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Albendazole resistance induced in *Ancylostoma ceylanicum* is not due to single-nucleotide polymorphisms (SNPs) at codons 167, 198, or 200 of the beta-tubulin gene, indicating another resistance mechanism

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Abstract

Mass drug administration has been implicated as the major cause of drug resistance in nematodes of ruminants. Single-nucleotide polymorphisms (SNPs) at codons 167, 198, and 200 of the beta-tubulin isotype 1 gene are associated with albendazole resistance mechanisms. Although drug resistance is suspected to occur in nematodes of the same order, at present, there is no evidence of a strong correlation between these canonical SNPs and albendazole resistance in hookworms. In the absence of a hookworm strain that is naturally resistant to albendazole, we produced an albendazole-resistant *Ancylostoma ceylanicum* strain by selective drug pressure. Restriction fragment length polymorphism-PCR (RFLP-PCR) was employed to identify the presence of SNPs previously associated with drug resistance in other nematodes. However, none of the benzimidazole resistance–associated SNPs known in other nematodes were found. A beta-tubulin isotype 1 gene mini-cDNA library was constructed to obtain the complete cDNA gene sequence for the analysis of the entire gene to identify distinct SNPs associated with resistance. Some SNPs were found, but the resulting sequences were not reproducibly detected among the different clones, preventing their association with the resistance mechanism. The parasitological and hematological parameters of the albendazole-resistant strain were characterized and compared to those of the sensitive strain. Although the albendazole-resistant strain was less adapted to its host, with fewer worms recovered, all other parameters analyzed were similar between both strains. The results of the present study indicate that the mechanism of albendazole resistance of the resistant strain described herein must differ from those that have previously been characterized. Thus, new mechanistic studies are needed in the future.

Keywords Anthelmintic resistance · Genetic structure · *Ancylostoma ceylanicum* · Beta-tubulin · Hookworms

Introduction

Hookworm infections are a major public health problem, especially in developing countries. An estimated 439 million people are infected by hookworms, resulting in a global disease burden of 3.5 million disability-adjusted life years (Murray et al. 2012). *Necator americanus*, *Ancylostoma duodenale*, and *Ancylostoma ceylanicum* hookworms infect the small intestine of humans, which may cause intestinal blood loss, iron deficiency anemia, and protein malnutrition in hosts (Pacanaro et al. 2014; Loukas et al. 2016). In addition, the zoonotic hookworms *Ancylostoma caninum* and *Ancylostoma braziliense* can cause cutaneous infections in humans (Hasslinger 1986).

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Based on overall reductions in the prevalence and intensity of infection, the main method of hookworm control is mass drug administration (MDA), primarily with benzimidazoles (Humphries et al. 2017). Although this strategy ensures that infection levels are maintained below those associated with morbidity, the frequent use of drugs may select for parasite strains resistant to treatment (Furtado et al. 2016).

Single-nucleotide polymorphisms (SNPs) in the beta-tubulin isotype 1 gene located at codons 167 (TTC/phenylalanine → TAC/tyrosine), 198 (GAG/glutamate → GCG/alanine), and 200 (TTC/phenylalanine → TAC/tyrosine) have been associated with benzimidazole resistance in some nematodes (Kwa et al. 1994; Prichard 2001; Ghisi et al. 2007). However, there are some reports that these common resistance-associated mutations are not found in populations of hookworms that are unresponsive to treatment (Albonico et al. 2004; Diawara et al. 2013), suggesting that other molecular mechanisms may be involved. To date, SNPs at codons 198 in *N. americanus* (Rashwan et al. 2016) and *A. braziliense* (Furtado et al. 2018), and 200 in *A. caninum* (Furtado et al. 2014) and *N. americanus* (Diawara et al. 2013; Zuccherato et al. 2018) have been reported. In addition, although not fully understood, genotypes related to anthelmintic resistance may be accompanied by phenotypic alterations that influence other nematode characteristics, such as the survival rate of eggs in the environment and the strain pathogenicity (Prichard 2001). In this context, the use of selective drug pressure to obtain a strain resistant to one of the drugs commonly used to control hookworms would provide a useful model for determining resistance mechanisms and phenotypic characteristics. In the present study, a drug-resistant strain was obtained by selective drug pressure, and the parasitological and hematological parameters related to its infectiveness in a host were analyzed. Furthermore, a different resistance mechanism, other than the well-established presence of SNPs in the beta-tubulin isotype 1 gene of ruminant parasites, was implicated, as those SNPs were not identified in this albendazole-resistant strain.

Methods

Animal ethics

All animal procedures were approved by the Animal Care Ethics Committee at the Universidade Federal de Minas Gerais (protocol number 328/12) and were performed under the guidelines of the Brazilian Council of Animal Experimentation (CONCEA), strictly following the Brazilian law for "Procedures for the Scientific Use of Animals" (11.794/2008).

Selection of an albendazole-resistant *A. ceylanicum* strain

An albendazole-resistant hookworm strain was obtained by selective drug pressure. The nematode *A. ceylanicum* was used as an experimental model in 4 and 6-week-old female hamsters (*Mesocricetus auratus*) maintained in a controlled environment. The albendazole-resistant strain was isolated by serial infection passages, followed by treatment as described below.

The hamsters were divided into three experimental groups containing seven animals each. All animals were infected with 100 third-stage larvae (L3) of *A. ceylanicum* at day 0 of the experiment. The control group received only distilled water for each treatment, and the other two groups were administered different dosages of an albendazole suspension, orally, at 21 DPI (days post-infection). At 30 DPI, all experimental animals were euthanized.

In a previous pilot study, a dosage of 0.2 mg albendazole per animal (1 mg of albendazole/0.6 kg bodyweight) eliminated 100% of the infection. Thus, the dosages of albendazole used for selective pressure were less than 0.2 mg albendazole per animal. For each passage, the animals were divided into three groups: a control group, which received no treatment, and two other groups, which were treated with gradually increasing doses of albendazole. Larvae obtained from treated groups that were resistant to treatment were used for the next passage. If two treated groups in the same passage were resistant to treatment, the larvae obtained from the group administered the highest treatment dosage were used to inoculate the next group. These passages were performed until a group that no longer showed an elimination of the infection after receiving a dose of 0.2 mg, which was initially considered lethal, was obtained. From 12 DPI to the day of euthanasia, the number of eggs per gram of feces (EPG) was estimated by analyzing one fecal pool sample from each group. The collections were performed in cages with a bottom border on a collecting container. This quantification was performed every 2 days by using the McMaster chamber (Gordon and Whitlock 1939) to observe the course of the infection (data not shown). Figure 1 shows the scheme used to select an albendazole-resistant *A. ceylanicum* strain as well as the dosage of albendazole received by each group, illustrating that four passages were necessary to obtain an albendazole-resistant strain.

Infection of hamsters with *A. ceylanicum*

Coprocultures were performed according to Roberts and O'sullivan (1950) by using the feces of infected hamsters, and the larvae were recovered by the Baermann–Moraes method (Moraes 1948). The larvae were collected in 15-ml Falcon tubes and centrifuged at 2400×*g* for 5 min at room temperature. The supernatant was discarded, and 0.85% saline

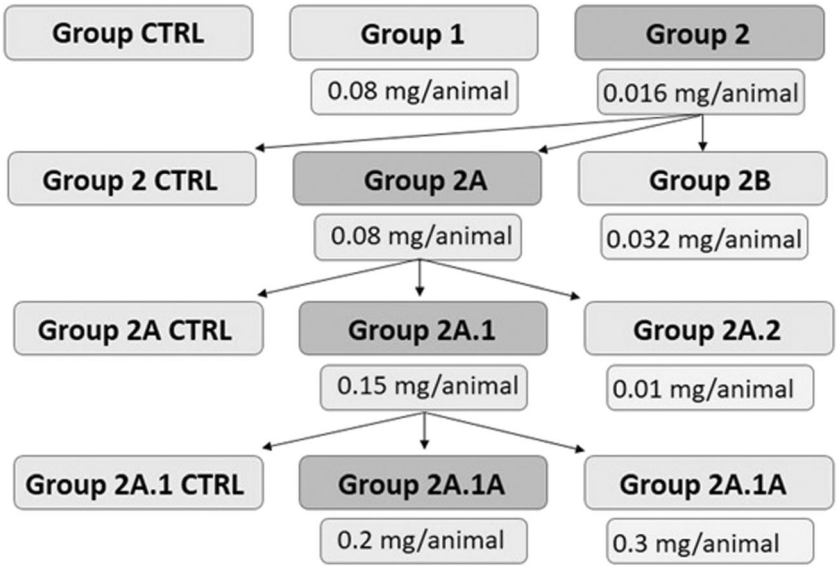

Fig. 1 Schematic illustration of the process used to select an albendazole-resistant *Ancylostoma ceylanicum* strain, indicating the albendazole dose received by each group

was added to a final volume of 10 ml. A stereomicroscope was used to observe motility, and the amount of L3 was estimated. The groups were infected orally with an average of 100 L3 by using a gavage needle.

