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Molecular diagnosis of natural fasciolosis by DNA detection in sheep faeces

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Abstract

Fasciolosis is an important parasitic zoonosis considered the most important helminth infection of ruminants in tropical countries. The aim of this study was to develop a PCR assay for the sensitive and specific detection of *F. hepatica* in formalin preserved sheep faeces. A 405-bp fragment of the cytochrome c oxidase subunit 1 gene of *F. hepatica* was amplified from stool samples of infected sheep. The PCR assay showed a detection limit of 20 pg of *F. hepatica* DNA. No cross-reactions were observed with samples containing coccidian oocysts or gastrointestinal nematodes eggs. Our PCR technique showed to be effective for specific detection of *F. hepatica* infections in sheep.

Keywords

Fasciola hepatica, PCR, faeces

Introduction

Fasciola hepatica Linnaeus, 1758 and *Fasciola gigantica* Cobbold, 1855 (Platyhelminth: Trematoda: Digenea) are common liver flukes in a range of animals and have a global geographical distribution. Fasciolosis is an important food-and water-borne parasitic zoonosis and is considered the most important helminth infection of ruminants in tropical countries with over 700 million production animals being at risk of infection and economic losses estimated at > US$ 2 billion per year worldwide (Spithill and Dalton 1998). The economic significance of fasciolosis lies in huge losses due to mortality, the number of confiscated livers, reduction in the production of milk and meat, reduced reproductive efficiency, secondary bacterial infections associated and high costs in deworming (Ntategize *et al.* 1993).

Several studies have shown that *F. hepatica* occurs in temperate areas, *F. gigantica* in tropical zones, and both species overlap in subtropical areas (Mas-Coma *et al.* 2005, 2009; Ashrafi *et al.* 2006).

In the last decades the prevalence of *F. hepatica* infections in domestic ruminants have been reported in Europe with values of 5% in Italy, 10% in Great Britain, 29.5% to 59% in Spain, 18.8% in Norway, 11.9% in Poland, 10.7% in Germany, 45% in Ireland, 29.3% in Turkey, and ranged from 11.2% to 25.2% in central France (Gonzalez-Lanza *et al.* 1989; Torgerson and Claxton 1999; Mage *et al*. 2002; Martínez-Valladares *et al.* 2013; Domke *et al.* 2013; Kozłowska-Łój and Łój-Maczulska 2013). The prevalence of fasciolosis in cattle has also been reported from endemic areas of Chile (94%), the USA (ranging from 5.2 to 68%), Peru (29%), Morocco (10.4%), Spain (33%), Switzerland (18%), India (15%) Cambodia (10%), New Zealand (8.5%) and Brazil (64.8% to 10.59%) (Taylor 1989; Serra-Freire *et al.* 1995; Torgerson and Claxton 1999; Rapsch *et al.* 2006; Tum *et al.* 2007; Choubisa and Jaroli 2013; Arias *et al.* 2013).

In the Americas, this helminthic disease is caused by the species *F. hepatica* (Mas-Coma *et al.* 2009). Argentina is a country of high livestock production, where sheep and cattle constitute important economic sources. Animal fasciolosis

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covers the whole country according to official slaughterhouse, and the most important hosts are cattle and sheep. Goats, horses, pigs and some wild animals (deer, vicuña, guanacos, llamas, rabbits, hares and capybaras) grazing in contaminated areas, may be infected by *F. hepatica* (Olaechea 1994, 1995).

Diagnostic methods of fasciolosis in animals are mainly based on coprology, immunodiagnosis and necropsy. In the countryside, in the presence of very cachectic animals, especially sheep, necropsy of some of them with observation of the liver, injuries and checking of adult parasites in the bile ducts (Boero 1976) is performed. This observation is the method used in routine veterinary inspection in abattoirs (SENASA, 1998).

