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Phenotypic and genotypic characterisation of *Haemonchus* spp. and other gastrointestinal nematodes resistant to benzimidazole in infected calves from the tropical regions of Campeche State, Mexico

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ABSTRACT

The aim of this study was to identify the presence of anthelmintic resistance to benzimidazole (BZ) in gastrointestinal nematodes (GIN) from naturally infected calves in the tropical regions of Campeche State of Mexico. The faecal egg count reduction test (FECRT) was conducted at 10 livestock farms localised in the Carmen, Candelaria, Champotón, Escárcega and Palizada municipalities of Campeche. The assessed anthelmintic was albendazole. The trial period was between August and November 2012. Infected calves were allocated into two groups, control and treated, on each farm. The number of eggs excreted per g of faeces was estimated by the McMaster technique at 0 and 14 days pre- and post- treatment, respectively. Recovered infective larvae (L_3) (pre- and post-treatment) were identified using taxonomic keys and a genomic DNA (gDNA) template from a pool of L_3 species prior to BZ treatment. Additionally, BZ-resistance polymorphisms in *Haemonchus* were determined by Allele Specific PCR (AS-PCR) at codon 200 and by end-point PCR at codons 200, 198 and 167 from isotype 1 of the β-tubulin gene. Morphological identification revealed *Haemonchus*, *Cooperia*, *Trichostrongylus*, *Ostertagia* and *Oesophagostumum* L_3 species before BZ treatment, and *Haemonchus* and *Cooperia* L_3 species after treatment. Additionally, of the GIN populations, three exhibited BZ resistance, and seven were BZ-susceptible by FECRT. Molecular analysis identified mutations in *Haemonchus* populations on nine farms at codon 200 (TTC to TAC) by AS-PCR, while no changes were observed at 167 (TTC to TAC) or 198 (GAA to GCA) codons in any population. In conclusion, resistance to BZ was determined in *Haemonchus* and *Cooperia* nematodes in infected cattle in five tropical regions of Campeche State.

1. Introduction

The benzimidazole (BZ) family has been recognised as an anthelmintic drug group with significant activity against the nematodes of domestic ruminants (Brown et al., 1961;

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Actor et al., 1967). However, the number of reports concerning BZ resistance in gastrointestinal nematodes (GIN) is increasing, and it is clear that production systems that are wholly reliant upon frequent chemotherapy/prophylaxis are not sustainable (Gasbarre et al., 2009; Nari-Henrioud, 2011).

Currently, three major anthelmintics are frequently used by farmers in a variety of grazing areas: (1) macrocyclic lactones, *i.e.*, ivermectin (IVM), doramectin, eprinomectin and moxidectin; (2) imidazothiazole/tetrahydropyrimidina, including levamisole and morantel tartrate; and (3) the BZ family, which primarily includes the drugs febendazole, oxfendazole and albendazole (Khöler, 2001; Forbes, 2013; Soll et al., 2013). In recent years, few cases of anthelmintic resistance in infected cattle have been reported; however, anthelmintic resistance detection and dispersion in livestock farms have required major efforts to confirm resistance problems in cattle, requiring knowledge of husbandry procedures, animal production and nematode biology, which are different from sheep management (Coles, 2002; Soutello et al., 2007; Demeler et al., 2009). To determine early anthelmintic resistance problems, different methods of diagnosis have been used in grazing areas, such as the faecal egg count reduction test (FECRT), and in the lab, such as *in vitro* assays, polymerase chain reaction (PCR) and more recently, pyrosequencing to monitor the allele frequency involved in BZ resistance (Coles et al., 2006; von Samson-Himmelstjerna, 2006; Beech et al., 2011; Martinez-Valladares et al., 2012).

