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Detection of *Lymnaea columella* Infection by *Fasciola hepatica* through Multiplex-PCR

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From complete mitochondrial DNA sequence of Fasciola hepatica *available in Genbank, specific primers were designed for a conserved and repetitive region of this trematode. A pair of primers was used for diagnosis of infected* Lymnaea columella *by* F. hepatica *during the pre-patent period simultaneously with another pair of primers which amplified the internal transcribed spacer (ITS) region of rDNA from* L. columella *in a single Multiplex-PCR. The amplification generated a ladder band profile specific for* F. hepatica*. This profile was observed in positive molluscs at different times of infection, including adult worms from the trematode. The Multiplex-PCR technique showed to be a fast and safe tool for fascioliasis diagnosis, enabling the detection of* F. hepatica *miracidia in* L. columella *during the pre-patent period and identification of transmission areas.*

Key words: *Fasciola hepatica* - *Lymnaea columella* - multiplex polymerase chain reaction - DNA mitochondrial - diagnosis

Fascioliasis is regarded to be one of the most important parasitic diseases in domestic ruminants of economical importance and its aethiological agent, *Fasciola hepatica* (Linnaeus, 1758), is a cosmopolitan trematode in areas of bovine, caprine, ovine, and buffaloes breading (Lessa et al. 2000). Great economical losses are believed to be caused by such parasitism, leading to the decrease of meat and milk production as well as to high mortality rates in several countries in the world (Saleha 1991). Human cases have been reported in the five continents (Mas-Coma et al. 2001), but man participates as an accidental host in the parasite life cycle.

F. hepatica intermediate host snails belong to the genus *Lymnaea* (Saleha 1991). Lymnaeidae are widely distributed fresh water hermaphrodite pulmonata with a dextral shell and without operculum. There are over 20 species of the genus and many of them are *F. hepatica* and *F. gigantica* transmitters. *F. hepatica* Brazilian intermediate hosts are: *L. columella* (Say, 1817) and *L. viatrix* (Orbigny, 1835). The distribution of *L. columella* in South America has been reported in Venezuela, Colombia, and Equator, to the Andes east (Paraense 1982a). Prepelitchi et al. (2003) reported the first evidence of natural infection of *L. columella* with *F. hepatica* in Argentina. In Brazil, the geographical distribution of *L. columella* is quite broad and it has been reported to be present in the following states: Rio Grande do Sul, Santa Catarina, Paraná, São Paulo, Rio de Janeiro, Minas Gerais, Goiás, Distrito Federal, Mato Grosso, Mato Grosso do Sul, Amazonas, and Bahia (Paraense 1982a, b, 1983, 1986). In Minas Gerais, in the municipality of Itajubá, Silva et al. (1995) remarked the first finding of *L. columella* naturally infected by *F. hepatica*.

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Traditional methods of *F. hepatica* detection in *L. columella* are usually performed by exposing the snails to light and/or posterior dissection, through which cercariae may be observed (Souza et al. 2002). Such method does not allow the infection diagnosis during the pre-patent period, compromising field trials.

Molecular techniques have been extensively used as diagnosis tools. Up to the present, most of the works have reported the use of radioactive probes which hybridise with a ribosomal RNA (ribonucleic acid) region from *F. hepatica* (Shubkin et al. 1992, Heussler et al. 1993, Rognlie et al. 1994, Kaplan et al. 1995), but such methods are time-consuming besides demanding manipulation of radioactive materials.

The Multiplex-PCR technique consists of using specific primers, simultaneously, in a single reaction, under high stringency conditions. It has been widely used for detection and identification of a wide range of organisms, including: yeast strains; measles virus from clinical samples; isomorphic species from the complex *Anopheles dirus*; free-living *Amoeba* in the environment; and snails from the group *Bulinus africans* (Fujita et al. 1993, Mosqueira et al. 2002, Pelandakis & Pernen 2002, Stohard et al. 2002).

The present work was aimed at designing a pair of primers for the conserved and repetitive region of mitochondrial DNA from *F. hepatica* that could be used to detect infections by this trematode. Those primers were simultaneously used in a Multiplex-PCR with another pair of primers that amplified the internal transcribed spacer (ITS) region of rDNA of the trematode and molluscs which worked as an internal control.

