Veterinary Parasitology 203 (2014) 217–221

Contents lists available at ScienceDirect

Veterinary Parasitology

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

Short Communication

Development of an egg hatch assay for the detection of anthelmintic resistance to albendazole in *Fasciola hepatica* isolated from sheep

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ARTICLE INFO

Article history:
Received 7 June 2012
Received in revised form 30 October 2013
Accepted 18 November 2013

Keywords:
Fasciola hepatica
Anthelmintic resistance
Egg hatch assay
Albendazole

ABSTRACT

The aim of this study was to develop an egg hatch assay (EHA) to detect the resistance of *Fasciola hepatica* to albendazole (ABZ). With this purpose, two different *F. hepatica* isolates were tested: (i) susceptible (Shrewsbury/South Gloucester strain) to ABZ (SA); (ii) resistant to ABZ (RA). A commercial formulation of ABZ (Sinvermin®) diluted in dimethyl sulfoxide (DMSO) was used at concentrations of 0.0002, 0.002, 0.02, 0.2, and 2 μg/ml. In the SA isolates, eggs from feces exposed to ABZ at the three highest concentrations showed significantly lower hatching percentages than the control well ($P < 0.01$); also for the two highest concentrations, the percentage of development was significantly lower than the control ($P < 0.01$). However, in the RA isolate, no differences among treatments and control were found in the percentage of hatched or developed eggs. On the other hand, the EHA was conducted over a 15 day period following treatment with ABZ for sheep infected by the RA isolate. On day 7 post-treatment (pt), no differences among treatments and controls were observed. On day 15 pt, the development and hatching rates exposed to the highest ABZ concentration were lower than at lower concentrations, suggesting susceptibility. The ovicidal effect of ABZ and the potential use of EHA to differentiate between ABZ susceptible and resistant isolates are confirmed in the current study. However, the use of this technique should be confirmed with a higher number of isolates.

1. Introduction

Fasciolosis is an important infection of sheep and cattle with increasing prevalence in recent years (Kenyon et al., 2009; van Dijk et al., 2010; Martínez-Valladares et al., 2013).

Among all flukicides, one of the most important chemical groups is the benzimidazoles (BZs). Of all compounds, albendazole (ABZ) is recommended for the control of fasciolosis, and, although its activity is restricted to flukes older than 12 weeks (McKellar and Scott, 1990), it was one of the most used drug in Spain for many years according a recent survey (Rojo-Vázquez and Hosking, 2013), removing up to 94–95% of adult flukes in sheep (Coles and Stafford, 2001).

Recent reports have indicated an increase in the prevalence of anthelmintic resistance (AR) to *F. hepatica* due to drug overuse; however, the mechanisms involved in its development are not yet well understood. AR to *F. hepatica* has not reached the levels seen in gastrointestinal nematodes (GIN) (Jackson and Coop, 2000). Overend and Bowen (1995) described the first case of resistance to triclabendazole (TCBZ) in Australia, and, since then, there have been

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http://dx.doi.org/10.1016/j.vetpar.2013.11.020

reports in Ireland (Lane, 1998; O'Brien, 1998), Scotland (Mitchell et al., 1998), Wales (Thomas et al., 2000), and the Netherlands (Moll et al., 2000; Gaasenbeek et al., 2001). In Spain, Álvarez-Sánchez et al. (2006) described for the first time the resistance to ABZ and TCBZ in sheep in the province of León.

In vivo and *in vitro* tests have been developed to identify AR. Among the *in vivo* tests, the fecal egg count reduction test (FECRT) is based on reduction of the number of eggs in feces following anthelmintic treatment (Coles et al., 1992). In relation to *in vitro* tests, the egg hatch assay (EHA) has been developed to detect BZ resistance in trichostrongyles and is based on the ovicidal properties of some BZs; eggs of resistant isolates embryonate and hatch at higher concentrations than do those of a susceptible isolate (Whitlock et al., 1980). Although the EHA was originally designed to detect AR in GIN, preliminary studies were carried out in *F. hepatica* by Álvarez et al. (2009) and Fairweather et al. (2012), who compared the hatching rate of eggs taken from the gall bladder and using various concentrations of TCBZ, ABZ, and their sulfoxide metabolites.

