Testing albendazole resistance in *Fasciola hepatica*: validation of an egg hatch assay with isolates from different sources

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SUMMARY

The main goal of the current work was to develop and validate an *in vitro* fluke egg hatch test, as a method for the detection of albendazole (ABZ) resistance in the liver fluke, *Fasciola hepatica*. Fluke eggs (200/mL, n=5) from six (6) different isolates named Cullompton, CEDIVE, INTA-Bariloche, Rubino, Cajamarca and Río Chico, were incubated (25 ºC) for a 12-h period in the presence of either ABZ or its sulphoxide metabolite (ABZ.SO) (5, 0.5 or 0.05 nmol/mL). Untreated eggs were incubated as control. Hatched eggs were evaluated using an optical microscope, and the ovicidal activity, was assessed for each fluke isolate. A very low ovicidal activity (≤13.4 %) was observed in the ABZ-resistant CEDIVE isolate for both, ABZ and ABZ.SO. Conversely, in the INTA-Bariloche and Río Chico isolates, suspected to be susceptible to ABZ, ovicidal activities ≥70.3 % were observed after incubation with ABZ (0.05 nmol/mL). Finally, the Cajamarca and Rubino isolates behaved as ABZ-resistant, since no ovicidal activity was observed after eggs were incubated with ABZ at 0.5 nmol/mL. Regarding the specific results obtained for each isolate under assessment, the egg hatch test described here may be a suitable method for detection of ABZ resistance in *F. hepatica*.

INTRODUCTION

Fasciolosis, caused by the trematode liver fluke, *Fasciola hepatica*, is the cause of considerable losses in sheep and cattle production systems all over the world (Fairweather, 2005). Fasciolosis is also emerging as a major zoonosis (Mas Coma *et al.* 2005) and is considered to be a serious health problem in some countries (Fairweather, 2005). There are a limited number of anthelmintics available to treat fasciolosis in ruminants. Benzimidazoles (BZD) are broad-spectrum anthelmintic compounds, widely used in human and veterinary medicine for controlling nematode, cestode and trematode infections (McKellar and Scott, 1990). The BZD compounds, currently marketed as anthelmintics, can be grouped as BZD thiazolyls, BZD methylcarbamates, pro-BZD and halogenated BZD thiols (Lanusse and Prichard, 1993). Only a few BZD compounds display activity against *F. hepatica*. The halogenated derivative triclabendazole (TCBZ) is the most effective because of its excellent activity against immature and mature adult flukes (Boray *et al.* 1983). Albendazole (ABZ) is the only BZD methylcarbamate recommended for the control of fasciolosis in domestic animals, despite its activity being restricted to flukes older than 12 weeks (McKellar and Scott, 1990). ABZ is not found in the bloodstream after its enteral administration to sheep (Marriner and Bogan, 1980) and cattle (Prichard *et al.* 1985). ABZ oxidations lead to more polar and less active metabolites, which are detected systemically as the sulphoxide (ABZ.SO) and sulphone ($ABZ.SO_2$) derivatives. In terms of binding to *Haemonchus contortus* ß-tubulin, ABZ is more potent than the active ABZ.SO, while $ABZ.SO_2$ is an inactive metabolite (Lacey, 1990; Lubega and Prichard, 1991).

The intensive use of TCBZ in endemic areas of fasciolosis has resulted in the development of liver flukes resistant to this compound (Overend and Bowen, 1995; Moll *et al.* 2000; Thomas *et al.* 2000; Olaechea *et al.* 2011a; 2011b; Ortiz *et al.* 2011), which is considered a major problem for veterinary therapeutics. Interestingly, ABZ has been shown to be effective against the TCBZ-resistant fluke isolate named Sligo (Coles and Stafford, 2001; Fairweather, 2011a). Conversely, a *F. hepatica* isolate resistant to ABZ and susceptible to TCBZ has recently been characterized (Sanabria *et al.* submitted).

