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

MIGNAQUI, A.C.[1†]; ALVAREZ, L.P.[2†]; SOLER, P.[3]; LARROZA, M.[3]

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.*

[†]These authors contributed equally to this work.
[1]Instituto de Investigaciones Forestales y Agropecuarias Bariloche (IFAB), Instituto de Tecnología Agropecuaria (INTA), CONICET, Grupo de Nanomedicina Veterinaria, Modesta Victoria 4450, (8400) San Carlos de Bariloche, Río Negro, Argentina.
[2]Instituto Andino Patagónico de Tecnologías Biológicas y Geoambientales (IPATEC), CONICET-UNCOMA, Av. de Los Pioneros 2350, (8400) San Carlos de Bariloche, Río Negro, Argentina.
[3]Instituto de Investigaciones Forestales y Agropecuarias Bariloche (IFAB), Instituto Nacional de Tecnología Agropecuaria (INTA), CONICET, Grupo de Salud Animal, Modesta Victoria 4450, (8400) San Carlos de Bariloche, Río Negro, Argentina.
Correo electrónico: larroza.marcela@inta.gob.ar

Received April 17th 2019// Accepted July 15th 2019 // Published online March 03rd 2021

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). Indeterminated 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 26ºC 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 miscrocope to determine the presence of trematode larvae, as previously reported, and conserved in 70% ethanol at -20ºC 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 Nº1) (Bonne *et al*., 2008). In the other, we cleaned the material only with ethanol 70% (protocol Nº2).

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 56ºC and then for 30 min at 95ºC in water bath. The mixture was then centrifuged at 13000 x g for 7 min. The supernatant was collected and stored at -20ºC. 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: 95ºC for 5 min, followed by 40 cycles of 94ºC for 30 s, 53ºC for 30 s, and 72ºC for 90 s, with a final step of extension at 72ºC 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 Nº1 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 Nº2 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;

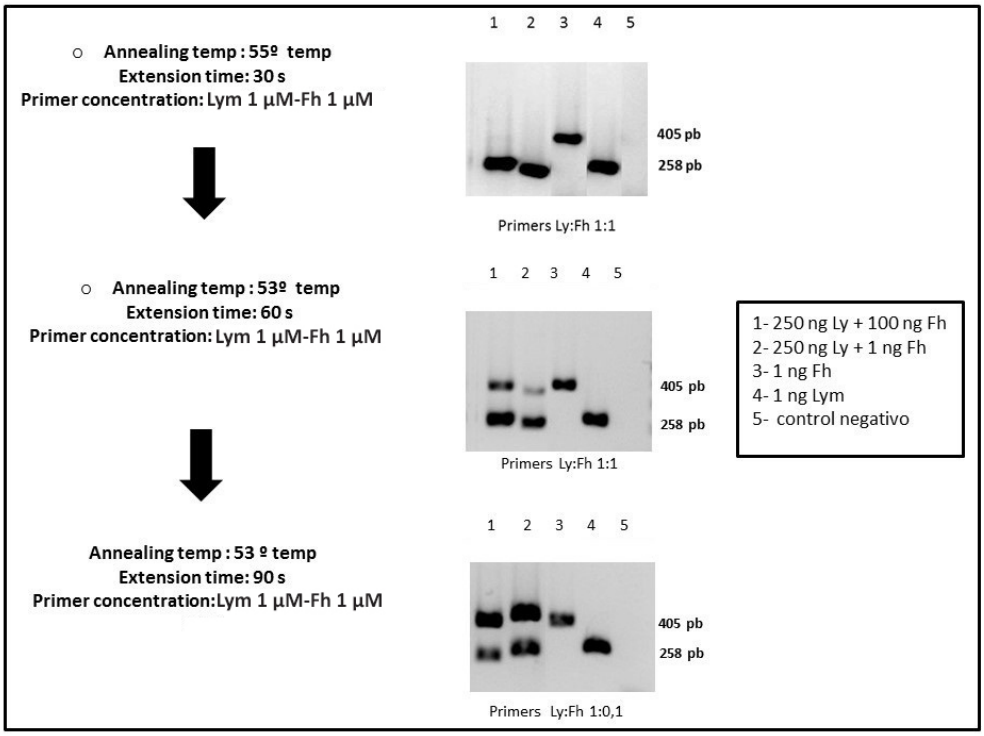

Figure 1. Duplex PCR set up.