Euthanasia, necropsy, and recovery of adult worms

Animal euthanasia was performed at 30 DPI via an intraperitoneal anesthetic overdose (45 mg/kg xylazine hydrochloride solution and 240 mg/kg ketamine). Each animal was then placed in a dorsal decubitus position, and the abdominal cavity was opened. The small intestine was removed and opened longitudinally, followed by scraping of the mucosa to detach the adult worms in a Petri dish with PBS (phosphate-buffered saline) solution, pH 7.4. The recovered worms were quantified and separated by sex.

Characterization of resistant and sensitive strains

The albendazole-resistant strain was selected, characterized, and compared to the sensitive strain. A new experiment was performed with three groups of hamsters (seven animals per group): an uninfected control group, a second group infected with the sensitive strain, and a third group infected with the albendazole-resistant strain. Each infected group received an inoculum of 75 L3, according to "Infection of hamsters with A. ceylanicum" section. The animals were euthanized at 25 DPI, according to "Euthanasia, necropsy, and recovery of adult worms" section.

Blood spoliation

At 0 and 25 DPI, two blood samples were collected from the orbital plexus of each animal by using a glass pipette after the application of an ocular anesthetic (proximetacaine hydrochloride 0.5%). One sample was collected with 0.5 M EDTA and the other sample was collected without anticoagulant. The EDTA blood sample was used for erythrogram evaluation, which was performed by using a volumetric impedance hematology analysis equipment (Abacus Júnior Vet, Austria). The EDTA-free blood sample was centrifuged at 14,000×*g* for 5 min, and the serum was used for iron quantification (Doles, Brazil).

Comparison of animal weight

Animal weight measurement of the groups was performed to compare infections of the resistant and sensitive strains. The weight of each animal was measured by using a semi-analytical balance on the day of infection and every 6 days thereafter until the day of euthanasia.

Comparison of the EPG, patency, and female fertility rate

Beginning at 12 DPI, the EPG was quantified every 2 days until the day of euthanasia, according to "Euthanasia, necropsy, and recovery of adult worms" section. This procedure was also performed to determine the patency between the two strains. On the day of euthanasia, stool was directly recovered from the rectum, and EPG determination was performed for each animal.

Female worms recovered from the intestine were placed in 12-well plates containing 10 ml of Roswell Park Memorial Institute (RPMI) culture medium and incubated in an oven for 24 h at 37 °C and 5% CO_2. Females from the same animal were placed in a single well. The worms were then removed from the plate, and the liquid from each well was transferred to a 15-ml Falcon tube and centrifuged at 10,000×*g* for 5 min.

The supernatant was discarded, the pellet was suspended in 1 ml of 10% formaldehyde, and all eggs present were stained with Lugol's iodine and quantified under an optical microscope. For the determination of the fertility rate, the total number of eggs in each well after 24 h of incubation was divided by the number of females present in the same well.

Comparison of hepcidin expression levels in the host

Total RNA extraction, treatment with DNase, and cDNA synthesis The left lobe of the liver from each hamster was collected and stored in RNA later (Thermo Fisher Scientific, USA) at − 20 °C until further use. The lobe removed from each animal used for the quantification of hepcidin gene expression was placed in a 2-ml Eppendorf tube individually, and total RNA was extracted with 1.0 ml of TRIzol (Invitrogen, USA) following the manufacturer's instructions. The quality and amount of extracted RNA were evaluated on 1% agarose gels and in an Epoch spectrophotometer apparatus (A260/280 and A260/230 ratios) (BioTek, USA). The extracted RNA was treated with DNase by using the TURBO DNA-free kit (Ambion, USA) to degrade trace amounts of genomic DNA. Subsequently, DNase was inactivated with the addition of the DNase inactivation reagent, and the RNA was again quantified by using an Epoch spectrophotometer apparatus (BioTek, USA). After treatment with DNase, total RNA was used as a template for cDNA synthesis with the High Capacity cDNA Reverse Transcription kit (Applied Biosystems, USA). Simultaneously, transcriptional controls were prepared without the reverse transcriptase enzyme as a negative control (RT −) to demonstrate the absence of genomic DNA.

Real-time PCR for hepcidin gene expression analysis All primers used in the present study were designed by using the Oligo Explorer 1.4 program (Gene link, USA) and are listed in Table 1. The primers used in the qPCR analysis were designed based on GenBank nucleotide sequences, under accession numbers XM_005081868.1 and XM_005085546.2 for the

Table 1 Primers designed in this work with their respective annealing temperatures and base substitutions (when applicable)

Primer (5′–3′)	Annealing temperature (°C)	Change
Fa167Ay: TGA GCT CGT CGA TAA CGT CC	57	
Fa198/200Ay: TGT TCC TAA AAA GGG GTC GGG	57	
Fc167Ay: CAG GTA TTT CGC AAC CGT GC	57	
Fc198/200Ay: GCA GTC CAC GTT CCT GCT TA	57	
FcDNA1: TTC TCG ACT GCA ATC ATG CG	56	
FcDNA2: ATC GGG TAT GGG AAC TCT C	54	
FHamp: CCT GTT TCT TGA TCC TCC TC	60	
FHPRT: TGG AGT GAT TAT GGA CAG GAC TGA	66	
Fsite198Ay: CTG TGC ACC AAT TGG TCG AGA ACA CAG **C**TG	64	A → C
Fsite200Ay: ACC AAT TGG TCG AGA ACA CAG ATG AGA C**G**T	63	C → G
Ftub1: TTC ATT TCT CGC GCT CGC T	57	
Ftub2: CAG TGG TGA ATC AGA TCT GC	54	
Ftub3: GCT TGT GAT CCT CGA CAT G	54	
Ra167Ay: TGA GGT CAT CCC CAG TTT GAC	57	
Ra198/200Ay: AAG CGA AGG CAG GTA GTG AC	57	
Rc167Ay: AGG AAC ACG ACC AGC GTT T	57	
Rc198/200Ay: ACC GGA CAT TGT TAC AGA CAC T	57	
RcDNA1: ACG GTG GTG GTC TAC TCC T	57	
RcDNA2: CAC GGA ACA TAG CAG CAA CA	55	
RcDNA3: ACG GTG GTG GTC TAC TCC T	56	
RHamp: CTG TAG TGC TTC AGG CTG TC	62	
RHPRT: ATG GCC TCC CAT CTC TTT CA	60	
Rm167Ay: AAC AGA G**T**A CGA GGA CAT AAT C	52	A → T
Rm198Ay: GAA GGT C**G**C ATC TGT GTT CTC	56	T → G
Rm200Ay: GAT ACA G**T**A GGT CTC ATC TGT	51	A → T
Rtub1: AAT TCT CTA CGT CGC ATC CT	54	
Rtub2: CAC GGA ACA TAG CAG CAA CA	56	

The bases that have been replaced are in bold

hepcidin and constitutive hypoxanthine phosphoribosyl transferase (HPRT) genes, respectively, both from the hamster species *Mesocricetus auratus*. Primers for *FHamp* and *RHamp* were used to amplify the hepcidin gene, producing an amplicon of 83 bp, and primers for *FHPRT* and *RHPRT* were used to amplify the constitutive HPRT gene, producing an amplicon of 64 bp.

The qPCR reactions were performed by using the Power SYBR Green PCR Master Mix Kit (Applied Biosystems, USA) and the StepOne Plus Real-Time PCR System (Applied Biosystems, USA) according to the manufacturer's instructions. The reactions were performed in triplicate in a final volume of 10 μl, which contained 2.0 μl of cDNA (20 ng of each sample), 0.3 μM of each primer, and 5.0 μl of SYBR Green. The amplification conditions were 95 °C for 10 min and 40 cycles of 95 °C for 15 s and 60 °C for 60 s. A negative control without the reverse transcriptase enzyme and a negative control without cDNA (replaced by Milli-Q water) were included in the plate to confirm the absence of genomic DNA amplification and to evaluate the presence of primer dimers or reagent contamination, respectively. The relative amount of target transcript in each sample was determined by using the $2^{-\Delta\Delta Ct}$ method (Livak and Schmittgen 2001).

