

Development of a duplex PCR for the identification of *Fasciola hepatica* in lymnaeid snails

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ABSTRACT

Fasciola hepatica is a parasitic trematode that causes fascioliasis, a disease that affects domestic livestock and humans. The complex life cycle of *F. hepatica* involves lymnaeid snails as intermediate hosts. Detection of *F. hepatica* in snails is a useful tool for the control of fascioliasis in livestock. Detection methods involve crushing the snails and microscopic observation, but have low sensitivity and are time-consuming. To overcome these disadvantages, researchers are developing molecular methods. In this work, we developed a duplex PCR that allows the detection of *F. hepatica* in snails as two single and bright bands: one corresponding to the parasite and one to the snail, the latter of which works as an internal control to detect PCR inhibitors. To avoid false-positive results, we also evaluated the method of disinfection of the material used for snail collection. The duplex PCR developed showed a sensitivity high enough to detect a single miracidium per snail, and significantly shortened the time required to analyze a large number of snails.

Keywords: fascioliasis, digenea, intermediate host, *Lymnaea* sp., PCR identification.

RESUMEN

Fasciola hepatica es un parásito trematodo que causa fasciolosis, una enfermedad que afecta al ganado doméstico y al ser humano. El complejo ciclo de vida de *F. hepatica* involucra a los caracoles lymnaeidos como huéspedes intermediarios. La detección de *F. hepatica* en caracoles es una herramienta útil para el control de la fasciolosis en el ganado. Los métodos de detección implican el aplastamiento de los caracoles y la observación microscópica, pero tienen baja sensibilidad y consumen mucho tiempo. Para superar estas desventajas, se encuentran en desarrollo métodos de diagnóstico molecular. En este trabajo, se desarrolló una PCR dúplex que permite la detección de *F. hepatica* en caracoles como dos bandas simples y brillantes: una banda corresponde al parásito y otra al caracol, funcionando esta última como control interno para detectar inhibidores de la PCR. Para evitar resultados falsos positivos, también evaluamos el método de desinfección del material utilizado para la manipulación de caracoles. La PCR dúplex desarrollada mostró una sensibilidad lo suficientemente alta como para detectar un solo miracidio por caracol y acortó significativamente el tiempo de trabajo de análisis de una gran cantidad de caracoles.

Palabras clave: fasciolosis, digeneo, huésped intermediario, *Lymnaea* sp., identificación por PCR.

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INTRODUCTION

Fasciola hepatica (liver fluke) is a parasitic trematode that causes fascioliasis, a worldwide distributed disease that affects domestic livestock and humans (Mas-Coma *et al.*, 2009). In livestock industry, fascioliasis causes important economic losses as it leads to a reduction in the production of meat, milk, or wool. The direct economic impact of the disease is increased condemnation of liver meat, but the far more damaging effects are a reduction in animal productivity, lower birth weight, and reduced growth of infected animals (Howell *et al.*, 2015; Khoramian *et al.*, 2014). *F. hepatica* is also an important human pathogen and fascioliasis is considered a re-emerging parasitic disease in many countries (Mas-Coma *et al.*, 2008).

The life cycle of *F. hepatica* involves lymnaeid snails as intermediate hosts and depends on the development of larval stages. Detection of *F. hepatica* in lymnaeid snails is a helpful tool to provide information on the level of pasture contamination and for prevalence studies, which are important issues in the control of fascioliasis in livestock (Caron *et al.*, 2008). Classical laboratory diagnosis for the identification of larval stages of *F. hepatica* in lymnaeid snails involves snail crushing and examination under an optical microscope (Caron *et al.*, 2008). This method is widely used because of its simplicity and the low cost of materials and equipment required. However, for a highly specific result, the method must be carried out by an experienced technician and its sensitivity is relatively low (Caron *et al.*, 2008).

To improve the identification and characterization of *F. hepatica* in snails, molecular techniques are increasingly being developed (Alba *et al.*, 2015; Caron *et al.*, 2011; Cucher *et al.*, 2006; Kozak and We, 2010; Magalhães *et al.*, 2004; Velusamy *et al.*, 2004). Although molecular techniques require specialized equipment, they may help overcome the specificity and sensitivity problems. However, the presence of false-positive and false-negative results is a key issue to be considered in the development of any reliable molecular technique (Burkardt, 2000; Victor *et al.*, 1993). Indeterminate results may be due to the contamination of negative samples or the presence of PCR inhibitors.

The aim of this work was to develop a multiplex PCR to detect *F. hepatica* in snails to improve the sensitivity and the specificity, to minimize false-negative and false-positive results.

