Veterinary Parasitology 271 (2019) 7–13

Contents lists available at ScienceDirect

Veterinary Parasitology

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

Research paper

The egg hatch test: A useful tool for albendazole resistance diagnosis in *Fasciola hepatica*

Laura Ceballos[a], Candela Canton[a], Cesar Pruzzo[b], Rodrigo Sanabria[b,c], Laura Moreno[a], Jaime Sanchis[d], Gonzalo Suarez[e], Pedro Ortiz[f], Ian Fairweather[g], Carlos Lanusse[a], Luis Alvarez[a,*], María Martinez-Valladares[h]

[a] *Laboratorio de Farmacología, Centro de Investigación Veterinaria de Tandil (CIVETAN), UNCPBA-CICPBA-CONICET, Facultad de Ciencias Veterinarias, Campus Universitario, Tandil, Argentina*
[b] *Facultad de Ciencias Veterinarias, Universidad Nacional de la Plata (UNLP), La Plata, Argentina*
[c] *INTECH, CONICET-UNSAM, Chascomus, Argentina*
[d] *Departamento de Parasitología, Universidad de la República (Regional Norte), Salto, Uruguay*
[e] *Área Farmacología, Facultad de Veterinaria, Universidad de la República (UDELAR), Montevideo, Uruguay*
[f] *Facultad de Ciencias Veterinarias, Universidad Nacional de Cajamarca (UNC), Cajamarca, Peru*
[g] *School of Biological Sciences, The Queen´s University of Belfast, Belfast, Northern Ireland, United Kingdom*
[h] *Instituto de Ganadería de Montaña (CSIC-Universidad de León), Department of Animal Health, Grulleros, León, Spain*

ARTICLE INFO

Keywords:
Fasciola hepatica
Albendazole
Resistance
Egg hatch test

ABSTRACT

In the current study, the egg hatch test (EHT) has been evaluated as an *in vitro* technique to detect albendazole (ABZ) resistance in *Fasciola hepatica*. The intra- and inter-assay variations of the EHT were measured by means of the coefficient of variation in different fluke isolates and over time; then, the results of the EHT were compared with the "gold standard" controlled efficacy test, which assesses the *in vivo* anthelmintic efficacy. The EHT was used later to evaluate the intra-herd variability regarding the level of ABZ resistance in calves infected by the same fluke isolate. Finally, several factors of the initial protocol were modified to improve the simplicity of the assay, including the incubation time of eggs with the drug and the use of eggs collected from faeces. The greatest uniformity between results within the assay and over time until 8 weeks after gallbladder collection (the deadline proposed for egg analysis) was obtained with an ABZ concentration of 0.5 μM. The length of exposure to ABZ was shown to be critical, as prolonged incubation (15 days) led to a change of ovicidal activity. The ABZ concentration of 0.5 μM is suggested as a possible discriminating dose to predict ABZ resistance, due to the close agreement between the results of the EHT at an ABZ concentration of 0.5 μM and those of the *in vivo* assays.

1. Introduction

Intensive use of anthelmintics to control the most important parasite infections that affect ruminants has resulted in the development of resistance. This has been observed in sheep and cattle, where anthelmintic-resistant gastrointestinal nematodes constitute a serious problem in different areas of the world (reviewed by Wolstenholme et al., 2004; Kaplan, 2004; Sutherland and Leathwick, 2011). It is well established that drug resistance in pathogenic nematodes such as *Haemonchus contortus* and *Trichostrongylus colubriformis* is a serious concern to livestock production. In the same way, resistance to flukicides is becoming a serious problem worldwide (Kelley et al., 2016). Fasciolosis, the disease produced by infection with *F. hepatica*, is the cause of considerable losses in sheep and cattle production systems all over the world (Fairweather, 2005), and it is also emerging as a major zoonosis (Mas Coma et al., 2018). Most reports of *F. hepatica* resistance are related to triclabendazole (TCBZ) (Kelley et al., 2016), the drug most used to control this trematode parasite. Reports of TCBZ resistance include unrelated geographical regions such as Northern Ireland, Scotland, Wales, Republic of Ireland, Australia, New Zealand, Spain, Peru and Argentina, among others (reviewed by Kelley et al., 2016). Albendazole (ABZ) is another benzimidazole (BZD) compound used against nematode and liver fluke infections, in both sheep and cattle. However, while TCBZ kills mature and immature stages of *F. hepatica* (Boray et al., 1983), ABZ only targets mature liver flukes (McKellar and Scott, 1990). Although the use of ABZ as a flukicide is not as widespread as that of TCBZ, reports of resistance to ABZ in liver flukes have increased in recent years (Alvarez-Sánchez et al., 2006; Sanabria et al., 2013; Novobilský et al., 2016).

* Corresponding author.
E-mail address: lalvarez@vet.unicen.edu.ar (L. Alvarez).

https://doi.org/10.1016/j.vetpar.2019.06.001
Received 27 February 2019; Received in revised form 30 May 2019; Accepted 1 June 2019

Clearly, there is a significant need for better management of drug use in helminth control (Kotze et al., 2014). A key point is to identify the presence of parasite populations resistant to specific drugs, in order to avoid ineffective treatment and to slow the selection for resistance. The development of accurate diagnostic methods is required by veterinarians to make correct drug-use decisions. The "gold standard" method for the determination of drug activity against *F. hepatica* in ruminants is the controlled efficacy test (CET) (Wood et al., 1995), in which efficacy is determined by comparison of the number of flukes in treated animals and in untreated controls. However, this methodology has the disadvantage of its relatively high cost and the time required to complete the study. The faecal egg count reduction test (FECRT) is potentially useful for the diagnosis of anthelmintic resistance in *F. hepatica.* The FECRT evaluates the number of *F. hepatica* eggs in faeces of infected animals, before and after treatment. The susceptibility of a given *F. hepatica* isolate is confirmed if a 95% reduction in faecal fluke egg counts at 14 days post-treatment (pt) is achieved (Mooney et al., 2009). However, the release of eggs stored in the gallbladder may produce false positive results, even when the flukes have been effectively removed by the drug treatment (Fairweather, 2011b). The coproantigen reduction test (CRT) (Flanagan et al., 2011a; b) and the "histological approach", which involves the evaluation of the morphological changes induced by drug treatment (Hanna et al., 2010), have been proposed as alternative methods for the diagnosis of drug efficacy and/or resistance.