Egg-hatch test for phenotypic resistance analysis of strains

On the day of euthanasia, approximately 3 g of feces was recovered from the end portion of the large intestine of each hamster. The feces were processed according to Sheather (1923) in a saturated sugar solution. The upper layer of the supernatant was transferred to a new 50-ml Falcon tube, which was filled with water and centrifuged at 14,000×*g* for 5 min. The sediment was recovered, analyzed under a microscope, and the *A. ceylanicum* egg concentration was adjusted to contain 50 eggs per 100 μl. The egg-hatch test was performed according to Humphries et al. (2013). Stock solutions of albendazole (Sigma, St. Louis, MO) were prepared in methanol (5 mg/ml) and diluted in distilled water. Egg suspensions (50 eggs/100 μl) isolated from each animal were added to the individual wells of a 96-well plate containing 100 μl of an albendazole solution (final concentrations = 0, 0.1, 1.0, 2.0, and 5 mg/ml). The plates were incubated at room temperature, and after 48 h, the number of hatched first-stage hookworm larvae was counted by using light microscopy.

Analysis of beta-tubulin isotype 1 gene codons 167, 198, and 200 in the albendazole-resistant *A. ceylanicum* strain

Although the *A. ceylanicum* genome is available in the NCBI database, it has been deposited as complete chromosome sequences. Therefore, to obtain the beta-tubulin cDNA sequence of this parasite, we performed a BLAST (https://blast.ncbi.nlm.nih.gov/Blast.cgi) search by using the beta-tubulin cDNA sequence from *A. caninum* (GenBank accession number DQ294930.1) against the *A. ceylanicum* genome. After the sequence was obtained, the alignment was edited to remove the intron sequences, according to canonical intron/exon nucleotide sites. Primers were designed based on this sequence (Table 1).

To standardize the molecular analyses, we first synthesized controls for the presence and absence of mutations for all codons, according to Furtado and Rabelo (2015). To construct a wild-type control allele for codon 167 (N167Ay), we performed an initial PCR amplification with the primers *Fa167Ay* and *Ra167Ay* (415 bp) by using genomic DNA from *A. ceylanicum*. Since codons 198 and 200 are close to each other in the genome, a single unmutated control was constructed for both codons by using the primers *Fa198/200Ay* and *Ra198/200Ay* (424 bp). Next, nested PCR was performed by using the primers *Fc167Ay* and *Rc167Ay* (306 bp, to codon 167) and the primers *Fc198/200Ay* and *Rc198/200Ay* (308 bp, to codons 198 and 200), and the obtained fragments were sequenced to confirm the absence of mutations in both amplified fragments. The fragments were subsequently cloned by using the pGEM™-T Easy Vector System (Promega, USA), transformed into XL1-blue™ cells (Phoneutria, Brazil), and recovered via miniprep (Wizard™ Plus Miniprep DNA Purification System; Promega, USA).

To construct mutated positive controls for codons 167 (M167Ay), 198 (M198Ay), and 200 (M200Ay), we performed site-directed mutagenesis by using the Megaprimer-PCR technique. Cloned N167Ay was used as a template for PCR amplification of codon 167 by using the primers *Fc167Ay* and *Rm167Ay* (176 bp), while cloned N198/200Ay was used as a template for PCR amplification of codons 198 and 200 by using the primer combinations *Fc198/200Ay* and *Rm198Ay* (132 bp) and *Fc198/200Ay* and *Rm200Ay* (138 bp), respectively. *Rm167Ay*, *Rm198Ay*, and *Rm200Ay* primers were designed to include a mismatch at position 8 of the 5′ end of the primer (see Table 1) to mimic the mutated sequence. The reaction products were subjected to electrophoresis on 1.0% agarose gels (*w*/*v*) (Midsci, USA) with 0.5× TAE buffer, and the gels were stained with GelRed™ (Biotium, USA). The fragments were gel excised and purified (Illustra GFX™ PCR DNA and Gel Band Purification Kit; GE Healthcare, UK), and their concentrations were determined. Approximately 20.0 ng of the first reaction product was used as a forward mega-primer in the second reaction in combination with the reverse *Rc167Ay* (306 bp, to codon 167) and *Rc198/200Ay* (308 bp to codon 198 and 200) primers. The products of the three codons were purified, sequenced, cloned, and recovered by using the same method as described for the negative controls. All PCR amplifications were performed by using GoTaq Green Master Mix (Promega, USA), with a final concentration of 0.2 μM for each primer. All amplification

steps were performed on a Mastercycler thermocycler (Eppendorf, Hamburg, Germany) according to the following program: 94 °C for 5 min; 30 cycles of 94 °C for 1 min, 57 °C for 1 min, and 72 °C for 1 min; and 72 °C for 8 min. In all amplification runs, a "blank" sample was included in which the DNA was replaced with water.

For the analysis of SNPs in codons 167, 198, and 200 of the beta-tubulin isotype 1 gene of *A. ceylanicum*, the restriction fragment length polymorphism-PCR (RFLP-PCR) technique was performed. The sequences were analyzed by using the NEBcutter V2.0 tool (http://www.labtools.us/nebcutter-v2-0/). The mutation at codon 167 created a site for the enzyme *RsaI*. Therefore, this enzyme was used to evaluate this codon by RFLP-PCR. However, there were no restriction sites that could differentiate the mutated from unmutated codons corresponding to amino acids 198 and 200. Thus, for the analysis of codons 198 and 200, site-directed mutagenesis was required to create a site for the restriction enzymes *DdeI* and *RsaI* to differentiate the respective genotypes by RFLP-PCR.

To evaluate the three codons that are commonly associated with resistance to benzimidazoles, we extracted DNA from 50 male worms of the albendazole-resistant *A. ceylanicum* strain according to a protocol described by Miranda et al. (2008) and Rabelo et al. (2017). For codon 167, an initial PCR using primers *Fa167Ay* and *Ra167Ay* (415 bp) was performed, followed by nested PCR with the primers *Fc167Ay* and *Rc167Ay* (306 bp). For codons 198 and 200, an initial PCR with the primers *Fa198/200Ay* and *Ra198/200Ay* (415 bp) was performed. For the introduction of the restriction sites, primers for the nested reaction were designed with an alteration in position 3 from the 3′ end for codon 198, which, in association with the remaining sequence, generated a site for the restriction enzyme *DdeI* for the unmutated allele. For codon 200, a mismatch was introduced in the primer *Fsite200Ay* at position 2 from the 3′ end that, in combination with the remaining sequence, generated an extra restriction site for the enzyme *RsaI* for the mutated allele. Figure 2 shows a representation of the methodology used for codons 198 and 200. From the product of this first reaction, nested PCR was performed for codon 198 with the primers *Fsite198Ay* and *Rc198/200Ay* (214 bp), and another nested PCR was performed for codon 200 with the primers *Fsite200Ay* and *Rc198/200Ay* (208 bp). The products of these three reactions were subjected to 1.0% (*w/v*) agarose gel electrophoresis (Midsci, USA) by using 0.5× TAE buffer and GelRed® staining (Biotium, USA) for confirmation of the amplicon sizes. Restriction enzyme digestion for analysis of all of the codons was performed with 5 U (codons 167 and 200) and 2 U (codon 198) of each enzyme, in individual reactions, according to the manufacturer's instructions. After digestion, the products were subjected to 15.0% (*w/v*) polyacrylamide gel electrophoresis in 0.5× TBE buffer, followed by GelRed® staining (Biotium, USA). The expected fragment sizes after the digestion of each genotype are listed in Table 2.