The emergence of drug-resistant liver flukes leads to the necessity of accurate diagnosis. The standard and established protocol for the determination of drug activity against *F. hepatica* in ruminants is the efficacy controlled test (Wood *et al.* 2005), in which efficacy is determined by comparison of the number of flukes in treated animals and in untreated controls. This methodology has the disadvantage of its relative high cost and length of time involved. The alternative is the use the faecal egg count reduction test (FECRT), where the efficacy of the treatment (or the susceptibility of the *F. hepatica* isolate) is claimed if a 95% reduction on faecal fluke egg counts at 14 days post-treatment is achieved. However, the release of eggs stored in the gall bladder may produce false positive results, even when the flukes have been effectively removed by drug treatment (Fairweather, 2011b). The coproantigen reduction test (Flanagan *et al.* 2011a; b) and the “histological approach” (Hanna *et al.* 2010; in press) which involves the evaluation of the histological changes induced by drug treatment, have been proposed as alternative methods for the

diagnosis of drug efficacy and/or resistance. The egg hatch test may have some potential to detect anthelmintic resistance in flukes. This test, used as a diagnostic method for the detection of BZD resistance in nematodes (Coles *et al.* 2006), is based on the capacity of BZD compounds, mainly the methylcarbamates, to affect parasite egg hatching. Previously, it has been shown under *in vitro* conditions that both ABZ and ABZ.SO have an excellent ovicidal activity against *F. hepatica* eggs (Coles and Briscoe, 1978; Alvarez *et al.* 2009). Thus, an egg hatch-based method potentially may be used for the detection of BZD resistance in *F. hepatica*. Using a high concentration of the TCBZ sulphoxide metabolite, the test has the potential to distinguish between TCBZ-susceptible and TCBZ-resistant fluke isolates, and may become a simple method of diagnosis of drug resistance (Fairweather *et al.* 2012). However, the use of such a methodology to detect ABZ resistance in liver flukes requires further investigation. The main goal of the work reported here was to develop and validate an *in vitro* fluke egg hatch test, for the detection of ABZ resistance in *F. hepatica*. The test was applied to assess ABZ ovicidal activity in fluke isolates obtained from different sources.

MATERIALS AND METHODS

Pure (≥99%) reference standards of ABZ and ABZ.SO were used in the current experimental assay. The solvent (methanol) used for drug dissolution was of analytical grade (Anedra, Buenos Aires, Argentina). *F. hepatica* eggs from six different isolates were assessed for ABZ/ABZ.SO susceptibility. Two of them (CEDIVE and Cullompton) were considered as Reference isolates, while the others were considered as Unknown isolates. Unfortunately, at the time of the

egg hatch test development there were no eggs from the Cullompton isolate available in our laboratory. Since the egg hatch assay previously published (Alvarez *et al.* 2009) was performed under the same experimental conditions as in the current study, the data relating to the inhibition of egg hatching of the Cullompton isolate by ABZ was included as a "positive control". Details of the different isolates with regard to ABZ susceptibility are given below:

Reference isolates

CEDIVE isolate. It was recovered from the bile ducts of two sacrificed sheep, and subsequently maintained in donor sheep and *Lymnaea viatrix* snails under laboratory conditions at the "Centro de Diagnóstico e Investigaciones Veterinarias" (CEDIVE), Facultad de Ciencias Veterinarias, Universidad Nacional de La Plata, Chascomús, Argentina. After two controlled efficacy tests, this isolate behaves as resistant to ABZ and susceptible to TCBZ (Sanabria *et al.*, submitted).

Cullompton isolate. It was first obtained (1998) from sheep slaughtered at an abbatoir in Cullompton, Devon, UK, and has been kept in Queens University, Belfast, UK, since 1999 (Fairweather, 2011a). In different *in vivo* and *in vitro* studies, it has been shown to be susceptible to TCBZ (Walker *et al.* 2004; McConville *et al.* 2009, Devine *et al.* 2010; 2012; Toner *et al.* 2010; Flanagan *et al.* 2011b) and ABZ (Buchanan *et al*. 2003; McConville *et al.* 2006; Alvarez *et al.* 2009).