The development of more accurate diagnostic methods is desirable. PCR assays of faeces are highly sensitive for the diagnosis of the infection due to platyhelminth parasites; i.e. *Echinococcus* spp., *Taenia* spp., *F. hepatica*, *F. gigantica*, using PCR assays of faeces (Bretagne *et al.* 1993; Dinkel *et al.* 1998; Naidich *et al.* 2006; Mathis and Deplazes 2006; Ai *et al.* 2010; Martínez-Pérez *et al.* 2012; Robles-Pérez *et al.* 2013). Previously developed PCR and nested-PCR methods to detect the presence of *F. hepatica* in faeces of sheep (Martínez-Pérez *et al.* 2012; Roble-Pérez *et al.* 2013) have employed fresh samples and commercial kits for DNA extraction.

We previously developed a PCR assay for the detection of *F. hepatica* in the intermediate host, amplifying a 405-bp fragment of the mitochondrial cytochrome c oxidase subunit 1 (*cox*1) gene (Cucher *et al.* 2006).

In order to improve the identification of *F. hepatica* DNA in sheep faeces, the aim of this study was to develop a PCR assay based on the mitochondrial *cox*1 gene employing DNA suitable for amplification isolated from formalin fixed stool samples.

Materials and Methods

Samples

Faecal samples from sheep were collected in the North area of Tinogasta department, Catamarca province, Argentina (latitude 27°20´00″S, longitude 67°34´00″W).

Stool samples (n = 70) were collected individually and fixed in 5% formalin saline solution. Faecal samples were examined for *F. hepatica* eggs by formalin-ether concentration (Telemann 1901) and positive samples were processed for eggs quantification (Stoll and Hausheer 1926). Briefly, 10 pellets of fixed faeces were homogenized with a glass rod and 10 ml were filtered through a funnel with double gauze to a conical 15-ml tube. 2 ml of ether were added and the tubes were centrifuged at 1000–1500 rpm for 5 minutes. Supernatant was removed and a drop of the pellet was observed under the light microscope for the presence of eggs. To determine the number of eggs per gram (EPG) in the positive samples, a Stoll flask was filled with 56 ml of 0.1 N NaOH solution and 4 ml of homogenized faeces. After glass beads (4 to 6) were added, the vessel was closed and shaken up and down for 1 minute. A volume of 150 µl of suspension from the centre of the flask was placed on a slide and covered with a cover slip. The number of eggs per gram was calculated as the counted eggs × 100 × 4 (correction factor for diluted samples).

Selected negative samples (n = 5) and samples with coccidian and/or gastrointestinal nematodes (n = 6), corresponding to *Eimeria sp.*, *Haemonchus* sp., *Trichostrongylus* sp. and *Nematodirus* sp., were also included.

Extraction of DNA from faecal samples

Two pellets of formalin preserved stool samples were resuspended in saline solution and processed by formalin-ether concentration as previously described.

Pellets from three positive individual animals were processed for DNA extraction under four different conditions. They included DNA isolation with two commercial kits corresponding to NucleoSpin DNA Tissue Kit (MACHEREY-NAGEL GmbH & Co. KG, Germany) according to the manufacturer´s instructions for mammalian cells and QIAamp DNA Stool Mini Kit (QIAGEN, USA). Two other manual protocols were also tested. The first one was performed as a previously described (Duenngai *et al.* 2013) for *Opistorchis viverrini* eggs, based on an alkaline eggs disruption associated with autoclaving. The forth method was a standard phenol–chloroform protocol. 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). The DNA was resuspended in 20 µl of bidistilled water and kept at –20ºC until use.

After the first results, the selected protocol was the manual phenol–chloroform extraction and all samples were processed under these conditions.

DNA from adult flukes extracted with NucleoSpin DNA Tissue Kit (MACHEREY-NAGEL GmbH & Co. KG, Germany) was also employed.

