. natalensis*, *Bythinia* sp. and *Melanopsis praemorsa* (Ismail and Abdelhafez, 1984). 2% of *M. tuberculata* were infected with pleurolophocerous cerceriae and described in same species, in other countries by Bogea *et al*. (2005) in Brazil. Other pleurolophocercous cercariae were described from other snail spp. in Egypt as from *Pirenella conica* (El-Gindy and Hanna, l963).

The comet assay was conducted after Singh *et al*. (1998) with modification after Grazefpe *et al*. (2008). The assay was performed with cells from hemolymph of *M. tuberculata*, *L. natalensis, B. truncatus* and *B. alexandrina*. Hemolymph of molluscs and crustaceans from the coelom of annelids was used for the comet assay (Lee and Steinert, 2003; Villela *et al*, 2006). Hemolymph is suggested as the more appropriate test tissue for low cell manipulation and a shorter slide preparation time. Hemocytes are exposed to environmental agents through their physiological roles in the transport of toxicants and in various defense mechanisms (Bolognesi *et al*, 2004). An advantage of using hemocytes of four tested species through a non-invasive technique: hemolymph is released by simply touching the foot surface, and a large number of cells can be obtained by previous stimulation by the same technique.

In the present study, DNA migration was evaluated by visual screening. Although image analysis is more accurate in the quantification of DNA damage, allowing measuring the fluorescence intensity and distribution of the DNA through the comet and giving a quantitative measure of DNA migration, visual scoring has the advantage of technical simplicity and low equipment costs. According to international guidelines and recommendations for comet assay, image analysis is preferred, but it was not required and visual scoring of comets remained a wheel-validated evaluation method used with high reliability (Burilnson *et al,* 2007).

The genetics of susceptibility to the different strains of snails to parasitic infection is complex and likely involve the interaction of several snail and parasite genes (Knight *et al*, 2000). On the other hand, there was no single morphological, biochemical or molecular marker that could reliably distinguish a susceptible from the non-susceptible snail. In controlled experimental conditions, where the influence of heterogeneous factors such as physical features of the water body (hydrodynamics, temperature, light intensity) and host size variation are limited. The infection pattern among the exposed freshwater snails to parasitic infection are determined by the level of host susceptibility resistance, and the basic cellular responses brought into play during the parasite development, as recently suggested (Kaset *et al*, 2010; Kozak and Wedrychowicz, 2010).

Several snail stocks in the present study showed a wide spectrum of host reaction to the parasite. In some snails, closer examination revealed small sporocysts in the mantle, collar, pseudobranch, or in the surface of the intestine. In the snails that developed footsporocysts, it seemed that the genetics of this phenotype probably involved multiple factors expressed in variable quantitative doses in the snail (Knight *et al*, 2000) between both isolates. This is the first time that a genetic variation is demonstrated between susceptible and resistant lymnaeid snails of *F. gigantica* infection in absence of experimental selection. By using RAPD-PCR analysis, Gutierrez *et al*. (2003) were

able to differentiate genetically defined lines of *Pseudosuccinea columella* selected for susceptibility and resistance to *F. hepatica*. RAPDs genetic variation was also detected for *B. glabrata* (Adema *et al*, 2010; Ittiprasert *et al*, 2011) and *B. tenagophila* (Ittiprasert *et al*, 2011) susceptible and resistant laboratory selected snails.

Conclusion

The molecular differentiation between the four species of Egyptian snails were analyzed for the presence of parasitic infection by using a multiplex PCR based on ITS-1 region and recognizing different types of cercariae using the crushing and emerging methods. The technique proved valuable for other snails of the zoonotic parasites

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References

Adema, CM, Hanington, PC, Lun, C, Rosenberg, GH, Aragon, AD, *et al*, 2010: Differential transcriptomic responses of *Biomphalaria glabrata* (Gastropoda, Mollusca) to bacteria & metazoan parasites, *Schistosoma mansoni* & *Echinostoma paraensei* (Digenea, Platyhelminthes). Mol. Immunol.47:49-60.