Unknown isolates

INTA-Bariloche isolate. It was isolated from naturally infected cattle on a Patagonian farm in Neuquén, Argentina, by Dr. Fermín Olaechea (INTA Bariloche, Bariloche, Argentina). Resistance to TCBZ has been determined by the faecal egg count reduction test (FECRT) (Olaechea *et al.* 2011a) and confirmed after TCBZ treatment of artificially-infected sheep (Olaechea *et al.* 2011b). The INTA-Bariloche isolate has been maintained under the same laboratory conditions as described for the CEDIVE isolate. No definitive data is available regarding potential susceptibility to ABZ. However, two sheep artificially infected with this isolate and orally treated with ABZ (7.5 mg/kg) 16 weeks after infection, became negative to *F. hepatica* eggs in faeces (Alvarez L., unpublished data), indicating its potential susceptibility to ABZ.

Rubino isolate. This isolate was obtained from naturally infected sheep from a farm near Salto, Uruguay. It was maintained under laboratory conditions at the DILAVE Laboratory, Montevideo, Uruguay. According to its previous history, this isolate is susceptible to closantel and nitroxynil, but there is no definitive information on ABZ susceptibility. However, *F. hepatica* eggs were recovered from faeces obtained from two sheep artificially infected with the Rubino isolate, fifteen days post-ABZ oral treatment (7.5 mg/kg)(Gayo V., unpublished data).

Cajamarca isolate. The Cajamarca isolate was obtained from faeces collected from one cow on a farm located in Cajamarca, Perú. It has been maintained under laboratory conditions at the Laboratorio de Diagnóstico Veterinario, Facultad de Ciencias Veterinarias, Universidad Nacional de Cajamarca, Cajamarca, Perú. This isolate behaves as TCBZ-resistant (Ortiz et al. 2011). No

175 data is available on ABZ susceptibility. However, in the area where the isolate
176 was recovered, the frequent use of different anthelmintics (including ABZ) to
177 control trematode and nematode infections is a common practice.
178
179 Río Chico Isolate. Eggs from this isolate were recovered from one sheep
180 sacrificed at the local abattoir in the area of Río Chico, Catamarca, Argentina.
181 No data on potential susceptibility/resistance to anthelmintics of this isolate has
182 been obtained. However, since no type of anthelmintic treatment is performed
183 at the farm where the sheep were bred, it is highly likely that the Rio Chico
184 isolate is susceptible to all flukicidal compounds, including ABZ.
185

186 In vitro *egg hatch assays*

187 The *in vitro* egg hatch assay used in the current experiment was as that
188 previously described by Alvarez *et al.* (2009). Briefly, fluke eggs (200/mL, n=5)
189 from each isolate (each isolate represents one experiment) were incubated (25
190 ºC) for a 12-h period with either ABZ or ABZ.SO, at a final concentration of 5,
191 0.5 or 0.05 nmol/mL. These are pharmacologically relevant concentrations
192 obtained from previous studies where the bile concentrations of these
193 compounds were measured after conventional treatments in sheep (Hennessy
194 *et al.* 1989; Alvarez *et al.* 2000) Untreated eggs were incubated as controls. The
195 low of number of *F. hepatica* eggs recovered from the Bariloche and Rubino
196 isolates prevented their incubation with ABZ.SO. Untreated and treated eggs
197 were gently washed with tap water (3x) to facilitate drug removal, and kept in
198 darkness at 25 ºC for 15 days. After this period, the trematode eggs were
199 exposed to daylight for 2 h. Immediately afterwards, 1 mL of 10 % (v/v) buffered

formalin was added to each tube in order to prevent further eggs from hatching. Hatched and unhatched eggs were evaluated using an optical microscope (40x magnification). Approximately 80-90 eggs were counted in order to estimate the proportion of hatched eggs in each tube. The “ovicidal activity” expressed as a percentage was estimated using the following formula:

Ovicidal activity (%) = [(% eggs hatched in control - % eggs hatched after drug incubation) / % eggs hatched in control] x 100

The percentages of egg hatch are reported as the arithmetic mean ± standard deviation (SD). Parametric ANOVA + Tukey test was used for the statistical comparison of the egg hatch data obtained from each experiment. A value of $P<0.05$ was considered statistically significant. The statistical analysis was performed using the Instat 3.0 Software (Graph Pad Software, CA, USA).