The egg hatch test (EHT) is another potentially useful approach to detect ABZ resistance in flukes. This test is based on the capacity of BZD compounds, mainly the methylcarbamates, to affect fluke egg development and hatching (Coles and Briscoe, 1978; Alvarez et al., 2009). The EHT appears to be able to discriminate between resistant and susceptible isolates (Canevari et al., 2014; Róbles-Pérez et al., 2014), and it was recently used in Sweden to test the efficacy of ABZ in sheep naturally infected with *F. hepatica* (Novobilský et al., 2016). However, the use of such methodology to detect ABZ resistance in liver flukes requires further development and standardisation. Therefore, the main goal of the work reported in this study was to validate an *in vitro* fluke EHT for the detection of ABZ resistance in *F. hepatica* isolates.

2. Materials and methods

2.1. Fluke isolates

Different *F. hepatica* isolates were used in the present study:

Cullompton isolate. It was first obtained (1998) from sheep slaughtered at an abattoir in Cullompton, Devon, UK, and has been kept in Queens University, Belfast, UK, since 1999 (Fairweather, 2011a). This isolate has been characterized as susceptible to both triclabendazole (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).

Cajamarca isolate. 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 resistant to TCBZ (Ortiz et al., 2013) and ABZ (Canevari et al., 2014).

Uru-mon isolate. It was kindly provided by Dr. Gonzalo Suarez, Montevideo, Uruguay. No data on the drug susceptibility/resistance of this isolate was available before the present study.

Spanish (SP) isolates. Eggs from the SP isolates were recovered from the gallbladders of calves naturally infected with *F. hepatica* at the slaughter house in León, Spain. The calves belonged to eight different herds (isolates SP1 to SP8). Prior to this study, their drug susceptibility/resistance status was unknown.

Argentinean (AR) isolates. Eggs from the AR isolates were obtained from faecal material of steers naturally infected with *F. hepatica* and belonging to four different farms located in the Entre Ríos Province, Argentina (isolates AR1 to AR4). Their drug susceptibility/resistance status was unknown before this study.

2.2. Description of the egg hatch test

The *in vitro* EHT described in the current study was based on a previous study (Alvarez et al., 2009). In brief, eggs of different fluke isolates were collected from gallbladders of infected animals by suctioning bile with a 10 mL syringe and a 19 G needle. After collection, the bile was washed several times using tap water and eggs were recovered by sedimentation. The eggs were identified and stored at 4 °C in darkness until required. Working solutions of ABZ were prepared by dissolution of ABZ (≥99% purity, Sigma–Aldrich, St. Louis, MO, USA) in pure methanol to reach final concentrations of 500, 50 and 5 μM. Fluke eggs (approximately 200) in 1 mL of water were incubated at 25 °C in darkness for a 12 h period with ABZ, at concentration of 5, 0.5 or 0.05 nmol/mL. Ten μL of each working solution or methanol was added to the egg suspension, reaching a final methanol concentration of 1% (v/v). In each assay, between 3 and 5 replicates were used for each drug concentration. Control eggs were incubated only with 10 μL of methanol in 1 mL of water. After incubation, all eggs were gently washed with tap water three times to facilitate drug removal, and kept in darkness at 25 °C for 15 days. After this period, eggs were exposed to light for 2 h to stimulate the hatching of miracidia. Immediately afterwards, 1 mL of 10% (v/v) buffered formalin was added to each tube in order to prevent further hatching of eggs. Hatched and unhatched (undeveloped) eggs were evaluated using an optical microscope (DM IL, Leica, Germany). The term "hatched eggs" includes hatched and embryonated eggs. In all test, embryonated eggs represent no more than 10% of total "hatched eggs". Approximately 90–110 eggs were counted in order to estimate the proportion of hatched eggs in each tube. The percentage of eggs hatched is presented as the arithmetic mean ± standard deviation (SD). The ovicidal activity, expressed as a percentage, was estimated for each dose using the following formula:

$$\text{Ovicidal activity (\%)} = \frac{\text{\% eggs hatched in control} - \text{\% eggs hatched after drug incubation}}{\text{\% eggs hatched in control}} \times 100$$

To compare several EHT results, a parametric test (Student's *t*-test or ANOVA + Tukey) was carried out using the Instat 3.0 Software (Graph Pad Software, CA, USA). A value of $P < 0.05$ was considered to be statistically significant.

2.3. Validation of the egg hatch test

2.3.1. Intra- and inter-assay variability

With the aim of validating the EHT technique (intra-assay variability), a total of 8 gallbladders were collected from naturally infected cows belonging to different herds (herd 1 to 8) at the slaughter house in León, Spain (isolates named SP1 to SP8). The gallbladders were transported at 4 °C to the laboratory, where the eggs were collected prior to carrying out the EHT as previously described (section 2.2). Within the same test, the intra-assay variation was measured by testing the same isolate in replicates of between 3 and 5; the coefficient of variation (CV) percentage was calculated for each concentration using the mean hatching rate and its SD. The CV was calculated as CV = (SD/mean) x 100.

The inter-assay variation over time was determined by evaluating the ovicidal activity in the same isolate at different time periods after the collection of gallbladders. Isolates SP1-SP4 were tested at 2, 4 and 8 weeks after collection (ac); isolates SP5-SP8 at 2 weeks and 6 months ac. Additionally, the isolate Uru-mon was tested on the day of collection and 45 days later. The CV between assays, ovicidal activity and its SD were calculated, after repeating the EHT at least three times over time.

2.3.2. Association between in vivo and in vitro tests

A comparison between the results of the EHT and the gold standard

test to assess the anthelmintic efficacy, the CET (Wood et al., 1995), was carried out. Eggs from a specific *F. hepatica* isolate (Uru-mon), maintained under laboratory conditions (Sanabria et al., 2013), were obtained from the gallbladder of one experimentally infected sheep (infected with 100 metacercariae), sacrificed by stunning followed by exsanguination 16 weeks post-infection (pi). The EHT was performed as previously described (section 2.2) and the ABZ ovicidal activity (%) was estimated. At the same time, a CET was carried out with 8 Corriedale sheep obtained from a farm located in an area free of *F. hepatica*. Eggs from the Uru-mon isolate were incubated (at 25 °C, for 15 days) to obtain miracidia and then lymnaeid snails were infected with hatched miracidia in order to produce metacercariae. The sheep were orally infected with 75 metacercariae each. At week 16 pi and after coprological confirmation of the infection (Ueno and Goncalves, 1988), animals were randomly distributed either to an untreated control group or to an ABZ-treated (7.5 mg/kg, Baxen 3.8%®, Tecnofarm, Argentina, intraruminal administration) group. At day 14 pt all animals were stunned and exsanguinated immediately and flukes were recovered and counted from all sheep following the protocol described by Wood et al. (1995). The efficacy of ABZ was determined by the comparison of fluke burdens in treated *versus* untreated control animals. The following equation expresses the percentage of efficacy against *F. hepatica* for the ABZ-treated group (T) when compared with the untreated control (C):

$$Efficacy\ (\%) = \frac{mean\ fluke\ burden\ in\ C - mean\ fluke\ burden\ in\ T}{mean\ fluke\ burden\ in\ C} \times 100$$

The criterion for efficacy was a statistically significant difference in fluke burdens between treated and untreated control groups with efficacy ≥90% (Wood et al., 1995). Liver fluke counts were compared by a non-parametric unpaired test (Mann-Whitney), using the Instat 3.0 Software (Graph Pad Software, CA, USA).

