PCR diagnosis of *Fasciola hepatica* in field-collected *Lymnaea columella* and *Lymnaea viatrix* snails

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Received 20 September 2005; received in revised form 31 October 2005; accepted 9 December 2005

Abstract

Fasciolosis, caused by the trematode *Fasciola hepatica*, is a zoonosis of economic importance in livestock that is emerging as a chronic disease in humans. The intermediate hosts are lymnaeid snails, in which diagnosis of infection is traditionally based on cercarial shedding, tissue sectioning and crushing.

We developed a PCR assay for the sensitive and specific detection of *F. hepatica* in field-collected *Lymnaea* sp. snails. A primer pair was designed to amplify a 405 bp fragment of the cytochrome c oxidase subunit 1 gene of *F. hepatica*.

The PCR assay showed a limit of detection of 10 pg of genomic *F. hepatica* DNA. No cross-reactions were observed with samples from other related trematode species or from the snail hosts *Lymnaea columella* and *Lymnaea viatrix*. DNA sequencing of the amplicon showed 100% homology with *F. hepatica*, and 75–89% homology with other trematodes on regions that did not include the entire set of primers.

Two samples from Argentina were analysed. For snails in sample 1 (n = 240), identified as *L. columella*, the infection rate was 17.5 and 51.3% by direct examination and PCR, respectively. For snails in sample 2 (n = 34), identified as *L. viatrix*, the infection rate was 2.9 and 61.8% by direct examination and PCR, respectively. Differences in infection rates between these diagnosis methods were significant for both samples.

Our PCR technique showed to be effective for detecting specific *F. hepatica* infections of low intensity in the intermediate host, and hence it could be used to study the epidemiological situation in a given area, as well as to assess host suitability for the parasite.

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Keywords: *Fasciola hepatica*; *Lymnaea* sp.; PCR

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1. Introduction

The common liver fluke, *Fasciola hepatica*, is the etiological agent of fasciolosis, a worldwide distributed disease that affects domestic livestock and humans (Hopkins, 1992; Rim et al., 1994). This disease causes important economic losses due to liver condemnation, higher mortality rates and reduced production of meat, milk and wool. In addition, human cases of fasciolosis have been increasing throughout the world, as documented between 1970 and 1990 (Mas-Coma et al., 2001).

The intermediate hosts involved in the transmission of the liver fluke are snails belonging to the Family Lymnaeidae. In Argentina, *Lymnaea columella* Say 1817 and *Lymnaea viatrix* Orbigny 1835, play an important epidemiological role as they have been found naturally infected with *F. hepatica*. The former is restricted to the northeast of the country (Castellanos and Landoni, 1981; Paraense, 1982; Prepelitchi et al., 2003), while the latter is more widely distributed (Paraense, 1982; Lombardero et al., 1979; Castellanos and Landoni, 1981; Rossanigo et al., 1983; Venturini and Fonrouge, 1985; Kleiman et al., 2004; Rubel et al., 2005).

The collection of accurate data on snail infection rates is crucial for estimating the potential infection risk of ruminants in endemic areas. The identification of *F. hepatica* in snails is usually based on morphological characteristics of mature cercariae. Sporocysts and rediae of *F. hepatica* and other trematodes belonging to the superfamily Echinocestoidea show a similar morphology, and therefore direct observation by microscopy may result in low sensitivity and/or specificity at early stages of infection (prepatent period). This highlights the importance of molecular techniques for the diagnosis of the parasite regardless its developmental stage. At present, most studies on the detection of *F. hepatica* in snails using hybridisation and/or amplification methods have been conducted in experimentally infected hosts (Shubkin et al., 1992; Rognlie et al., 1994; Kaplan et al., 1995; Magalhaes et al., 2004), and only a few ones dealt with natural infections (Rognlie et al., 1996; Kaplan et al., 1997).

The goal of this work was to develop a PCR assay allowing a sensitive and specific detection of *F. hepatica* in naturally infected *Lymnaea* sp. snails by means of an optimised DNA extraction protocol.