RESULTS

The results of egg hatching for each isolate are shown in Table 1. The mean egg hatch percentage obtained for untreated eggs in the different *F. hepatica* isolates ranged between 67.0 (CEDIVE) and 94.8 % (Río Chico). ABZ affected egg hatch in the Cullompton, INTA-Bariloche and Río Chico isolates at all concentrations tested. Conversely, ABZ (at 5, 0.5 and 0.05 nmol/mL) did not affect egg hatch in the CEDIVE isolate. A similar behaviour after ABZ incubation was observed in the Rubino and Cajamarca isolates, in which egg hatch was inhibited only at the highest concentration of 5 nmol/mL but, at lower concentrations (0.5 and 0.05 nmol/mL), the drug fail to inhibit egg hatch. In the Cullompton and Cajamarca isolates, egg hatch reduction with ABZ.SO was lower than that observed for ABZ.

The ovicidal activity (%) of ABZ on eggs obtained from different *F. hepatica* isolates is compared in Figure 1. A very low ovicidal activity (≤13.4 %) was observed in the ABZ-resistant CEDIVE isolate for both ABZ parent compound and its sulphoxide metabolite, even at the highest concentration tested (5 nmol/mL)(Figure 1). Conversely, in the INTA-Bariloche and Río Chico isolates, suspected to be susceptible to ABZ, ovicidal activities ≥70.3 % were observed after incubation with ABZ at the lowest concentration tested (0.05 nmol/mL) (Figure 1). This finding correlates with that previously described for the ABZ-susceptible Cullompton isolate. Finally, in the Cajamarca and Rubino isolates no ovicidal activity was observed after incubation of eggs with ABZ at 0.5 nmol/mL (Figure 1).

DISCUSSION

In ABZ-susceptible *F. hepatica* isolates, such as the Cullompton isolate, ABZ showed excellent ovicidal activity (Alvarez *et al.* 2009), even at concentrations as low as 0.05 nmol/mL (Figure 1). In this isolate, a high ovicidal efficacy was also observed for the active ABZ.SO metabolite, in spite of its lower anthelmintic potency compared to the parent drug. However, in the well-characterized ABZ-resistant CEDIVE isolate (Sanabria *et al.* submitted), both ABZ and ABZ.SO failed to prevent the egg hatch, demonstrating that the method is suitable to detect ABZ resistance in *F. hepatica*. It is important to highlight that in CEDIVE ABZ-resistant isolate egg hatching was not inhibited even at the highest ABZ/ABZ.SO concentration tested (5 nmol/mL) (Figure 1).

Regarding the “Unknown isolates”, the INTA-Bariloche isolate showed susceptibility to ABZ in the *in vitro* assay, with ovicidal activities ranging between 71.2 % (0.05 nmol/mL) and 96.4 % (5 nmol/mL)(Figure 1). This is a TCBZ-resistant isolate (Olaechea *et al.* 2011a; b) obtained from a farm where all anthelmintic treatments used in cattle are mainly directed against the liver fluke (and involve TCBZ and closantel), with sporadic treatment of gastrointestinal nematodes using ivermectin. The lack of a BZD methylcarbamate selection pressure may help to explain the potential susceptibility of the INTA-Bariloche isolate to ABZ observed in the current work. It may be assumed that the INTA-Bariloche isolate behaves in a similar way to the Sligo isolate, which has been previously characterized as resistant to TCBZ and susceptible to ABZ (Coles and Stafford, 2001). The present *in vitro* finding was partially validated by an *in vivo* study in which two sheep, artificially infected with this isolate, were treated with ABZ (7.5 mg/kg) 16 weeks after infection. There were no fluke eggs in the faeces 15 days after treatment, which would indicate a good ABZ efficacy (Alvarez L., unpublished data). Although this *in vivo* trial is not definitive, the result may support the usefulness of the *in vitro* method in detecting ABZ resistance in liver flukes.