Construction of a beta-tubulin isotype 1 gene cDNA mini-library for *A. ceylanicum*

To evaluate other potential SNPs in the *A. ceylanicum* beta-tubulin isotype 1 gene, we constructed a cDNA mini-library. RNA was extracted from a pool of 100 male *A. ceylanicum* worms obtained from seven hamsters experimentally infected with the resistant strain. RNA extraction, treatment with DNase, and cDNA synthesis was performed as described in "Comparison of hepcidin expression levels in the host" section. After cDNA synthesis, an initial PCR with the primers *FcDNA1* and *RcDNA1* (1.373 bp) was performed to amplify the entire gene of interest. The conditions and reaction volumes of all PCRs were the same as those described

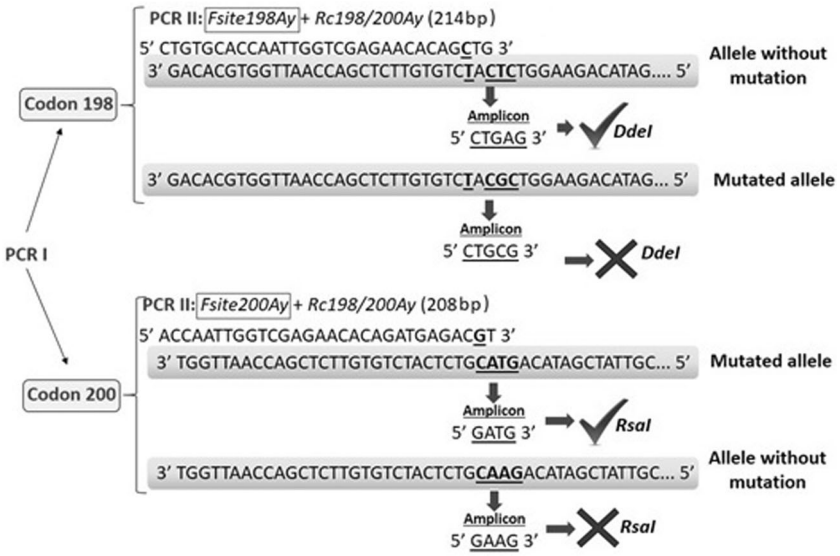

Fig. 2 Representation of the methodology used to analyze codons 198 and 200 of the beta-tubulin isotype 1 gene from *Ancylostoma ceylanicum*

Table 2 Amplicon sizes after RFLP-PCR for the analysis of codons 167, 198, and 200 of the β-tubulin isotype 1 gene of *Ancylostoma ceylanicum*

Codon	Wild-type	Mutated
167	162 + 95 + 49	138 + 95 + 49 + 24
198	186 + 28	214
200	137 + 71	137 + 41 + 30

in "Analysis of beta-tubulin isotype 1 gene codons 167, 198, and 200 in the albendazole-resistant *A. ceylanicum* strain" section. The product was subjected to 1.0% (*w*/*v*) agarose gel electrophoresis (Midsci, USA) by using 0.5× TAE buffer and GelRed® staining (Biotium, USA) for confirmation of the amplicon size. After confirmation, cloning was performed by using the pGEM-T Easy Vector System (Promega), followed by transformation into XL1-blue cells (Phoneutria Bio, Brazil) and recovery via miniprep (Wizard Plus Miniprep DNA Purification System; Promega). Sanger sequencing was performed for 15 clones (Sanger 1977), as described by Mello et al. (2018). As the gene is long (1373 bp), four primers were used to cover the entire region of interest. External primers, which annealed to the plasmid [M13 forward (5′-GTA AAA CGA CGG CCA G-3′) and reverse (5′-GGA AAC AGC TAT GAC CAT G-3′)], and internal primers *FcDNA2* and *RcDNA2* were used. After generating the cDNA sequences, the amino acid sequences were obtained by using the Transeq tool (www.ebi.ac.uk/Tools/st/emboss_transeq/). The amino acid sequences obtained from the albendazole-resistant strain were compared to the reference sequence by using the Clustal Omega tool (www.ebi.ac.uk/Tools/msa/clustalo/). Moreover, the complete sequence of the beta-tubulin isotype 1 gene was obtained for five individual worms of the wild-type *A. ceylanicum* strain. For the sequencing of the exonic region, an initial PCR with the *Ftub1* and *Ra167Ay* (1326 bp) primers was performed on the same DNA sample, followed by secondary reactions with the primers *Ftub1* and *Rtub1* (712 bp) and *Ftub2* and *Rc167Ay* (760 bp). For the second part of the gene, an initial PCR was performed using primers *Fa198/200Ay* and *RcDNA3* (1370 bp), followed by secondary reactions with *Fc198/200Ay* and *Rtub2* (628 bp) and *Ftub3* and *RcDNA3* (698 bp). The products of these PCRs were purified (Illustra GFX™ PCR DNA and Gel Band Purification Kit; GE Healthcare, UK) and Sanger sequenced (1977).

Statistical analysis

Statistical analysis was performed by using Graph Pad Prism 5 software. The Kolmogorov–Smirnov test was used to verify the data distribution. Analyses between two groups were performed by using paired or unpaired *t* tests (parametric data) statistical methods. For analyses of three or more groups, the data were subjected to ANOVA or repeated-measures ANOVA, followed by Tukey's test (parametric data). Grubbs' test was used to detect outliers. All tests were considered significant when they presented a value of $p \leq 0.05$.

Results

Analysis of beta-tubulin isotype 1 gene codons 167, 198, and 200 in *A. ceylanicum*

The analysis of beta-tubulin isotype 1 gene codons 167, 198, and 200 in 100 individual worms by RFLP-PCR showed no mutated codon in any of the analyzed samples. Figure 3 shows a representative polyacrylamide gel image of the RFLP-PCR.

Analysis of the mini-library of the beta-tubulin isotype 1 gene from *A. ceylanicum*

Fifteen clones from a beta-tubulin isotype 1 gene cDNA library from an albendazole-resistant *A. ceylanicum* strain were sequenced, and two mutated samples were observed, one of which had two mutations. One sample with a single mutation presented an alteration at codon 177, an exchange of an adenine with a guanine (leading to the substitution of an aspartic acid (GAC) with glycine (GGC)). One sample with two mutations presented alterations at codons 110 and 390, with both mutations showing a cytosine-to-thymine substitution (resulting in the replacement of alanine (GCT) with valine (GTT) at codon 110 and arginine (CGC) with cysteine (TGC) at codon 390). Sequencing of the full beta-tubulin isotype 1 gene showed that three of the five samples from the sensitive strain analyzed showed a change that did not occur in either the albendazole-resistant strain or wild-type samples. None of the polymorphisms were associated with albendazole resistance.

Characterization of albendazole-resistant and sensitive *A. ceylanicum* strains

The two strains were compared with respect to their parasitological characteristics (Fig. 4). The patency of the two strains occurred at 14 DPI. However, for all time points analyzed, the sensitive strain released a larger number of eggs than the albendazole-resistant strain (Fig. 4a). No statistical analysis was performed because the stool collection was performed by group. However, on the day of euthanasia, EPG determination was performed with feces obtained directly from the rectum of each hamster, enabling the observation of significant differences in the EPG between the two strains (Fig. 4b).

There were more male and female worms recovered from the euthanized animals inoculated with the L3 sensitive strain than those inoculated with the albendazole-resistant strain (Fig. 4c), and consequently, the highest number of total worms was recovered from the wild-type strain (Fig. 4d). However,

