Preventive Veterinary Medicine 185 (2020) 105162

Contents lists available at ScienceDirect

Preventive Veterinary Medicine

journal homepage: www.elsevier.com/locate/prevetmed

Haemonchus sp. in beef cattle in Brazil: species composition and frequency of benzimidazole resistance alleles

Flávia C. Fávero [a], Larissa B. dos Santos [a], Flábio R. de Araújo [b], Sabrina Ramünke [c], Jürgen Krücken [c], Georg von Samson-Himmelstjerna [c], Fernando de A. Borges [a,*]

[a] School of Veterinary Medicine and Animal Science, Federal University of Mato Grosso do Sul, Campo Grande, Brazil
[b] Brazilian Agricultural Research Corporation, Embrapa Gado de Corte, Campo Grande, Brazil
[c] Institute for Parasitology and Tropical Veterinary Medicine, Freie Universität Berlin, Berlin, Germany

ARTICLE INFO

Keywords:
Haemonchus contortus
Haemonchus placei
Larvae
Pyrosequencing
Ruminants
β-Tubulin

ABSTRACT

The aim of the present study was to investigate the occurrence of *Haemonchus contortus* and *Haemonchus placei* in beef cattle and the frequency of single nucleotide polymorphisms associated with benzimidazole (BZ)-resistance in *Haemonchus* spp. in Brazil. For such, fecal samples were collected from 61 beef cattle ranches in 11 Brazilian states. Third-stage larvae (L3) were produced for morphological species identification and DNA extraction. PCR was performed for the analysis of the isotype 1 β-tubulin gene and the products were sequenced to confirm the presence of *H. placei* and *H. contortus*. For each field population, pyrosequencing assays were performed to quantify the frequency of the F167Y, E198A and F200Y polymorphisms in the isotype-1 β-tubulin gene. The results of the morphometric analysis of 2345 larvae showed that *H. placei* was present on all ranches. The analysis of the isotype 1 β-tubulin gene confirmed 100% prevalence for *H. placei* and 23.7% for *H. contortus*. Pyrosequencing assays demonstrated single-nucleotide polymorphisms (SNPs) associated with BZ-resistance in all three codons (F167Y, E198A and F200Y) of the isotype 1 β-tubulin gene in *H. placei* field populations. Frequencies of resistance-associated alleles above background (≥ 15%) were found for at least one codon in 11.4% of the field isolates and maximum frequencies of 30, 21 and 29% were found for codons 167, 198 and 200, respectively, on individual ranches. This study confirms the presence of *H. contortus* in beef cattle in the major livestock farming states in Brazil and demonstrates that genotypes associated with BZ resistance are present in field populations of *Haemonchus* spp..

1. Introduction

Haemonchus spp. are among the most important ruminant gastrointestinal nematodes in Brazil (Amarante et al., 2017), as demonstrated by the high prevalence and pathogenicity, causing severe reductions in bovine productivity (Borges et al., 2013). The host specificity of *Haemonchus* spp. in domestic ruminants is not strict, which is in contrast to the traditional concept that *Haemonchus contortus* (Rudolphi, 1803; Cobb, 1898) can be classified as a parasite of sheep and goats, whereas *Haemonchus placei* (Place, 1893) and *Haemonchus similis* (Travassos, 1914) are parasites of cattle. However, *H. similis* occurs with lower intensity and prevalence than the other two species (Santos et al., 2010). Artificial infections in cattle with *H. placei* and *H. contortus* revealed that the latter species has a shorter pre-patent period (Bremner, 1955; Riggs, 2001; Fávero et al., 2016a) and is more fertile (Jacquiet et al., 1998; Fávero et al., 2016a). There are reports of natural mixed infections involving both species in cattle raised on pastures throughout the world (Roberts and Bremmer, 1955; Santiago, 1968; Borba, 1988; Lichtenfels et al., 1994; Achi et al., 2003; Hoberg et al., 2004; Gasbarre et al., 2009; Chaudhry et al., 2015), including Brazil (Amarante et al., 1997; Brasil et al., 2012). The host adaptation and pathogenicity of these species has been confirmed in experimental infections of cattle (Basseto et al., 2011; Fávero et al., 2016ab). In addition to mixed infections, the recent field evidence of interspecies hybridization between *H. contortus* and *H. placei* in small ruminants may indicate a new form of transmission of anthelmintic-resistance mutations (Chaudhry et al., 2015).

