THE DIAGNOSIS OF FASCIOLOSIS IN FAECES OF SHEEP BY MEANS OF A PCR AND ITS APPLICATION IN THE DETECTION OF ANTHELMINTIC RESISTANCE IN SHEEP FLOCKS NATURALLY INFECTED

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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.

Key words: *Fasciola hepatica*; anthelmintic resistance; PCR; FECRT; CRT.
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 *F. hepatica* eggs in feces of sheep with suspected infection is only useful to identify chronic infections (Burger, 1992), 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 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 to 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; Mathis and Deplazes, 2006; 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 *Fasciola 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; Novobilský 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. Material 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 Martinez-Perez 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:

\[
\% = \frac{\text{Mean epg day 0} - \text{Mean epg day pt}}{\text{Mean epg day 0}} \times 100
\]

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 MgCl₂, 0.4 mM dNTPs and 0.05 units/μl Ampliqon Taq polymerase), 0.5 μM specific primers (ITS2F: 5´GTGCCAGATCTATGGCGTTT3´ and ITS2R: 5´ACCGAGGTCAGGAAGACAGA3´) 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 of 30 s at 95 °C, 30 s at 63 °C, and 45 s at 72 °C and a final extension step at 72 °C for 10 min. A negative control was included. The amplification products were analyzed by electrophoresis in 1.5% agarose TBE gel and stained with Gel Red. Then, the bands were excised, purified, and sent to the Laboratorio de Técnicas Instrumentales at the University of León to be sequenced.

2.5. Sandwich ELISA
The copro-antigen was measured in the naturally infected sheep from the SV flock on days 0, 7, 15, and 30 pt.

To measure the copro-antigen in fecal samples, 1 g of fresh feces from each sheep was added to 2 mL of PBS-Tween 20. Fecal eluates were stored 48 h at 4 °C and centrifuged for 10 min at 1,000 x g. The supernatants were collected and stored at -20 °C until analysis. The specifications indicated by the Bio-X Bovine *F. hepatica* Antigenic ELISA Kit (BIO K 201) were followed. Only samples with optical density (OD) values higher than the cut-off value of 0.150 at 450 nm were considered positives. All samples were analyzed in duplicate. The OD values are expressed as arithmetic mean and standard deviation.

3. Results

The diagnosis of an experimental infection by *F. hepatica* was carried out in weeks 1-8 pi by a PCR technique using specific primers for the amplification of a 292 bp fragment of the ribosomal ITS2 sequence. The PCR product identified showed 100% identity with the ITS2 of *F. hepatica* (GQ231547.1) (Fig. 1). The detection of *F. hepatica* infection was possible from the second week pi (Fig. 2).

To confirm the specificity of the technique, a PCR with fecal samples from uninfected sheep and from those experimentally infected with GINs, *T. circumcincta* and *H. contortus*, was also carried out (Fig. 3). The absence of amplification product in these PCRs confirmed the specificity of the technique.

After developing a PCR to diagnose the infection, we used the technique to detect AR in naturally infected sheep. The PCR was carried out on fecal DNA samples of sheep on days 0, 7, 15, and 30 after the treatment with ABZ, CL, and TCBZ. The PCR products before and after anthelmintic treatment are shown in the Figs 4 and 5 for SV and CR.
flocks, respectively. We confirmed a reduction in the number of positive animals in both flocks throughout the study for all drugs, with all samples being negative on day 30 pt TCBZ.

The number of sheep positive by PCR, relative to the number of sheep analyzed, is shown in Table 1 as along with the percent positive for each day pt.

Results obtained by PCR for the diagnosis of AR were compared to those of other *in vivo* and *in vitro* techniques, FECRT and CRT. For FECRT, the percent reduction of eggs is shown in Table 2.

To confirm the results of PCR and FECRT, a sandwich ELISA was carried out in the SV flock to compare all techniques. The number of positive sheep each sampling day is shown in Table 1 and the mean OD value in Table 3. The day of treatment administration, the mean OD value was greater than 0.150 in all sheep and groups, confirming that all animals were infected. On days 7, 15, and 30 pt, the mean OD values were lower than 0.150 only after treatment with TCBZ.

When comparing the three diagnostic methods individually (Table 1), we observed that the most sensitive technique was PCR followed by ELISA and sedimentation.