Studies on BZ-resistance mechanisms have provided evidence of the nucleotide point mutations localised at codons 167 and 200 (T*T*C to T*A*C) and 198 (G*A*A to G*C*A) in GIN populations in isotype 1 of the β-tubulin gene, the target of BZ derivatives (Silvestre and Cabaret, 2002; Ghisi et al., 2007; Von Samson-Himmelstjerna et al., 2007; Blackhall et al., 2011; Barrère et al., 2012). In addition, some mutations identified in the nematode genome were found to be associated with BZ and with IVM (*avr*-14, *avr*-15, and *glc*-1) (Dent et al., 2000). Therefore, the presence of multiple anthelmintic resistance problems threatens the health of domestic animals, such as goat, sheep, cattle and horses, and the farmers' economy (Coles, 2002; Gasbarre et al., 2009; Pape et al., 2003).

In contrast, the features of tropical and template regions, *i.e.*, temperature, humidity, high grass and grazing-cattle exposure, provide suitable environmental conditions for promoting the favourable development of GIN in the grasslands, thereby increasing nematode prevalence and affecting animal health and production (Vázquez-Prats et al., 2004; Bianchin et al., 2007). Nematode infection is becoming a major concern in young cattle worldwide, especially in light of the resistance of anthelmintics to BZ and macrocyclic lactone (Anziani et al., 2004; Perri et al., 2011; Bartley et al., 2012; Charlier et al., 2012; Borges et al., 2013). The state of Campeche, situated in the southern Mexican tropic, is considered to be an important geographic area for livestock production in the country. Because of the high prevalence of GIN species, anthelmintic control is necessary. However, in recent years, the presence of anthelmintic resistance to IVM has been reported (Encalada-Mena et al., 2008, 2009), and the undocumented inefficacy of BZ in calves (observed by farmers and veterinarians) make it necessary to investigate the current state of anthelmintic resistance in nematodes to the available anti-parasitic drugs.

In addition, the nematicidal efficacy of IVM has recently been questioned due to a report from the Yucatan State, also localised in southeastern Mexico, adjoining Campeche. The authors mentioned the consecutive use of IVM from 1 to 11 years, (Canul-Ku et al., 2012), a situation that causes difficulties in the control of GIN and perhaps in the control external parasites such as ticks. Ivermectin and other MLs are the main anthelmintic drugs used by farmers, which has reduced their efficacy. As a result, the high anthelmintic efficacy of BZ could offer an opportunity as an alternative drug to control GIN in grazing calves. Therefore, it is important to assess BZ toxicity against GIN in livestock farms in Campeche State, as this may help the sustainability of the BZ-susceptible worm population in the livestock of Campeche, thereby ultimately promoting the animals' health.

2. Materials and methods

2.1. Geographic localisation

Two hundred yearling heifers naturally infected with GIN species were selected from 10 livestock producers localised in the Southeast of Campeche State, Mexico. Campeche is bordered to the north by Yucatán, to the south by Tabasco State and the Guatemala Republic, to the east by Quintana Roo State of Mexico and Belize and to the west by the Gulf of Mexico (Fig. 1). This study was carried out after the rainy season in southeastern Campeche State (between August and November 2012) in farms from five main livestock municipalities (Carmen, Candelaria, Champotón, Escárcega and Palizada). Four of them (Carmen, Escárcega, Candelaria and Champotón) are classified as (Aw) tropical wet climate, located at latitude 18°50′11″N at 500 m above sea level. Palizada municipality is classified as (Am) tropical wet climate, located at latitude 90°24′12″W. The annual mean temperature of Campeche in 2012 was approximately 28 °C.

2.2. Faecal egg count reduction test

The FECRT was carried out following the method described by the World Association for the Advancement of Veterinary Parasitology (Coles et al., 2006). Farmers from 10 different livestock farms of Campeche State of Mexico were invited to participate and agreed to collaborate.

Calves infected with GIN were selected based on the number of young cattle per farm with at least 20 grazing calves from 3 to 6 months up to 1 year of age (male and female). Additionally, the selection of grazing cattle was considered based on the previous frequency of deworming to avoid residual anthelmintic effects (from 30 to 60 days before the experimental assays were performed). Infected calves with more than 200 eggs per gram (EPG) determined by the McMaster technique were selected (MAFF, 1986). Briefly, faecal samples were individually collected from the