Aldhoun, JA, Faltýnková, A, Karvonen, A, Horák, P, 2009: Schistosomes in the north: A unique finding from a prosobranch snail using the molecular tools. Parasitol. Int. 58, 3:314-7.

Bargues, MD, Vigo, M, Horak, P,

Dvorak, J, Patzner, RA, *et al*, 2004: Gastropoda: Intermediate hosts of trematodiases, based on nuclear ribosomal DNA ITS-2 sequences. Infect. Genet. Evol. 1:85-107.

Bargues, MD, Vigo, M, Horak, P, Dvorak, J, Patzner, RA, *et al*, 2001: European Lymnaeidae (Mollusca: Gastropoda), intermediate hosts of trematodiases, based on nuclear ribosomal DNA ITS-2 sequences. Infect. Gen. Evol. 1, 2:85-107.

Birnboom, HC, Doly, J, 1979: A rapid alkaline extraction procedure for screening recombinant plasmid DNA. Nucleic Acids Res. 7:1513-23.

Bernatchez, L, 2002: Phylogeographic congruence between mtDNA and rDNA its markers in brown trout. Mol. Biol. Evolut. 19:1261-75.

Bogéa, T, Cordeiro, FM, Gouveia, J S, 2005: *Melanoides tuberculatus* (Gastropoda: Thiaridae) as intermediate host of Heterophyidae (Trematoda: Digenea) in Rio de Janeiro metropolitan area. Brazil. Rev. Inst. Med. Trop. de Sao Paulo, 47, 2:87-90.

Bolognesi, A, Buschini, E, Branchi, P, Carboni, M, Furlini, A, Martino, M, Monteverde, P, Rossi, C, 2004: Comet and micronucleus assays in zebra mussel cells for genotoxicity assessment of surface drinking water treated with three different disinfectants. Sci. Total Environ. 333:127-36.

Brandt, RA, 1974: The non-marine aquatic mollusca of Thailand. Archiv für Molluskenkunde, 105:1-423.

Bunnag, T, Thirachandra, S, Impand, P, Vorasanta, P, Imlarp, S, 1983: *Schistosoma incognitum* and its zoonotic potential role in Phitsanulok and

Phichit provinces, northern Thailand. Southeast Asian J. Trop. Med. Publ. Hlth. 14:163-70.

Burch, JB, Upatham, ES, 1989: Medical important mollusks of Thailand. J. Med. App. Malacol. 1:1-9.

Burlinson, PR, Tice, G, Peit, E, Agurell, SY, Brendler-Schwaab, AR, *et al*, 2007: Fourth International Work group on Genotoxicity testing results of the *in vivo* comet assay workgroup. Mutat. Res. 627:31-5.

Charoenchai, A, Tesana, S, Pholpark, M, 1997: Natural infection of trematodes in *Lymnaea* (Radix) *auricularia rubiginosa* in water reservoirs in Amphoe Muang, Khon Kaen Province, Southeast Asian. J. Trop. Med. Publ. Hlth. 28:209-12.

Christian, F, 2003: The indigenous fauna of trematodes. http://www.uni-bielefeld.de/biologie/Didaktik/zoologie/html-eng/trematoden-eng.html.

Cribb, TH, Bray, RA, Olson, PD, Littlewood, DT, 2003: Life cycle evaluation in the digenea: A new prespective from phylogeny. Adv. Parasitol. 54: 197-254.

El-Gindy, MS, Hanna, A, 1963: Larval trematodes found in snails *Pirenella conica* and *Melania tuberculata* with special reference to the heterophyiasis. Bull. Dis. 5:33-58.

El-Sabah AA, El-Metwally MT, Abozinadah, NY, 2011: Hepatitis C and B virus in schistosomiasis patients on the oral or parenteral treatment. J. Egypt. Soc. Parasitol. 41, 2:307-14.

Faltýnková, A, Haas, W, 2006: Larval trematodes in freshwater molluscs from the Elbe to Danube rivers (Southeast Germany) before and to-day. Parasitol. Res. 99:572-82.