MATERIALS AND METHODS

Field-collected snails and parasite materials

Adult *F. hepatica* flukes were obtained from the liver of a naturally infected sheep. Eggs of *F. hepatica* were recovered from fecal samples of naturally infected animals and incubated in the dark at 26°C for 14 days to isolate miracidia. Embryonated eggs were observed under a microscope until the release of miracidia. Each miracidium was preserved individually in tubes with 70% alcohol at -20°C until DNA extraction.

Snails were collected from bodies of water in the province of Neuquén, Argentina, during summertime. The snails were

examined under the microscope to determine the presence of trematode larvae, as previously reported, and conserved in 70% ethanol at -20°C until use (Prepelitchi *et al.*, 2003).

To compare the duplex PCR and microscopic examination, these techniques were conducted in parallel in field-collected snails. Also, we compared both techniques in two sets of snails (n=50), following two disinfection protocols for the material used to manipulate snails. In one case, we cleaned all the material with bleach, water, and ethanol 70%, as previously reported to avoid carry-over contamination in molecular techniques (protocol N°1) (Bonne *et al.*, 2008). In the other, we cleaned the material only with ethanol 70% (protocol N°2).

DNA isolation

DNA was isolated as described by Caron *et al.* with some modifications using Chelex-100® (Bio-Rad) chelating resin (Caron *et al.*, 2011). Tubes containing the snails' debris after snail crushing, miracidia of *F. hepatica* flukes were centrifuged at 13000 x g for 1 min and washed twice with 200 µl of distilled water to eliminate ethanol traces. The supernatant was discarded and 150 µl of 5% Chelex-100® (Bio-Rad) was added. The mixture was vortexed three times for 30 s and incubated for 1 h at 56°C and then for 30 min at 95°C in water bath. The mixture was then centrifuged at 13000 x g for 7 min. The supernatant was collected and stored at -20°C. DNA concentration and purity (260/280 wavelength ratio) were measured with a spectrophotometer (Thermo Scientific, NanoDrop 2000).

Amplification by PCR

PCR was performed using the specific primers FhCO1F: 5'-TAT GTT TTG ATT TTA CCC GGG-3' and FhCO1R: 5'-ATG AGC AAC CAC AAA CCA TGT-3', which amplify a 405-bp fragment in *F. hepatica*, and the primers LymF: 5'-TCC TAC TTG GAT AAC TGT GGC A-3' and LymR: 5'-TTA CAA ACA TGG TAG GCA TAT C-3', which amplify a 258-bp fragment in snails (Cucher *et al.*, 2006; Duffy *et al.*, 2009). DNA from *F. hepatica* flukes and *Lymnaea viatrix* were used as positive controls. To confirm that snails were not infected with *F. hepatica*, they were analyzed by microscopic examination and tested with the PCR for *F. hepatica* reported by Cucher *et al.*, 2006. DNA from free-living parasites and water were used as negative controls. The PCR reaction mixture consisted of Buffer 1X (PB-L, Argentina), 250 µM of each dNTP (PB-L), 1 µM of FhCO1 primers, 0.1 µM of Lym primers (Life Technologies, USA), 3 mM of MgCl₂, 0.02 U of Taq DNA polymerase (PB-L) and 100 – 500 ng of DNA in a final volume of 25 µl. The amplification parameters were: 95°C for 5 min, followed by 40 cycles of 94°C for 30 s, 53°C for 30 s, and 72°C for 90 s, with a final step of extension at 72°C for 5 min. PCR products were resolved by 1.5% agarose gel electrophoresis stained with GelRed (Biotium, USA). Amplified bands were viewed under a UV transilluminator and the image of the gel was captured using the FOTO/Analyst® Investigator/Eclipse System (Fotodyne, USA).

RESULTS

PCR setup

To set up the duplex PCR, it was necessary to prepare in-house reference standards in the same matrix as field samples (Burkardt, 2000; Victor *et al.*, 1993). For that purpose, we measured the average amount of DNA obtained from one medium-sized snail (250 ng/μl) and prepared in-house standards with that amount of snail DNA and different amounts of *F. hepatica* DNA. Then, to set up the optimal conditions for the duplex PCR, we evaluated different amplification reaction mixtures and cycling parameters. Parameters such as primer concentration and extension time were critical to obtain two defined bands of the expected sizes on a mix of 250 ng of *L. viatrix* DNA and 100 ng up to 1 ng of *F. hepatica* DNA (fig. 1).

We determined the analytical sensitivity of the duplex PCR by mixing 250 ng of snail DNA and serial dilutions of *F. hepatica* DNA. The analytical sensitivity for *F. hepatica* was 1 ng/μl. We analyzed a total of eight miracidia and measured the average DNA obtained from one miracidium, which was 2.5 ng/μl. Thus, we confirmed that this duplex PCR was able to detect one miracidium in one snail (fig. 2). The specificity of the set of primers used for *F. hepatica* detection was already tested by Cucher *et al.* (2006). Also, we performed the duplex PCR on DNA extracted from snails infected with free-living larvae observed in the analyzed snails and no bands were obtained (data not shown).