MATERIALS AND METHODS

Parasites, snails, and experimental infections - Adult worms and eggs from *F. hepatica* (Uruguai strain) were obtained from experimentally infected rabbit gall bladder. The eggs were incubated at 27°C in distilled water in the dark for 12-14 days and then exposed to artificial light for approximately 2 h, at 28 ± 1ºC, for miracidia eclosion. Miracidia were collected with a micropipette under a stereomi-

croscope and then transferred to cell culture plate with 24 wells of the 2.5 ml liquid capacity. Into each well one *L. columella* snail was introduced together with five new-ecloded miracidia and the total volume was completed with distilled water. The dishes were covered with a lid for approximately 2 h in order to ensure that the snails were totally immersed in distilled water. The snails were killed at different times of infections (1, 5, 7, 10, 15, 20, and 34 days). The specimens of *L. columella*, measuring approximately 3-5 mm length, were reared and kept at the Snail Rearing of Centro de Pesquisas René Rachou-Fiocruz, according to Souza and Magalhães (2000). *F. hepatica* adult worms were frozen and kept at –70°C. The species *Cercaria macrogranulosa* and *Cercaria caratinguensis*, obtained from snails collected at Barreiro de Cima, city of Belo Horizonte, state of Minas Gerais, were also included in this study.

DNA extraction - Total DNA was extracted from the snails body, a cercariae pool, and adult worms, using the Genomic DNA Purification Kit Wizard (Promega). Briefly, the material was mechanically disrupted in 200 µl of nucleic lysis solution and incubated overnight with 50 µg/ml proteinase K. Thereafter, 80 µl of protein precipitation solution was added to the initial mix. The mixture was placed in vortex for 10-20 s and centrifuged at 14,000 rpm for 5 min. The supernatant was transferred to a microcentrifuge tube containing 300 µl of isopropanol at room temperature precipitation. The mixture was gently mixed by inversion for 20 min and centrifuged at 14,000 rpm for 5 min. The DNA pellet was washed with 300 µl of 70 % ethanol and centrifuged for 10 min. The pellet was treated with 50 µl of DNA rehydration solution for 30 min at 65°C and stored at –20°C.

Primers design - From the complete mitochondrial DNA sequence of *F. hepatica* (Le et al. 2001), available in NCBI (National Center for Biotechnology Information) Genbank, specific primers were designed for the conserved and repetitive region of mitochondrial DNA of the trematode, which consisted of identical tandem repeats of 85 nucleotides, rich in G and C. The designed primer pair was: FASCR (5' CCA AAT AAA TAG ATC AGC CC 3') and FASCF (5' ATA TTA AGA GTT GTG CCC C 3'). Annealing temperature was set to be 56°C.

Multiplex-PCR and PCR product analysis - Multiplex-PCR consisted of using simultaneously 2 pairs of primers in a single reaction, under high stringency conditions. The primers FASCR and FASCF flanked a region of mtDNA from *F. hepatica,* while the other pair, ETTS1 and ETTS2 (Kane & Rollison 1994), flanked the ITS region of the trematode and the mollusc, which worked as an internal control. PCR amplification was carried out in a final volume of 10 µl, with 1ng target DNA, 5 pmol of each primer, 200 µM each desoxyribonucleotide triphosphate (dNTP- PROMEGA), 0.8 units *Taq*DNA polymerase (Cenbiot RS) in a buffer containing 10 mM Tris - HCl, pH 8.5, 50 mM KCl, 1.5 mM MgCl2. Afterwards, each reaction tube was covered with 20 µl of mineral oil in order to avoid evaporation during PCR cycles. The samples were amplified in thermocycler M J Research, Inc; model PTC-100 (Programmable Thermal Controller). The program used involved 26 cycles: denaturation step at 95°C for 3 min, annealing at 56°C for 1 min and extension at 72°C for 1 min, followed by 25 cycles with denaturation step at 95°C for 45 s, annealing at 56°C for 1 min and extension at 72°C for 1 min, and the last cycle extension period time was changed to 5 min. A negative control (no DNA) was included in all the experiments. PCR products were visualized on 6% silver stained polyacrylamide gels. The gels were photographed using a digital camera.