2.3.3. Intra-herd variability using the egg hatch test

The variability of the resistance to ABZ among animals infected by the Cajamarca isolate was measured by the EHT described in this study. Eight cows were experimentally infected with metacercariae obtained from the Cajamarca isolate (400 metacercariae/animal) at the facilities of the Cajamarca University (Perú). *F. hepatica* eggs were obtained directly from the gallbladder (Section 2.2) after sacrifice of animals at the local abattoir. Eggs collected from individual animals were transported at 4 °C in darkness to the Pharmacology Laboratory in Tandil, Argentina, to perform the EHT, following the protocol previously described (Section 2.2) but using only the two highest ABZ concentrations (5 and 0.5 µM). The EHT was performed using *F. hepatica* eggs obtained from the gallbladder of each animal (eight different tests).

2.4. Calculation of the resistance ratio by means of the Egg Hatch Test

The resistance ratio was calculated for the Uru-mon isolate (Section 2.3) as its EC50 (the concentration required to inhibit 50% of the viable eggs) divided by the EC50 of an ABZ-susceptible isolate (Cullompton). For calculating the EC50 in both isolates (Cullompton and Uru-mon), two Corriedale sheep were orally infected with 100 *F. hepatica* metacercariae each, one with the Cullompton isolate and the other with the Uru-mon isolate. At week 16 pi, and after a 24 h fasting period, both animals were stunned and exsanguinated immediately. Gall bladders were processed and EHTs were carried out as previously described (Section 2.2), but with the following final concentrations of ABZ: 5, 0.5, 0.05, 0.005 or 0.0005 µM. EC50 values were determined by plotting the concentration of each isolate *versus* the percentage of ovicidal activity. Data was analysed using non-linear regression with GraphPad Prism software (GraphPad Software Inc., USA). EC50 values and 95% confidence intervals (CI) were calculated for each isolate. Significant differences in IC50 between isolates were based on overlap of 95% CI.

2.5. Modifications of the standard protocol for the Egg Hatch Test

Several factors in the initial protocol were modified to improve the simplicity of the assay.

2.5.1. Incubation time with ABZ

The effect of increasing the incubation time of eggs with ABZ was tested. The EHT was performed following the previous protocol (Section 2.2) or incubating the eggs without ABZ removal (15 days of egg-drug exposure), using eggs from the Uru-mon isolate (Section 2.3). The mean (± SD) percentage of hatched eggs and the ABZ ovicidal activity (%) was estimated.

2.5.2. Eggs isolated from faeces

In order to explore the utility of the EHT for the diagnosis of ABZ resistance in *F. hepatica* in live animals, the EHT was evaluated using eggs recovered by sedimentation (MAFF, 1986) from faeces of naturally infected steers (14–17 months old) belonging to four commercial farms located in the Entre Ríos province, Argentina (AR1 to AR4 isolates). The result of the EHT was compared with the results obtained with the *in vivo* FECRT, in order to compare ABZ resistance in liver flukes estimated by both methods. In each farm, 20 steers naturally infected with *F. hepatica* were included in the FECR study. *F. hepatica* infection was confirmed in all steers by measuring the number of *F. hepatica* eggs per gram (EPG) by a coprological sedimentation method (Ueno and Goncalves, 1988). On each farm, steers were randomly assigned into two groups (n = 10), a group treated orally with ABZ (10 mg/kg, Valbazen® 10% suspension, Zoetis, Argentina), and an untreated control group. Faecal samples were individually collected from the rectum of each steer the day before treatment (day -1) and on day 15 pt (for climatic reasons the samples were collected on day 15 pt instead day 14 pt). The efficacy of ABZ was assessed according to the formula recommended by the WAAVP (Coles et al., 1992):

$$FECRT\ (\%) = 100 \times (1 - [T \div C])$$

where T is the arithmetic mean EPG count in the treated group at 15 days pt, and C is the arithmetic mean EPG count in the control group at 15 days pt. The 95% confidence intervals were calculated as described by Coles et al. (1992). The EPGs, expressed as arithmetic means (± SD), were compared by the non-parametric Kruskal–Wallis test using the Instat 3.0 Software (Graph Pad Software, CA, USA).

For the EHT, faecal samples from each farm (approx. 500 g) were collected from animals of each control group, and maintained at 4 °C during transport to the laboratory. Liver fluke eggs were isolated from the faeces by sequential filtration procedures. Briefly, aliquots of 100 g of faecal material were put in a mortar with 250 mL of distilled water; the material was broken down and 50 mL of additional water was added. The mixture was poured sequentially through a series of through meshes with apertures of 250, 150 and 50 µm. *F. hepatica* eggs were retained in the 50 µm mesh. Retained eggs were recovered in a glass (250 mL) by flushing water (in reverse direction) through the mesh. Collected eggs were maintained at 4 °C in tap water until the EHT was performed, within 15 days after collection. The EHT was performed on the samples from the four farms as previously described (Section 2.2). According to Canevari et al. (2014), when the ovicidal activity was higher than 70% or lower than 40%, the isolate was considered to be resistant or susceptible, respectively; intermediate values indicate suspicions of resistance.

2.6. Ethical issues

Animal procedures and management protocols were carried out in accordance with the Animal Welfare Policy (Act 087/02) of the Faculty of Veterinary Medicine, Universidad Nacional del Centro de la Provincia de Buenos Aires (UNCPBA), Tandil, Argentina (http://www.vet.unicen.edu.ar).

Table 1
Percentage of hatched eggs (mean ± SD) and ovicidal activity (%) obtained for different *Fasciola hepatica* isolates (SP1 to SP4) following incubation in albendazole (ABZ) according to the week of analysis after collection (ac) of gallbladders.

