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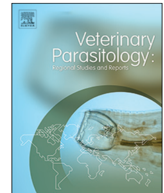

Original article

# Molecular analysis of benzimidazole-resistance associated SNPs in *Haemonchus contortus* populations of Uruguay

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ABSTRACT

*Haemonchus contortus* is one of the most important parasite nematodes of small ruminants around the world and causes great economic losses in livestock production. Control of gastrointestinal nematode infections, like haemonchosis, relies mainly on anthelmintic drugs, but its excessive and inappropriate use has caused serious drug resistance issues in many countries, including Uruguay, where sheep production occupies an important place in the country's economy. Benzimidazole (BZ) anthelmintics have been used for decades to treat sheep against *H. contortus* infection and resistance to this anthelmintic group has been widely described. Molecularly, BZ resistance in *H. contortus* has been correlated with single nucleotide polymorphisms (SNPs) of the β-tubulin isotype 1 gene at codon 200 and 167 (both TTC to TAC, F167Y and F200Y) and at codon 198 (GAA to GCA, E198A).The aim of this work was to explore the presence of these tubulin SNPs in *H. contortus* adult worms recovered from sheep abomasa from a slaughterhouse in Uruguay. The mean resistant allelic frequencies at positions F167Y and F200Y were 20.25 and 47.45%, respectively, for worms recovered from naturally infected sheep slaughtered in 2013, while those that were slaughtered in 2014 presented only F200Y SNP with a frequency of 86.89%. Also *H. contortus* Kirby adult worms (anthelmintic- susceptible McMaster isolate), recovered from artificially infected sheep, were analyzed as reference for comparative purposes This analysis showed susceptible genotype at 167 and 198 position, and a low level of the resistance allele at the 200 position (3.66%). This is the first study for the presence of SNPs in the isotype-1 β-tubulin gene of *H. contortus* populations in Uruguay, which is consistent with the previous epidemiological studies carried out through the method of fecal egg count reduction test (FECRT), thus confirming the serious resistance levels to BZ anthelmintics also in this country.

## 1. Introduction

Gastrointestinal nematodes represent a real threat to the sheep industry because the intensive use of anthelmintic drugs has led to widespread resistance to all current commercial products (Martin et al., 2016). Surveys conducted in three of the largest sheep producing countries, namely Australia, South Africa and Uruguay, ranked nematode parasites as the most important of all the infectious diseases in sheep (Waller, 2006).Particularly in South America, anthelmintic resistance has been explored mainly in southern countries of the continent. Here, Argentina, Brazil, Uruguay, and Paraguay, are the countries where anthelmintic resistance seems to be more prevalent and it is common to find sheep flocks with multiple resistant worms (Torres-Acosta et al., 2012).

A survey carried out between 1994 and 1995 showed that in Uruguay, 92% of sheep- producing establishment showed anthelmintic resistance, and 86% of those were resistant to anthelmintic benzimidazoles (BZ), 71% to imidazothiazoles and 1% to ivermectin (Nari et al., 1996). A more recent report showed that the 100% of sheep-producing establishment presented resistance to BZ anthelmintics, 91.2% to imidazothiazoles, 94.4% to, moxidectin and 6.1% to the new anthelmintic monepantel, which shows an alarming tendency towards the expansion of anthelmintic resistance in Uruguay (Mederos et al., 2016).

*H. contortus* is the most prevalent and resistant nematode in these infections, affecting small ruminants (Kotze and Prichard, 2016). This is a species of high pathogenicity, due to its hematophagous action and biotic potential, being infections often severe and fatal (Lambert et al., 2017).

Benzimidazole anthelmintics have been used for decades to treat sheep against gastrointestinal nematode infections and the phenomenon of resistance to this anthelmintic group has been widely described

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Veterinary Parasitology: Regional Studies and Reports 13 (2018) 110–114

(Furtado et al., 2016; Kaplan, 2004). This group of drugs exerts their action interrupting the dynamic equilibrium of microtubules, inhibiting their polymerization through the binding to β-tubulin (Lacey, 1988).

Characteristic single nucleotide polymorphisms (SNP) at codons 167, 198 and 200 of the β-tubulin isotype-1 gene are involved in the development of benzimidazole resistance in most, if not all, trichostrongylid nematode species. The first discovered SNP associated to BZ-resistance was a substitution that caused a change in codon 200 (TTC to TAC) from phenylalanine to tyrosine (F200Y) (Kwa et al., 1994). Later on, two more SNPs were discovered in different nematode species at codons167 (F167Y) (Silvestre and Cabaret, 2002) and 198 (E198A) (Ghisi et al., 2007) in the β-tubulin isotype-1 gene. Since the molecular basis of BZ-resistance is well characterized, the identification of these three genetic markers by PCR- based detection methods could be used to detect benzimidazole resistance (Von Samson-Himmelstjerna et al., 2007, 2009).

The present study aimed to perform a preliminar evaluation of the genotypes present in *H. contortus* pooled worms recovered from abomasa of naturally infected sheep, from a national slaughterhouse in Uruguay. Although there were already epidemiological surveys about BZ- resistance in Uruguay there was no molecular data on the genotype of the resistant populations. We analyzed by PCR and sequencing the three previously shown SNPs associated to BZ- resistance, and allowed an estimation of the relative frequency of each allele *in H. contortus* populations in Uruguay. As a control, we performed the analysis of these SNPs in *H. contortus* Kirby adult worms (anthelmintic- susceptible McMaster isolate) recovered from artificially infected sheep.