Reaction sensitivity to detect F. hepatica in L. columella - To establish the reaction sensitivity for *F. hepatica* detection in *L. columella,* amplification of mtDNA was performed. DNA was extracted from 1 to 10 miracidia of *F. hepatica* and 1 negative *L. columella*. DNA from such organisms was quantified by spectrophotometry. DNA from a variable number of miracidia was mixed to a constant amount of total DNA from negative snails, in order to simulate the infection. Thus, DNA from negative snails (7 ng/µl) was mixed with DNA extracted 10 miracidia from *F. hepatica* (9.8 ng/µl), 5 miracidia (4.8 ng/µl), 3 miracidia (3.1 ng/µl), and 1 miracidia (0.8 ng/µml). Afterwards, PCR was performed as described above. PCR products were visualised on 6% silver stained polyacrylamide gels.

RESULTS

Multiplex-PCR - The designed primers generated a ladder band profile, specific for *F. hepatica* (Fig. 1 lane 2). Simultaneous use of species-specific primers directed to *F. hepatica* and the ITS region allowed us to diagnose infected *L. columella* with the trematode after: 1 day of infection (Fig. 1, lanes 4, 5); 5 days (Fig. 1, lanes 6, 7), 10 days; (Fig. 1, lane 8); 15 days (Fig. 1, lane 9); 20 days (Fig. 1, lane 10), and 34 days after infection (Fig. 1, lane 11). The characteristic ladder observed in Fig. 1 shows bands with intervals of 85 nucleotides. Such pattern mirrors the amplification of a tandem repeat DNA region, and it was ob-

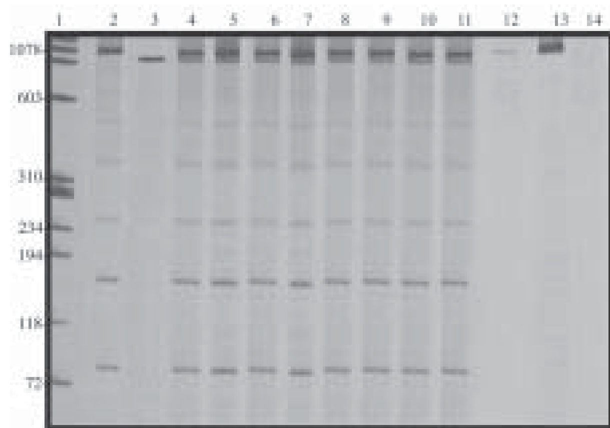

Fig. 1: 6% silver stained polyacrylamide gels showing mtDNA amplification of *Fasciola hepatica* by Multiplex-PCR. Lanes - 1: molecular weight pattern (ØX174 restriction enzyme *Hae* III digestion); 2: adult worm of *F. hepatica*; 3: negative *L. columella*; 4, 5: *L. columella* after 1 day of infection; 6, 7: *L. columella* after 5 days of infection; 8: *L. columella* after 10 days of infection; 9: *L. columella* after 15 days of infection; 10: *L. columella* after 20 days of infection; 11: *L. columella* after 34 days of infection; 12: *Cercaria macrogranulosa*; 13: *C. caratinguensis*; 14: negative control (no DNA)

served in all infected snails (Fig. 1, lanes 4 - 11), in adult worms from *F. hepatica* (Fig. 1, lane 2) and it was absent in non-exposed snails to miracidia (Fig. 1, lane 3). In this lane, only the ITS of rDNA from *L. columella* could be visualised (1200 pb). In positive snails, the ITS of rDNA from *F. hepatica* (1300 pb) and *L. columella* (1200 pb), and the specific bands generated by amplification of mtDNA from *F. hepatica* can be observed.

Reaction sensitivity of F. hepatica detection in L. columella **-** The reaction showed to be sensitive, detecting 1 miracidia (0.8 ng/µl) from *F. hepatica* (Fig. 2, lanes 10, 11) in the presence of the snail DNA (7 ng/µl).

