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Case Report

First report of multiple anthelmintic resistance in goat farm in Cuba

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Summary

This study determined the efficacies of four classes of anthelmintics (AH) in a goat flock where anthelmintic resistance (AR) to ivermectin was suspected. We selected and randomly distributed 105 animals with >500 eggs per gram of feces into seven groups of 15 animals: an untreated control group and groups treated with ivermectin, eprinomectin, albendazole sulfoxide, albendazole, levamisole, and closantel. The goats were individually weighed and treated with the recommended dose. Fecal samples were collected 14 days post-treatment to determine the fecal egg counts and for a fecal egg count reduction test (FECRT). Coprocultures were also performed for identifying any resistant genera. A molecular assay (polymerase chain reaction, PCR) was used to confirm benzimidazole resistance. The FECRTs for the ivermectin, eprinomectin, albendazole sulfoxide, and albendazole treatments were <90 %, indicating multiple anthelmintic resistance, all in *Haemonchus* spp. Levamisole had a FECRT confidence interval <90 %, indicating a moderate level of AR. The PCR detected the ß-tubulin alleles responsible for benzimidazole resistance, confirming AR. This study is the first monospecific report of AR in goats in Cuba, with a total failure of macrocyclic lactones anthelmintic class.
Keywords: goats; multiple anthelmintic resistance; anthelmintics; *Haemonchus* spp.

Introduction

Anthelmintic treatment has been the main method for controlling gastrointestinal parasites of small ruminants in Cuba for decades. Most producers generally use anthelmintics liberally without any rational strategy, although Cuba has a national anthelmintic program for all farm animals, under the jurisdiction of the National Center for Parasitology. This situation has led to the development of anthelmintic resistance (AR), until now with low prevalence (Mencho *et al*., 2013) and mainly for one anthelmintic class. Multiple AR is common throughout the world (Almeida *et al*., 2010; Van den Brom *et al*., 2015), mostly in regions with intensive systems of small-ruminant production.

Cuba does not have highly intense systems of small-ruminant production. Most production is based on a small economy or small systems of subsistence production (Borroto *et al*., 2011); however, under these circumstances we can expect conditions for the development and spread of the AR to the most available anthelmintics. Either it is due to an easy access to antiparasitic drugs, or limited availability of different active ingredients. The selection pressure by these anthelmintics has increased in the last decade, in part due to the lack of an appropriate training program for parasite control. The objective of the present study was thus to determine the efficacy of six anthelmintics from four classes in a goat flock in Matanzas, Cuba. In addition, the AS-PCR reaction was performed to confirm failure of benzimidazole treatment.

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Materials and Methods

Animals

One hundred five goats were selected from a flock of 156 cross-bred lactating goats based on fecal egg counts (FECs) >500 eggs per gram (EPG) of feces

Farm characteristics

This farm, along with the other 10, was selected as model for a study aimed at the evaluation of an integral strategy for gastrointestinal parasite control. The animals grazed continuously in a semi-extensive 58.3-ha system for about nine hours daily on native *Dichrostachys cinerea* and *Acacia farnesiana* shrubs (90 % of the grazing area) and a complex of naturalized *Dichanthium* spp. and *Bothriochloa* spp. grasses. The animals were confined at night. The goats were milked twice a day, and the kids were artificially fed. The anthelmintic treatments on the farm had been administered arbitrarily without a rational control strategy; during the dry season and first lactating days, goats were used to deworm every 21 – 30 days. The anthelmintic selected depended on availability on the farm, without a rotational strategy using different active ingredients. Fifty animals had been introduced in the flock a few months before our tests without a quarantine routine, and the former owner suspected the presence of AR to ivermectin (personal communication). The goats were never weighed for deworming, and the producer used the dose recommended by Jackson *et al.* (2012).

Fecal egg count reduction (FECR) test

The study followed the recommendations of the World Association for the Advancement of Veterinary Parasitology (Coles *et al*., 1992, 2006). Briefly, at the first farm visit faecal samples were collected directly from the rectum of each animal. The samples were processed in laboratory within 24 h after faecal collection and examined by use of a modified McMaster procedure. Subsequently, animals with EPG below 500 were excluded. Fifteen animals with FECs >500 EPG were randomly distributed into each treatment group which corresponds with the anthelmintic used (Table 1) and a control (untreated) group. A second coprological sample was collected 14 days post-treatment for determining the FEC for calculating the FECR. Coprocultures were performed to identify the genera of the parasites in each group following the method by Roberts and O´Sullivan (1950).

Extraction of genomic DNA

Infective larvae pooled from the post-treatment samples were washed with 40 % sucrose and unsheathed with 0.187 % sodium hypochlorite. Genomic DNA (gDNA) was isolated with a commercial kit for extracting DNA from tissue (DNeasy Blood & Tissue Kit®, QIAGEN, Hilden, Germnay) following the manufacturer's protocol. The extracted gDNA was quantified in a Nanodrop (Waltham, Massachusetts, USA) and stored at -20 °C.

