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Case Report

Genotypic profile of benzimidazole resistance associated with SNP F167Y and F200Y beta-tubulin gene in Brazilian populations of *Haemonchus contortus* of goats

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ABSTRACT

Benzimidazoles are the most common anthelminthic used for control of gastrointestinal nematodes of goats in the Brazilian semi-arid region. Resistance to these compounds in the nematode *Haemonchus contortus* has been associated with single nucleotide polymorphisms (SNP) in codons 167 (F167Y) and 200 (F200Y) on the β-tubulin isotype 1 gene. To determine the resistance profile to benzimidazoles of populations of *H. contortus* of goats of Brazilian semi-arid region, larvae of 29 populations of these nematodes were individually genotyped by real time PCR using a Taqman assay. The percentage of larvae homozygous (RR) for SNP F200Y was relatively low (18.9%), particularly when compared to SNP F167Y (32.7%), indicating that the latter has more relevance in this region. However, the associations between these two SNP demonstrate percentages of resistance ranging from 34.7% to 100% between populations, being the highest percentages for homozygous individuals resistant for the mutation 167 and susceptible to mutation 200 (RR-F167Y/F200Y-SS: 26.7%), followed by combination of heterozygous for both mutations (F167Y-SR/F200Y-SR: 22.8%). These results indicate high levels of resistance in populations of *H. contortus* of goats in the Brazilian semi-arid region, and thus ineffective antiparasitic control with the use of benzimidazoles in the region.

1. Introduction

The semi-arid region of northeastern Brazil is characterized by a hot, dry climate, with scarce, irregular rainfall and the *Caatinga* biome, a type of arborescent, xerophytic vegetation (Koeppen, 1948). In this region, the climate generates prolonged cyclical droughts, bringing negative socio-economic consequences. Due to lack of conditions for agriculture, the breeding of goats and sheep is the main mean of subsistence of small farmers, since these animals are well adapted to local edaphoclimatic conditions. Despite its importance, this activity is limited by failures in the health management and feeding of livestock, leading to the development of gastrointestinal helminths and, consequently, problems in animal health and production (Cunha Filho et al., 2010).

Among the parasites of major importance in livestock production, *Haemonchus contortus* has featured in many regions of the world, because it is a species of high pathogenicity, due to its hematophagous action and biotic potential. Although it is originally a tropical parasite, *H. contortus* currently is prevalent in regions of both the southern and northern hemispheres, reaching the Arctic Circle (Falzon et al., 2013). The severity of infection frame is related to the existing parasitic load, which is often severe and can be fatal.

Benzimidazoles (BZs) are often used for the control of ruminant infections with gastrointestinal nematodes. However, resistance to this drug class has been observed by means of *in vivo* and *in vitro* assays in goats and sheep in the Brazilian semi-arid region (Coelho et al., 2010; Rodrigues et al., 2007). BZ resistance in various ruminates nematodes parasites (*Haemonchus contortus*, *H. placei*, *Teladorsagia circumcincta*, *Ostertagia ostertagi*, *Cooperia onchophora*, *Trichostrongylus colubriformis*) has been associated with three single nucleotide polymorphisms (SNP) in codons 167, 198 and 200 of the β-tubulin isotype 1 gene (Ghisi et al., 2007; Kwa et al., 1993; Silvestre and Cabaret, 2002), leading to the substitution of amino acids and consequently to a loss of the binding

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receptor (Chaudhry et al., 2015; Ramünke et al., 2016; von Samson-Himmelstjerna et al., 2007).

Among these mutations, the one at codon 200 (F200Y) is still considered the most frequently associated with resistance to BZ in *Haemonchus* species (Chaudhry et al., 2015; Saunders et al., 2013). The mutation at residue 167 (F167Y) has been increasingly identified in the determination of the resistance profiles of populations of nematodes, while the E198A mutation seems to be less common, mainly in the Americas (Barrère et al., 2013; Brasil et al., 2012; Santos et al., 2014). The parasites with a resistant phenotype can either be homozygous for one of these SNPs or even heterozygous for more than one SNP (Barrère et al., 2012, 2013).

The identifying of the mutations present in these three SNPs mentioned above is the basis for the molecular diagnosis of BZs resistance currently available, being used conventional PCR (Silvestre and Humbert, 2000; Winterrowd et al., 2003), Real-time PCR (Alvarez-Sanchez et al., 2005; Walsh et al., 2007) as well as pyrosequencing (Demeler et al., 2013; Ramünke et al., 2016).

In nematodes with high genetic diversity, such as *Haemonchus contortus*, the rapid selection of resistant individuals, whose survival favors the spread of alleles with such characteristics to their progeny, has been observed. Moreover, the intensity of treatments and management practices contribute to increased selection pressure for the following generations, increasing the frequency of alleles for resistance, and the consequent worsening of this problem (Kwa et al., 1994; Papadopoulos, 2008; Prichard, 2001). For goats, in particular, it still is needed to emphasize the unavailability in the chemical industry of BZ commercial formulations with the effective dose for this species, so that it is used the dose for sheep, which represents lower bioavailability of the product, therefore reduced efficacy in goats (Bogan et al., 1987; Hennessy et al., 1993; Sangster et al., 1991), allowing nematodes homozygous dominant and some heterozygotes to survive, accelerates the development of anthelmintic resistance (Jackson et al., 2012; Silvestre et al., 2001).

Due to the global occurrence of anthelmintic resistance to BZ, it is important to constantly monitor populations of parasites to enable adequate control, especially in regions with low efficiency of production systems, such as the semi-arid region of northeastern Brazil. Genotyping the β-tubulin gene allows to analyze the frequency of mutations in this gene, the possibility of real efficacy of the BZs based anthelmintic drugs and the situation of field populations of nematodes for a faster and more accurate resistance diagnostic (Beech et al., 2011).