Diagnosis by PCR

In order to amplify a portion of 405 bp of the *cox*1 gene (complete mitochondrial DNA of *F. hepatica* GenBank accession number X15613.1), the primer set FhCO1F (5´-TATGTTTTGATTTTACCCGGG-3´) and FhCO1R (5´-ATGAG CAACCACAAACCATGT-3´) (Cucher *et al.* 2006) was employed. PCR assays were carried out in a MyCycler thermocycler (Biorad, USA) in a final volume of 50 µl. The PCR mixture consisted of 10 mM Tris-HCl (pH 8.8), 50 mM KCl, 0.08% (v/v) Nonidet P40, 100 mM (each) dNTP, 2.5 mM MgCl2, 0.1 mM of each primer, 1.0 U of Taq DNA polymerase (Fermentas International Inc.), and 0.048% BSA. The

template used for each reaction corresponds to 2 µl of purified DNA from each sample. A second round of amplification was added employing 5 µl of the products of the first round, under the same cycling conditions.

The amplification products were analyzed by electrophoresis in a 1.5% agarose gels and stained with ethidium bromide.

In order to ascertain the presence of an inhibitor as the cause of a failure to detect parasite DNA, *F. hepatica* DNA (1 ng) was added to the negative samples and the PCR test was repeated. A positive PCR test result confirmed that the sample was free of inhibitory substances and the sample was regarded as negative for *F. hepatica* DNA.

The sensitivity was determined using parasite purified DNA. Negative DNA samples were spiked with 2 ng, 1 ng, 0.5 ng, 0.2 ng, 0.1 ng, 50 pg, 20 pg, 10 pg, 5 pg, 2 pg, 1 pg, 0.5 pg, 0.2 pg, 0.1 pg of *F. hepatica* DNA and run through PCR.

Results

Out of the 70 analyzed samples, 18 resulted positive for *F. hepatica* eggs (25.71%). Faecal egg counts of positive *F. hepatica* samples reached values that ranged from 40 to 400 EPG. When three positive faeces were processed for DNA extraction under different conditions, no amplification products were obtained for samples isolated with NucleoSpin DNA Tissue Kit, QIAamp DNA Stool Mini Kit, and the manual protocol based on alkaline disruption/autoclaving (Duenngai *et al.* 2013). Amplification was only observed in samples processed by standard phenol–chloroform extraction. After these results, the selected protocol was the manual phenol–chloroform extraction and all samples were processed under these conditions.

The PCR assay detected 5 pg of purified genomic *F. hepatica* DNA but this minimum detection level was diminished to 20 pg by the presence of DNA extracted from negative stool samples (Fig. 1).

All samples containing *F. hepatica* eggs resulted positive by the double PCR assay, showing an amplification fragment of 405 bp expected size, independently from egg count. No cross-reactions were observed with samples containing coccidian oocysts or gastrointestinal nematodes eggs (Fig. 2). No amplification was detected with DNA from uninfected stool samples.

Primers specificity was also tested by sequence comparison with the information available at the GenBank (http://www.ncbi.nlm.nih.gov/genbank/) for different trematodes of veterinary importance for domestic sheep including