Allele-specific polymerase chain reaction (AS-PCR)

The AS-PCR was designed to detect the TAC polymorphism at codon 200 in *Haemonchus contortus* ß-tubulin, which causes resistance to benzimidazoles in nematodes, following the methodology proposed by Silvestre and Humbert (2000) and Winterrowd *et al.* (2003). The AS-PCR was carried out in two separate reactions. The first reaction was performed in a volume of 20 µL using 100 ng of gDNA, nuclease-free water, and 1 µl (20 µM) of each of the following oligonucleotides: Pn1 (5' GGCAAATATGTCCCACGTGC 3') and Pn2 (5' GAAGCGCGATACGCTTGAGC 3'). The product of the first PCR (Pn1-Pn2) was used as the template for a second (nested) PCR using 20 µM of each of the following oligonucleotides in separate reactions: Fw-P1 (5' GGAACGATGGACTCCTTTCG 3'), Rv-P2 (5' GATCAGCATTCAGCTGTCCA 3'), and resistance-specific Fw-P3 (5' CTGGTAGAGAACACCGATGAAACATA 3') to obtain a fragment of 250 bp; and FW-P1, Rv-P2, and susceptibility-specific Rv-P4 (5' ATACAGAGCTTCGTTGTCAATACAGA 3') to obtain a fragment of 550 bp. A commercial PCR master mix (GoTaq® Green Master Mix, Promega, Madison, USA) was used for all PCR assays. We used the AS-PCR reaction conditions described by Encalada-Mena *et al.* (2014). All reactions were carried out in a C1000 Touch Thermal Cycler (Bio-Rad Technologies, Hercules, USA) using the program: denaturation at 94 °C for 5 min, followed by 33 cycles of denaturation at 94 °C for 55 s, annealing at 60 °C for 55 s, extension at 72 °C for 55 s, and a final extension at 72 °C for 10 min. The PCR products were visualized using 4 % agarose gels stained with ethidium bromide (Sigma-Aldrich, St Louis, USA) in a UV-light photodocumenter.

Statistical analysis and determinations

Drug efficacy was determined by the faecal egg count reduction test (FECRT) and the percentage reduction was calculated according to Coles *et al*. (1992). The efficacy of the anthelmintics

Table 1. Commercial name, active ingredient, dose, and route of administration of the anthelmintics used in the study.

Commercial name	Active ingredient	Dose and administration route
Labiomec® (LBM)	Ivermectin	0.30 mg/kg BW. SC
Labiozol® (LBZ)	Albendazole sulfoxide	10 mg/ kg BW. SC
Ricobendazol® (RIC)	Albendazole	10 mg/ kg SC. Oral
Eprinomex® (EPR)	Eprinomectin	0.75 mg/ kg BW. Pour on
Belaclosan® (CLO)	Closantel	5 mg/kg BW. SC.
Levamisol 10® (LEV)	Levamisole hydrochloride	12.0 mg/kg BW. IM

BW – body weight; SC – subcutaneous; IM – intramuscular

Table 2. Mean eggs per gram of faeces (EPG), faecal egg count reduction percentages (FECR%) and 95% confidence intervals (CI) of the six anthelmintics in lactating goats.

	Control	Eprinomex®	Labiomec®	Labiozol®	Ricobendazol®	Levamisol 10®	Belaclosan®
n	15	15	15	15	15	15	15
Arithmetic mean EPG Day 14	1300	1015	963	571	608	100	58
FECR (%)		22	26	56	53	94	96
CI max.		71	69	82	82	97	99
CI min.		0	0	0	0	84	91
Diagnosis		Resistant	Resistant	Resistant	Resistant	Low resistant	Susceptible
Resistant species				*Haemonchus* spp.			-

was analyzed using the RESO 2.01 Analysis Software (Wursthorn & Martin 1990).

Anthelmintic resistance was declared if: 1) the FECR was <95 % and 2) the confidence intervals were <90 % (Coles *et al*., 1992; 2006). If only one of the two criteria was met, the gastrointestinal parasite population was considered "suspect" of AR and it was considered "susceptible" to any given anthelmintic class when none of the criteria above-mentioned was satisfied (Herrera-Manzanilla *et al*., 2017)

Results

Table 2 shows the results of the *in vivo* efficacy test. Four of the anthelmintics from two classes had low efficacies (<95 %), confirming the presence of resistant parasites. The FECRs did not exceed 26 % for the macrocyclic lactones (ivermectin and eprinomectin) and were <57 % for the benzimidazoles (albendazole sulfoxide and albendazole). The post-treatment coprocultures confirmed the dominant presence of *Haemonchus* spp for these anthelmintic-treatment groups. Levamisole had an efficacy of 94 %, but the lowest confidence interval was 84 %, which confirmed the inability of this drug to adequately control *Haemonchus* spp. Closantel reduced FECs by 96 %, with a confidence interval >90 %.