### 4. Discussion

The development of sensitive, rapid, and precise techniques for routine diagnosis of infections in flocks is critical. The first aim of this study was to develop a PCR technique for the diagnosis of fasciolosis in sheep and, further, to use this technique to detect the presence of AR in naturally infected flocks. The development of new diagnostic methods for the detection of AR, avoiding the sacrifice of animals, is important and necessary for animal welfare considerations.
We developed a PCR to detect the presence of *F. hepatica*, amplifying a 292 bp fragment of the ITS2 in sheep. The infection was detected at week 2 pi. We confirmed the specificity of the technique with DNA fecal samples from uninfected sheep and those infected with GIN. Results were in accordance with those of Martínez-Perez et al. (2012) who detected the infection in feces from week 2 pi using Nested-PCR to amplify a 423 bp fragment of the cytochrome C oxidase 1 gene (Cox1). However, the PCR described in the present study is a less time consuming method than Nested-PCR. With coprological techniques, studies in experimentally infected sheep have reported detection later: at week 7 pi (Paz-Silva et al., 2002), between weeks 8 and 10 pi (O'Neill et al., 2000; Martínez-Valladares et al., 2010a), and after 10 weeks pi (Duménigo and Mezo, 1999).

The benzimidazole compound TCBZ is the only drug effective against both adult and immature flukes (Boray et al., 1983). Treatment with ABZ and CL is only effective against adult stages (McKellar and Scott, 1990; Geurden et al., 2012) having limited efficacy against immature flukes (Fairweather and Boray, 1999). With FECRT, in both the SV and CR flocks, adult flukes showed resistance to ABZ and CL on day 7 pt. Eggs detected on days 15 and 30 pt had likely been produced by resistant adults and immature flukes that had reached the mature stage. With respect to TCBZ, in the SV flock the reduction of eggs was higher than 95% on the sampling days, indicating that all parasite stages were susceptible to the drug. However, in the CR flock resistance to TCBZ was shown in adult flukes on day 7 pt. Since the epg in feces in both flocks at the beginning of the assay was only approximately 30 epg for most of the groups, we confirmed the infection by other techniques.

To compare the FECRT and the PCR results, we calculated the percent of positive animals each sampling day. In both flocks, the percent of positive sheep was
consistently higher by PCR than by sedimentation, confirming that PCR is a more sensitive method of diagnosis and, therefore, of detecting resistance in infected animals. Dinkel et al. (1998), in a study of foxes infected with *Echinococcus multilocularis*, also discarded coprological techniques and opted for coprodiagnosis by PCR as an alternative to necropsy. The results obtained in our study indicate that FECRT is not able to accurately detect the level of AR due to low sensitivity of the coprological techniques.

Use of the commercial kit Bio X Diagnostics makes detection of *F. hepatica* coproantigen in feces possible 4-5 and 2 weeks earlier than using egg detection in experimental *F. hepatica* and *Fasciola gigantica* sheep infections, respectively (Valero et al., 2009). This method has also been employed by other authors (Flanagan et al., 2011, Gordon et al., 2012) to detect the presence of AR in infected animals. Flanagan et al. (2011) defined the absence of coproantigens in collected fecal samples at 14 day pt as successful treatment. In the current study, comparing the percent positive animals with PCR and sandwich ELISA, in flock SV, after treatment with ABZ, the percent of positive animals was the same, although slightly higher on day 30 pt by PCR. After the administration of CL, all percentages were the same (42.9%) by both techniques. With TCBZ, the percent of positive sheep was higher by PCR on days 7 and 15 pt (62.5 and 12.5%, respectively). Therefore, we confirmed that the PCR technique was more sensitive than sandwich ELISA, and therefore the presence of AR could be detected more accurately. Lahmar et al. (2007) also carried out a comparison between sandwich ELISA and PCR in feces to diagnose the infection of *E. granulosus* in dogs. They observed ELISA to detect prepatent infections with a high sensitivity (82.8%), but a detection rate of only 25.9% during an equivalent time period with PCR.
On the other hand, Flanagan et al. (2011) compared CRT and FECRT to determine TCBZ efficacy in 4 isolates of *F. hepatica*. Methods produced similar results: 3 isolates were susceptible and 1 showed resistance to TCBZ. In the present study, in the SV flock, on days 7 and 30 pt with ABZ, differences were evident: 3 animals were positive by sandwich ELISA but not by sedimentation. However, 7 pt with TCBZ, one animal was positive by sedimentation but negative by ELISA. It may be that fluke eggs can also be detected in feces as false positives, due to their presence in the gall bladder some days after successful treatment (Flanagan et al., 2011). This phenomenon was also observed by Novobilský et al. (2012) who reported that the number of positive animals was higher by sedimentation than when using the kit Bio-X K201 *Fasciola* copro-antigen.

5. Conclusion

The early detection of fasciolosis is possible by week 2 pi using PCR that amplifies a fragment of the ITS2 subunit. This technique was used to diagnose the AR in two naturally infected sheep flocks. When comparing FECRT, PCR, and CRT, the most sensitive technique to detect resistant flocks was shown to be PCR. Our study is the first to compare three diagnostic techniques to determine the presence of AR.

Acknowledgements

This study has been funded by the national projects INIA-MEC, RTA2010-00094-C03-02. The work of María Martínez-Valladares has been supported by a postdoctoral Jae-Doc contract from the Consejo Superior de Investigaciones Científicas (CSIC) and co-funded by the European Social Fund.
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