DIAGNOSIS OF *FASCIOLA GIGANTICA* IN SNAIL USING THE POLYMERASE CHAIN REACTION (PCR) ASSAY

BY

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Abstract

The 124-bp repetitive and highly abundant DNA sequence -used as a specific probe for the detection of *Fasciola hepatica* infection in snails- was tested in the detection of *F. gigantica* infection in *Lymnaea natalensis*. The probe did not show any positive PCR results with *Lymnaea natalensis*, *Physa acuta*, *Biomphalaria alexandrina* and *Bulinus truncatus* or with *Schistosoma mansoni*, *S. haematobium* and *Echinostoma liei*. However, the probe was found capable to detect *F. gigantica* infection within *L. natalensis* at very early stages of the prepatent period and at very low concentrations. Thus, the present assay is specific and sensitive for the detection of *F. gigantica* within its intermediate host. It confirmed the idea that 124-bp repetitive and highly abundant DNA sequence in *Fasciola* sp. genome could be used as an epidemiological tool for examination of fasciolosis intermediate host. The nucleic acid-based assay could eliminate both inherent uncertainties and lengthy periods of time required for visual examination of the snails. Also, the assay is valuable in epizootiology of *F. gigantica*, vector suitability and host-parasite relationship.

Introduction

The bovine liver fluke, *Fasciola hepatica*, is an endemic parasite of herbivores in most temperate areas of the world.
In tropical regions the giant liver fluke, *Fasciola gigantica*, occurs instead of *F. hepatica*, whereas both species occur in subtropical climates (Kramer and Schnieder, 1998). Certain species of pulmonate snails belonging to the genera *Lymnaea*, *Pseudosuccinea* or *Stagnicola* are the usual vectors for these liver flukes (Shubkin et al., 1992). In Egypt, *Lymnaea natalensis* is considered as the susceptible snail of *F. gigantica* (Mohamed et al., 1998). The most common methods for identifying infected snails are cercarial release, dissection or crushing and examining microscopically snails. These methods are limited in sensitivity and practicality, especially if the prevalence of infection in snails is low or if the fluke is in its early stage of the prepatent period (Ronglie et al., 1994 and Kaplan et al., 1995). The use of nucleic acid-based techniques has become an important tool that overcome the shortcomings of traditional methods in the detection of parasitic organisms in intermediate and/or final hosts (Shubkin et al., 1992; Heussler et al., 1993; Ronglie et al., 1994; Kaplan et al., 1995; Touré et al., 1997; Ramzy et al., 1997; Hamburger et al., 1998; Kirvar et al., 2000). Despite the increasing popularity of these techniques, research devoted to the molecular detection of *F. gigantica* in its intermediate hosts has been minimal.

The goal of this study is to design an assay based on DNA-oligonucleotide probe that sensitively and specifically detects *F. gigantica*-infected snails, particularly at early stages of intramolluscan development of the parasite.

**Materials and Methods**

Adult worms of *Fasciola gigantica* were obtained from livers of Egyptian buffaloes (*Bubalus bubalis*), cows (*Bos taurus*) and sheep (*Ovis aries*). The worms were incubated for 6 hrs in 0.85% NaCl at room temperature to remove adherent host cells and empty intestinal caeca. *Schistosoma mansoni* and *S. haematobium* as well as *Biomphalaria alexandrina* and *Bulinus truncatus* were kindly supplied by the Schistosome Biological Supply Program (SBSP) at Theodore Bilharz Research Institute, Giza. *Echinostoma liei* adults were obtained from experimentally infected *Rattus norvegicus*.
according to Jeyarasasingam et al. (1972). All flukes were stored at -70°C until used. Lymnaea natalensis and Physa acuta were collected from irrigation canals at Abu-Rawash, Giza and transferred to the laboratory for breeding.

Eggs of F. gigantica recovered from the bile of infected bovine livers were washed several times and incubated in dechlorinated tap water at 26°C for 2 weeks in darkness for miracidial development. Following incubation eggs were exposed to light to stimulate hatching. L. natalensis were individually placed in 24-well tissue culture plates along with freshly hatched miracidia and 0.5 ml dechlorinated tap water. The snails were left overnight at room temperature to ensure maximum penetration, then removed from wells and reared in culture.

To determine the specificity of the primers, genomic DNA extracted from F. gigantica isolated from sheep, buffaloes and cows, E. liei, S. mansoni, S. haematobium, non-infected L. natalensis, B. alexandrina, B. truncatus and P. acuta were subjected to the PCR reaction. Additionally, 1 µl of DNA from L. natalensis infected by a single miracidium of F. gigantica for 24 hrs was diluted with sterile distilled water as follows 1:5, 1:10, 1:15, 1:20 and 1:25 to perform the PCR.

