Veterinary Parasitology 197 (2013) 277–282

Contents lists available at SciVerse ScienceDirect

Veterinary Parasitology

journal homepage: www.elsevier.com/locate/vetpar

The diagnosis of fasciolosis in feces of sheep by means of a PCR and its application in the detection of anthelmintic resistance in sheep flocks naturally infected

D. Robles-Pérez [a], J.M. Martínez-Pérez [a], F.A. Rojo-Vázquez [a,b,*], M. Martínez-Valladares [b]

[a] Faculty of Veterinary Medicine, University of León, Campus de Vegazana, 24071 León, Spain
[b] Instituto de Ganadería de Montaña (CSIC-ULE), Finca de Marzanas, 24346 Grulleros, León, Spain

ARTICLE INFO

Article history:
Received 9 January 2013
Received in revised form 1 May 2013
Accepted 3 May 2013

Keywords:
Fasciola hepatica
Anthelmintic resistance
PCR
FECRT
CRT

ABSTRACT

The aim of this study was to develop a PCR for the diagnosis of *Fasciola hepatica* infection in feces of sheep based on the ribosomal internal transcribed spacer. Detection of infection was possible from the second week post-infection in experimentally infected sheep by amplification of a 292 bp fragment. This PCR was employed for the detection of anthelmintic resistance (AR) in naturally infected sheep flocks, and results were compared with techniques such as the fecal egg count reduction test (FECRT) and the copro-antigen reduction test (CRT). The FECRT was carried out in two flocks, Santillan de la Vega (SV) and Corullón (CR), with sheep treated with albendazole (ABZ), clorsulon (CL), or triclabendazole (TCBZ). Feces were collected from individuals on days 0, 7, 15, and 30 post-treatment (pt). The FECRT showed adult *F. hepatica* to be resistant to ABZ and CL in both flocks. All parasite stages in the SV flock were susceptible to TCBZ, while in the CR flock, adult flukes showed resistance and immature forms were susceptible to the treatment. To compare FECRT and the PCR results, we calculated the percent of positive sheep on day 1 pt. In both flocks, the percent positive sheep was consistently higher by PCR than by sedimentation, confirming that the PCR is a more sensitive method of diagnosing infection and therefore to detect the resistance in infected animals. The CRT was carried out in the SV flock using a sandwich ELISA kit. The percent of sheep found positive by PCR was higher than with ELISA. Comparison of FECRT, CRT, and PCR for the detection of AR showed PCR to be the most sensitive.

1. Introduction

Fasciolosis is a cosmopolitan helminthosis that is an important limiting factor in sheep and bovine production (Spithill and Dalton, 1998). The most significant economic losses are due to chronic disease with consequences such as weight loss, decrease in the conversion index, and reduction in milk production as well as liver condemnation.

Detection of *Fasciola hepatica* eggs in feces of sheep with suspected infection is only useful to identify chronic infections, since eggs appear in the feces only after the fluke reaches sexual maturity, at around 9 weeks of infection (Martínez-Valladares et al., 2010a), when liver damage has already occurred. The conventional method of identifying eggs is sedimentation, however, the sensitivity of this technique is estimated to be only 30% (Happich and Boray, 1969), leading to false negatives.

Another diagnostic method is the detection of specific antigens in feces by sandwich ELISA. Mezo et al. (2004) reported sensitivity of this test to be 100% in sheep and cattle, even when sheep were infected with only a single

* Corresponding author. Tel.: +34 987291338; fax: +34 987291304.
E-mail address: francisco.rojo@unileon.es (F.A. Rojo-Vázquez).

0304-4017/$ – see front matter © 2013 Elsevier B.V. All rights reserved.
http://dx.doi.org/10.1016/j.vetpar.2013.05.006

fluke. Moreover, through the detection of copro-antigen, diagnosis of the infection can be made earlier (Moustafa et al., 1998): about 1 week post-infection (pi) (Almazán et al., 2001) compared to 4–5 weeks pi with sedimentation (Rodríguez-Osorio et al., 1998; Flanagan et al., 2011; Martínez-Pérez et al., 2012). Molecular methods are highly sensitive for the diagnosis of diseases. Several authors have confirmed infection by parasites such as *Echinococcus* spp., *Toxocara* spp., and *F. hepatica* using PCR assay of feces (Bretagne et al., 1993; Dinkel et al., 1998; Martínez-Pérez et al., 2012).

In recent years, there has been a reported increase in the prevalence of anthelmintic resistance (AR) in *F. hepatica* due to the misuse of drugs for fluke control. Several cases have been described in Scotland (Mitchell et al., 1998), Wales (Thomas et al., 2000), Netherlands (Moll et al., 2000), Ireland (Mooney et al., 2009), Spain (Álvarez-Sánchez et al., 2006; Martínez-Valladares et al., 2010b), and Argentina (Olaechea et al., 2011). Anthelminthic resistance limits the use of many drugs for controlling infection.