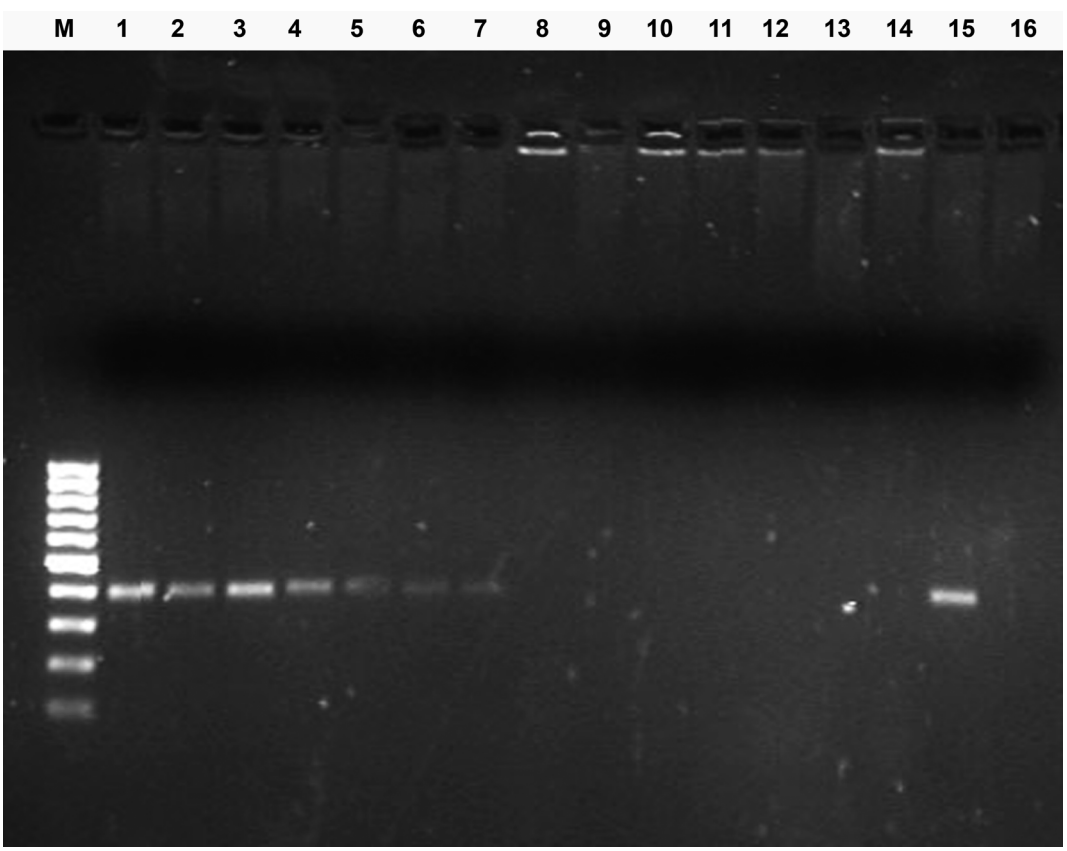

Fig. 1. Limit of detection of the PCR assay by agarose gel electrophoresis of amplification products using as template DNA from negative stool sample spiked with *F. hepatica* DNA. Lane M, molecular size marker 100 bp ladder; lanes 1 to 14, sample spiked with 2 ng, 1 ng, 0.5 ng, 0.2 ng, 0.1 ng, 50 pg, 20 pg, 10 pg, 5 pg, 2 pg, 1 pg, 0.5 pg, 0.2 pg, 0.1 pg, respectively of *F. hepatica* DNA; lane 15, positive control (2 ng *F. hepatica* DNA); lane 16, reaction mixture