Haemonchus spp. have distinct morphological characteristics that enable the identification of adults or infective larvae, the latter being a

* Corresponding author at: Faculty of Veterinary Medicine and Animal Science, Federal University of Mato Grosso do Sul. Senador Filinto Müller Avenue 2443, Postal Code: 79074-460, Campo Grande, MS, Brazil.
E-mail address: fernando.borges@ufms.br (F.A. Borges).

https://doi.org/10.1016/j.prevetmed.2020.105162
Received 2 December 2019; Received in revised form 23 September 2020; Accepted 24 September 2020
Available online 9 October 2020

simple, economical method for the classification of helminths in host feces. Furthermore, the identification of infective larvae is considered to be more precise in comparison to the measurement of spicule lengths on males (Santos et al., 2014b). The mean total length and sheath tail length ranges of infective larvae of *H. placei*, *H. contortus* and *H. similis* were described by Keith (1953), Santiago (1968), van Wyk (2004) and Santos et al. (2014b).

In Brazil, *Haemonchus* spp. in cattle are highly resistant to macrocyclic lactones (Soutello et al., 2007; Souza et al., 2008; Borges et al., 2013; Borges et al., 2015), whereas the resistance to benzimidazoles (BZ) is considered to be very low (Soutello et al., 2007; Souza et al., 2008; Brasil et al., 2012; Neves et al., 2014). However, BZ resistance status in Brazil may be underestimated, since previous studies were based on methods such as a phenotypic evaluation using the fecal egg count resistance test (FECRT), which has limited sensitivity (Martin et al., 1989).

The analysis of the BZ-resistance status of a worm population based on the genotyping of individual larval or adult worms is laborious and expensive, which underscores the need for the application of molecular tests that have been evaluated for use with field samples pooled on the population (ranch) level. Therefore, the use of DNA from pools of larvae is appropriate for routine molecular resistance tests (von Samson-Himmelstjerna, 2006; Forte and Molento, 2013; Ramünke et al., 2016). The identification of isotype 1 β-tubulin alleles associated with BZ resistance in DNA extracted from pools of nematode larvae can be performed using the conventional qualitative PCR, quantitative real-time PCR or pyrosequencing. These techniques are sensitive, reliable tests for evaluating the presence (and frequency) of alleles associated with BZ resistance in populations of *Haemonchus* spp. in the field (von Samson-Himmelstjerna et al., 2009; Ademola et al., 2015; Redman et al., 2015; Chaudhry et al., 2016).

In comparison to other anthelmintics, the mechanisms of resistance to BZs are currently better characterized using three single nucleotide polymorphisms (SNPs) described as largely responsible for resistance in gastrointestinal nematodes (von Samson-Himmelstjerna et al., 2007; Kotze et al., 2014). These SNPs lead to structural modifications in β-tubulin that decrease the affinity for BZs (Ghisi et al., 2007). The first identified SNP occurs at position 200 of the isotype 1 β-tubulin gene, where Phe (TTC) and Tyr (TAC) are encoded in susceptible and resistant worms, respectively (Kwa et al., 1995). Similarly, a TTC/TAC polymorphism at codon 167 (Silvestre and Cabaret, 2002) and a Glu to Ala (GAA/GCA) polymorphism at codon 198 were found to be associated with BZ-resistance (Ghisi et al., 2007).

Successful *Haemonchus* spp. control is directly related to the effective diagnosis of the etiology and anthelmintic resistance status (Santos et al., 2014a). It is therefore essential to study the frequency of isotype 1 β-tubulin SNPs associated with BZ resistance in populations of *Haemonchus* spp. in cattle in Brazil, which will enable the early diagnosis of resistance through the use of sensitive and specific diagnostic tools. To date, there have been few extensive studies quantifying the frequency of different isotype 1 β-tubulin SNPs associated with BZ resistance in *Haemonchus* spp. in cattle. A low frequency of resistance-associated SNPs i.e. F200Y but no F167Y and E198A polymorphisms was found in *H. placei* in the USA (Chaudhry et al., 2014; Ali et al., 2019), whereas no of SNPs associated with BZ resistance were detected in Nigeria (Ademola et al., 2015).