Fernandez-Tajes, J, Mendez, J, 2007: Identification of the razor clam species *Ensis arcuatus*, *E. siliqua*, *E. directus*; *E. macha* and *Solen marginatus* using PCR-RFLP analysis of 5Sr DNA regions. J. Agric. Food Chemist. 55:7278-82.

Fernandez-Tajes, J, Freire, R, Mendez, J, 2010: A simple one-step PCR method for the identification between European and American razor clams species. Food Chemistry, 118: 995-8.

Ferte, H, Depaquit, J, Carre, S, Villena, I, Leger, N, 2005: Presence of *Trichobilharzia szidati* in *Lymnaea stagnalis* and *T. franki* in *Radix auricularia* in Northeastern France: Molecular evidence. Parasitol. Res. 95:150-4.

Frandsen, F, Christensen, NQ, 1984: An introductory guide to the identification of cercariae from African freshwater snails with special reference to cercariae of trematode species of medical and veterinary importance. Acta Trop. 41:181-202.

Fried, B, Graczyk, TK, Tamang, L, 2004: Food-borne intestinal trematodiases in humans. Parasitol. Res. 93:159-70.

Ghobadi, H, Farahnak, A, 2004: A faunistic survey on the cercariae of *Bellamya* (*Viviparous*) *bengalensis* snail and their zoonotic importance. Iran. J. Publ. Hlth. 33:38-42.

Grazeffea, V, Tallarico, L, Kawanoa, T, Sujuki, M, Nakano, E, 2008: Establishment of the comet assay in the fre-

shwater snail *Biomphalaria glabrata* (Say, 1818). Mut. Res. 654:58-63.

Gutierrez, A, Pointier, JP, Fraga, J, Jobet, E, Modat, S, *et al*, 2003: *Fasciola hepatica*: identification of molecular markers for resistant and susceptible *Pseudosuccinea columella* snail hosts. Exp. Parasitol. 105:211-8.

Hamburger, J, He, NA, Xin, XY, Ramzy, RM, Jourdane, J, *et al*, 1998: A polymerase chain reaction assay for detecting snails infected with bilharzias parasites (*Schistosoma mansoni*) from very early prepatency. Amer. J. Trop. Med. Hyg. 59:872-6.

Heath, DD, Rawson, PD, Hilbish, TJ, 1995: PCR-based nuclear markers identify alien blue mussel (*Mytilus* spp.) genotypes on the west coast of Canada. Canad. J. Fish. Aquat. Sci. 52:2621-7.

Hubendick, B, 1951: Recent Lymnaeidae, their variation, morphology, taxonomy, nomenclature and distribution. Kungl Svenska Vetenskaps akademiens Handlingar, 3:1-223.

Ismail, NS, Abdel-Hafez, SK, 1984: Two new cercariae from *Melanopsis praemorsa* (Thiaridae) snails in Azraq Oasis, Jordan. J. Parasitol. 33:353-9.

Ittiprasert, W, Myers, J, Odoemelam, EC, Raghavan, N, Lewis, F, *et al*, 2011: Advances in the Genomics and Proteomics of the Freshwater Intermediate Snail Host of *Schistosoma mansoni*, *Biomphalaria glabrata*. *Biomphphalaria* Snails and Larval Trematodes. pp. 191-213.

Jannotti-Passos, LK, Vidigal, TA, Dias-Neto, E, Pena, SDJ, Simpson, AJG, *et al*, 1997: PCR amplification of the mitochondrial DNA minisatellite region to detect *Schistosoma mansoni* infection in the *Biomphalaria glabrata* snails. J. Parasitol. 83:395-9.

Kaset, C, Eurstthichal, V, Viyanornt, V, Grams, R, 2010: Rapid identification of lymnaeid snails and their infection with *Fasciola gigantica* in Thailand. Exp. Parasitol. 126:482-8.

Knight, M, Ongele, E, Lewis, FA, 2000: Molecular studies of *Biomphalaria glabrata*, an intermediate host of *Schistosoma mansoni*. Int. J. Parasitol. 30:535-41.

Kolářová, L, Rudolfová, J, Hampl, V, Skírnisson, K, 2006: *Allobilharzia visceralis* gen. nov. sp. nov. (Schistosomatidae: Trematoda) from *Cygnus cygnus* (L.) (Anatidae). Parasitol. Int. 55: 179-86.