To detect infected snails during pre-patent period, 2 groups of 30 laboratory-raised L. natalensis were infected with a single & 5-7 miracidia respectively. Five snails from each group were removed from the culture tanks on 0 day (an hour post-infection, pi.), 1, 3, 9, 15 & 25 days pi. for PCR reaction.

The PCR technique was performed in three steps: DNA extraction, then DNA amplification followed by the detection of the amplified products by agarose gel electrophoresis. Genomic DNA from flukes and snails was extracted as described by Yap and Thompson (1987) but, with some modifications. The tissues were homogenized individually in 1.5 ml of lysis buffer (8% Triton X-100, 0.25 M sucrose, 50 mM Tris-HCl, 50 mM EDTA, pH 7.5). Then, freshly prepared proteinase K (1 mg/ml) was added and the homogenate was incubated at 65°C in a water bath for 2 hrs. To precipitate the genomic DNA, 1.0 ml of a sterile 2% CTAB solution was added to the homogenate and centrifuged at 1500 g. The supernatant was discarded and the precipitate was dissolved in
0.5 ml of 2.5 M NaCl, 10 mM EDTA, pH 7.7 and diluted with 1.0 ml of 40 mM Tris-HCl, 2 mM EDTA, pH 7.7. Two volumes of chloroform were added to the mixture and centrifuged at 12000 g for 10 min. Afterwards, the DNA was precipitated in absolute ethanol, incubated at -20°C overnight and centrifuged at 12000 g for 10 min. The DNA pellet was washed with 70% ethanol and dissolved in 30 μl of TE buffer (10 mM Tris, 1 mM EDTA, pH 8.0). The DNA was stored at -20°C until used for amplification. PCR mixtures consisted of 15 mM MgCl₂, 1 μl of 10 mM dNTPs, 2.5 μl of 50 pm of the two specific primers (Forward 5'-ATTCAACCATTCTGT-TAGTCC-3'; reverse 5'-ACTAGGCTTAAAGGCGTCC-3') and sterile Millipore water to a final volume of 50 μl. The design of the primers is based on 124 base-pair (bp) highly repeated sequence of DNA specific for *Fasciola* sp. (Kaplan et al., 1995 and Kramer and Schnieder, 1998). The PCR reactions were carried out in DNA thermal cycler (T-personal, Biometra) with the following thermocycling pattern: 94°C for 3 min (1 cycle); 94°C for 1 min (denaturing), 65°C for 1 min (annealing), 72°C for 1 min (primer extension) (35 cycles) and 72°C for 10 min (1 cycle). After amplification, 10 μl of PCR product were separated by electrophoresis on 2% agarose in TAE buffer. The gels were stained with ethidium bromide and visualized under UV irradiation.

Results

Genomic DNA extracted from three Egyptian isolates of *F. gigantica* was tested through polymerase chain reaction using the specific primers. The results of the reaction gave repeated fragments of DNA ladder, the size of the smallest one is about 114 bp. No variations were noticed among the 3 isolates of *F. gigantica* (Fig. 1A). The same results were found when the infected *L. natalensis* was subjected to the PCR amplification. To ensure the specificity of the primers, the PCR assay was tested in three species of other Digenea, *S. gigantica* specific primers (Fig. 1B). DNA extracted from *L. natalensis* infected with two different doses of miracidia (a single miracidium and 5-7 miracidia) at 0, 1, 3, 9, 15, and 25 days pi. were subjected to PCR amplification. *mansonii, S. haematobium* and *E. liei*
Fig. 1A: PCR products of 4 species of trematodes. Lane 1=F. gigantica from sheep, lane 2=F. gigantica from cow, lane 3=F. gigantica from buffalo, lane 4=S. mansoni, lane 5=S. haematobium and lane 6=E. hel. M = 100 bp DNA ladder size marker.

Fig. 1B: +ve=F. gigantica-infected L. natufensis, lane 1= non-infected L. natufensis, lane 2= B. alexandrina, lane 3= B. truncatus, lane 4= P. gracilis and lane 5= -ve control. M = 100 bp DNA ladder size marker.

Fig. 2A: PCR products of DNA extracted from L. natufensis infected with a single miracidium of F. gigantica at 0, 1, 3, 9, 15 and 25 days post-infection (Lanes from 1 to 6 respectively). M= 100 bp DNA ladder size marker.

Fig. 2B: PCR products of DNA extracted from L. natufensis infected with a single miracidium at different dilutions: 1:5, 1:10, 1:15, 1:20 and 1:25 (Lanes 2-6). Lane 1=-ve control. M= 100 bp DNA ladder size marker.
using the specific primer of *F. gigantica* and no PCR products were noticed (Fig. 1A). Negative results were obtained with non-infected *L. natalensis*, *B. alexandrina*, *B. truncatus* and *P. acuta*, using *F*. All snails gave positive results even those infected with a single miracidium and tested an hour post-penetration, indicating the high sensitivity of the assay (Fig. 2A). Several dilutions of snails genomic DNA infected with one miracidium (a day pi) were prepared and tested by PCR to detect PCR sensitivity. Dilution up to 1:20 gave optimum positive results with *Fasciola* specific primers (Fig. 2B).