PCR on field-collected snails

When protocol N°1 was used, *F. hepatica* was detected in two out of the 50 snails by both microscopic examination and PCR and only one sample presented no bands (table 1). When protocol N°2 was used, all samples were positive for snail, and *F. hepatica* was detected in one out of the 50 snails by both microscopic examination and PCR. *F. hepatica* was detected in six out of the 50 snails only by duplex PCR.

DISCUSSION

The traditional diagnosis of *F. hepatica* infestation by microscopic examination is simple and affordable. However, it requires experienced laboratory technicians and has low sensitivity for early stages, especially if snails die before cercarial release (Kaplan *et al.*, 1995; Kaplan and Reed, 1997). Therefore, sensitive methods for rapid and accurate identification of *F. hepatica* are needed for epidemiological surveys and infection control. Molecular diagnosis based on DNA detection by PCR is a promising tool to detect *F. hepatica* DNA in snails, since this technique is rapid and sensitive and no fresh samples are required (Alba *et al.*, 2015, Caron *et al.*, 2011, 2008; Cucher *et al.*, 2006; Magalhães *et al.*, 2004).

Some authors have attempted to detect *F. hepatica* in snails by PCR. However, the multiplex PCRs developed until now are based on repetitive regions of *F. hepatica* DNA, which generates several bands, or can detect the parasite in a specific snail host (Alba *et al.*, 2015, Caron *et al.*, 2011;

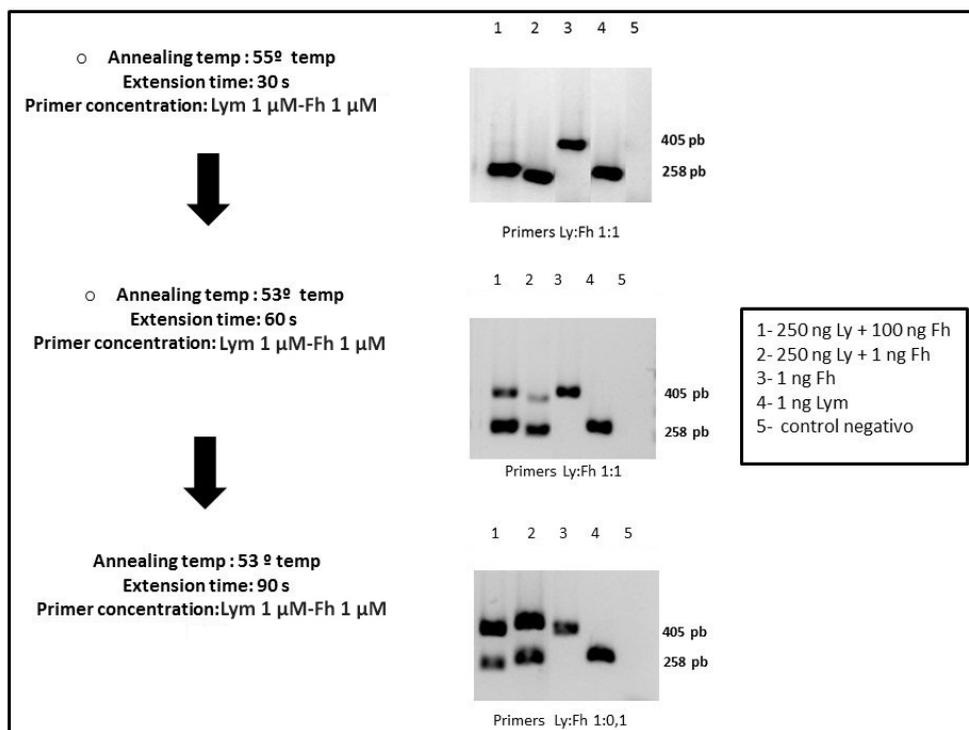


Figure 1. Duplex PCR set up.

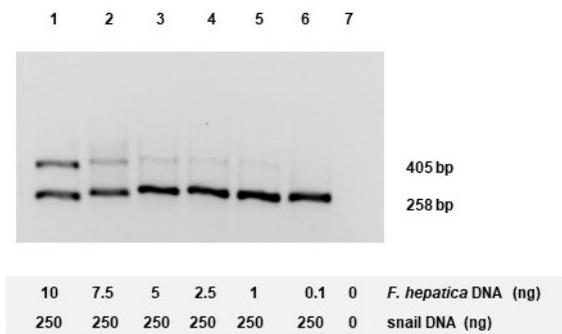


Figure 2. Duplex PCR sensitivity.

