SCIENTIFIC PAPER

Diagnosis of resistance to Albendazole sulfoxide in sheep and goats, in the Matanzas province

Javier Arece-García*[1], Yoel López-Leyva[1], Uriel Flores-Aguilar[2], David Ramon Ventura[2], Emmanuel Macedo-Porcayo[2] and Rolando Rojo-Rubio[2]

[1]*Estación Experimental de Pastos y Forrajes Indio Hatuey. Universidad de Matanzas. Ministerio de Educación Superior. Central España Republicana, CP 44280, Matanzas, Cuba*

[2]*Centro Universitario UAEM Temascaltepec, Universidad Autónoma del Estado de México, México*

**Corresponding author:* arece@ihatuey.cu

ABSTRACT: In order to evaluate the efficacy of Albendazole sulfoxide in the control of parasite infestation in sheep and goats, *in vivo* and *in vitro* studies were conducted in three sheep herds and a goat herd, in the Matanzas province (Cuba). In each herd, a fecal egg count reduction (FECR) trial and an egg hatching inhibition essay were carried out. For the *in vivo* essay 30 animals per herd with high nematode egg counts were selected, and two treatments with 15 animals each were randomly assigned: Albendazole sulfoxide (Labizol®, Labiofam, Cuba) in the recommended dose for sheep and goats, and a control group which was not dewormed. Eleven days post-treatment the animals were sampled to evaluate the FECR. For the *in vitro* trial feces were collected, anaerobically stored and the egg hatching inhibition was evaluated against serial concentrations of Albendazole sulfoxide, from 75 to 0,07 mg/mL. The FECR was calculated and the mean effective concentration (EC_{50}) was estimated. In the four herds Labiozol® could not reduce the egg count, with values that indicated resistance of the parasites to this drug (FECR lower than 95 % and/or its confidence intervals lower than 90 %) and absolute predominance of *Haemonchus contortus*. The egg hatching inhibition study showed high variability in the EC_{50}, with values up to 1 000 times higher with regards to the lowest EC_{50}. It is concluded that in the studied herds there was evidence of the resistance of *H. contortus* to Albendazole sulfoxide.

Keywords: hatching, *Haemonchus contortus*, eggs, nematodes

INTRODUCTION

The resistance of ruminant parasites to antiparasitic drugs constitutes a global problem of unpredictable and scarcely quantified consequences. In Cuba, the first reports of nematode resistance to antiparasitic drugs started to appear in the early 21st century (Rodríguez, 2002; Arece *et al*., 2004), and at present there is an increasing interest in knowing the efficacy status of these drugs, because there is general consensus between farmers and veterinarians on the fact that they do not work anymore.

In the country there are antiparasitic drugs –produced by the LABIOFAM enterprise– from three pharmacological families or groups: Ivermectine (Labiomec® 1 %), Albendazole (Albendazol®/Labiozol®) and Imidazotiazoles (Levamisole 10 %). They share different action mechanisms and could be inserted in rotation plans which prevent the development of resistance; however, sometimes their low availability leads to the use of the same product on consecutive occasions. This is in addition to the lack of knowledge, by most farmers, of the main factors that are involved in the appearance of resistance, as well as the tradition of doing "preventive" treatments on all the stock. Even when it is estimated that the prevalence of resistance is low, knowing the status of its dispersion is of special interest in order to select the group of drugs to be used (Cudekova *et al*., 2010).

Among the drugs used in Cuba, Labiozol® (injectable presentation, Albendazole sulfoxide) and Albendazol® (oral presentation) are highly accepted, because of their margin of therapeutic safety and their short-term result, besides their effect (in other doses) against *Fasciola hepatica*. This has propitiated the excessive use of these drugs in regimes of arbitrary treatments and without epizootiological bases, which has caused the suspicion, by the farmers themselves, of the drug failure in parasite control.

The increasing interest about resistance needs standardized methods for its diagnosis, and today several methods are used for that purpose. The most disseminated one is an *in vivo* study which determines the fecal egg count reduction (Coles *et al*., 2006); nevertheless, other *in vitro* essays have also been validated (Várady *et al*., 2007) and constitute a practical and useful tool to know the current status of drugs in certain regions. Thus, it has been proven that the egg hatching inhibition essay has a solid basis for the clinical diagnosis of resistance to albendazole (Varady *et al*., 2015) and its use under field conditions.