Studies that identify natural infections by *Haemonchus* spp. in cattle in tropical areas contribute to knowledge on the etiology of gastrointestinal nematodes and the impact on anthelmintic resistance. Thus, the aim of the present study was to investigate the occurrence of *H. placei* and *H. contortus* in Brazilian beef cattle as well as the frequency of β-tubulin isotype 1 SNPs associated with BZ resistance in these parasites.

2. Materials and Methods

2.1. Field populations of *Haemonchus* spp. in beef cattle in Brazil

Fecal samples from beef cattle were collected from January to April 2015 on 61 different ranches located in different regions of Brazil: Rondônia (1), Pará (10), Tocantins (2), Maranhão (1), Alagoas (1), Distrito Federal (1), Goiás (1), Mato Grosso (7), Mato Grosso do Sul (9), Minas Gerais (6), São Paulo (8), Paraná (6) and Rio Grande do Sul (8). The number of herds per geographic area was determined based on the total number of herds and animals in each area (IBGE, 2016) and ranches within each geographical area were selected by convenience. However, the following criteria were considered to allow the inclusion of a ranch: at least 100 male calves in the post-weaning age group, *Bos taurus indicus* or *Bos taurus taurus* x *Bos taurus indicus* breed and only animals without anthelmintic treatment in the 90 days prior to the study. The latter criterion was chosen because it was not possible to perform fecal egg counts to identify positive animals for nematode infection.

Fecal samples were collected individually from approximately 100 calves on each ranch, stored in refrigerated boxes and sent to the laboratory, where coprocultures were carried out for ten days. Fecal samples were pooled on the ranch level and 10-15 g of feces from each animal were included in each pool (Roberts and ÓSullivan, 1950). Third-instar larvae (L3) were then quantified, identified to the genus level (Keith, 1953) and aliquots of 1,000 L3 were stored in 2% formalin for morphological identification of the species based on van Wyk et al. (2004). The remaining larvae were washed in distilled water and stored at −20 °C for the molecular studies.

2.2. Morphological identification of *Haemonchus* spp

If the number of *Haemonchus* spp. in the pool was low, all larvae were measured. Larvae of the genus *Haemonchus* were identified and discriminated from other parasitic nematodes based on the distance between the posterior end of the larva and the end of the tail, which was determined using an ocular micrometer (Zeiss®) (van Wyk et al., 2004). The following were the criteria for the morphological identifications: *H. placei* – a minimum sheath tail length of 80 µm (van Wyk et al., 2004) and a maximum of 119 µm (Keith, 1953; van Wyk et al., 2004); *H. contortus* – a minimum length of 57.15 µm and a maximum of 82.55 µm (Santos et al., 2014b); *H. similis* – a minimum length of 56.5 µm and a maximum length of 58.1 µm (Santiago, 1968). Size ranges for the sheath and resulting diagnoses are summarized in Table 1.

2.3. Extraction of genomic DNA

Genomic DNA was extracted from the pools of larvae obtained in the coprocultures from each ranch using the Wizard® Genomic DNA Purification Kit (Promega) following the manufacturer's instructions. The DNA was stored at −20 °C until it was transported to Freie Universität

Table 1
Morphometric characters of sheath tail lengths used for identification of third stage larvae of *Haemonchus* species

Sheath tail length (µm)	Species	References
56.5 to <57.15	*H. similis* (HS)	Santiago (1968), Santos et al.(2014b)
57.15 to 58.1	*H. similis* or *H. contortus* (HC/HS)	Santiago (1968), Santos et al.(2014b)
>58.1 to <80.0	*H. contortus*(HC)	Santos et al.(2014b), Santiago (1968), van Wyk et al. (2004)
80.0 to 82.55	*H. contortus* or *H. placei* (HP/HC)	Santos et al.(2014b), van Wyk et al. (2004)
>82.55 to 119	*H. placei* (HP)	Santos et al.(2014b), van Wyk et al. (2004), Keith (1953)

Berlin in Germany at room temperature for 24 hours followed by storage at -20 °C until processing.