In this context, the aim of this study was to develop an EHA to determine the resistance of *Fasciola hepatica* isolates to ABZ. EHAs were designed using eggs from feces to test a formulation of ABZ with susceptible and resistant *F. hepatica* isolates.

2. Materials and methods

2.1. Fasciola hepatica isolates

Two *Fasciola hepatica* isolates differing in resistance and susceptibility to ABZ were used. The susceptible isolate (SA) was the Shrewsbury/South Gloucester strain (Ridgeway Research Ltd Company, UK). On the other hand, a field isolate from Santillán de la Vega, (Palencia, Spain), resistant to ABZ (RA), was also tested. With the aim to determine the resistance in the isolate RA the FECRT was carried out by Robles-Pérez et al. (2013). In this study, after the administration of ABZ, the reduction of eggs in feces was 50.1% on day 7 post treatment (pt) and 31.2% on day 15 pt.

2.2. EHA

The formulation used in this study was the commercial of ABZ (Sinvermin®) diluted in DMSO to guarantee its complete solubilization.

With the aim to assess the resistance to ABZ, a stock solution (400 μg/ml) of a commercial formulation of ABZ (Sinvermin®) (28.5 mg/ml) diluted with DMSO was used to guarantee the complete solubilization of the drug. Dilutions of 0.04, 0.4, 4, and 40 μg/ml were prepared to obtain a final concentration in the wells of 0.0002, 0.002, 0.02, 0.2, and 2 μg/ml after adding 10 μl of each solution to a total volume of 2 ml. In all EHAs, control wells with 10 μl of DMSO, without drug, were included.

The concentration of ABZ in the stock solution was confirmed as a function of the respective peak area carried out by HPLC chromatography following the protocol previously described by Ragno et al. (2006).

Eggs from feces of infected sheep by both isolates were obtained by a fecal sedimentation method (MAFF, 1986).

For the EHA, a 24 well cell culture plate was used and all anthelmintic concentrations were tested in duplicate. Into each well, 1890 μl of water, 100 μl of water with 30–50 eggs, and 10 μl of each dilution were placed. A control well containing 10 μl of DMSO was also used. Plates were incubated for 14 days at 25 °C in darkness. They were then placed under light for 2 h to stimulate hatching of the miracidia. The number of eggs hatched, embryonated, and unembryonated were counted.

All EHA assays were repeated five times for each isolate.

With the aim to detect AR to ABZ using the EHA after an *in vivo* treatment, eggs from feces of six sheep infected by the isolate RA were recovered. The EHAs were carried out on days 0, 7, and 15 after administration of a single oral dose of ABZ (7.5 mg/kg, Sinvermin®).

2.3. Data and statistical analysis

The percentage of hatched and developed eggs was calculated for each isolate, using the following formula:

$$\text{Percentage of development} = \left(\frac{\text{number of developed eggs}}{\text{total number of eggs}}\right) \times 100$$

$$\text{Percentage of hatching} = \left(\frac{\text{number of hatched eggs}}{\text{total number of eggs}}\right) \times 100$$

The number of developed eggs is the sum of hatched (operculum opened without miracidium) and embryonated eggs (miracidium inside the egg). The number of eggs is the sum of hatched, embryonated, and unembryonated eggs (egg in morula stage, without miracidium).

To compare two EHAs differing in hatch rates, a ratio of the results of each concentration to the control was calculated, using the following formula:

$$\text{Ratio} = \left(\frac{\%\,\text{hatching of each concentration}}{\%\,\text{hatching of control}}\right) \times 100$$

The data were analyzed using the statistical computer package for social sciences SPSS. A one-way ANOVA was used to assess differences. To confirm significant differences between concentrations and the control group, the Dunnett test was carried out. Differences of less than 5% were considered significant ($P < 0.05$).

3. Results

The results of the percentage of hatched and developed eggs in the SA and RA isolates are shown in Fig. 1. For the SA isolate, the hatching rate increased at lower drug concentrations, from 8 to 29%, and 33% in the control. The percentage of developed eggs ranged from 21 to 70% for the respective concentrations and was 72% for the control. The hatching rate of the control was significantly higher than that of the three highest concentrations ($P < 0.01$), and the percentage of developed eggs was significantly