2. Materials and methods

2.1. Parasite materials

Adult *F. hepatica* flukes obtained from the livers of naturally infected bovines at a local abattoir were washed three times with 0.01 M phosphate-buffered saline, pH 7.2, and stored at −70 °C until processing. In order to extract parasite DNA, seven flukes were mechanically disrupted and 2 ml of 100 mM Tris–HCl, pH 8.0, were added. The homogenate was filtered through two layers of gauze and then centrifuged at 6000 × g for 3 min. The pellet was resuspended in 600 μl lysis buffer (100 mM Tris–HCl, pH 8.0, 100 mM EDTA, pH 8.0, 2% SDS, 150 mM NaCl and 200 μg/ml proteinase K), and incubated for 2 h at 56 °C followed by an overnight incubation at 37 °C. The DNA was purified by a standard phenol–chloroform extraction procedure and ribonuclease A treatment (20 μg/ml) (Maniatis et al., 1989). The final pellet was resuspended in 100 μl TE (10 mM Tris–HCl, pH 7.4, 1 mM EDTA, pH 8). The analysis of purified DNA was performed by electrophoresis in ethidium bromide-stained 1% agarose gel, and intensity was compared to quantitative standards.

2.2. Field-collected snails

Snails collected from water bodies located in different endemic areas of Argentina were taxonomically identified according to Paraense (1976, 1984). Sample 1 (*n* = 240), composed of *L. columella* specimens from Berón de Astrada (27°33’S and 57°32’W), Corrientes province, was collected in May 2003. Sample 2 (*n* = 34), composed of *L. viatrix* snails from La Toma (33°03’S and 65°36’W), San Luis province, was collected in March 2003.

All snails were measured from the apex to the anterior margin of the shell. To compare infection rates among different age classes, individuals in sample 1 were grouped into shell length intervals of 0.99 mm. In order to balance the number of snails in each interval, the smallest and largest sizes were joined together in intervals ranging 1.9–4.87 and 8.88–
12.87 mm, respectively. Finally, they were examined microscopically for the presence of trematode larvae as previously reported (Prepelitchi et al., 2003).

2.3. Snail DNA isolation

Following microscopic analysis, whole snail bodies were kept in 70% ethanol at 4 °C until processing. After ethanol evaporation, each sample was incubated in 300 µl of lysis buffer (100 mM Tris–HCl, pH 8, 100 mM EDTA, pH 8, 2% SDS and 150 mM NaCl) and 10 µl of proteinase K (20 mg/ml) for 3 h at 56 °C. After overnight incubation at 37 °C, 10 µl of proteinase K were added again and the samples were incubated for another 3 h at 56 °C. This was followed by a standard phenol–chloroform DNA extraction procedure (Maniatis et al., 1989) with an additional step to remove inhibitors (Kamenetzky et al., 2000). Briefly, each sample was resuspended in 1 ml of 60% GuSCN, 50 mM Tris–HCl, pH 6.4, and two washes with 0.5 ml of 60% (w/v), GuSCN, 50 mM Tris–HCl, pH 6.4, and two washes with 0.5 ml of 70% ethanol were performed by 1-min centrifugation at 12,000 × g. After drying the pellet, nucleic acids were eluted by a 10-min incubation at 56 °C in 50 µl of 5 mM Tris–HCl, pH 8. The eluted DNA was clarified by centrifugation at 12,000 × g during 2 min.

2.4. Primers design

The complete mitochondrial DNA sequence of F. hepatica, available at the GenBank Data Base (accession number X15613.1), was used to design the primer pair employed in this study. The positions were selected to amplify a 405 bp region of the cytochrome c oxidase subunit 1 gene (gene position corresponds to nucleotides 1888–3420 of X15613.1). The forward primer, named FhCO1F (5’-tattgttggattt-tacccggg-3’), and the reverse primer, named FhCO1R (5’-atggcacaaccacaacactt-3’), were based on nucleotides 2596–2616 and 3000–2980, respectively, of the above-mentioned sequence. Although data Bank search displayed no homologies with other parasites for this set of primers, an additional specificity assay was performed.