2.4. Molecular identification of Haemonchus contortus and Haemonchus placei

To diagnose the presence of *H. placei* and *H. contortus* in the samples, PCR reactions were used for amplification of a 330-331 bp fragment of the isotype 1 β-tubulin gene locus in *Haemonchus* spp. using the forward primer Haem_BetatubulinHRM-For 5'-CTG GAT CTG GAA TIG GCA CTT-3' and the reverse primer Haem_BetatubulinHRM-Rev 5'-AAG CAG ATA TCA TAC AGR GCT TCG TT-3'. Each reaction contained 0.2 µM of forward primer, 0.36 µM of reverse primer and 2 µL of each genomic DNA sample in 25 µL 1× GoTaq® qPCR Master Mix (Promega). After initial denaturation at 95 °C for 2 min, amplification occurred during 45 cycles of denaturation at 95 °C for 15 s, annealing at 62 °C for 30 s and extension at 72 °C for 30 s. Positive and negative controls were included in each PCR analysis and the products were analyzed using electrophoresis in 1.5% agarose gels.

PCR products were purified using the DNA Clean & Concentrator™-5 Kit (Zymo Research) and submitted to Sanger sequencing at LGC Genomics (Berlin, Germany). DNA sequences were analyzed using BLASTn (Altschul et al., 1990) against GenBank®. Multiple sequences from GenBank were aligned and compared with individual reads from the field samples using MUSCLE (Edgar, 2004) implemented in MEGA version 6 (Tamura et al., 2013). The diagnosis of mixed samples of *H. contortus* and *H. placei* was performed by manually by comparing chromatograms with the sequences available in GenBank to identify double peaks in an intron region containing 16 polymorphisms between both species, as detailed in Fig. S1.

2.5. Pyrosequencing assays for determination of frequencies of isotype 1 β-tubulin alleles

For each sample, the *Haemonchus* spp. isotype 1 β-tubulin gene was amplified from genomic DNA by PCR. The forward primer HcPy2PCR-For 5'-GAC GCA TTC ACT TGG AGG AG-3' was used together with the biotinylated reverse primer HcPy2PCR-Rev 5'-Biotin-CAT AGG TTG GAT TTG TGA GTT-3' (von Samson-Himmelstjerna et al., 2009). This assay was developed for *H. contortus* but has been used for *H. placei* as well (Ademola et al., 2015) and determines SNP frequencies on the genus level but not the species level. PCR and pyrosequencing were performed essentially as described by Ademola et al. (2015). Initial denaturation was performed at 98 °C for 30 s, followed by 40 cycles of denaturation at 98 °C for 10 s, annealing at 56 °C for 30 s and extension at 72 °C for 30 s. Finally, a single elongation step was performed at 72 °C for 5 min. Positive and negative controls were included in each PCR analysis and the products were analyzed using electrophoresis in 1.5% agarose gels.

The products of these PCRs were submitted to pyrosequencing assays to determine the frequencies of the SNPs. The pyrosequencing assays were conducted using the PyroMark Q24 MD System (QIAGEN) following the manufacturer's protocol and the PyroMark Gold Q24 (QIAGEN) reagents with the sequencing primers HcPyIso1_167 (5'-ATA GAA TTA TGG CTT CGT-3') and HcPyIso1_198_200 (5'-GGT AGA GAA CAC CGA TG-3') for the measurement of the allele frequencies at codons 167 and codons 198 and 200, respectively (von Samson-Himmelstjerna et al., 2009; Demeler et al., 2013; Ademola et al., 2015). All samples were analyzed in duplicate.

Frequencies above 15% in at least one of the isotype 1 β-tubulin SNPs associated with BZ resistance were considered the criterion for the identification of samples with an increased frequency of resistance alleles. This threshold was adopted to avoid false positives in samples with a low frequency of resistance-associated SNPs due to technical noise.