At present there is discrepancy between the *in vivo* and *in vitro* essays and even of the substances used in each case. In the *in vivo* trials the effects of drugs on the adult stages of the parasites are evaluated; while in the *in vitro* essays, the exogenous stages of the life cycles of parasites (eggs and larvae) are analyzed. There are also other trials, such as the larval development test (useful against macrocyclic lactones and levamisole) and the larval migration inhibition essay.

For the diagnosis of resistance, the fecal egg count reduction trial has been the most studied and recommended by the World Association for the Advancement in Veterinary Parasitology –WAAVP– (Coles *et al*., 1992). It has the disadvantage of being costly and requiring time for its execution, because it is necessary to have a minimum of 15 animals per drug to be evaluated and to perform two samplings (day zero and day 14 post-treatment). In contrast, the egg hatching inhibition essay is simple and cheap when the minimum laboratory conditions exist. Both show low sensitivity, but can detect resistance when 25 % of the population is already resistant (Fortes and Molento, 2013).

The objective of this study was to determine the efficacy of Albendazole sulfoxide through paired essays of fecal egg count reduction and *in vitro* egg hatching inhibition.

MATERIALS AND METHODS

Location. The study was conducted under production conditions, in three herds of sheep and one of goats of the Matanzas province. In these herds, habitually, antiparasitic treatments are carried out arbitrarily and the existing drug at that moment is used.

Selection of the animals, treatments and drug doses. In each unit all the animals were sampled. Feces were directly extracted from the rectum of each animal, and were transported to the parasitology laboratory of the EEPF Indio Hatuey for the determination of the individual infestation level, through the McMaster technique (Arece *et al*., 2002). From the results of the fecal egg counts (fec) 30 animals were selected, with fec higher than 50 eggs per gram of feces (epg); from them 15 for the control group (which did not receive antiparasitic treatment) and 15 that received Albendazole sulfoxide (Labiozol®, LABIOFAM, Cuba, injectable presentation) in the dose recommended by the manufacturer (3,5 mg/kg of live weight) for sheep; while in the goats a dose of 11,5 mg/kg of live weight was used (Jackson *et al*., 2012).

In vivo essay (fecal egg count reduction, FECR)

Experimental procedure. The methodology for drug evaluation proposed by the WAVVP (Coles *et al*., 1992) was used. Thus, feces samplings were made directly from the rectum of each animal to diagnose the parasite rate through the modified McMaster technique.

Eleven days post-treatment a second feces sampling was made to determine the fec, as well as the FECR. Stool cultures were made to define the parasite species present in each group (Roberts and O'Sullivan, 1952).

In vitro essay (egg hatching test)

Experimental procedure. In the pre-treatment sampling feces were taken, which were mixed and anaerobically preserved to perform the hatching inhibition essay (Coles *et al*., 1992). Eleven concentrations of Albendazole sulfoxide were used from serial dilutions from 75 to 0,07 mg/mL, and distilled water was used as negative control.

Strongyle eggs were collected through the technique proposed by Hubert and Kerboeuf (1992) and were deposited in 24-well cell culture plates, to be faced with each of the concentrations, based on a completely randomized design, with six replications per treatment. They were incubated during 48 h, and after that time hatching was stopped with 10 µL of Lugol solution. The larvae and the eggs in 20 aliquots of 10 µL, and the hatching percentage was determined.

Statistical analysis. In the *in vivo* study the FECR and the upper and lower confidence intervals were determined through the RESO© software (Wursthorn and Martin, 1990). These variables were the criteria for the determination of the drug susceptibility or resistance; thus, a drug was considered resistant if: i) the FECR (%) was lower than

95 %, and/or ii) its confidence intervals were lower than 90 % (Coles *et al*., 1992).

In the *in vitro* study a Probit regression analysis was made through the statistical package SAS version 9.0, in order to determine the mean effective concentration (EC_{50}) for egg hatching, in which the values of the positive control group were used for the correction of the results following this model (González-Garduño *et al*., 2014):

Pr (response) = C + (1-C)F(X'β) = C+ (1-C Φ (b0+ b1x log10 (Dose))

Where:

- β: vector of estimated parameters
- F: accumulative distribution function (normal)
- X: vector of explicative variables
- Pr: probability of a response
- C: natural response rate (proportion of individuals that respond to dose zero)

RESULTS AND DISCUSSION

At present, the *in vivo* phenotypic essay is the method by choice and, also, the one recommended by the WAAVP for the resistance diagnosis (Kaplan and Vidyashankar, 2012); nevertheless, it is little sensitive for such purpose. To perform this essay a previous feces sampling should be made and one after the antiparasitic treatment, and there should be a minimum number of animals with a minimum infestation rate; in addition, the fecal counts are individually performed.