2.5. PCR procedure

Amplifications were performed in a total volume of 50 µl. The PCR mixture consisted of 50 mM KCl, 10 mM Tris–HCl (pH 9.0), 0.1% Triton X-100, 200 µM (each) dNTP, 2.5 mM MgCl2, 0.1 µM of each primer and 1.0 U of Taq DNA polymerase. The template used for each standard reaction corresponds to 10% of the total purified DNA from each snail (5 µl). The PCR procedure consisted of 60 s of denaturation at 95 °C, followed by 60 s of annealing at 56 °C and 90 s of extension at 72 °C, for 30 cycles. An initial step of 3 min at 98 °C and a final step of 3 min at 72 °C were included.

Fifteen microlitres of the amplification products were electrophoretically resolved in 1.2% agarose gels and stained with ethidium bromide.

All negative samples were also tested by PCR with the addition of 0.048% BSA in the reaction mix to inactivate possible remaining inhibitors.

2.6. Specificity and sensitivity assays

To test the specificity of the assay, we used trematode species other than F. hepatica which had been found parasitizing Lymnaea sp., namely Cotylurus sp., Echinoparyphium sp., Echinostoma sp., and some specimens of Echinostomatidae of unknown genus. Uninfected laboratory-reared L. columella and L. viatrix were also tested using the same DNA isolation and PCR protocols as described above for field-collected samples.

The sensitivity was determined using parasite-purified DNA. Background DNA samples comprising 10% of the total extracted from an infection-free Lymnaea sp. snail were spiked with 2.5 ng, 1 ng, 500 pg, 250 pg, 100 pg, 10 pg or 1 pg of F. hepatica DNA and run through PCR. Sensitivity was also tested with 2.5 ng, 500 and 100 pg in the presence of 20% of the DNA snail sample.

2.7. DNA sequencing

To confirm the sequence of the amplification product, we used one sample negative by standard
PCR protocol but positive after BSA addition. Amplicon of the right size was purified from an agarose gel with the NucleoSpin Extract Kit (Macherey-Nagel). DNA sequencing of the PCR product was performed using an ABI 377 Automated DNA Sequencer (Perkin-Elmer) with the ABI PRISM Big Dye™ Terminator Cycle Sequencing Ready Reaction Kit (Perkin-Elmer Applied Biosystems). Sequence similarity was analysed using the Blast program of the National Center for Biotechnology Information (www.ncbi.nlm.nih.gov), and multiple alignment employing the ClustalW 1.8 free software.

2.8. Statistical analysis

The \( \chi^2 \) test was used to compare overall infection rates between the PCR assay and direct examination in the two samples, as well as relative infection rates obtained by both methods within each shell length interval in sample 1.

The kappa statistic (calculated at a 99% confidence interval) was used in both samples to determine the concordance between direct examination and the molecular method (Cohen, 1960). The comparison of kappa value among shell length intervals (test for kappa homogeneity) was performed for sample 1.

All analyses were done using the STATS and EPIDAT softwares.

3. Results

3.1. Sensitivity and specificity of the PCR technique

The PCR assay detected 10 pg of purified genomic \( F. hepatica \) DNA. This minimum detection level was not diminished by the presence of 10% of the total DNA extracted from an infection-free \( Lymnaea \) sp. snail, but it decreased to 100 pg when 20% of snail DNA was added (Fig. 1).

No cross-reactions were observed with samples from \( Cotylurus \) sp., \( Echinoparyphium \) sp., \( Echinostoma \) sp., or other Echinostomatidae. Likewise, no amplification was detected with DNA from uninfected laboratory-reared \( L. columella \) and \( L. viatrix \) (Fig. 2).

Fig. 1. Limit of detection of the PCR assay by agarose gel electrophoresis of amplification products using purified DNA of \( F. hepatica \) and \( Lymnaea \) sp. as templates. Lane M, molecular size marker (100 bp ladder); lanes 1–7, 2.5 ng, 1 ng, 500 pg, 250 pg, 100 pg, 10 pg and 1 pg of \( F. hepatica \) DNA, respectively; lanes 8–14, samples of \( F. hepatica \) DNA as described for lanes 1–7, with the addition of 10% of the DNA extracted from a whole \( Lymnaea \) sp. snail; lanes 15–17, 2.5 ng, 500 pg and 100 pg of \( F. hepatica \) DNA, respectively, with the addition of 20% of the DNA extracted from a whole \( Lymnaea \) sp. snail; lane 18, reaction mixture.