2.6. Statistical analysis

To evaluate the hypothesis that the presence of *H. contortus* could be a risk factor for BZ resistance on a ranch, Fisher's exact test was applied to a 2×2 contingency table, considering the presence or absence of *H. contortus* and the occurrence or non-occurrence of resistance using GraphPad Prism version 6.00. Differences in the level of BZ resistance among geographic regions were also calculated using Fisher's exact test. The Wilson score interval method was used to calculate confidence intervals for proportions of genera classified by morphology or molecular methods (Dean et al., 2013).

3. Results

3.1. Morphological identification of Haemonchus species

Fecal cultures from all 61 ranches were positive for trichostrongyloid larvae. However, two coprocultures had very low numbers of recovered larvae and were therefore not submitted to morphological classification in order to save the material for the molecular tests.

The relative and absolute numbers of *H. placei*, *H. contortus* or *H. similis* for the different Brazilian regions are shown in Table 2 and the frequency of each species in individual farms are shown in Fig. 1. The results of the morphometric analysis of 2345 larvae showed 100% prevalence for *H. placei* in beef cattle in Brazil. *H. similis* was found in 10/59 farms, corresponding to a prevalence of 16.94%, however, the average frequency of larvae of this species in fecal cultures was very low (0.85%), which demonstrates the low relevance of this species in cattle and beef in Brazil. Based on the morphometric criteria, no larvae could be classified with absolute certainty as *H. contortus*. However, 1.8% of the total *Haemonchus* spp. larvae had lengths compatible with *H. contortus*. of which, 1.4% showed sheath length in the overlap region of size ranges for *H. contortus* and *H. placei* (1.4%) and 0.4% for *H. contortus* and *H. similis*. (Table 2). This result was observed on 12/59 of the ranches located in the states of Paraná (three ranches), Rio Grande do Sul (five ranches), Mato Grosso (one ranch), Minas Gerais (one ranch), Alagoas (one ranch) and Rondônia (one ranch). There was a predominance of *H. placei* larvae (97.3%) and a low number of *H. similis* (0.85%).

3.2. Molecular identification of Haemonchus species

The manual analysis of the isotype 1 β-tubulin gene of the 59 populations revealed the occurrence of *H. placei* (>99% identity to *H. placei* in GenBank) on all ranches evaluated (prevalence: 100%), whereas *H. contortus* was found on 14 (23.7%) of the ranches (Table 3) according to the manual identification of *H. contortus*-specific (mostly minor) peaks in the chromatograms. Remarkably, only four of these samples originated from the same ranches on which larvae were identified as morphometrically compatible with this species.

3.3. Pyrosequencing assays for determination of frequency of β-tubulin alleles in Haemonchus spp

The mean of two replicates was used to classify a population as showing an increased SNP frequency in a certain codon. In general, in 92% of the samples, the difference between two technical replicates was 3% or lower. At least low frequencies of isotype 1 β-tubulin SNPs associated with BZ resistance were detected in all of the 61 populations evaluated in this study. However, considering the conservative criterion of ≥15% SNP frequency, seven (11.4%) of the 61 populations were classified as resistant (Fig. 2, Table S1). In six of the seven farms, both technical replicates were above 15% while in one of the farms individual measurements of 13% and 17% leading to a mean 15% were obtained. Using a cut-off frequency of 10%, 37/61 (60.6%) ranches were above this threshold.

Table 2
Results of morphological identification of *Haemonchus placei, H. contortus* and *H. similis* larvae obtained from coprocultures of beef cattle from different regions of Brazil.