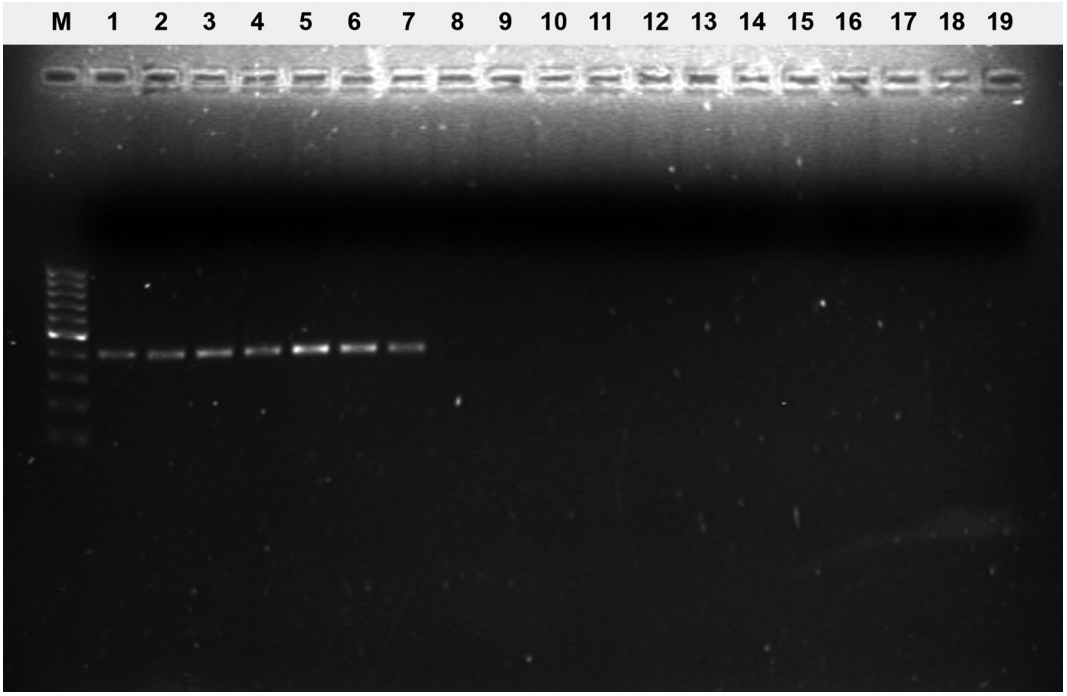

Fig. 2. Specificity analysis of the PCR assay by agarose gel electrophoresis of the amplification products. DNA from sheep stool samples was used as templates. Lane M, molecular size marker 100 bp ladder; lanes 1 to 7, positive stool samples for *F. hepatica* by light microscopy; lanes 8 to18, negative stool samples for *F. hepatica* (lanes 8 to10 with gastrointestinal nematodes eggs, lanes 11 to13 with coccidian oocysts, 14 to18 without parasites); lane 19, reaction mixture

Fasciola gigantica, Paramphistomidae, *Fascioloides magna*, *Dicrocoelium dendriticum* (GenBank Accesssion Numbers GU112472.1, KF475773.1, EF534998.1 and JF690758.1), respectively. For the first three ones, which eggs morphology is similar to *F. hepatica*, identities were lower that 95%. For the last one, with operculated eggs smaller than *F. hepatica* ones, less than 90% of one of the two primers of the set was covered by the available sequences.

Discussion

The diagnosis of fasciolosis in sheep begins by the observation of specific signs and symptoms. But sometimes, animals suffering from fasciolosis do not show specific clinical signs, or symptoms are present when the infection is due to another disease, or the course of fasciolosis is subclinical (Rojo–Vázquez *et al.* 2012).

F. hepatica infection is usually determined by finding eggs in faeces with direct methods. Given the high specific weight of the eggs (1050–1100) are preferable methods of enrichment by sedimentation (Locatelli *et al.* 1984; Boch and Supperer 1988); however, the sensitivity of this technique is estimated to be only 30% (Happich and Boray 1969), leading to false negatives. However, the coproparasitological techniques are not valid during the acute phase of the disease and if eggs are accumulated in the gallbladder with little release to the intestine (Locatelli *et al.* 1984). Due to the long prepatent period in ovine, coprological methods are only sensitive from 8 weeks post-infection (wpi) onwards (Zimmerman *et al.* 1982; Rodríguez-Pérez and Hillyer 1995; O'Neill *et al.* 2000; Martínez-Valladares *et al.* 2010), when liver damage has already occurred.

During the last three decades, most investigations were directed to use the ELISA assay and its variants for detection of antibodies against *F. hepatica* in animals. The different methods initially developed employed somatic extracts, and then used the excretory-secretory products, partially purified or recombinant antigens (Pfister *et al.* 1984; Hillyer and Soler de Galanes 1991; Cornelissen *et al.* 1992; El-Bahi *et al.* 1992; Rickard, 1995; Gorman *et al.* 1995; Martínez *et al.* 1996; Anderson *et al.* 1999; Castro *et al.* 2000; Carnevale *et al.* 2001). The advantage of these assays is that it is possible to detect 2–3 weeks post- infection, reaching a peak at 8–10 weeks, being applicable during acute and latent phases of the disease (Santiago and Hillyer 1988; Reichel 2002). There has also been some progress in the detection of circulating parasite antigens and coproantigens (Langley and Hillyer 1989; Rodriguez-Perez and Hillyer 1995; Dumenigo and Mezo 1999; Dumengio *et al.* 2000). In addition to its advantage in the early diagnosis of infection, these methods provide the ability to process multiple samples simultaneously, speeding up diagnosis times and being able to apply at large scales.