Isolate	ABZ Concentration (μM)	Eggs hatched (%)									Ovicidal activity (%)					
		2 weeks ac			4 weeks ac			8 weeks ac			weeks ac			Mean	SD	CV
		Mean	SD	CV	Mean	SD	CV	Mean	SD	CV	2	4	8			
SP1	5	0.6	0.1	11.7	2.3	0.4	16.1	2.1	0.7	**31.9**	99.3	97.6	97.4	98.1	0.9	0.9
	0.5	0.9	0.1	7.8	2.7	0.3	10.5	3.6	0.3	7.1	99.0	97.3	96.3	97.5	1.1	1.1
	0.05	24.8	2.9	11.9	13.1	0.4	3.1	93.5	0.4	0.4	71.2	86.6	4.1	54.0	35.8	**66.3**
	control	86.0	6.3	7.3	97.5	0.2	0.2	97.5	1.2	1.3						
SP2	5	5.1	0.6	11.1	1.8	0.5	**25.0**	1.9	0.5	**23.9**	94.4	98.4	97.6	96.8	1.7	1.8
	0.5	31.5	4.4	13.9	53.8	1.7	3.2	38.8	2.5	6.5	65.4	43.2	57.1	55.2	9.2	16.6
	0.05	75.6	5.0	6.6	83.9	1.3	1.5	82.4	2.1	2.5	16.9	11.4	8.7	12.3	3.4	**27.7**
	control	91.0	0.4	0.4	94.7	0.5	0.6	90.3	0.9	1.0						
SP3	5	16.4	2.7	16.2	14.0	0.5	3.4	11.4	0.2	1.9	82.4	84.9	87.8	85.0	2.2	2.6
	0.5	19.1	3.0	15.9	12.8	0.8	6.5	10.9	0.0	0.4	79.5	86.1	88.3	84.6	3.7	4.4
	0.05	50.2	4.3	8.5	77.2	1.4	1.8	22.0	3.7	16.6	46.0	16.4	76.5	46.3	24.5	**53.0**
	control	92.9	3.1	3.3	92.3	0.4	0.4	93.4	1.6	1.7						
SP4	5	4.5	1.5	**34.3**	4.6	0.2	3.4	2.8	0.4	15.8	94.0	94.9	96.8	95.2	1.1	1.2
	0.5	4.5	0.5	11.0	5.4	0.8	15.2	2.4	0.4	17.6	94.7	94.1	97.2	95.3	1.4	1.4
	0.05	13.7	2.3	17.1	51.0	1.6	3.1	7.1	1.2	16.7	84.1	43.4	91.9	73.2	21.3	**29.1**
	control	86.3	1.2	1.4	90.1	10.2	11.4	88.0	0.9	1.0						

CV = coefficient of variation (CV values > 20% are in bold).

3. Results

3.1. Validation of the EHT

The intra-assay variability was measured according to the percentage of eggs hatched in each EHT for 9 different isolates (Tables 1 and 2). The intra-assay variability (expressed as CV) did not exceed 20% in most of the tests within 8 weeks after gallbladder collection. In some cases and when the egg hatching was low (from 0.3% to 4.5%), CV values higher than 20% were observed (Tables 1 and 2). The ovicidal activity observed after incubation with ABZ (0.5 μM) decreased at 6 months ac compared to that observed at 2 weeks ac (isolates SP6 to SP8) (Table 2).

In the Uru-mon isolate, the CV was low at both determinations (day 0 and day 45 ac) using the two highest drug concentrations (5 and 0.5 μM, Table 2).

With the aim of determining the inter-assay variability over time, the percentage of ovicidal activity was calculated at different weeks ac in isolates SP1-SP4 (Table 1). In this case, CV values for the ovicidal activity over time, until 8 weeks ac, were low when the EHT was carried out at 5 and 0.5 μM, ranging from 0.9 to 16.6%. However, in all isolates, the variability was increased when the eggs were exposed to an ABZ concentration of 0.05 μM, with CV values between 27.7 and 66.3%. According to the criteria of Canevari et al. (2014) and the current EHT results, all SP isolates, with the exception of SP2 could be described as being ABZ-susceptible. Since the ABZ ovicidal activity

Table 2
Percentage of hatched eggs (mean ± SD) and ovicidal activity (%) obtained for different *Fasciola hepatica* isolates (SP5-SP8, Uru-mon) following incubation in albendazole (ABZ) according to the week of analysis after collection (ac) of gallbladders.

Isolate	ABZ Concentration (μM)	Eggs hatched (%)						Ovicidal activity (%)	
		2 weeks ac			6 months ac			2 weeks ac	6 months ac
		Mean	SD	CV	Mean	SD	CV		
SP5	5	0.5	0.4	**71.0**	0.6	1.1	**200.0**	99.3	99.3
	0.5	0.3	0.5	**141.4**	2.0	1.5	**73.0**	99.6	97.6
	0.05	0.6	0.5	**71.9**	33.2	23.4	**70.3**	99.2	60.3
	control	78.6	3.1	4.0	83.7	2.4	2.9		
SP6	5	16.7	1.6	9.5	29.9	12.8	**43.0**	79.3	47.6
	0.5	9.7	1.9	19.6	54.7	7.6	13.8	81.9	4.1
	0.05	30.2	4.1	13.5	54.2	12.1	**22.3**	62.5	4.9
	control	80.6	0.9	1.1	57.0	6.0	10.5		
SP7	5	0.0	0.0	0.0	47.0	8.6	18.4	100.0	47.3
	0.5	0.0	0.0	0.0	75.7	11.7	15.5	100.0	15.1
	0.05	81.1	5.9	7.3	84.6	7.0	8.3	2.8	5.1
	control	83.4	0.0	0.0	89.1	2.1	2.4		
SP8	5	3.1	0.6	18.4	90.3	3.4	3.8	96.4	2.9
	0.5	5.2	0.9	17.9	86.6	2.5	2.8	94.0	6.8
	0.05	56.9	2.2	3.8	92.2	2.2	2.4	34.7	0.8
	control	87.1	4.9	5.6	92.9	4.3	4.7		
		day 0			day 45 ac			day 0	day 45 ac
		Mean	SD	CV	SD	Mean	CV		
Uru-mon	5	91.2	0.14	0.2	93.1	0.14	0.2	2.6	0.6
	0.5	94.6	0.12	0.1	94.7	0.12	0.1	0	0
	control	93.6	5.4	5.8	93.7	5.4	5.8		

CV = coefficient of variation (CV values > 20% are in bold).

Table 3
Percentage of hatched eggs (mean ± SD) and ovicidal activity (%) following incubation in albendazole (ABZ) obtained for different cows experimentally infected with the same *Fasciola hepatica* isolate, the ABZ-resistant Cajamarca isolate.