Region / State	Number of ranches	Absolute number of L3 measured, percentage (95%CI)					
		Total	HP	HP/HC	HC	HC/HS	HS
NORTH							
Pará	9	225	225 100 (98.3-100)	0	0	0	0
Rondônia	1	47	46 97.9 (88.9-99.6)	0	0	1 2.1 (0.3-11.1)	0
Tocantins	2	30	30 100 (88.6-100)	0	0	0	0
NORTHEAST							
Maranhão	1	61	61 100 (94-100)	0	0	0	0
Alagoas	1	25	22 88 (70-95.8)	3 12 (4.1-29.9)	0	0	0
WEST CENTRAL							
Mato Grosso	7	160	156 97.5(93.7-99)	2 1.25 (0.3-4.4)	0	0	2 1.25 (0.3-4.4)
Mato Grosso do Sul	9	376	376 100 (98.9-100)	0	0	0	0
Goiás	2	116	116 100 (96.7-100)	0	0	0	0
SOUTHEASTERN							
Minas Gerais	5	171	166 97 (93.3-98.7)	2 1.2 (0.3-4.1)	0	0	3 1.8 (0.6-5)
São Paulo	8	398	396 99.5 (98.1-99.8)	0	0	0	2 0.5 (0.1-1.8)
SOUTH							
Paraná	6	341	310 90.9 (87.3-93.5)	14 4.1 (2.4-6.7)	0	7 2 (1-4.1)	10 3 (1.6-5.3)
Rio Grande do Sul	8	395	379 96 (93.5-97.4)	12 3 (1.7-5.2)	0	1 0.2 (0.04-1.4)	3 0.8 (0.2-2.2)
TOTAL	59	2345	2,283 97.3 (96.6-97.9)	33 1.4 (1-1.9)	0	9 0.4 (0.2-0.7)	20 0.85 (0.5-1.3)

HP: *Haemonchus placei,* HC: *Haemonchus contortus,* HS: *Haemonchus similis*
* morphological identification of *Haemonchus* spp. per farm is show in Fig.1

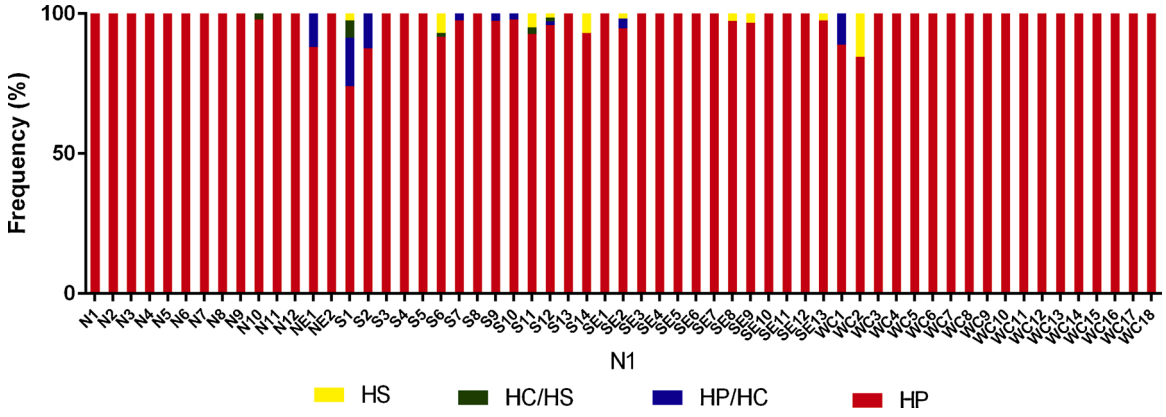

Fig. 1. Frequency of *Haemonchus placei* (HP), *H. contortus* (HC) and *H. similis* (HS) of third-stage larvae obtained from the fecal cultures per farm per region (North: N, Northeast: NE, South: S, Southeastern: SE, West Central: WC) in Brazil.

On some ranches, high frequencies of isotype 1 β-tubulin alleles associated with BZ resistance were found for one of the three codons (F167Y, E198A and F200Y) (Fig. 2). However, these frequencies did not occur simultaneously in the three codons. In the two populations with mixed *H. placei* and *H. contortus* infection (samples 36 and 54), one had a codon 200 TAC frequency of 17% and the other had a codon 198 GCA frequency of 19% (Table S1). In three populations containing exclusively *H. placei*, codon 198 GCA frequencies were between 15% and 21%, whereas two other populations had TAC frequencies between 26 and 30% at codons 167 and 200 simultaneously (Table S1). However, the sum of the frequencies of isotype 1 β-tubulin alleles associated with BZ resistance in both cases was only 59% (27% for 167TAC + 6% for 198GCA + 26% for 200TAC) and 65% (30% for 167TAC + 6% for 198GCA + 29% for 200TAC) and thus well below 100%, suggesting that they did not necessarily occur in the same allele.