In vivo and *in vitro* tests to reveal AR have been developed. The fecal egg count reduction test (FECRT) is an *in vivo* assessment based on reduction in the number of eggs in feces following anthelmintic treatment (Coles et al., 1992). The egg hatch test (EHT) was developed to detect, *in vitro*, AR to benzimidazoles (BZs) used against trichostrongylids, and recently Robles-Pérez et al. (unpublished data) have carried out EHT for the detection of AR to triclabendazole (TCBZ) and albendazole (ABZ) in *F. hepatica* isolates. Currently the detection of *F. hepatica* antigens in feces is being applied in infected sheep to detect AR to ABZ and TCBZ (Flanagan et al., 2011; Novolbilský et al., 2012).

The primary goal of our study was to develop a PCR based on the ribosomal internal transcribed spacer 2 (ITS2) for the detection of *F. hepatica* in sheep and to use this technique to detect AR in naturally infected sheep flocks. We compared the results to those of FECRT and the copro-antigen reduction test (CRT).

2. Materials and methods

2.1. Fecal DNA samples from experimentally infected sheep

DNA from pooled fecal samples of sheep experimentally infected with *F. hepatica* were used. The samples were collected from the initiation of infection until 8 weeks pi. Fecal DNA samples from two uninfected sheep and five sheep experimentally infected with gastrointestinal nematodes (GIN), three with *Teladorsagia circumcincta* and two with *Haemonchus contortus*, were also used in the analysis. All samples were those used by Martínez-Pérez et al. (2012).

2.2. Naturally infected sheep

Naturally infected sheep were randomly selected from two flocks with a history of fasciolosis. The flocks were located in Santillán de la Vega (SV) Province, Palencia, and Corullón (CR) Province of León, both located in the Autonomous Community of Castilla y León, Northwest Spain.

The predominant breeds of sheep were Assaf in SV and Churra in CR, reared for production of milk and meat, respectively. At both farms, flocks were grazed on irrigated pastures from 6 to 8 h per day. Both flocks contained grazing animals at the time of the study, and the most recent anthelmintic treatment had been carried out at least two months prior to the beginning of the study.

Infection by *F. hepatica* was confirmed in both flocks by analyzing four pools of fecal samples from 20 sheep selected at random *via* a coprological test. Feces were processed by sedimentation (MAFF, 1986) using a McMaster chamber (Thienpont et al., 1986). The farms were selected for the current study because sheep showed mean eggs per gram (epg) in feces greater than 45.

2.3. FECRT

To carry out the FECRT, sixty sheep were individually sampled to select those positive for *F. hepatica*. The selected sheep were separated into three groups to be treated with ABZ (Sinvermin®; 7.5 mg/kg), clorsulon (CL) (Ivomec-F®; 2 mg/kg), or TCBZ (Fasinex®; 10 mg/kg). Feces were collected on days 0, 7, 15, and 30 post-treatment (pt). The number of sheep per treatment group ranged from 7 to 9 sheep.

Individual feces samples were processed by sedimentation. The level of resistance present in each group and farm was determined with the following formula:

$$\% = \left[\frac{\text{mean epg day 0} - \text{mean epg day pt}}{\text{mean pg day 0}}\right] \times 100$$

According to the WAAVP guidelines for Trichostrongylidae (Coles et al., 1992), resistance can be confirmed when the percent fecal egg count reduction after treatment is lower than 90%. When the percent ranged from 90 to 95%, the flock was considered borderline between susceptibility and resistance, and, when higher than 95%, susceptible.

All values of epg in feces are expressed as the arithmetic mean with standard deviation.

2.4. DNA extraction and PCR

DNA extraction was carried out on 0.5 g of feces from each naturally infected sheep on days 0, 7, 15, and 30 pt.

The extraction of DNA from feces followed specifications of the commercial kit SpeedTools Tissue DNA Extraction Kit (*Biotools*). DNA samples were resuspended in 60 μL of buffer BBE and stored at −20 °C until use. To improve the quality of the DNA, samples were subjected to ethanol precipitation and finally resuspended in 40 μL of buffer.

PCR reaction was based on a 25 μL volume containing 12.5 μL of Taq DNA Polymerase Master Mix (2.0 mM $MgCl_2$, 0.4 mM dNTPs and 0.05 units/μl Ampliqon Taq polymerase), 0.5 μM specific primers (ITS2F: 5′-GTGCCAGATCTATGGCGTTT-3′ and ITS2R: 5′-ACCGAGGTCAGGAAGACAGA-3′) and 4 μL of DNA diluted ten times. Primers were based on the sequence GQ231547.1 of *F. hepatica* encoding the ribosomal internal transcribed spacer 2 (ITS2). The thermocycler used (Biorad) was set to 2 min at 95 °C, followed by 40 cycles