The pre-treatment coprocultures revealed that *Haemonchus* spp. was found the dominant genus (95 %) followed by *Oesophagostomum spp.* (3 %) and *Trichostrongylus spp.* (2 %). After treatment, only *Haemonchus* spp. was found in each anthelmintic groups, while the same composition was found in the second fecal sample in the control group.

The agarose gel of the electrophoresed AS-PCR reactions is shown in Figure 1. The 250-bp fragment indicated the presence of benzimidazole-resistance alleles in the pooled larvae. The lack of a visible 550-bp fragment indicated either the absence or a low frequency of the susceptible allele.

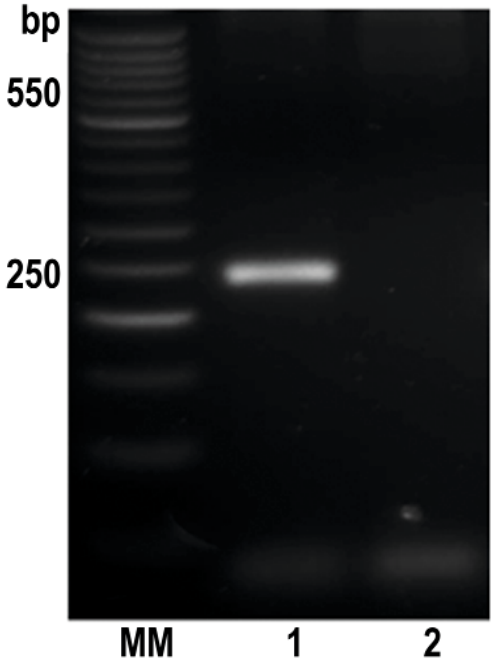

Fig. 1. Agarose gel showing the electrophoresed PCR reactions. Lane 1, resistance allele; lane 2, susceptibility allele; MM – molecular marker; bp – base pairs.

Discussion

Our results is the first report in Cuba about the resistance to three classes of anthelmintics (macrocyclic lactones, benzimidazoles, and imidazothiazoles) in the same flock, demonstrating the need for the establishment of parasite control strategies. This amount of resistance is due to the concomitance of several factors. The lack of strategic treatment plans and the use of mass-drenching regimes (Arece *et al*., 2016) are the most important. The use of the same dose for sheep and goats has also likely increased the selection pressure; goats require higher doses than sheep for most anthelmintics (Jackson *et al*., 2012).

Finding eprinomectin resistance was unexpected, mainly because this product had never been used before in this flock. De Souza-Chagas *et al*. (2007) and Murri *et al*. (2014) reported similar results in Brazil and Switzerland, respectively. This resistance could be because eprinomectin is a macrocyclic lactone (Lespine *et al*., 2012) that likely shares the same mechanism of action as ivermectin, which has been the most widely used drug for controlling gastrointestinal parasites in small ruminants during the last deca-

de. Cross-resistance between albendazole and macrocyclic lactones may also occur; nucleotide changes in ivermectin-resistant *H. contortus* have been observed in the gene coding for β-tubulin isotype 1 (codons 200Tyr or 167Tyr), which are responsible for resistance to benzimidazoles (Eng et al., 2006; Mottier and Prichard, 2008), even though ivermectin and benzimidazoles have different mechanisms of action.

FECR for levamisole was >95 %, but the confidence interval was slightly less than 90 %. The introduction of Labiomec® and Labiozol® in Cuba has likely displaced the use of Levamisol-10® in goat flocks due to the better safety margin for these two drugs than for levamisole, which was the most widely used drug until the beginning of the new millennium.

The diagnosis of AR to benzimidazoles and macrocyclic lactones is currently very complex in Cuba, where producers tend to prefer these drugs because of their safety and appreciable short-term results. The prevalence of AR is aggravated by the complexity of the Cuban production systems for small ruminants, where no clear trends toward comprehensive strategies for parasite control have been established and where a single antiparasitic is generally used throughout the year (Delgado-Fernández, 2016).

The AS-PCR assay was based on the search for mutations at codon 200 (TTC to TAC) in the gene encoding β-tubulin isotype 1 (Silvestre and Cabaret, 2002; Ghisi *et al*., 2007; von Samson-Himmelstjerna *et al*., 2007, Blackhall *et al*., 2011; Barrère *et al*., 2012). The presence of these mutations in a population causes resistance of the parasites to benzimidazoles. The lack of a visible 550-bp fragment indicated either the absence or a low frequency of the susceptible allele, and was related with a failure of the benzimidazole treatment. The susceptibility-specific primers used in our study were previously tested on known benzimidazole-susceptible worms (Encalada-Mena *et al*., 2014).

This study is the first report of monospecific AR in goats in Cuba, with a total failure of macrocyclic lactones. It indicates that AHs must be used rationally to minimize the development and spread of resistant nematode populations, and that the replacement of mass treatment schemes of entire flocks by more complex integrated programs is necessary to prevent the development of multiple AR.

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