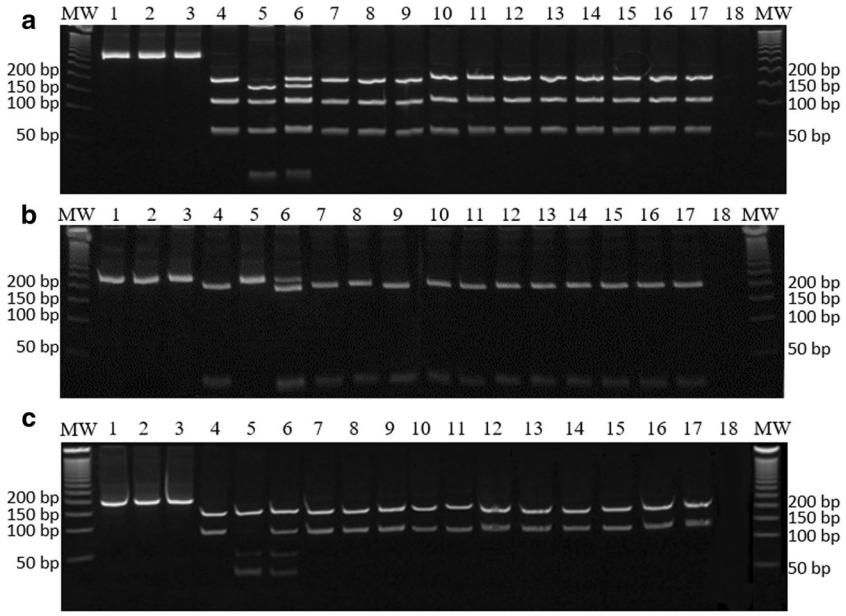

Fig. 3 Representative RFLP-PCR results from the analysis of codons 167 (**a**), 198 (**b**), and 200 (**c**) of the *Ancylostoma ceylanicum* beta-tubulin isotype 1 gene using the *RsaI* enzyme for codons 167 and 200 and the *DdeI* for codon 198. Lanes *1* to *3* contain undigested PCR products using the plasmid controls (*1* unmutated plasmid, *2* mutated plasmid, *3* mutated and unmutated plasmid mix). Lanes *4* to *6* contain PCR digestion products using the control plasmids (*4* unmutated plasmid, *2* mutated plasmid, *3* mutated and unmutated plasmid mix). Lanes *7* to *17* contain PCR products using DNA from *A. ceylanicum*. Lane *18* is the reaction blank. Each image is a polyacrylamide gel (15%) that was stained with GelRed™ (Biotium, USA). MW: 50-bp molecular weight ladder. Expected fragment sizes: codon 167—unmutated: 162, 95, and 49 bp; mutated: 138, 95, 49, and 24 bp; codon 198—unmutated: 186 and 28 bp; mutated: 214 bp; and codon 200—unmutated: 137 and 71 bp; mutated: 137, 41, and 30 bp

the females from both strains had a similar fertility rate (data not shown). The egg-hatch test showed that at concentrations of 0.1, 1, and 2 μg/ml albendazole, there was greater survival of larvae coming from the albendazole-resistant strain group (Fig. 4e).

The results of the erythrogram (Fig. 5) showed a significant difference for the total erythrocyte count, hemoglobin, hematocrit, mean corpuscular volume (VCM), and corpuscular hemoglobin concentration (CHCM) parameters between the infected groups and the uninfected control group, but no differences were observed between groups infected with wild-type and albendazole-resistant strains. No significant differences were observed between any of the groups for the red cell width distribution (RDW) or platelet parameters.

Although there were no significant differences between the infected groups with respect to their red blood cell parameters, other more sensitive markers, such as the expression analysis of the hepcidin hormone in the host, could potentially show a difference in the spoliation between the strains since differences in worm recovery were observed. This hormone is considered a biomarker for the regulation of iron hemostasis in the host, so that when the oxygen supply is inadequate, as occurs in anemia, there is a decrease in the level of hepcidin expression (Meynard et al. 2014). The expression levels of hepcidin in the livers of uninfected control hamsters as well as in those infected with the susceptible strain and the albendazole-resistant strain at 25 DPI are shown in Fig. 6a. Even with the use of this marker, no difference was observed between the two infected groups; however, a difference was observed between the infected groups and the uninfected control group, in accordance with the findings of the erythrocyte series. Once hookworms feed on blood, serum iron stores are consumed by the worms. Figure 6b shows the serum iron concentrations of uninfected control hamsters as well as of those infected with the susceptible strain and the albendazole-resistant *A. ceylanicum* strain at 25 DPI; no differences were observed between the two infected groups, but a difference was observed between the infected groups and the uninfected control group, consistent with the results found for the hepcidin and erythrocyte analyses.

The bodyweight variation of groups shows that there were no differences between the two infected groups; however, there was a difference between the infected groups and the uninfected control group beginning at 12 DPI (data not shown).

Discussion

Treatment with anthelmintic is the main means of helminth control in general; however, the indiscriminate use of these drugs selects for naturally resistant parasites in the target population that are capable of surviving exposure to the drug and can produce resistant offspring strains (Elard et al. 1998;

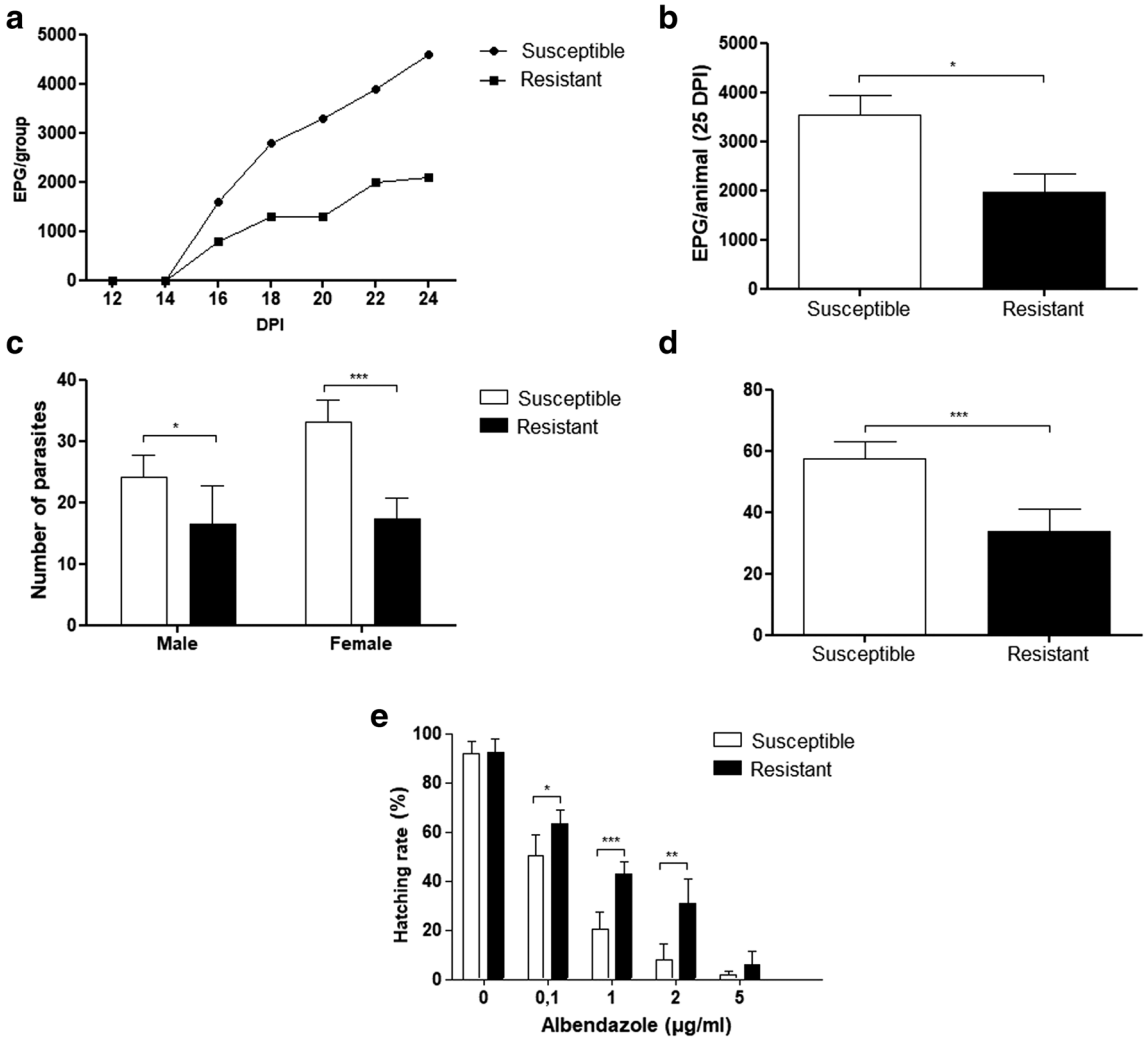

Fig. 4 Parasitological comparison of the albendazole-susceptible and albendazole-resistant *Ancylostoma ceylanicum* strains. (**a**) Comparison of the number of eggs per gram of feces (EPG) analyzed in pool from groups of hamsters infected with the susceptible and resistant strains, from the 12 DPI (days post-infection) to 24 DPI. (**b**) Comparison of the number of eggs per gram of feces (EPG) collected from the rectum of each individual animal. (**c**) Numbers of male and female worms per group. (**d**) Total number of worms per group. (**e**) Egg-hatch test. $N = 7$. $^{*}p < 0.05$; $^{**}p < 0.01$; $^{***}p < 0.001$. CTRL = control

Prichard et al. 2012). The occurrence of benzimidazole resistance has been associated with SNPs in codons 167, 198, and 200 of the beta-tubulin isotype 1 gene of several helminths (Kwa et al. 1994; Prichard 2007). In the present study, we selected a hookworm strain resistant to a benzimidazole and performed genotypic and phenotypic characterizations.