Table 1 shows the result of the *in vivo* efficacy test of Albendazole sulfoxide in the four units. In all the cases resistance of the parasite to this drug was observed, by the FECR as well as the confidence intervals. In Cuba, this is the first time in which resistance to this drug is reported in small ruminants.

In 2004 the first evaluation study of the efficacy of Albendazole sulfoxide was conducted in two Cuban productive organizations, from which it was stated that its efficacy was adequate; this was ascribed to the limited distribution of this drug, because its commercialization in Cuba in injectable presentation was just starting (Arece *et al*., 2004). Today the situation is completely different, because albendazole in its two current presentations (oral and injectable), along with Labiomec® (Ivermectine 1 %), constitutes the most used drug in the country.

Nevertheless, when the selection pressure to which a drug is subject is low, the efficacy is achieved in acceptable ranges, as reported by Hernández *et al*. (2015) in Cuba, who, with these same products, found efficacy of 99,2 % of the fecal egg count reduction. Under the conditions of the country it is possible to reduce the selection pressure to which antiparasitic drugs are subject, as long as integral strategies are established for parasite control. First, the stereotype assumed for years of carrying out preventive or suppressive antiparasitic treatments in stock (to 100 % of the herd) should be eliminated, because it has been proven that treating all the animals when less than 20 % of them is known and they host more than 80 % of the parasites constitutes a mistake; second, the rotation of drugs per families and incentivizing the culture of weighing the animals when they are dewormed are highly important. Another aspect to be considered is the implementation of selective treatments through an integral study of the ocular mucosa color (FAMACHA method), the body condition and the general status of the animal, among other aspects.

There were differences in the mean lethal concentration of Albendazole sulfoxide in each unit (table 2). The highest values were found in the goat herd, which were more than 100 times higher than the ones found in a sheep herd and more than 1 000 times more than those observed in another. Such results constitute a sound proof of the level of nematode resistance to this drug.

At international level Tiabendazole (purity higher than 99 %) is used, instead of albendazole, to

Table 1. Fecal egg count reduction (FECR) and confidence intervals (CI), in four herds

Unit	FECR (%)	Upper CI (%)	Lower CI (%)
Goats	56	82	0
Sheep 1	44	78	0
Sheep 2	76	91	41
Sheep 3	52	72	5
Resistant species	H. contortus	H. contortus	H. contortus

Table 2. Mean lethal concentration (EC_{50}) and confidence intervals (CI) in four herds

Unit	EC_{50} (mg/mL)	Lower CI (mg/mL)	Upper CI (mg/mL)
Goats	3,38	0,32	11,77
Sheep 1	0,097	0,09	0,104
Sheep 2	0,003	0,001	0,006
Sheep 3	12,35	5,23	23,36

determine resistance through the egg hatching inhibition test; in which every EC_{50} value higher than 0,1 mg/mL (Coles *et al*., 1992) is established as cut-off value for resistance. Nevertheless, Kumar *et al*. (2014) used Albendazole sulfoxide and maintained the same cut-off value for resistance suggested by the WAAVP. Taking these elements into consideration, from the four studied herds, two showed EC_{50} higher than the resistance limit and another one was close to that value, but its upper confidence interval exceeded it. The hatching study is a test that can be considered for the resistance diagnosis, as reported by Várady *et al*. (2015).

It has been recently proven, through molecular tools, that the *in vitro* essays, mainly the egg hatching inhibition test, are useful practices for the diagnosis of resistance to benzimidiazoles, with high reliability (Várady *et al*., 2015). They also have, as advantage, the fact that random samplings can be performed on the animals and form pool, which implies only one visit to the farm; on the other hand, they allow to make samplings in several productive farms.

Until now isolated cases of resistance to some drugs have been reported, among which Ivermectine stands out (Labiomec®, LABIOFAM, Cuba), but with low dispersal level in the studied sheep herds, because it was found in only one herd from a total of 15 studied ones (Mencho-Ponce *et al*., 2013). Similar situation occurs in sheep, because in a study conducted in the Camagüey province it was reported that this same drug was efficacious in the 20 selected herds, with an efficacy higher than 97 % (Guerra-Llorens *et al*., 2014). From the results it was proven that there is resistance of *H. contortus* to Albendazole sulfoxide in sheep and goats, in *in vivo* and *in vitro* essays.

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