Cows	Eggs hatched (%)			Ovicidal activity (%)	
	Control	ABZ 0.5 µM	ABZ 5 µM	ABZ 0.5 µM	ABZ 5 µM
1	85.5 ± 2.62	85.5 ± 2.62	91.1 ± 2.62	0.0	0.0
2	91.1 ± 2.43	92.7 ± 2.43	93.6 ± 2.43	0.0	0.0
3	80.7 ± 3.11	90.6 ± 3.11	nd	0.0	nd
4	83.4 ± 1.81	82.0 ± 1.81	83.5 ± 1.81	1.7	0.0
5	75.6 ± 2.22	81.7 ± 2.22	81.7 ± 2.22	0.0	0.0
6	83.7 ± 2.34	88.2 ± 2.34	88.2 ± 2.34	0.0	0.0
7	86.5 ± 1.61	86.3 ± 1.61	86.3 ± 1.61	0.2	0.2
8	88.4 ± 2.14	86.3 ± 2.14	86.3 ± 2.14	2.4	2.4

nd: not determined. No statistical differences ($P > 0.05$) were observed in egg hatching among calves.

observed for the SP2 isolate ranged from 43.2 to 65.4 (ABZ concentration 0.5 µM), ABZ resistance could be suspected.

With the aim of comparing the *in vivo* (CET) and *in vitro* (EHT) assays to determine the level of resistance, the Uru-mon isolate was characterized. After the CET, the same number of flukes were recovered in the ABZ-treated group (range: 6–25; mean: 18.3 ± 8.5) as in the control group (range: 13–21; 18.5 ± 3.6). ABZ efficacy against mature liver flukes was 1.1%, showing the high resistance level of this isolate. Moreover, after the EHT, the ovicidal activity was 1.7 or 0% at a drug concentration of 0.5 µM or 5 µM, respectively. Therefore, the Uru-mon isolate is highly ABZ-resistant, as shown by the similar results with both assays.

The intra-herd variability of the EHT was also evaluated. No significant differences were observed between the ABZ ovicidal activities using the two highest drug concentrations (0.5 and 5 µM) among the infected cows (Table 3).

3.2. Resistance ratio by means of the egg hatch test

For calculating the resistance ratio in the Uru-mon isolate, the level of resistance of an ABZ-susceptible isolate (Cullompton) was taken as reference. The EC50 values were 0.16 and 8.66 µM for the Cullompton and Uru-mon isolates, respectively (Fig. 1), with a resistance ratio of 54.1.

3.3. Modifications of the standard protocol for the Egg Hatch Test

3.3.1. Incubation time with ABZ

With the Uru-mon isolate, incubation of eggs in ABZ until the end of the assay led to a change in the ovicidal activity, from 0 to 1.7% (resistant status) to 92.6–95.2% (susceptible status). Significant differences were observed in the percentage of eggs hatched and ovicidal activity between the two different incubation conditions ($p < 0.01$) (Table 4).

3.3.2. Eggs isolated from faeces

ABZ efficacy against liver fluke assessed by the FECRT showed that ABZ resistance was present on all farms, with efficacies between 0 and 45% (Table 5). When the EHT was carried out in each farm using eggs collected from the faeces of naturally infected steers, similar results were observed using a drug concentration of 0.5 µM (Table 5).

4. Discussion

In the current study, the EHT has been validated and evaluated as an *in vitro* technique to determine ABZ resistance in *F. hepatica*. Although there have been a few studies describing this test, the standardisation of

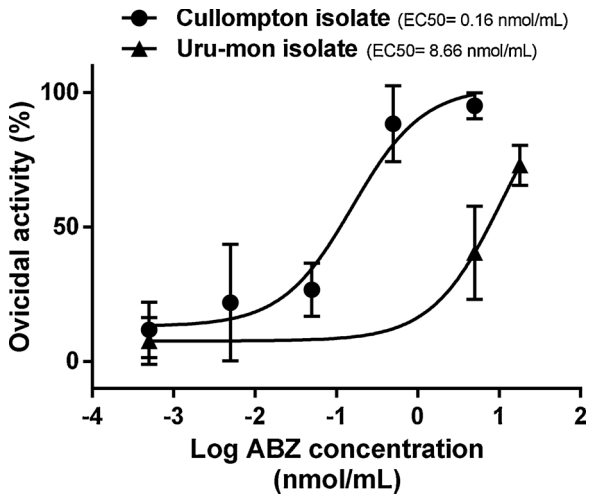

Fig. 1. Dose–response curves and EC50 values obtained for the albendazole (ABZ)- susceptible *Fasciola hepatica* isolate (Cullompton) and the ABZ-resistant isolate (Uru-mon). The resistance ratio calculated for the Uru-mon isolate was 54.1.

the technique has not yet been fully determined, especially with regards to establishing certain guidelines. The EHT was initially designed to measure the ovicidal activity of BZD compounds against gastrointestinal nematodes (Coles et al., 2006), and was later applied in surveys as a tool to study the prevalence of anthelmintic resistance (Martínez-Valladares et al., 2013). However, to date the potential of this technique has not been fully explored to determine the prevalence of ABZ resistance in *F. hepatica* due to the lack of any standardisation.

An EHT carried out by Alvarez et al. (2009) demonstrated that ABZ exerts an ovicidal effect on *F. hepatica* egg development. A later study carried out by Canevari et al. (2014) evaluated the ABZ resistance of six isolates by means of the EHT, one isolate from the United Kingdom and the rest from South America. In that study, the authors characterized the isolates using ABZ and its sulphoxide metabolite (ABZ.SO) at the same concentrations as used in the current study (that is, 0.05, 0.5 and 5 µM).

In this study, the possible intra- and inter-assay variability was assessed, with the aim of determining how long the liver fluke eggs could be stored before testing. In general, CV ≤ 20% were observed among replicates within the same EHT, when the assays were carried out within 8 weeks after egg collection and using the highest ABZ concentrations (0.5 or 5 µM). Higher intra-assay variability was seen on occasion: it was related to eggs showing a low mean hatching rate (ranging from 0.3 to 4.5%). Likewise, the ovicidal activity was similar (CV ≤ 20%) at 2, 4 or 8 weeks ac, after incubation of ABZ at concentrations of 0.5 and 5 µM. However, the ovicidal activity of ABZ observed after incubation at the lowest concentration (0.05 µM) shows higher variability (CV > 20%). When the ovicidal activity was measured after a longer storage period (6 months), it decreased significantly in 3 out of 4 isolates (SP5-SP8). Therefore, for eggs incubated with ABZ at 0.5 or 5 µM, a storage limit of 8 weeks (˜2 months) is recommended.