The absence of SNPs commonly related to benzimidazole resistance in the selected albendazole-resistant strain suggests the involvement of other mechanisms in the benzimidazole resistance process of hookworms (Furtado et al. 2016). Indeed, SNPs in other regions of the beta-tubulin isotype 1 gene have been associated with resistance to benzimidazoles in fungi (Robinson et al. 2004; Ma et al. 2005). These changes may occur in nematodes, given that the drug has the same mechanism of action in different species. In addition, the absence of resistance-linked SNPs in members of the Ancylostomatidae family isolated from patients who experienced treatment failure (Albonico et al. 2004) supports the hypothesis that other SNPs or even other mechanisms are involved in this process.

Some non-synonymous mutations were found via a cDNA analysis of the albendazole-resistant strain samples; however, these SNPs were not reproducible in different samples. Thus, although it seems unlikely that the polymorphisms identified through the mini-library analysis are related to albendazole resistance, this possibility cannot be ruled out. However, the low frequency and lack of reproducibility of these SNPs suggest that if these polymorphisms are related to the process, they are not the main mechanism of resistance in *A. ceylanicum*. The presence of other SNPs in the gene from the sensitive strain corroborates the idea that these

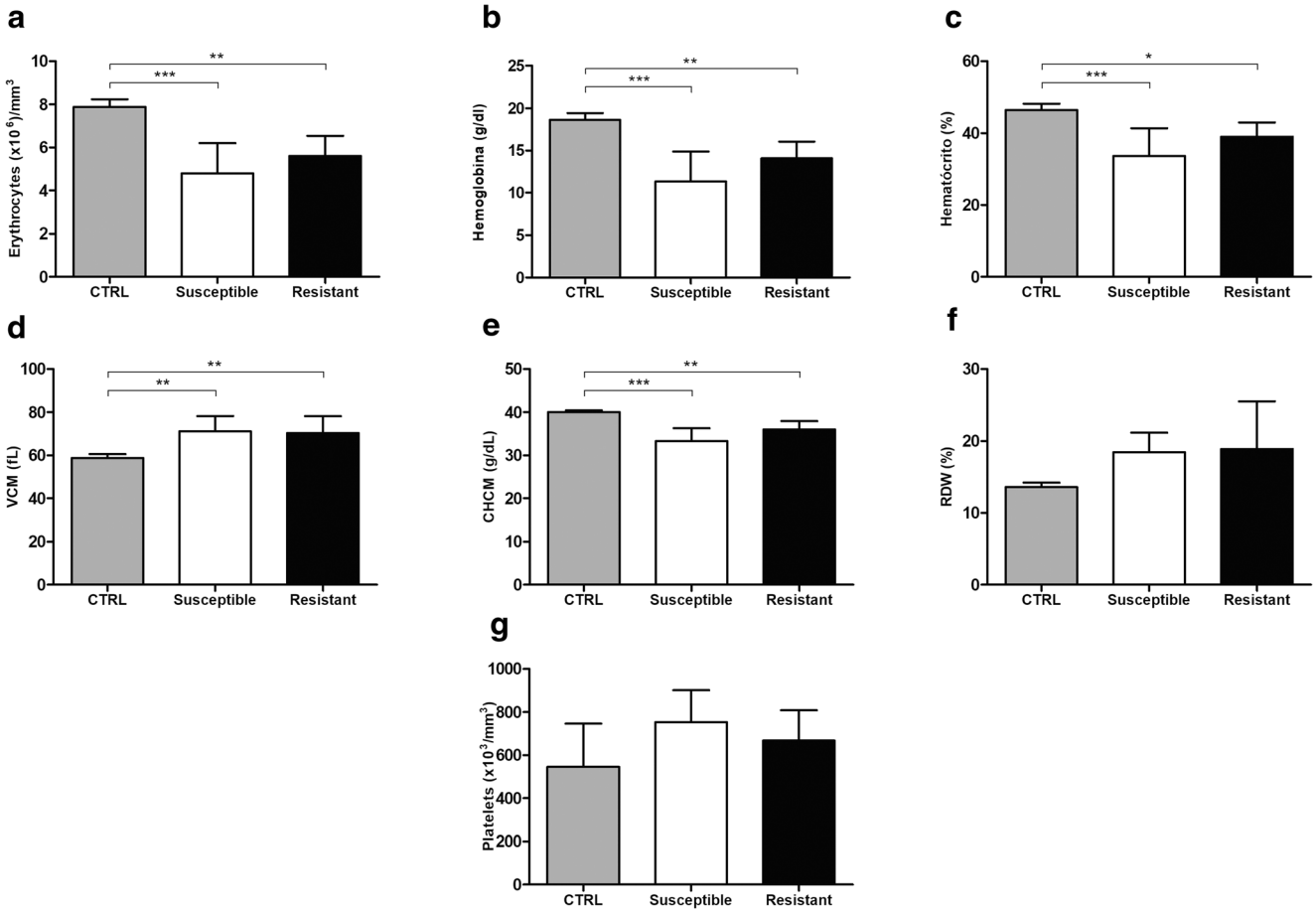

Fig. 5 Erythrogram performed on the 25th day post-infection in the uninfected control group, infected with the susceptible strain and infected with the resistant *Ancylostoma ceylanicum* strain, with total erythrocyte count (**a**), hemoglobin (**b**), hematocrit (**c**), mean corpuscular volume (VCM) (**d**), mean corpuscular hemoglobin concentration (CHCM) (**e**), red cell width distribution (RDW) (**f**), and platelet count (**g**). Each bar represents the mean ± standard deviation of the group ($n = 7$). *$p < 0.05$; **$p < 0.01$; ***$p < 0.001$. CTRL = control

SNPs are not related to the resistance process; rather, these alterations may constitute random mutations or even amplification mistakes.

Blackhall et al. (2008) observed polymorphisms in a gene encoding a P-glycoprotein, a cell membrane transport protein with a high affinity for ivermectin from a thiabendazole-resistant *Haemonchus contortus* strain. These authors suggested that other mechanisms independent of the drug-binding target may be involved in the anthelmintic resistance process. In tumor cells, a specific type of post-transcriptional regulation, including altered microRNA expression or activity (miRNA), has been increasingly implicated in drug resistance

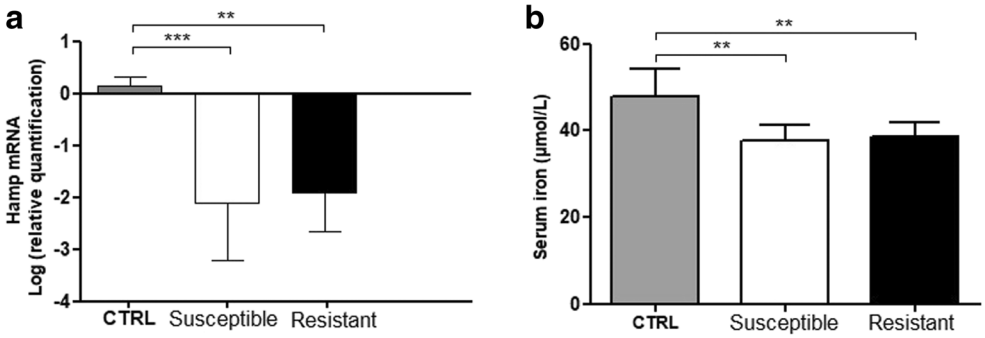

Fig. 6 Comparison of iron spoliation between albendazole-susceptible and albendazole-resistant *A. ceylanicum* strain. (**a**) Expression levels of hepcidin in the livers of uninfected control hamsters as well as in those infected with the susceptible strain and the resistant strain at 25 days post-infection. (**b**) Serum iron concentrations at 25 days post-infection in the uninfected control group as well as in hamsters infected with the tsusceptible strain and the resistant strain. Each bar represents the mean ± standard deviation of the group ($n = 7$). **$p < 0.01$. CTRL = control

(Devaney et al. 2010). As noted by Simon (2008), miRNAs in *Caenorhabditis elegans* may play a role in drug resistance mechanisms. Since drug resistance in nematodes may result from a variety of different mechanisms, such as altered levels of target or non-target gene expression, including drug transporters or enzymes, changes in P-glycoprotein genes or changes related to miRNAs are likely present in the albendazole-resistant strain selected in the present study; further studies are needed to corroborate these hypotheses. Other studies evaluating parasites of veterinary importance, such as *Teladorsagia circumcincta*, did not detect mutations in the canonical SNPs related to resistance that underwent treatment (Martínez-Valladares et al. 2012; Esteban-Ballesteros et al. 2017). These data support the idea of the involvement of another mechanism in the process of resistance to treatment, independent of the drug-binding target.