The development of new, sensitive, rapid, and precise techniques for routine diagnosis of fasciolosis is necessary. In this way we developed a PCR to detect the presence of *F. hepa-*

tica in sheep amplifying a 405 bp fragment of the *cox*1 gene employing DNA isolated from formalin fixed stool samples. We confirmed the specificity of the technique with DNA extracted from faecal samples of uninfected sheep and those with coccidian or gastrointestinal nematodes infection. We also confirmed primers specificity by sequence analysis using information available at the GenBank related to trematodes that infect sheep, including *Fasciola gigantica*, Paramphistomidae, *Fascioloides magna*, *Dicrocoelium dendriticum*, especially those whose eggs are similar.

Recently, PCR and nested-PCR methods have been developed to detect for the presence of *F. hepatica* in faeces of sheep (Martínez-Pérez *et al.* 2012; Roble-Pérez *et al.* 2013), based on the amplification of the coding region of *cox*1 gene, regions of the *cox*1 gene and the large ribosomal RNA subunit, or the ribosomal internal transcribed spacer 2 in the mitocondrial DNA. Martínez-Pérez *et al.* (2012) compared different techniques for the early diagnosis of the infection by *F. hepatica* in experimentally and naturally infected sheep, including coprological method a commercial immunoassay, standard PCR and nested PCR. Their results showed that *F. hepatica* infection was detected from 3 wpi with a standard PCR, and from 2 wpi with a nested-PCR, while eggs were detected by 9 weeks pos infection (wpi) and the sandwich-ELISA was positive by 4 wpi in the 57.1% experimentally infected, concluding that the sensitivity of the nested-PCR was higher than the detection of eggs in faeces as well as a commercial immunoassay. Roble-Pérez *et al.* (2013) developed a PCR and employed it for detection of anthelmintic resistance in naturally infected sheep flocks showing that PCR was the most sensitive method compared with the fecal egg count reduction test and the coproantigen reduction test. All these methods have been developed using DNA extracted from stool samples employing comercial kits.

The PCR technique has been also used to detect other platyhelminth parasites in animal and/or human faeces, such as *Echinococcus* spp., *O. viverrini*, *Schistosoma mansoni*, *Schistosoma japonicum*, *Clonorchis sinensis* (Bretagne *et al.* 1993; Dinkel *et al.* 1998; Pontes *et al.* 2002; Sandoval *et al.* 2006; Naidich *et al.* 2006; Xia *et al.* 2009; Rahman *et al.* 2011; Costa de Carvalho *et al.* 2012; Cho *et al.* 2013) but all these approaches employed stool samples that were fresh or preserved in 70% ethanol.

Although many studies have shown problems of DNA damaged with formalin-fixed specimens reducing, particularly, the efficiency of extraction (Chang and Loew 1994**;** Skage and Schander 2007; Kuk *et al.* 2012), there are also other studies showing that it is possible to employ preserved samples with good results in amplification (Lelièvre *et al.* 2010; Richter *et al.* 2010). We previously demonstrate the possibility of DNA extraction with formalin fixed stool samples and paraffin-embedded tissues employing this material for PCR of coccidian and/or microsporidia, requiring in this case the use of a second round of amplification in a double or a nested PCR (Velásquez *et al.* 1996, 2005, 2011; Carnevale *et al.* 2000). The most widely used methods for extracting DNA from formalin-fixed samples is based on phenol/chloroform extraction and in this paper we showed that this was the most useful procedure, coupled to a double PCR protocol in order to increase the sensitivity of the amplification.

Although further work should be done in order to assay our PCR method in different situations including early diagnosis and anthelmintic resistance evaluation, our findings clearly show the advantage of our double PCR method. The DNA sources were formalin-fixed stool samples. Although these samples can provide high molecular weight DNA sufficiently intact, it can be degraded resulting in a less robust amplification substrate than DNA from fresh samples. In this way, a target sequence with an amplification product of 405 bp resulted in a good choice for this assay, instead of amplicons over 1000 bp commonly used in the first round of nested PCR techniques, showing that a shorter sequence is preferable as target in formalin-fixed specimens.

The detection of DNA from *F. hepatica* in the host faeces and the specificity of the PCR technique confirmed that formalin-preserved stool specimens can be employed with good diagnostic results.

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