Fig. 2. Specificity analysis of the PCR assay by agarose gel electrophoresis of amplification products. Lane M, molecular size marker (100 bp ladder); lanes 1–5, template DNA extracted from \( Echinoparyphium \) sp., \( Echinoparyphium \) sp., \( Echinostoma \) sp., Echinostomatidae and \( Cotylurus \) sp., respectively; lanes 6 and 7, uninfected laboratory-reared \( L. columella \) and \( L. viatrix \); lane 8, 2.5 ng of \( F. hepatica \) DNA; lane 9, reaction mixture.
3.2. Comparison of infection rates between direct examination and PCR assay

According to direct observation, the overall infection prevalence in *L. columella* (sample 1) was 17.5% (42/240) (Table 1). *F. hepatica* was accurately identified by the morphology of cercariae in 12 of the infected snails (28.6%), while rediae were present in the remainder. In *L. viatrix* (sample 2) only 1 out of 34 snails (2.9%) was infected (shell length: 9.01 mm), but the taxonomical identity of the parasite could not be established because only rediae were found.

Due to the morphological characteristics of the rediae found and the fact that no other trematodes within the superfamily Echinostomatoidea were present in the samples, we considered them as *F. hepatica* rediae. For further analysis, snails hosting any of the larval stages were considered as positive for *F. hepatica* by microscopic observation.

The presence of inhibitors in sample 1 was inferred from some snails positive for *F. hepatica* by direct observation but negative by the standard PCR procedure. To overcome this problem, the PCR mix was modified by the addition of 0.048% BSA. This procedure was successful, since 32.0% (55/172) and 45.8% (11/24) of the negative snails from samples 1 and 2, respectively, turned out to be positive. On this basis, we reported results of prevalence by PCR after inhibitor removal.

For sample 1, the overall prevalence of *F. hepatica* infection by the PCR technique was 51.3% (123/240) (examples in Fig. 3). Only one snail positive by direct observation was negative by PCR. Out of 123 PCR-positive snails, 82 (66.7%) were not detected by direct observation (Table 1). All immature infections previously attributed to *F. hepatica* were confirmed by PCR.

In sample 2, the PCR technique showed 61.8% (21/34) of positive snails, including the one detected by direct observation.

3.3. Statistical analysis

The overall prevalences of infection were significantly different (*p* < 0.005) between the PCR assay and direct examination for samples 1 and 2.

![Fig. 3. Agarose gel electrophoresis of amplification products showing the feasibility of PCR assay for detecting *F. hepatica* in field-collected snail samples. Lane M, molecular size marker (100 bp ladder); lane 1, 2.5 ng of *F. hepatica* DNA; lane 2, reaction mixture; lanes 3–12, template DNA from 10 specimens of sample 1; lanes 13–19, specimens from sample 2.](image)
Fig. 4. Multiple sequence alignment of the PCR product (aFhCO1) with sequences of cytochrome oxidase subunit 1 (CO1) gene from different trematode species. Dots indicate base identical to aFhCO1 and horizontal lines indicate gaps inserted for optimal alignment or end of the available sequence. Primer positions are underlined. Each sequence is named by its GenBank accession number. Homologies with the amplicon sequence are presented in brackets. X15613.1, Fasciola hepatica mitochondrial genome (100%); AF025823, Echinostoma revolutum partial CO1 gene (89%); AF025824, Echinostoma revolutum (89%); AF025829, Echinostoma caproni Madagascar (87%); AB189980, Isthmiophora hortensis (85%); AY626537, Cercaria shikokuensis lineage PL3 haplotype 2 (81%); AF159596, Paragonimus mexicanus strain Ecuador (79%); U82264.1, Schistosoma japonicum (75%).
In sample 1, shell length intervals showed significant differences in infection rates between PCR and direct examination (Table 1), except for the last one (largest sizes).