Kozak, M, Wedrychowicz, H, 2010: The performance of PCR assay for field studies on the prevalence of *Fasciola hepatica* infection in *Galba truncatula* intermediate host snails. Vet. Parasitol. 168:25-30.

Lee, RF, Steinert, S, 2003: Use of the single cell gel electrophoresis/comet assay for detecting DNA damage in aquatic (marine and freshwater) animal. Mutat. Res. 544:43-64.

Mas-Coma, K, Esteban, JG, Bargues, R, 1999: Epidemiology of Human Fascioliasis: A review and proposed new classification. Bull. WHO, 77, 4: 340-6.

Nicholas, KB, Nicholas, HB, Deerfield, DW, 1997: GeneDoc: analysis and visualization of genetic variation. EMB net News 4, 2:1-4.

Presa, P, Pardo, BG, Martınez, PG, Puslednik, L, Ponder, WF, Dowton, M, Davis, AR, 2009: Examining the phylogeny of the Australasian Lymnaeidae (Heterobranchia: Pulmonata: Gastropoda) using mitochondrial, nuclear and morphological markers. Mol. Phylog. Evolut. 52, 3:643-59.

Rashed, AA, Khalil, HH, Morsy, AT, 2010: Zoonotic ectopic fascioliasis: A review and discussion. J. Egypt. Soc. Parasitol. 40, 3:591-608.

Readev, V, Nollen, PM, Gold, D, 1999: Life history and identification of *Philophthalmus lucipeuts* from Israel. J. Parasitol. 5:291-4.

Rozen, S, Skaletsky, HJ, 2000: Primer3 on the WWW for general users and for biologist programmers. In: Bioinformatics Methods and Protocols: Methods in Molecular Biology. Krawetz S, Misener S (eds); Humana Press, Totowa, NY.

Rysavy, B, Brgens, R, Crosoheft, J, Yousif, F, *et al*, 1975: Larval trematodes stages in water snails from the Area of Warak El Arab (A.R.E.). Vest. Cs. Spol. Zod. 39:135-53.

Sangpradub, N, Boonsoong, B, 2007: Identification of freshwater invertebrates of the Mekong River and its tributaries. The Technical Publications, Mekong River Commission, Vientiane.

Sharif, M, Daryani, A, Karimi, SA, 2010: A faunistic survey of cercariae isolated from *Lymnaeid* snails in central areas of Mazandaran, Iran. Pak. J. Biol. Sci. 13:158-163.

Singh, NP, McCoy, MT, Tice, RR, Schneider, EL, 1988: A simple technique for quantitation of low levels of DNA damage in individual cells. Exp, Cell Res. 175:184-91.

Soliman, MFM, 2008: Epidemiological review of human and animal fascioliasis in Egypt. J. Infect. Dev. Countries 2:182-98.

Srihakim, S, Pholpark, M, 1991: Problem of fascioliasis in animal husbandry in Thailand. Southeast Asian J. Trop. Med. Publ. Hlth. 22:352-5.

Thompson, JD, 1997: The CLUSTAL X windows interface: flexible strategies for multiple sequence alignment aided by quality analysis tools. Nucl. Acids Res. 25:4876-82.

Ukong, S, Krailas, D, Dangprasert, T, Channgarm, P, 2007: Studies on the morphology of cercariae obtained from freshwater snails at Erawan waterfall, Erawan national park, Thailand. Southeast Asian J. Trop. Med. Publ. Hlth. 38:302-12.

Villela, IV, Oliveira, IM, Silva, J, Henriques, JAP, 2006: DNA damage and repair in haemolymph cells of golden mussel (*Limnoperna fortunei*) exposed to environmental contaminants, Mutat. Res. 605:78-86.

Yousif, F, Ibrahim, A, Elbardisy, S, Sleem, S, Ayoub, M, 2010: Morphology of new eleven cercariae procured from *Melanoides tubercultata* snails in Egypt. Aust. J. Basic Appl. Sci. 4: 1482-94.