The Rio Chico isolate behaves as susceptible to ABZ and/or ABZ.SO, since a marked ($P<0.05$) egg hatch reduction was observed at the three concentrations assayed compared to the untreated control (Table 1). The ABZ ovicidal activity ranged between 70.3 % (0.05 nmol/mL) and 84 % (5 nmol/mL). Similarly, the efficacy of ABZ.SO ranged between 73.8 % and 81.2 % (Figure 1). Río Chico is

located in the Catamarca province, Argentina, in a semi-arid region where nematode parasites are not prevalent. Most farmers graze sheep under a very low density of animals, and do not use anthelmintics in their sanitary management. As a consequence, it is not surprising that the isolate behaves as susceptible to ABZ. Interestingly, the ovicidal activity against the Río Chico isolate appears to be slightly lower than that described for the Cullompton isolate. Differences in drug susceptibility between isolates have been previously described (Fairweather, 2011a). For instance, the Cullompton isolate is more sensitive to nitroxynil than either the Sligo, Oberon or Fairhurst isolates (McKinstry *et al.* 2009), which may be related to biological differences among the fluke isolates (Walker *et al.* 2004).

Similar findings were observed after drug incubation with *F. hepatica* eggs from the Cajamarca and Rubino isolates. Interestingly, following incubation with ABZ at the highest concentration (5 nmol/mL), both isolates behave as susceptible to ABZ. Ovicidal efficacies were 90.5 % (Cajamarca) and 99.3 % (Rubino) (Figure 1). However, at lower concentrations (≤ 0.5 nmol/mL), the drug failed to inhibit egg hatching, which suggests that these isolates may be resistant to ABZ, but probably at a lower degree than that observed for the CEDIVE isolate. Unfortunately, this hypothesis could not be fully tested under *in vivo* conditions. However, in a preliminary field efficacy trial, *F. hepatica* eggs were recovered after ABZ treatment (7.5 mg/kg) in 2 sheep artificially infected with the Rubino isolate (Gayo V., unpublished data). Although, a controlled efficacy test is needed to corroborate these findings, the preliminary data described here may

299 demonstrate that flukes belonging to the Rubino isolate may possess some
300 degree of resistance to ABZ.

301

302 Concerning animal welfare, *in vitro* methods constitute an alternative to clinical
303 efficacy tests such as “dose and slaughter” trials (where a large number of
304 animals need to infected and sacrificed after treatment). Egg collection for this
305 type of test could eventually be performed directly from faecal material, making
306 any animal sacrifice unnecessary. The use of the egg hatch test as a tool to
307 detect ABZ resistance should be corroborated with *in vivo* trials, in order to
308 establish the *in vitro*/*in vivo* relationship, and its applicability with eggs isolated
309 from faecal material (sheep and/or cattle) and from different animal categories
310 (that is, young animals, adult animals). Therefore, the described *in vitro* egg
311 hatch test appears to be a suitable method for detection of ABZ resistance in *F.*
312 *hepatica*. The key reference ABZ concentration to be used in the test appears
313 to be 0.5 nmol/mL, assuming susceptibility with efficacies ≥70% and resistance
314 with efficacies ≤40 % (Figure 1). The area between 40 to 70 % of egg hatch
315 inhibition represents an area where resistance/susceptibility would be
316 suspected. The correct adjustment of this “scale” needs further research to be
317 much more conclusive. However, the data described here clearly demonstrate
318 the value of the egg hatch test as a suitable method to detect ABZ resistance in
319 *F. hepatica*.

320

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501 **Figure caption**

502

503 Figure 1: Albendazole (ABZ) ovicidal activity (%) on eggs obtained from
504 different *Fasciola hepatica* isolates. The "key" ABZ concentration to be
505 used in the test appears to be 0.5 nmol/mL, assuming susceptibility with
506 efficacies ≥70 % and resistance with efficacies ≤40 %. The area between
507 40-70 % of egg hatch inhibition represents an area where
508 resistance/susceptibility may be suspected.[1]Data obtained from Alvarez
509 *et al.* (2009).