Kelly et al. (1978) suggest that there are physiological differences between resistant and sensitive strains, highlighting the possibility that albendazole-resistant strains may present heightened pathogenic characteristics. However, the results observed in the present study do not support this hypothesis. Considering the number of worms recovered for each strain, the low recovery observed for the albendazole-resistant strain indicates that these worms were somewhat less adapted to the host, which contradicts the findings of Leignel and Cabaret (2001), who observed no significant differences in the survival of resistant and sensitive adult worms of *T. circumcincta*. Prichard (2001) observed that resistant strains of *C. elegans* have dysfunctions in motility.

The higher recovery of sensitive strain worms could indicate an increased spoliation in the animals infected with this strain, which would consequently lead to lower bodyweight increases. However, no significant differences in bodyweight were observed between the two infected groups, the resistant and sensitive strains, at any point during the infection, but a difference was observed between the two infected groups compared to the uninfected control group. Serafim et al. (2014) observed a difference in bodyweight between the uninfected control and the group infected with an inoculum of 75 *A. ceylanicum* L3 in hamsters. Although the degree of pathogenesis is directly proportional to the number of parasites harbored (Hotez et al. 2006), the difference in the number of worms recovered between the susceptible and resistant strains was not sufficient to result in a bodyweight difference between the two groups.

Since the number of EPG was lower in hamsters infected with the albendazole-resistant strain, a lower rate of recovery of albendazole-resistant strain worms was expected. In addition, there was no significant difference in the female fecundity rate between the two strains. Maingi et al. (1990) also observed that a benzimidazole-susceptible *H. contortus* strain had a higher net egg output; however, the per-capita egg output was also equal between these worms and a resistant strain. This finding is in contrast with the results of Prichard (2001), who found that resistant *C. elegans* strains exhibit dysfunctional oviposition.

For the hematological analyses, significant differences were not observed between the groups infected with the albendazole-resistant strain and those infected with the sensitive strain for the hemoglobin, hematocrit, and total erythrocyte number parameters; however, a difference was observed between the infected groups and the control uninfected group. Hookworms feed on blood and consequently iron, and these organisms constantly change their positions in the small intestine, resulting in lacerations and blood loss (Loukas and Prociv 2001). Although there was a difference in the number of worms recovered for each of the infected groups, this difference was not sufficient to cause disparity in the hemoglobin, hematocrit, total erythrocyte number, serum iron concentration, or hepcidin expression levels. Thus, in terms of pathogenicity, these two strains present a similar profile, contrary to the findings of Kelly et al. (1978), who observed differences in the pathogenicity of benzimidazole-susceptible and benzimidazole-resistant *H. contortus* strains.

In the egg-hatch test, albendazole concentrations of 0.1, 1.0, and 2.0 mg/ml resulted in significant differences between the two strains, suggesting that the process of obtaining the albendazole-resistant strain was successful. In addition, the standardization of this test for *A. ceylanicum* is important for the detection of resistance in this species.

Conclusion

Although there have been several reports of treatment failure for hookworm infections, the genetic basis of benzimidazole resistance has not been elucidated. Despite the successful selection of an albendazole-resistant *A. ceylanicum* strain, the molecular analyses performed herein did not determine the mechanisms involved in this process. SNPs commonly associated with benzimidazole resistance in other helminths were not found in the beta-tubulin isotype 1 gene of the selected strain. In addition, through a mini-library analysis, other SNPs present in this gene were identified. Are these SNPs involved in the resistance process in hookworms? Is the genetic diversity of P-glycoprotein involved in this process? Could variations in gene expression or miRNA activity be involved in hookworm benzimidazole resistance? To answer these questions, new studies involving molecular analyses of new targets must be performed, which may help determine the molecular mechanisms involved in resistance. Phenotypic analyses of the albendazole-resistant strain in the present study facilitated the examination of the parasite–host relationship as well as the biology of the strain as a whole, suggesting that this strain is less adapted to the host. This same situation may occur in humans as well, but further studies are needed to verify this idea.

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References

Albonico M, Wright V, Bickle Q (2004) Molecular analysis of the beta-tubulin gene of human hookworms as a basis for possible benzimidazole resistance on Pemba Island. Mol Biochem Parasitol 134(2): 281–284. https://doi.org/10.1016/j.molbiopara.2003.12.008

Blackhall WJ, Prichard RK, Beech RN (2008) P-Glycoprotein selection in strains of *Haemonchus contortus* resistant to benzimidazoles. Vet Parasitol 152(1–2):101–107. https://doi.org/10.1016/j.vetpar.2007.12.001

Devaney E, Winter AD, Britton C (2010) microRNAs: a role in drug resistance in parasitic nematodes? Trends Parasitol 26(9):428–433. https://doi.org/10.1016/j.pt.2010.05.003

Diawara A, Halpenny CM, Churcher TS, Mwandawiro C, Kihara J, Kaplan RM, Streit TG, Idaghdour Y, Scott ME, Basáñez MG, Prichard RK (2013) Association between response to albendazole treatment and β-tubulin genotype frequencies in soil-transmitted helminths. PLoS Negl Trop Dis 7(5):e2247. https://doi.org/10.1371/journal.pntd.0002247

Elard L, Sauve C, Humbert JF (1998) Fitness of benzimidazole-resistant and -susceptible worms of *Teladorsagia circumcincta*, a nematode parasite of small ruminants. Parasitology 117:571–578. https://doi.org/10.1017/S0031182098003436

Esteban-Ballesteros M, Rojo-Vázquez FA, Skuce PJ, Melville L, González-Lanza C, Martínez-Valladares M (2017) Quantification of resistant alleles in the β-tubulin gene of field strains of gastrointestinal nematodes and their relation with the faecal egg count reduction test. BMC Vet Res 13(1):71. https://doi.org/10.1186/s12917-017-0992-9.

Furtado LF, Rabelo EM (2015) Development of a new amplification-refractory mutation system for detection of a single nucleotide polymorphism linked to drug resistance in *Ancylostoma caninum*. Genet Mol Res 14(2):5103–5111. https://doi.org/10.4238/2015.May.12.13

Furtado LF, Bello AC, dos Santos HA, Carvalho MR, Rabelo ÉM (2014) First identification of the F200Y SNP in the β-tubulin gene linked to benzimidazole resistance in *Ancylostoma caninum*. Vet Parasitol 206(3–4):313–316. https://doi.org/10.1016/j.vetpar.2014.10.021

Furtado LF, de Paiva Bello AC, Rabelo ÉM (2016) Benzimidazole resistance in helminths: from problem to diagnosis. Acta Trop 162:95–102. https://doi.org/10.1016/j.actatropica.2016.06.021

Furtado LFV, Alves WP, Moreira TB, Costa LM Junior, Miranda RRC, Rabelo EML (2016) Standardization and application of the tetraprimer ARMS-PCR technique for screening of the E198A SNP in the betatubulin gene of hookworm populations in Brazil. Vet Parasitol 224:65–67. https://doi.org/10.1016/j.vetpar.2016.05.013P

Furtado LFV, Magalhães JGS, Rabelo ÉML (2018) Standardization and application of a modified RFLP-PCR methodology for analysis of polymorphisms linked to treatment resistance in *Ancylostoma braziliense*. Parasit Vectors 11(1):540. https://doi.org/10.1186/s13071-018-3125-9

Ghisi M, Kaminsky R, Mäser P (2007) Phenotyping and genotyping of *Haemonchus contortus* isolates reveals a new putative candidate mutation for benzimidazole resistance in nematodes. Vet Parasitol 144:313–320. https://doi.org/10.1016/j.vetpar.2006.10.003

Gordon H, Whitlock HV (1939) A new technique for counting nematode eggs in sheep feces. J Counc Sci Ind Res 12:50–52

Hasslinger MA (1986) Helminths of carnivores relevant to veterinary practice. Tierarztl Prax 14(2):265–273