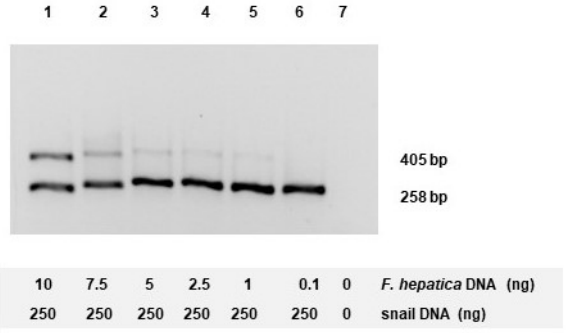

Figure 2. Duplex PCR sensitivity.

	Bleach, ethanol and water	Etanol
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 Nº1 was used. The application of a rigorous disinfection protocol to clean the material between snail collection was critical to achieve these results. When protocol Nº2 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.

ACKNOWLEDGEMENTS

This work was supported by INTA (grants Nº 1115054, 1281101, and 1281103).

We thank Vet. Fernando Raffo, Vet. Catalina Lauroua and Mr. Raul Cabrera for their assistance during field sampling.

REFERENCES

ALASAAD, S.; SORIGUER, R.C.; ABU-MADI, M.; EL BEHAIRY, A.; JOWERS, M.J.; BANOS, P.D.; PIRIZ, A.; FICKEL, J.; ZHU, X.Q. 2011. A TaqMan real-time PCR-based assay for the identification of *Fasciola spp*. Vet. Parasitol. 179, 266-271.

ALBA, A.; VÁZQUEZ, A.; HERNÁNDEZ, H.; SÁNCHEZ, J.; MARCET, R.; FIGUEREDO, M., SARRACENT, J.; FRAGA, J. 2015. A multiplex PCR for the detection of *Fasciola hepatica* in the intermediate snail host *Galba cubensis*. Vet Parasitol 211, 195-200.

BONNE, N.; CLARK, P.; SHEARER, P.; RAIDAL, S. 2008. Elimination of false-positive polymerase chain reaction results resulting from hole punch carryover contamination. J Vet Diagn Invest 20, 60-63.

BURKARDT, H. 2000. Standardization and quality control of PCR analyses. Clin Chem Lab Med. 38, 87-91.

CARON, Y.; RIGHI, S.; LEMPEREUR, L.; SAEGERMAN, C.; LOSSON, B. 2011. An optimized DNA extraction and multiplex PCR for the detection of *Fasciola sp*. in lymnaeid snails. Vet. Parasitol. 178, 93-99.

CARON, Y.; RONDELAUD, D.; LOSSON, B. 2008. The detection and quantification of a digenean infection in the snail host with special emphasis on *Fasciola sp*. Parasitol. Res. 103, 735-744. doi:10.1007/s00436-008-1086-1

CUCHER, M.; CARNEVALE, S.; PREPELITCHI, L.; LABBÉ, J.; WISNIVESKY-COLLI, C. 2006. PCR diagnosis of *Fasciola hepatica* in field-collected *Lymnaea columella* and *Lymnaea viatrix* snails. Vet Parasitol 137, 74-82.

DUFFY, T.; KLEIMAN, F.; PIETROKOVSKY, S.; ISSIA, L.; SCHIJMAN, A.G.; WISNIVESKY-COLLI, C. 2009. Real-time PCR strategy for rapid discrimination among main lymnaeid species from Argentina. Acta Trop. doi:10.1016/j.actatropica.2008.08.003

HENEGARIU, O.; HEEREMA, N. 1997. Multiplex PCR: critical parameters and step-by-step protocol. Biotechniques 23, 504-11.