	Bleach, ethanol and water	Ethanol
Microscopic examination	2	1
PCR	2	7
Total samples	50	50

Table 1. Comparison of duplex PCR and microscopic examination of field-collected snails after two different disinfection protocols (protocol 1: 10% bleach, rinsed in water and 70% ethanol, and protocol 2: 70% ethanol). The number of positive samples is shown.

Magalhães *et al.*, 2004). Other authors have developed Real-Time PCRs protocols using specific probes, all of which are expensive and some of which only detect *F. hepatica* DNA, but not the snail's DNA (Alasaad *et al.*, 2011; Schweizer *et al.*, 2007).

The duplex PCR developed in this work was able to detect *F. hepatica* in snails as two single and bright bands, with only two primer pairs in one step. The band corresponding to the snail works as an internal control that guarantees the presence of DNA and avoids false-negative results. Besides, the snail PCR amplifies a polymorphism located within the helix E10-1 of the variable region V2 of the 18S rRNA gene and if it is sequenced and aligned with GenBank sequences (Gen Bank accession numbers: AY057089, EU241866, EU728668, and EU241865) it can be used to identify the snail's species (Duffy *et al.*, 2009).

Interestingly, the snail band was detected in all the reaction mixtures and cycling parameters evaluated to set up the duplex PCR, while the *F. hepatica* band was only amplified after increasing the extension time and reducing the concentration of primers specific for the snail band. This is in agreement with previous reports that suggest performing these modifications in multiplex PCRs when long products are weak or absent (Henegariu and Heerema, 1997).

The detection limit was 1 ng of *F. hepatica* in the presence of *Lymnaea* spp. DNA, which is optimal since it allows the identification of one miracidium (2.5 ng/μl) in one

snail, which is the biological unit intended to detect (Kaplan and Reed, 1997).

Once the duplex PCR was set up, we evaluated how to process the snails considering the same sample was going to be analyzed in the parasitology and molecular biology laboratory. Right after collection, snails were crushed and analyzed under microscopic observation in the parasitology laboratory, and snails' debris were collected in tubes with ethanol for conservation at -20°C, until DNA extraction.

During the parasitological analyses, snails are handled with stainless steel clamps and glass Petri dishes. In contrast, during molecular biology analyses, extreme care is taken to avoid false-positive results, such as the use of disposable material and the physical separation of reagents and materials before the PCR reaction. Thus, to obtain accurate results, and considering what was already reported by Bonne *et al.* (2008), we compared two disinfection protocols of the stainless steel clamps and glass Petri dishes.

The duplex PCR developed in this work showed results similar to those obtained by microscopic examination when protocol N°1 was used. The application of a rigorous disinfection protocol to clean the material between snail collection was critical to achieve these results. When protocol N°2 was used, only one snail was positive under microscopic examination and PCR while six snails were positive only by duplex PCR.

Interestingly, some of the snails that were positive only by PCR were processed right after the only positive snail detected by microscopic examination and the amplicon obtained showed a subsequently decreased in the signal, suggesting carry-over contamination. Although we cannot confirm that the six positive results only by PCR achieved with protocol N°2 are false-positive results, we strongly recommend using protocol N°1 to clean the material used in the parasitology laboratory to handle snails previously to DNA extraction.

Previous studies have shown a higher level of detection of *F. hepatica* by PCR than by microscopic examination (Caron *et al.*, 2011; Cucher *et al.*, 2006; Kozak and We, 2010). Cucher *et al.* (2006) analyzed two samples from Corrientes and San Luis, Argentina. Snails were identified as *L. columella* and 17.5% of snails were positive for *F. hepatica* by microscopic observation while 51.3% were positive by PCR in samples from Corrientes. Snails were identified as *L. viatrix* and 2.9% of snails were positive for *F. hepatica* by microscopic observation while 61.8% were positive by PCR in samples from the province of San Luis (Cucher *et al.*, 2006). Kozak and We (2010) evaluated the performance of a PCR assay for the detection of *F. hepatica* in *Galba truncatula* snails in four geographical areas of Eastern Poland and obtained an overall prevalence rate of 26.6%, which varied from 21% to 84% according to the region. However, in these reports the authors did not clearly state which measures were taken to avoid carry-over contamination.

With our duplex PCR, only one sample showed no bands. This may be due to the traces of bleach or ethanol present

in the sample. Thus, we confirm the importance of the snail band, since, when this band is absent, the result cannot be taken into account. In conclusion, the duplex PCR developed in this work considerably shortened the working time, decreased the number of false-positive results, can detect one miracidium, and can be performed by a technician with no experience in parasitology.

Although analysis of a greater number of samples is needed to validate this PCR, these results suggest that this duplex PCR is a promising tool to estimate the potential infection risk of ruminants in areas endemic for fascioliasis. The duplex PCR is very convenient because it is a sensitive technique that allows the detection of *F. hepatica* in snails, significantly shortens the working time, and has an acceptable cost.

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