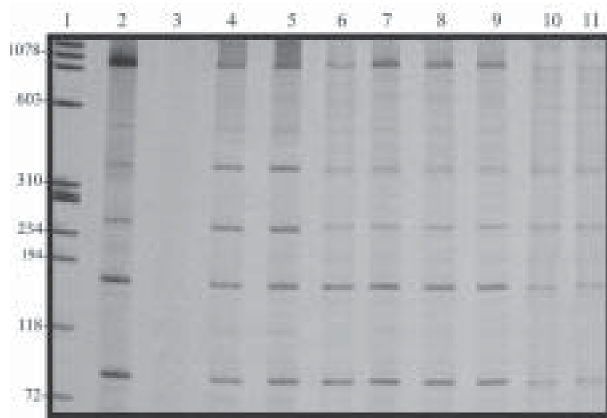

Fig. 2: 6% silver stained polyacrylamide gels, showing the sensitivity of the primers FASCR and FASCF for detecting *Fasciola hepatica* through polymerase chain reaction amplification. Lanes - 1: molecular weight pattern (ØX174 restriction enzyme *Hae* III digestion); 2: adult worm of *F. hepatica*; 3: negative snail DNA; 4, 5: negative snail mixed with 1 miracidia; 6, 7: negative snail with 3 miracidia; 8, 9: negative snail with 5 miracidia; 10, 11: negative snail with 10 miracidia

DISCUSSION

In the present work, primers were designed in order to detect the presence of *F. hepatica* in *L. columella* snails in the pre-patent period of infection by Multiplex-PCR, enabling a fast, sensitive and safe diagnosis of the trematode into its invertebrate host.

Diagnosis of *L. columella* infection by *F. hepatica* is routinely performed either by exposing the snails to light in order to observe cercariae or by dissecting them to visualize cercariae, sporocysts, and redia, mainly when located into the digestive gland. When the sporocysts are young and located in the cephalopodal region, or when the snails are collected in the field and probably infected with different trematode (Loker et al. 1982), it is impossible to diagnose the trematode through those methods due to the similarity of young stages. Another difficulty arises when the snails collected in the field die before their arrival in laboratory, precluding any chance of trematode detection. Such remarks are quite important once the snails should be kept in laboratory and periodically observed, in order to obtain reliable results. In addition, during the maintenance period, the snails may come to death before starting eliminating cercariae, and the diagnosis is once again compromised.

To overcome such constraints, molecular techniques have been used as auxiliary tools to detect *F. hepatica* in the invertebrate host. Other investigations, carried out so far, have used radioactive probes that hybridize with the ribosomal DNA region from *F. hepatica* (Shubkin et al. 1992, Heussler et al. 1993, Rognlie et al. 1994, Kaplan et al. 1995). These methods are long lasting and there is manipulation of radioactive material. Kaplan et al. (1997) used labeled probes and a chemiluminescent system to detect *F. hepatica.*

Here, we employed Multiplex-PCR, using 4 primers simultaneously in a single reaction, under high stringency conditions, enabling an specific DNA amplification from the snail and the trematode. This method was also highly sensitive, detecting specific *F. hepatica* infection in early pre-patent periods. Multiplex-PCR, besides being specific, provides an efficient internal control for the amplification of DNA from the mollusc.

The designed primers were specific for *F. hepatica* detection as demonstrated by the band profiles obtained from snails infected with this trematode and other ones (*C. macrogranulosa* and *C. caratinguenses*, for instance), commonly found in *L. columella*.

The profile with specific bands obtained through Multiplex-PCR was observed in positive snails at the different times of infection proposed here, including adult worms. This methodology has been applied to detect and differentiate several other organisms (Mosquera et al. 2002, Patsoula et al. 2003, Rivera et al. 2003, Fettene & Temu 2003). The high sensitivity of the test showed that the detection threshold of the parasite DNA was 1 miracidia and it may be used to detect infections during the pre-patent period, as demonstrated by the time intervals studied here. This methodology will be useful in field trials, providing a more realistic picture of the epidemiology of the disease.

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