Therefore, the aim of this study was the determination of the two BZ resistance associated SNPs F167Y and F200Y in different *H. contortus* populations of goats in the semi-arid region of Brazil.

2. Materials and methods

2.1. Study area and sampling

In total, 29 populations of *H. contortus* were studied in goat flocks of household production in semi-arid regions of the state of Bahia, northeastern Brazil, that comprised the following municipalities: Anagé (ANG - n = 2), Bom Jesus da Lapa (BJL - n = 2), Cansanção (CAN - n = 8), Curaçá (CUR - n = 1), Entre Rios (ETR - n = 1), Ipirá (IPI - n = 2), Jequié (JEQ - n = 2), Morro do Chapéu (MCH - n = 2), Remanso (REM - n = 1), Santa Inês (STI - n = 6), Uauá (UAU - n = 1) e Xique-Xique (XIX - n = 1) – (Fig. 1).

The sampled area is entirely located within the "drought polygon" (SUDENE, 2011), and the chosen municipalities have a representative production of goats for the semiarid region. This is an area with a hot semi-arid climate (BSh) (Koeppen, 1948), with annual rainfall ranging from 73 mm to 750 mm and average temperatures of 16 °C to 35 °C, with the *Caatinga* as the predominant vegetation type.

Studied flocks consisted of 15 to 140 goats raised extensively or semi-intensively, where the animals were kept in communal areas of native vegetation throughout the day (this being its food base), and housed in the sheepfolds overnight, most of these spaces (90%) being of hard soil, unpaved. In 69% of the properties, there were no proper veterinary assistance, being the use of veterinary products recommended by local merchants or other farmers and not by professionals duly qualified to do so. The deworming was done in all animals, semi-annually (41%) and fortnightly (17%), without weighing the animals for measurement for the correct dose of the medication. On all farms, BZ was used at some point for the deworming of goats. However, for the last reported deworming, 36% of the flocks was treated with BZs, another 36% with imidazothiazoles and 28% with macrocyclic lactones.

Faecal samples were collected from 15 to 30 animals/flock without deworming for at least 90 days between October de 2012 and July 2015. To obtain 3rd-stage larvae (L3) of gastrointestinal nematodes, coprocultures were performed (Coles et al., 1992) with pooled flock samples. All the flocks showed mixed infection by *Haemonchus* and *Tricostrongilus*. Nevertheless, the target specie (*H. contortus*) represented at least 40% of the sample in all of them, making it possible to use the samples for study. From each flock, 35 to 50 L3 of *H. contortus* were identified with base on morphological characteristics and individually separated for genotyping.

2.2. DNA extraction and genotyping F200Y and F167Y polymorphism

To obtain the genotypic and allelic frequencies, the L3 were individually subjected to DNA extraction using a commercial kit (PureLink® Genomic DNA – Invitrogen, Carlsbad, USA). Genotyping of polymorphisms F200Y and F167Y of the β-tubulin gene was performed by real-time PCR (7500 Real Time PCR System, Applied Biosystems, Foster City, EUA), using Taqman® methodology. For SNP F200Y genotyping LNA (Locked Nucleic Acids) probes and primers were used, as described by Walsh et al. (2007), while for SNP F167Y a new set of primers and probes were designed, using β-tubulin DNA sequences from Genbank (X80046.1; X67489.1; JF784609.1) - Primer F167Y-F: 5′-AAAAATTCGTGAAGAGTACCCTGATAG-3′; Primer F167Y-R: 5′-AAAAAGCAAAGATTAACACGATCTCA-3′; Probe F167Y-S: 5′-/5HEX/ATTATGGCTTCGTTCTC/3BHQ/-3′ and probe F167Y-R: 5′-/FAN/TATGGCTTC GTACTC/3BHQ/3′.

PCRs were conducted with a final volume of 20 μL using PCR buffer (1×), 3.0 mM $MgCl_2$, 0.2 mM of each dNTP, 0.1% BSA, 10 nM of each primer, 1 U Taq DNA Platinum (Invitrogen, Carlsbad, USA) and 10 ng of DNA. For genotyping reactions of the F200Y SNP, 50 nM of the LNA-S probe, 150 nM of the LNA-R probe were used, while for the F167Y SNP 50 nM of each probe were used. For amplification of these SNPs, the following conditions were used: 60 °C for 1 min, 95 °C for 2 min, followed by thirty amplification cycles at 95 °C 30 s, 64 °C (for F200Y) or 56 °C (for the F167Y) for 1 min, and final extension of 60 °C for 1 min. End-point analysis in triplicates for classification of larvae in homozygous or heterozygous for the resistance and susceptibility alleles in the respective mutations were performed (Following Genotyping Experiments by Applied Biosystems 7500 PCR Real Time). Lastly, 10% of the samples were subjected to bidirectional capillary sequencing (ABI 3130 Platform - Applied Biosystems, Foster City, USA) for full confirmation of the data.

The Brazilian isolates S-IVM (UNESP, Botucatu campus- Echevarria et al., 1991) and Embrapa 2010 (EMBRAPA CPPSE - Chagas et al., 2013) were used as susceptibly and resistant controls, respectively.

2.3. Data analysis

Parasites were considered phenotypically resistant to BZ when one of the following combinations of genotypes was detected (F167Y/F200Y): SS/RR; RR/SS; SR/SR; RR/SR; SR/RR or RR/RR, as suggested by Barrère et al. (2012, 2013).

Differences in genotypic and allelic frequencies within and between populations were analyzed by chi-square tests using Statistical Package for Social Science for Windows (SPSS). The association