HOWELL, A.; BAYLIS, M.; SMITH, R.; PINCHBECK, G.; WILLIAMS, D. 2015. Epidemiology and impact of *Fasciola hepatica* exposure in high-yielding dairy herds. Prev. Vet. Med. 121, 41-48. doi:10.1016/j.prevetmed.2015.05.013

HURTREZ-BOUSSÈS, S.; MEUNIER, C.; DURAND, P.; RENAUD, F. 2001. DYNAMICS OF HOST – parasite interactions : the example of population biology of the liver fluke (*Fasciola hepatica*). Microbes Infect. 3, 841-849.

KAPLAN, R.; DAME, J.; REDDY, G.; COURTNEY, C. 1995. A repetitive DNA probe for the sensitive detection of *Fasciola hepatica* infected snails. Int. J. Parasitol. 25, 601-610.

KAPLAN, R.M.; REED, W. 1997. The prevalence of *Fasciola hepatica* in its snail intermediate host determined by DNA probe assay. Int. J. Parasitol. 27, 1585-1593.

KHORAMIAN, H.; ARBABI, M.; MAHAMI OSQUI, M.; DELAVARI, M.; HOOSHYAR, H.; ASGARI, M. 2014. Prevalence of ruminants fascioliasis and their economic effects in Kashan, center of I ran. Asian Pac. J. Trop. Biomed. 4, 918-922. doi:10.12980/APJTB.4.2014APJTB-2014-0157

KOZAK, M.; WE, H. 2010. The performance of a PCR assay for field studies on the prevalence of *Fasciola hepatica* infection in Galba truncatula intermediate host snails. Vet. Parasitol. 168, 25-30. doi:10.1016/j.vetpar.2009.10.014

MAGALHÃES, K.G.; KONOVALOFF, L.; PASSOS, J.; CARVALHO, S. 2004. Detection of *Lymnaea columella* Infection by *Fasciola hepatica* through Multiplex-PCR. Mem Inst Oswaldo Cruz 99, 421-424.

MAS-COMA, S.; VALERO, M.; BARGUES, M. 2008. Effects of climate change on animal and zoonotic helminthiases. Rev Sci Tech. 27, 443-457.

MAS-COMA, S.; BARGUES, M.; VALERO, M. 2009. Chapter 2. Fasciola, lymnaeids and human fascioliasis with a global overview on disease transmission, epidemiology, evolutionary genetics, molecular epidemiology and control. Adv Parasitol. 69, 41-146.

PREPELITCHI, L.; KLEIMAN, F.; PIETROKOVSKY, S.M.; MORIENA, R.A.; RACIOPPI, O.; ALVAREZ, J.; WISNIVESKY-COLLI, C. 2003. First Report of *Lymnaea columella* Say. 1817 (Pulmonata : Lymnaeidae) Naturally Infected with *Fasciola hepatica* (Linnaeus, 1758) (Trematoda : Digenea) in Argentina. Mem Inst Oswaldo Cruz 98, 889-891.

SCHWEIZER, G.; MELI, M.L.; TORGERSON, P.R.; LUTZ, H.; DEPLAZES, P.; BRAUN, U. 2007. Prevalence of *Fasciola hepatica* in the intermediate host *Lymnaea truncatula* detected by real time TaqMan PCR in populations from 70 Swiss farms with cattle husbandry. Vet. Parasitol. 150, 164-169. doi:10.1016/j.vetpar.2007.08.006

VELUSAMY, R.; SINGH, B.P.; RAINA, O.K. 2004. Detection of *Fasciola gigantica* infection in snails by polymerase chain reaction. Vet. Parasitol. 120, 85-90.

VICTOR, T.; JORDAAN, A.; DU TOIT, R.; VAN HELDEN, P. 1993. Laboratory experience and guidelines for avoiding false positive polymerase chain reaction results. Eur J Clin Chem Clin Biochem. 31, 531-535.