Among the seven ranches with *Haemonchus* sp. populations for which we found isotype 1 β-tubulin allele frequencies indicative of BZ resistance (i.e. > 15% in one of the codons), only two presented mixed infections with *H. contortus* (Table 4). It is noteworthy that the presence of *H. contortus* detected by PCR/sequencing in cattle on a ranch was not a risk factor for BZ resistance ($p = 1.0$, Fisher's exact test). Moreover, no significant difference in the frequencies of β-tubulin alleles associated with BZ resistance on ranches with resistance was found among the geographic regions (Table S1).

Table 3
Haemonchus species identified by sequencing genomic DNA of larval pool obtained from coprocultures of beef cattle in different regions of Brazil.

Region / State	Number of ranches	Positive ranches for species researched by sequencing	
		H. placei	*H. contortus*
NORTH			
Pará	10	100%	50%
Rondônia	1	100%	100%
Tocantins	2	100%	0%
NORTHEAST			
Maranhão	1	100%	0%
Alagoas	1	100%	0%
WEST CENTRAL			
Mato Grosso	7	100%	42.9%
Mato Grosso do Sul	8	100%	12.5%
Goiás	2	100%	0%
SOUTHEASTERN			
Minas Gerais	6	100%	16.7%
São Paulo	8	100%	12.5%
SOUTH			
Paraná	6	100%	16.7%
Rio Grande do Sul	7	100%	14,3%
TOTAL	59	100%	23,7%

4. Discussion

This study offers an extensive survey of the occurrence of *H. placei* and *H. contortus* in beef cattle from all regions of Brazil as well as the isotype 1 β-tubulin SNPs associated with BZ resistance for the species. The findings confirm the natural infection of cattle by *H. contortus*, albeit with low intensities.

Although advantageous due to its practicality, speed and ease of obtaining samples, the traditional morphometric species identification of *Haemonchus* spp. using L3 is based on subtle differences (Amarante et al., 2017) and an important limitation of this technique is the overlapping of measurements (Corticelli and Lai, 1964; van Wyk et al., 2004; Santos et al., 2014b), leading to some inconclusive results. In addition to overlapping ranges of sheath-tail-extension length, even considering the lower limit of 85 µm for *H. placei*, there is a proportion of outliers (less than 1%) that could confound species differentiation (Santos et al., 2014b). Therefore, the sheath-tail-extension length found in 1.4% of the *Haemonchus* spp. larvae in this study are only suggestive of *H. contortus*, because the observed measurements have rarely been reported by other authors for *H. placei* (Niec, 1968; Van Wyk et al., 2004; Santos et al., 2014; Silva, 2014).

Even without a precise diagnosis of *H. contortus* by morphometry, the sequencing of the PCR product of the β-tubulin isotype 1 gene enabled the identification of this species on 23.7% of the ranches evaluated, indicating the higher sensitivity of the molecular approach, as described elsewhere (Zarlenga et al., 1994, Santos et al., 2014a). Additionally, only four of the twelve samples with suspected *H. contortus* presence based on morphometric analysis were confirmed by the molecular analysis, which could demonstrate higher specificity. However, the evidence that mitochondrial or nuclear classic markers for the diagnosis of Trichostrongyloidea species may not be accurate, due to the high intra-species variation or other factors (Ali et al., 2018; Ramünke et al., 2018) underscores the need for species identification performing both morphometric and molecular approaches.

The present results confirm the occurrence of natural *H. contortus* infections in cattle in Brazil. This infection occurred in four out of the five geographic regions evaluated and, although it was not found to be a risk factor for the presence of an increased frequency of isotype 1 β-tubulin SNPs associated with BZ resistance in this study, it may be considered an aggravating factor for the increase of anthelmintic resistance in cattle, considering the genetic hybridization between parasitic nematode species supposed by Chaudhry et al. (2015) and demonstrated under field conditions in sheep in Brazil (Almeida et al., 2018). The