**Discussion**

Traditional methods for the detection of trematode infections of snails were summarized into three methods by Kaplan *et al.* (1995). The first method depends on the observation of cercarial shedding, which has been used routinely in the study of schistosome infection rates in field populations of snails (Sturrock and Karamsadkar, 1979). The second method was the microscopic dissection, which has been used frequently for studying *F. hepatica* infection rates in snails (Smith, 1981). This technique has several problems: the snail dissection is tedious and time consuming; the intramolluscan stages of different trematodes are difficult to distinguish prior to cercarial development which requires stained histological sections to confirm the identification; furthermore, the detection of early stages prior to the release of rediae from the sporocyst is inadequate even with careful dissection. The third method includes snail crushing for identifying rediae or cercariae (Khallaayoune *et al.*, 1991). Although this technique is quick and simple but it fails to detect the prepatent infection. Due to these drawbacks of traditional techniques, parasitologists utilized recent nucleic acid technology for the detection of parasites within their intermediate and final hosts. Some efforts were directed towards the molecular detection of *F. hepatica* within its intermediate host but rare or no similar work has been done for *F. gigantica*. Shubkin *et al.* (1992) developed a nucleic acid-based probe that specifically detects *F. hepatica* as ssrRNA. The probe was examined closely in northern blots.
Heussler et al. (1993) developed a DNA probe based on *F. hepatica* repetitive DNA sequence. They were able to detect individual *F. hepatica*-infected snails in squash blots. At this assay, the sensitivity of the probe was not reported and snails were not tested at various times of the parasite's development. Rognlie et al. (1994) used the reverse transcriptase-polymerase chain reaction (RT-PCR) to amplify specifically a region of *F. hepatica* small-subunit rRNA, followed by hybridization to a *F. hepatica*-specific probe. The assay does not cross-react with trematodes outside Fasciolidae but detect *Fascioloides magna* rRNA. Additionally, the assay detects individual infected snails immediately after miracidial exposure. Kaplan et al. (1995) described a specific DNA probe for the detection of developmental stages of *F. hepatica* in snails. They identified the 124-bp repetitive and highly abundant DNA sequence that represents about 15% of the whole *F. hepatica* genome. Kramer and Schnieder (1998) examined *F. hepatica* and *F. gigantica* specimens from five continents using the repetitive DNA sequence described by Kaplan et al. (1995) as a specific probe. They reported that the degree of intraspecific identity ranged from 79 to 99% in *F. hepatica*, 93 to 99% in *F. gigantica* and the interspecific identity ranged from 81 to 100%. They suggested that this DNA probe sequence could be used worldwide as an epidemiological tool for the examination of intermediate snails infected with. The highly repetitive DNA constituents are present in multiple copies; the relative abundance of this repetitive DNA enables very sensitive detection. Additionally, these sequences evolve more rapidly than gene coding sequences, making them excellent candidates for species-specific probes for eukaryotic organisms (McLaughlin et al., 1987). DNA probes, using highly repetitive sequences, have been developed for the identification of various species of parasites. Barker et al. (1986), Buening et al. (1990), Sirisinha et al. (1991), Ramzy et al. (1997) and Hamburger et al. (1998) used these sequences for the detection of *Plasmodium falciparum*, * Babesia bigemina*, *Opithorchis viverrini*, *Wucheraria bancrafi* and *Schistosoma mansoni*, respectively.
So, the PCR primers used in the present investigation were designed as based on the 124-bp repetitive and highly abundant DNA sequence (Kaplan et al., 1995 and Kramer and Schnieder, 1998). These primers did not show any positive PCR reaction with the pulmonate snails and other trematodes examined. Moreover, the present study detected the parasite at very early stages of the prepatent period and at very low concentrations. Thus, it is concluded that the present assay is specific and sensitive for the detection of *F. gigantica* within their intermediate host. Also, this project has recon-firmed the idea that 124-bp repetitive and highly abundant DNA sequence could be used worldwide as an epidemiological tool for the examination of the snails in fasciolosis (Kramer and Schnieder, 1998). The nucleic acid-based assay could eliminate both inherent uncertainties and lengthy periods of time required for visual examination of the snail vector of *F. gigantica*. Moreover, with this assay novel and valuable research into epizootiology of *F. gigantica*, vector suitability and the host-parasite relationship will become possible in Egypt and other parts of the world where *F. gigantica* is prevalent.

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