The least variable results were obtained with an ABZ concentration of 0.5 µM, both within the assay and over the recommended time period to determine *in vitro* resistance. This finding agrees with that of Canevari et al. (2014), who recommended this concentration as a cut-off point to measure the resistance status; the authors assumed drug susceptibility when the ovicidal activity was higher than 70%; resistance with values lower than 40%; and a suspicion of resistance when activity was between these values at the 0.5 µM concentration. According to the authors, for the correct adjustment of these values, comparison between *in vivo* and *in vitro* assays requires further research. In order to carry this out in the current study, ABZ resistance by the EHT was compared with the gold standard method to determine drug

Table 4
Percentage of hatched eggs (mean ± SD) and ovicidal activity (%) of the Uru-mon *Fasciola hepatica* isolate after albendazole (ABZ) incubation for a 12-h or 15-day period.

ABZ incubation period	Eggs hatched (%)			Ovicidal activity (%)	
	control	0.5 μM	5 μM	0.5 μM	5 μM
12 h	83.4 ± 3.6	82.0 ± 2.8	83.5 ± 3.4	1.7	0
15 days	86.4 ± 3.7	6.40 ± 3.00*	4.11 ± 1.10*	92.6	95.2

* Statistical differences between groups ($P < 0.05$).

efficacy, namely, the CET (Wood et al., 1995). However, in the current study the sheep were experimentally infected with a lower number of metacercariae than the dose suggested by Wood et al. (1995) due to limited availability at the time of the experiment. The efficacy of ABZ against the Uru-mon isolate after the clinical trial was 1.1%, a value similar to that obtained with the EHT at 0.5 μM (1.7%), thus supporting the use of 0.5 μM as the discriminating concentration of ABZ to determine its resistance. In gastrointestinal nematodes from sheep, Coles et al. (2006) suggested the use of a discriminating dose to simplify the EHT; the hatching rate using a discriminating dose would indicate the percentage of BZD resistant eggs in the sample.

The possible variability of the level of resistance within the same herd was tested by the EHT with eggs collected from cows infected with the same ABZ-resistant isolate (the Cajamarca isolate). No inter-individual animal differences were observed, which may indicate that the EHT is not affected by physio-pathological conditions of animals (*i.e.* immunological status).

The EHT described in this study involves the collection of eggs after the slaughter of animals, but this is not always possible. This drawback could be solved by collecting eggs from the faeces. Robles-Pérez et al. (2014) described an EHT using eggs from the faeces of sheep to determine the efficacy of ABZ against *F. hepatica*; the authors suggested that the method of egg recovery is an important factor that may influence the results of the EHT, since low hatching rates could be obtained due to the presence of impurities. In the current study, the results of an EHT with eggs collected from the faeces of steers and carried out on four different farms was compared with the ABZ resistance level measured by the FECRT. The resistance status of fluke populations on each farm observed after the *in vivo* assay (FECRT) was similar to that when the EHT was carried out at an ABZ concentration of 0.5 μM. In a study on ABZ resistance carried out on 3 farms in Sweden, Novobilský et al. (2016) also observed agreement between the results of different tests, in this case the FECRT, CRT and EHT, using eggs collected from faeces. Flukes on 2 of the farms were shown to be ABZ-resistant, with EC50 values (0.947 and 1.171 nmol/mL) 10-fold higher than the value (0.087 nmol/mL) obtained for the isolate on the third farm, which was deemed to be ABZ-susceptible (Novobilský et al., 2016). In our study, the EC50 value (0.16 nmol/mL) for the ABZ-susceptible Cullompton isolate was similar to that reported in the study by Novobilský et al. (2016). It is important to note that for comparing the resistance level among isolates, more studies are needed to determine the EC50 in well-characterized ABZ-susceptible and -resistant isolates and establish a reference value to predict ABZ resistance. For instance, the ABZ-resistant status of the Uru-mon isolate was determined for the first time in the current work, since the ED50 was 54.1-fold higher than the value obtained in the ABZ-susceptible Cullompton isolate.

With the aim of simplifying the test, ovicidal activity was assessed by incubating eggs of the Uru-mon isolate with ABZ for a period of 15 days. This modification of the protocol led to an increase in the ovicidal activity from 1.7% (12 h incubation) to 92.6% (15 days incubation). This changes the interpretation of the EHT result, since an originally ABZ-resistant *F. hepatica* isolate could be diagnosed as susceptible. Therefore, removing ABZ after 12 h of incubation is a critical step for the EHT.

5. Conclusions

According to the validation carried out in the present study, the EHT could be a valuable technique to predict ABZ resistance in *F. hepatica* when the assay is carried out within 8 weeks after egg collection. Using an ABZ concentration of 0.5 μM in the EHT resulted in low variability between results. This dose is proposed as a discriminating dose to predict ABZ resistance, since similar results were observed between the results of the EHT at 0.5 μM and those of the *in vivo* assays. The EHT could be also used with eggs collected from faeces, although in this case further research in order to standardize the protocol is needed.

CRediT authorship contribution statement

Laura Ceballos: Conceptualization, Data curation, Formal analysis, Investigation, Project administration, Resources, Software, Software, Visualization, Writing - review & editing. **Candela Canton:** Conceptualization, Formal analysis, Investigation, Validation, Visualization. **Cesar Pruzzo:** Conceptualization, Formal analysis,

Table 5
Albendazole (ABZ) resistance in *Fasciola hepatica* assessed on four different farms (farms 1–4) by the faecal egg count reduction test (FECRT) and the egg hatch test (EHT).

Farm /isolate	FECRT[1]				Egg Hatch Test					
	Epg[2] day -1	Epg[2] day 15	Efficacy	Isolate status	Eggs hatched (%)			Ovicidal activity		Isolate status
					control	0.5 μM	5 μM	0.5 μM	5 μM	
1 / AR1	4(1-14)	2.2(1-4)	45 %(25-70)	R	85.5 ± 3.2	69.9 ± 2.6	38.7 ± 2.4	18.2 %	54.7 %	R
2 / AR2	23.1(1-78)	17.3(4-50)	25 %(3-47)	R	89.2 ± 2.5	70.6 ± 4.2	15.9 ± 3.1	20.9 %	82.2 %	R
3 / AR3	8.3(3-24)	17.4(2-55)	0 %(0-0)	R	88.9 ± 2.8	87.5 ± 2.5	85.8 ± 3.1	1.6 %	3.5 %	R
4 / AR4	3.1(1-11)	3.7(2-9)	0 %(0-0)	R	76.2 ± 2.8	82.8 ± 2.5	85.8 ± 3.1	0.0 %	0.0 %	R

Mean faecal egg counts (S.E.M.) [range], % reduction and 95% confidence intervals (C.I.) from faecal egg count reduction tests carried out.