Hotez PJ, Bethony J, Bottazzi ME, Brooker S, Diemert D, Loukas A (2006) New technologies for the control of human hookworm infection. Trends Parasitol 22:327–331. https://doi.org/10.1016/j.pt.2006.05.004

Humphries D, Simms BT, Davey D, Otchere J, Quagraine J, Terryah S, Newton S, Berg E, Harrison LM, Boakye D, Wilson M, Cappello M (2013) Hookworm infection among school age children in Kintampo north municipality, Ghana: nutritional risk factors and response to albendazole treatment. Am J Trop Med Hyg 89(3): 540–548. https://doi.org/10.4269/ajtmh.12-0605

Humphries D, Nguyen S, Kumar S, Quagraine JE, Otchere J, Harrison LM, Wilson M, Cappello M (2017) Effectiveness of albendazole for hookworm varies widely by community and correlates with nutritional factors: a cross-sectional study of school-age children in Ghana. Am J Trop Med Hyg 96(2):347–354. https://doi.org/10.4269/ajtmh.16-0682

Kelly JD, Whitlock HV, Thompson HG, Hall CA, Martin IC, Le Jambre LF (1978) Physiological characteristics of free-living and parasitic stages of strains of *Haemonchus contortus*, susceptible or resistant to benzimidazole anthelmintics. Res Vet Sci 25(3):376–385

Kwa MS, Veenstra JG, Roos MH (1994) Benzimidazole resistance in *Haemonchus contortus* is correlated with a conserved mutation at amino acid 200 in beta-tubulin isotype 1. Mol Biochem Parasitol 63(2):299–303

Leignel V, Cabaret J (2001) Massive use of chemotherapy influences life traits of parasitic nematodes in domestic ruminants. Funct Ecol 15: 569–574. https://doi.org/10.1046/j.0269-8463.2001.00567.x

Livak KJ, Schmittgen TD (2001) Analysis of relative gene expression data using real-time quantitative PCR and the 2(−Delta Delta C(T)) method. Methods 25:402–408. https://doi.org/10.1006/meth.2001.1262

Loukas A, Prociv P (2001) Immune responses in hookworm infections. Clin Microbiol 14(4):689–703. https://doi.org/10.1128/CMR.14.4.689-703.2001

Loukas A, Hotez PJ, Diemert D, Yazdanbakhsh M, McCarthy JS, Correa-Oliveira R, Croese J, Bethony JM (2016) Hookworm infection. Nat Rev Dis Primers 2:16088. https://doi.org/10.1038/nrdp.2016.88

Ma Z, Yoshimura MA, Holtz BA, Michailides TJ (2005) Characterization and PCR-based detection of benzimidazole-resistant isolates of *Monilinia laxa* in California. Pest Manag Sci 61:449–457. https://doi.org/10.1002/ps.982

Maingi N, Scott ME, Prichard RK (1990) Effect of selection pressure for thiabendazole resistance on fitness of *Haemonchus contortus* in sheep. Parasitology 2:327–335. https://doi.org/10.1017/S0031182000061345

Martínez-Valladares M, Donnan A, Geldhof P, Jackson F, Rojo-Vázquez FA, Skuce P (2012) Pyrosequencing analysis of the beta-tubulin gene in Spanish *Teladorsagia circumcincta* field isolates. Vet Parasitol 184(2–4):371–376. https://doi.org/10.1016/j.vetpar.2011.09.009

Mello ÉM, Furtado LFV, Rabelo ÉML, Pinto HA (2018) DNA barcoding of metacestodes found in the *Guerlinguetus ingrami* (Rodentia: Sciuridae) reveals the occurrence of *Hydatigera taeniaeformis* sensu stricto (Cyclophyllidea: Taeniidae) in the Americas. Parasitol Int 67 (2):115–18. https://doi.org/10.1016/j.parint.2017.10.005

Meynard D, Babitt JL, Lin HY (2014) The liver: conductor of systemic iron balance. Blood 123(2):168–176. https://doi.org/10.1182/blood-2013-06-427757

Miranda RR, Tennessen JA, Blouin MS, Rabelo EM (2008) Mitochondrial DNA variation of the dog hookworm *Ancylostoma*

caninum in Brazilian populations. Vet Parasitol 151(1):61–67. https://doi.org/10.1016/j.vetpar.2007.09.027

Moraes RG (1948) Contribuição para o estudo do *Strongyloides stercoralis* e da estrongiloidose no Brasil. Rev Serv Esp Saude Publica 1:507–624

Murray CJ, Vos T, Lozano R, Naghavi M, Flaxman AD et al (2012) Disability-adjusted life years (DALYs) for 291 diseases and injuries in 21 regions, 1990–2010: a systematic analysis for the Global Burden of Disease Study 2010. Lancet 380:2197–2223. https://doi.org/10.1016/S0140-6736(12)61689-4

Pacanaro CP, Dias SR, Serafim LR, Costa MP, Aguilar E, Paes PR, Alvarez-Leite JI, Rabelo EM (2014) Evaluation of biochemical, hematological and parasitological parameters of protein-deficient hamsters infected with *Ancylostoma ceylanicum*. PLoS Negl Trop Dis 8(9):e3184. https://doi.org/10.1371/journal.pntd.0003184

Prichard RK (2001) Genetic variability following selection of *Haemonchus contortus* with anthelmintics. Trends Parasitol 17: 445–453. https://doi.org/10.1016/S1471-4922(01)01983-3

Prichard RK (2007) Markers for benzimidazole resistance in human parasitic nematodes? Parasitology 134(8):1087–1092. https://doi.org/10.1017/S003118200700008X

Prichard RK, Basáñez MG, Boatin BA, McCarthy JS, García HH, Yang GJ, Sripa B, Lustigman S (2012) A research agenda for helminth diseases of humans: intervention for control and elimination. PLoS Negl Trop Dis 6(4):e1549. https://doi.org/10.1371/journal.pntd.0001603

Rabelo ÉM, Miranda RR, Furtado LF, Redondo RA, Tennessen JA, Blouin MS (2017) Development of new microsatellites for the hookworm *Ancylostoma caninum* and analysis of genetic diversity in Brazilian populations. Infect Genet Evol 51:24–27. https://doi.org/10.1016/j.meegid.2017.03.008

Rashwan N, Bourguinat C, Keller K, Gunawardena NK, de Silva N, Prichard R (2016) Isothermal diagnostic assays for monitoring single nucleotide polymorphisms in *Necator americanus* associated with benzimidazole drug resistance. PLoS Negl Trop Dis 10(12): e0005113. https://doi.org/10.1371/journal.pntd.0005113

Roberts FHS, O'sullivan PJ (1950) Methods for egg counts and larval cultures for strongyles infecting the gastro-intestinal tract of cattle. Aust J Agric Res 1:99–102. https://doi.org/10.1071/AR9500099

Robinson MW, McFerran N, Trudgett A, Hoey L, Fairweather I (2004) A possible model of benzimidazole binding to beta-tubulin disclosed by invoking an inter-domain movement. J Mol Graph Model 23: 275–284. https://doi.org/10.1016/j.jmgm.2004.08.001

Sanger F, Nicklen S, Coulson AR (1977) DNA sequencing with chain-terminating inhibitors. Proc Natl Acad Sci 74:5463–5467

Serafim LR, da Silva JP, de Paiva NC, dos Santos HA, da Quintão Silva MG, Carneiro CM, Dias SR, Rabelo ÉM (2014) The crowding effect in *Ancylostoma ceylanicum*: density-dependent effects on an experimental model of infection. Parasitol Res 113(12):4611–4621. https://doi.org/10.1007/s00436-014-4151-y

Sheather AT (1923) The detection of intestinal protozoa and monge parasites by a flotation technique. J Comp Pathol 36:266–275

Simon DJ (2008) The microRNA miR-1 regulates a MEF-2-dependent retrograde signal at neuromuscular junctions. Cell 133:903–915. https://doi.org/10.1016/j.cell.2008.04.035

Zuccherato LW, Furtado LF, Medeiros CDS, Pinheiro CDS, Rabelo ÉM (2018) PCR-RFLP screening of polymorphisms associated with benzimidazole resistance in *Necator americanus* and *Ascaris lumbricoides* from different geographical regions in Brazil. PLoS Negl Trop Dis 12(9):e0006766. https://doi.org/10.1371/journal.pntd.0006766