## 2. Materials and methods

### 2.1. *H. contortus* adult stage (Hc U, Hc S)

*H. contortus* nematodes (third stage, L3) from the anthelmintic-susceptible McMaster isolate (Kirby) were kindly provided by Dr. A. Kotze and Dr. M. Knox (CSIRO McMaster Laboratory, Armidale, NSW). The artificial infection (10,000 L3 per sheep) was maintained at the Campo Experimental de Higiene de Facultad de Medicina, UdelaR, as it was described (Munguía et al., 2015; 2017).*H. contortus* adult susceptible worms (*Hc S*) were recovered from sheep abomasa (21 days after infection) by manual picking. The parasites were washed with PBS and pooled in 50 specimens per sample. At least three samples per infected sheep were analyzed.

Animal protocols complied with Uruguayan Law No. 18.611. Experimental protocol no 071140–001021-11 of the study was reviewed and approved by IACUC of Facultad de Medicina – UdelaR, Uruguay.

Additionally, *H. contortus* adult worms of naturally infected animals from sheep farms (*Hc U),*obtained from a national slaughterhouse (Frigorifico Las Moras, Chiadel S.A., La Paz, Canelones, Uruguay) were recovered from abomasa by manual picking and were processed as it was described for *Hc S* (50 specimens per sample, at least three pooled samples per infected sheep were analyzed).

### 2.2. Preparation of genomic DNA, PCR and sequencing

Pooled *Hc S* or *Hc U* adult worms were cut with scalpel and then homogenized with a pellet pestle in lysis buffer from DNeasy Blood and Tissue kit (Cat No69504,QiagenInc). Genomic DNA was extracted using Proteinase K protocol following kit instructions.

PCR primers were synthesized to amplify an 812 pb fragment from *H. contortus* beta- tubulin isotype 1 gene (Ghisi et al., 2007). The forward primer for amplification was 5′-GCCTGGAACGATGGACTCCGT-3′ and the reverse primer was 5′-GGCTAACTTGCGAAGATCAGCAT-3′. PCR conditions for DNA samples were as follow: 95 °C for 3 min, followed by 32 cycles of 95 °C for 40 s, 60 °C for 40 s, 72 °C for 40 s, followed by 72 °C for 7 min, using KAPA 2G robust PCR kit (Cat No KK5023, Kapa Biosystems). PCR products were examined on 1% agarose gels.

The PCR products were purified using PureLink PCR purification kit (Cat NoK310001, Invitrogen Thermo Fisher Scientific). Sequencing reactions were performed by direct sequencing (Macrogen Korea) across SNP positions of isotype 1beta-tubulin (aminoacid positions 167, 198 and 200), using the following primers: forward 5′-GCTGAAGGTTGTG ATTGCC-3′ and reverse 5′-CCAAGGTGGTTGAGATCTC-3′.

### 2.3. Determination of allele frequencies

Sequence traces were examined using ChromasPro 2.6.4 free trial version software (Technelysium co.), focusing on F167Y (TTC/TAC), E198A (GAA/GCA) and F200Y (TTC/TAC) SNPs. Their frequencies were estimated by relative peak heights and when two peaks were present the height of the nucleotide known to be associated with resistance was expressed as a percentage of the combined height of the two peaks (Von Samson-Himmelstjerna et al., 2007).

## 3. Results

Molecular analysis of the Kirby isolate *(Hc S)* showed susceptible genotype (TTC) at aminoacid position 167 and 198, although a low level of the resistant allele (TAC) at position 200 was detected (Table 1) (Fig. 1A).

Adult worms recovered from abomasa of naturally infected sheep (Hc U) were grouped according to the day they were slaughtered. Worms recovered from animals in the year 2013 showed significant levels of resistant alleles F167Y and F200Y (Table 2) (Fig. 1B), while animals slaughtered in 2014 only presented the F200Y allele but at significantly higher levels than 2013 animals (one way ANOVA, Tukey's post test, $p < 0.05$).

We want to point out that the frequency values were consistently higher for most of reverse direction analysis, which could be due to different nucleotide incorporation efficiencies under our experimental conditions as it was previously described (Parker et al., 1995). More recently, this observation was also reported (Hancock et al., 2005).

## 4. Discussion

There has been a fast increase in BZ-resistance in Uruguay in the last

**Table 1**
Resistance allele frequency for the F200Y SNP in isotype 1 beta-tubulin from pooled adult samples of *Hc S* isolates of *H. contortus*.

| Date of necropsy (one animal per date) | % Resistant allele (mean ± SEM)[a] | |
|---|---|---|
| | Forward | Reverse |
| 11/16/2016 | 1.65 ± 0.35 | 7.25 ± 4.85 |
| 09/07/2016 | 4.30 ± 4.30 | 9.40 ± 1.00 |
| 03/09/2016 | 1.70 ± 0.10 | 7.65 ± 4.45 |
| 12/14/2015 | 3.03 ± 1.61 | 8.00 ± 0.80 |
| 11/16/2015 | 3.77 ± 0.94 | 5.30 ± 2.58 |
| 09/23/2015 | 7.75 ± 0.15 | 6.95 ± 1.25 |
| 06/25/2015 | 3.43 ± 0.88 | 2.47 ± 2.47 |
| Mean ( ± SEM)[b,c] | 3.66 ± 0.78 | 6.72 ± 0.85 |

SEM: standard error of the mean.

[a] Means of at least 3 independent pooled samples of adult worms, per sheep. For each animal the forward and reverse sequencing data were not significantly different (Unpaired *t*-test, two tailed, $p < 0.05$).

[b] Means of percentage of resistant allele for forward sequencing were not significantly different between different animals (One way ANOVA, Tukey post test, $p < 0.05$).

[c] Means of percentage of resistant allele for reverse sequencing were not significantly different between different animals (One way ANOVA, Tukey post test, $p < 0.05$).

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