[1] FECRT: estimated according to Coles et al. (1992).

[2] Epg: mean faecal egg per gram counts (range) at day -1 and day 15 post-ABZ treatment (10 mg/kg) and efficacy (lower and upper 95% confidence intervals [95% CI]). No statistical differences ($P > 0.05$) were observed in epg counts between day -1 and day 15 post-treatment. EHT: Percentage of hatched eggs (mean ± SD) and ovicidal activity (%). Isolate status: resistant (R) or susceptible (S).

Methodology, Investigation. **Rodrigo Sanabria:** Conceptualization, Data curation, Formal analysis, Investigation, Methodology, Validation, Writing - review & editing. **Laura Moreno:** Conceptualization, Formal analysis, Investigation, Methodology, Validation, Visualization. **Jaime Sanchis:** Conceptualization, Data curation, Formal analysis, Investigation, Methodology, Validation. **Gonzalo Suarez:** Conceptualization, Investigation, Methodology. **Pedro Ortiz:** Conceptualization, Formal analysis, Investigation, Methodology, Supervision. **Ian Fairweather:** Conceptualization, Formal analysis, Investigation, Supervision, Writing - review & editing. **Carlos Lanusse:** Conceptualization, Funding acquisition, Supervision, Writing - review & editing. **Luis Alvarez:** Conceptualization, Data curation, Formal analysis, Funding acquisition, Investigation, Methodology, Resources, Software, Validation, Visualization, Writing - original draft, Writing - review & editing. **María Martinez-Valladares:** Conceptualization, Data curation, Formal analysis, Funding acquisition, Investigation, Methodology, Project administration, Resources, Software, Supervision, Validation, Visualization, Writing - original draft, Writing - review & editing.

Acknowledgment

This study was partially supported by CONICET and Agencia Nacional de Promoción Científica y Técnica (ANPCyT), from Argentina, and by the Spanish "Ramón y Cajal" Programme, Ministry of Economy and Competitiveness (Ministerio de Economía y Competitividad) (MMV, RYC-2015-18368).

References

Alvarez, L., Moreno, G., Moreno, L., Ceballos, L., Shaw, L., Fairweather, I., Lanusse, C., 2009. Comparative assessment of albendazole and triclabendazole ovicidal activity on *Fasciola hepatica* eggs. Vet. Parasitol. 164, 211–216.

Alvarez-Sánchez, M.A., Mainar-Jaime, R.C., Pérez-García, J., Rojo-Vázquez, F.A., 2006. Resistance of *Fasciola hepatica* to triclabendazole and albendazole in sheep in Spain. Vet. Rec. 159, 424–425.

Boray, J.C., Crowfoot, P.D., Strong, M.B., Allison, J.R., Schellenbaum, M., Von Orelli, M., Sarasin, G., 1983. Treatment of immature and mature *Fasciola hepatica* infections in sheep with triclabendazole. Vet. Rec. 113, 315–317.

Buchanan, J.F., Fairweather, I., Brennan, G.P., Trudgett, A., Hoey, E.M., 2003. *Fasciola hepatica*: surface and internal tegumental changes induced by treatment *in vitro* with the sulphoxide metabolite of albendazole ("Valbazen"). Parasitology 126, 141–153.

Canevari, J., Ceballos, L., Sanabria, R., Romero, J., Olaechea, F., Ortiz, P., Cabrera, M., Gayo, V., Fairweather, I., Lanusse, C., Alvarez, L., 2014. Testing albendazole resistance in *Fasciola hepatica*: validation of an egg hatch test with isolates from South America and the United Kingdom. J. Helminthol. 88, 286–292.

Coles, G.C., Bauer, C., Borgsteede, F.H.M., Geerts, S., Klei, T.R., Taylor, M.A., Waller, P.J., 1992. World Association for the Advancement of Veterinary Parasitology (W.A.A.V.P.) methods for the detection of anthelmintic resistance in nematodes of veterinary importance. Vet. Parasitol. 44, 35–44.

Coles, G.C., Briscoe, M.G., 1978. Benzimidazoles and fluke eggs. Vet. Rec. 103, 360–361.

Coles, G.C., Jackson, F., Pomroy, W.E., Prichard, R.K., von Samson-Himmelstjerna, G., Silvestre, A., Taylor, M.A., Vercruysse, J., 2006. The detection of anthelmintic resistance in nematodes of veterinary importance. Vet. Parasitol. 136, 167–185.

Devine, C., Brennan, G.P., Lanusse, C.E., Alvarez, L.I., Trudgett, A., Hoey, E., Fairweather, I., 2010. Inhibition of cytochrome P450-mediated metabolism enhances *ex vivo* susceptibility of *Fasciola hepatica* to triclabendazole. Parasitology 137, 871–880.

Devine, C., Brennan, G.P., Lanusse, C.E., Alvarez, L.I., Trudgett, A., Hoey, E., Fairweather, I., 2012. Potentiation of triclabendazole action *in vivo* against a triclabendazole-resistant isolate of *Fasciola hepatica* following its co-administration with the metabolic inhibitor, ketoconazole. Vet. Parasitol. 184, 37–47.

Fairweather, I., 2005. Triclabendazole: new skills to unravel an old(ish) enigma. J. Helminthol. 79, 227–234.

Fairweather, I., 2011a. Liver fluke isolates: a question of provenance. Vet. Parasitol. 176, 1–8.

Fairweather, I., 2011b. Reducing the future threat from (liver) fluke: realistic prospect or quixotic fantasy? Vet. Parasitol. 180, 133–143.

Flanagan, A.M., Edgar, H.W.J., Forster, F., Gordon, A., Hanna, R.E.B., McCoy, M., Brennan, G.P., Fairweather, I., 2011a. Standardisation of a coproantigen reduction test (CRT) protocol for the diagnosis of resistance to triclabendazole in Fasciola hepatica. Vet. Parasitol. 176, 34–42.

Flanagan, A., Edgar, H.W.J., Gordon, A., Hanna, R.E.B., Brennan, G.P., Fairweather, I., 2011b. Comparison of two assays, a faecal egg count reduction test (FECRT) and a coproantigen reduction test (CRT), for the diagnosis of resistance to triclabendazole in *Fasciola hepatica* in sheep. Vet. Parasitol. 176, 170–176.