Concordance between the PCR technique and direct examination was non-acceptable with kappa statistics of 0.3194 (99% C.I. = 0.2030–0.5358) and 0.0368 (99% C.I. = −0.0589 to 0.1325) for samples 1 and 2, respectively. When kappa values were calculated for each shell length interval of sample 1, they showed non-acceptable concordance between both methods (kappa < 0.4), and the test for kappa homogeneity showed non-significant differences (p = 0.6768) among shell length intervals.

3.4. Sequence analysis

The nucleotide sequence showed that the amplification product is a 405 bp element. The data Bank search displayed 100% homology with the region of the cytochrome c oxidase subunit 1 gene of F. hepatica, contained in the mitochondrial DNA (GenBank accession number X15613.1). Homologies with other trematodes varied from 89 to 75%, but all of them were on regions that did not include the entire set of primers (Fig. 4).

4. Discussion

Traditionally, the diagnosis of F. hepatica infection in snails has been carried out by checking for cercarial shedding and/or microscopic examination of developmental stages of the parasite with or without snail crushing (Olsen, 1944; Khallaayoune et al., 1991). As already mentioned, this procedure has low specificity and sensitivity for early intramolluscan stages.

If field-collected snails die either before arriving at the laboratory or before cercarial release, diagnosis of F. hepatica by direct examination is seriously threatened and hence DNA-based tests become the most suitable diagnostic tool. In addition, our study provides the first PCR assay with a high specificity for F. hepatica in field-collected intermediate hosts, thus overcoming the disadvantages of the traditional method.

Our results showed that PCR detected a larger number of infected snails than direct observation, confirming our previous assumption that all rediae found belonged to F. hepatica.

The lack of significant differences in the infection rates of the largest-sized individuals between methods might be explained by a longer time of exposure to the parasite.

The PCR methodology developed in this study should be considered appropriate for an accurate identification of any F. hepatica larvae during the prepatent period of the infection, since the nucleotide sequence of the amplification product showed total homology with the region of the cytochrome c oxidase subunit 1 gene of F. hepatica, and no cross-reactions were detected with other trematode species.

The PCR showed a minimal detection level of 10 pg of F. hepatica DNA, either in absence or presence of 10% Lymnaea sp. DNA. Regarding that the amount of DNA in the host depends on its total biomass, which varies markedly among individuals, we performed an assay employing 20% of the total extracted DNA.

A detection level of 100 pg was then obtained, ruling out the possibility of inhibition due to template excess.

According to Kaplan et al. (1995), a detection threshold of 1 ng is enough to recognize the presence of one miracidium by 4 h post infection. On this basis, our assay may have the sensitivity required for the detection of the parasite at early developmental stages in the intermediate host.

The high sensitivity of our PCR technique should be evaluated under experimental conditions, because abortive or recently resolved infections might lead to false positive results (van der Knaap and Loker, 1990).

In the present study, we made an attempt to avoid the interference of inhibitors in the samples using an optimised DNA extraction procedure. If 0.048% of BSA had not been added to the reaction mixture, the action of inhibitors could have produced misleading results. In this sense, prevalence of infection would have been 28.3% (68/240) instead of 51.3% (123/240) for sample 1 and 29.4% (10/34) instead of 67.8% (21/34) for sample 2. It must be stressed that the presence of inhibitors was not taken into account in previous works on experimental infections with F. hepatica in L. columella (Magalhaes et al., 2004).
As PCR detects parasite invasion and infection and microscopic examination only reveals successful infections in snails, both techniques could be used together to achieve a more comprehensive understanding of the epidemiological situation in a given area and to assess host suitability for the parasite, as suggested by Hamburger et al. (2004).

On the other hand, the use of the PCR test to detect *F. hepatica* infection in snail populations may indirectly provide information on the level of pasture contamination, an important issue concerning the control of fasciolosis in livestock.

Acknowledgements

We kindly thank Florencia Kleiman and Margarita Ostorowski for providing samples of different trematode species. We acknowledge Marta Cabrera and Jorge Velásquez for their participation in collecting sample 2, Sergio Miguel for helping with the DNA sequencing and Marcelo Rodríguez for his comments on the statistical analysis.

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