Hanna, R.E.B., Edgar, H.W.J., McConnell, S., Toner, E., McConville, M., Brennan, G.P., Devine, C., Flanagan, A., Halferty, L., Meaney, M., Shaw, L., Moffett, D., McCoy, M., Fairweather, I., 2010. *Fasciola hepatica*: histological changes in the reproductive structures of triclabendazole (TCBZ)-sensitive and TCBZ-resistant flukes after treatment *in vivo* with TCBZ and the related benzimidazole derivative, compound alpha. Vet. Parasitol. 168, 240–254.

Kaplan, R.M., 2004. Drug resistance in nematodes of veterinary importance: a status report. Trends Parasitol. 20, 477–481.

Kelley, J.M., Elliott, T.P., Beddoe, T., Anderson, G., Skuce, P., Spithill, T.W., 2016. Current threat of triclabendazole resistance in *Fasciola hepatica*. Trends Parasitol. 32, 458–469.

Kotze, A.C., Hunt, P.W., Skuce, P., von Samson-Himmelstjerna, G., Martin, R.J., Sager, H., Krücken, J., Hodgkinson, J., Lespine, A., Jex, A.R., Gilleard, J.S., Beech, R.N., Wolstenholme, A.J., Demeler, J., Robertson, A.P., Charvet, C.L., Neveu, C., Kaminsky, R., Rufener, L., Alberich, M., Menez, C., Prichard, R.K., 2014. Recent advances in candidate-gene and whole-genome approaches to the discovery of anthelmintic resistance markers and the description of drug/receptor interactions. Int. J. Parasitol. Drugs Drug Resist. 4, 164–184.

MAFF (Ministry of Agriculture, Fisheries and Food), 1986. Manual of Veterinary Parasitological Laboratory Techniques. Her Majesty's Stationery Office, London.

Martínez-Valladares, M., Martínez-Pérez, J.M., Robles-Pérez, D., Cordero-Pérez, C., Famularo, M.R., Fernández-Pato, N., Castañón-Ordóñez, L., Rojo-Vázquez, F.A., 2013. The present status of anthelmintic resistance in gastrointestinal nematode infections of sheep in the northwest of Spain by *in vivo* and *in vitro* techniques. Vet. Parasitol. 191, 177–181.

Mas Coma, S., Bargues, M.D., Valero, M.A., 2018. Human fascioliasis infection sources, their diversity, incidence factors, analytical methods and prevention measures. Parasitology 145, 1665–1699.

McConville, M., Brennan, G.P., McCoy, M., Castillo, R., Hernández-Campos, A., Fairweather, I., 2006. Adult triclabendazole-resistant *Fasciola hepatica*: surface and sub-surface tegumental responses to *in vitro* treatment with the sulphoxide metabolite of the experimental fasciolicide compound alpha. Parasitology 133, 195–208.

McConville, M., Brennan, G.P., Flanagan, A., Edgar, H.W.J., Hanna, R.E.B., McCoy, M., Gordon, A.W., Castillo, R., Hernández-Campos, A., Fairweather, I., 2009. An evaluation of the efficacy of compound alpha and triclabendazole against two isolates of Fasciola hepatica. Vet. Parasitol. 162, 75–88.

McKellar, Q.A., Scott, E.W., 1990. The benzimidazole anthelmintic agents – a review. J. Vet. Pharmacol. Ther. 13, 223–247.

Mooney, L., Good, B., Hanrahan, J.P., Mulcahy, G., de Waal, T., 2009. The comparative efficacy of four anthelmintics against a natural acquired *Fasciola hepatica* infection in hill sheep flock in the west of Ireland. Vet. Parasitol. 164, 201–205.

Novobilský, A., Solis, N.A., Skarin, M., Höglund, J., 2016. Assessment of flukicide efficacy against *Fasciola hepatica* in sheep in Sweden in the absence of a standardised test. Int. J. Parasitol. Drugs Drug Resist. 6, 141–147.

Ortiz, P., Scarcella, S., Cerna, C., Rosales, C., Cabrera, M., Guzmán, M., Lamenza, P., Solana, H., 2013. Resistance of *Fasciola hepatica* against triclabendazole in cattle in Cajamarca (Peru): a clinical trial and an *in vivo* efficacy test in sheep. Vet. Parasitol. 195, 118–121.

Robles-Pérez, D., Martínez-Pérez, J.M., Rojo-Vázquez, F.A., Martínez-Valladares, M., 2014. Development of an egg hatch assay for the detection of anthelmintic resistance to albendazole in *Fasciola hepatica* isolated from sheep. Vet. Parasitol. 203, 217–221.

Sanabria, R., Ceballos, L., Moreno, L., Romero, J., Lanusse, C., Alvarez, L., 2013. Identification of a field isolate of *Fasciola hepatica* resistant to albendazole and susceptible to triclabendazole. Vet. Parasitol. 193, 105–110.

Sutherland, I.A., Leathwick, D.M., 2011. Anthelmintic resistance in nematode parasites of cattle: a global issue? Trends Parasitol. 27, 176–181.

Toner, E., Brennan, G.P., McConvery, F., Meaney, M., Fairweather, I., 2010. A transmission electron microscope study on the route of entry of triclabendazole into the liver fluke, Fasciola hepatica. Parasitology 137, 855–870.

Ueno, H., Goncalves, P., 1988. Manual De Laboratorio Para El Diagnóstico De Helmintos En Rumiantes, 2nd ed. Japan International Cooperation Agency, Tokyo, Japan.

Walker, S.M., McKinstry, B., Boray, J.C., Brennan, G.P., Trudgett, A., Hoey, E.M., Fletcher, H., Fairweather, I., 2004. Response of two isolates of *Fasciola hepatica* to treatment with triclabendazole in vivo and in vitro. Parasitol. Res. 94, 427–438.

Wolstenholme, A.J., Fairweather, I., Prichard, R., von Samson-Himmelstjerna, G., Sangster, N.C., 2004. Drug resistance in veterinary helminths. Trends Parasitol. 20, 469–476.

Wood, I.B., Amaral, N.K., Bairden, K., Duncan, J.L., Kassai, T., Malone Jr., J.B., Pankavich, J.A., Reinecke, R.K., Slocombe, O., Taylor, S.M., Vercruysse, J., 1995. World Association for the Advancement of Veterinary Parasitology (W.A.A.V.P.) second edition of guidelines for evaluating the efficacy of anthelmintics in ruminants (bovine, ovine, caprine). Vet. Parasitol. 58, 181–213.