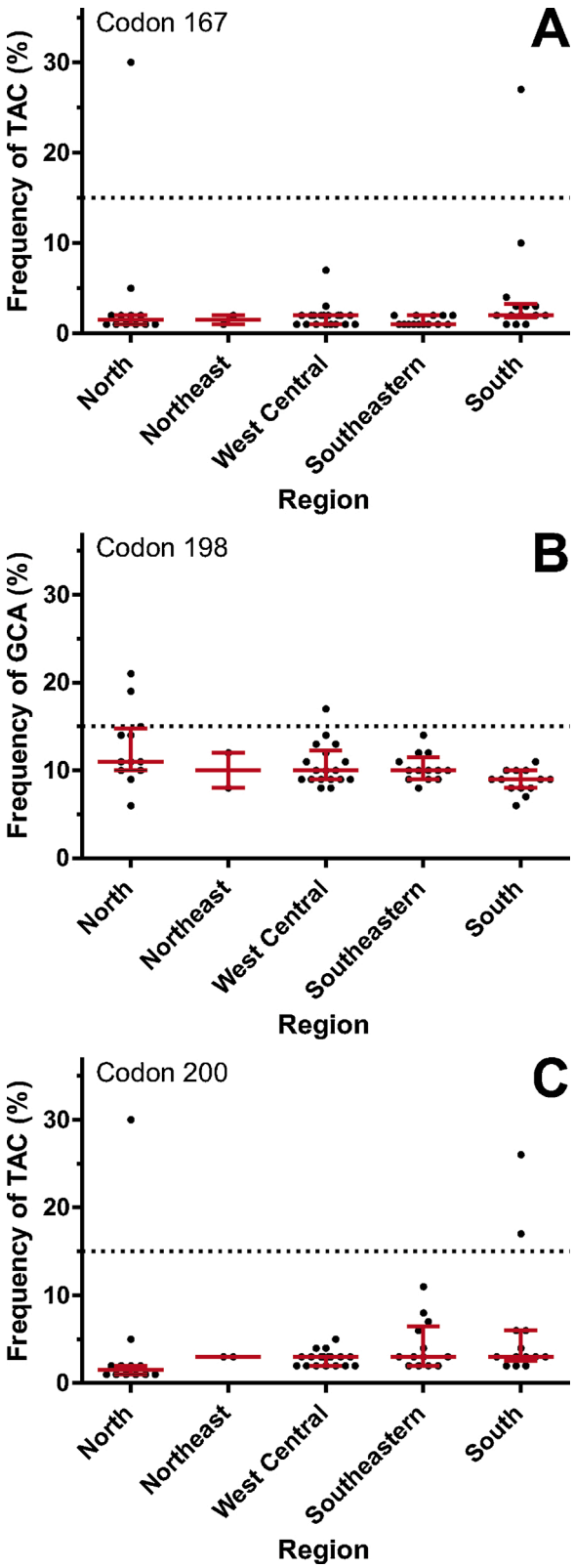

Fig. 2. Frequencies of benzimidazol resistance-associated single nucleotide polymorphism in codon 167 (A), codon 198 (B) and codon 200 (C) of the β-tubulin isotype 1 gene. Dots represent the mean of two technical replicates from a farm. In addition, the medians with inter-quartile ranges are shown. The horizontal dotted lines indicates the 15% frequency threshold. Only frequencies above this value were considered to represent truly elevated while lower values were considered to be due to technical background.

Table 4
Frequencies of resistance SNPs for codons F167Y, E198A and F200Y in β-tubulin isotype 1 gene in pools of larvae of *Haemonchus* spp. from beef cattle in different regions of Brazil. Only farms with frequencies of at least 15% in one of the codons are shown.

Sample identification	HP or HP/HC	Region/State	Codon 167	Codon 198	Codon 200
19	HP	NORTH/Pará	1	21	4
25	HP	NORTH/Pará	1	15	5
40	HP	NORTH/Pará	30	6	29
54	HP/HC	NORTH/Pará	2	19	6
16	HP	WEST CENTRAL/Mato Grosso do Sul	1	17	3
36	HP/HC	SOUTH/Rio Grande do Sul	10	7	17
39	HP	SOUTH/Rio Grande do Sul	27	6	26

HP: population exclusively with *H. placei*. HP/HC: population with both *H. placei* and *H. contortus*

method used for species identification in the present study is not able to identify potential hybrids, since the analyses were only performed on pools of larvae rather than individual worms. Thus, the presence of peaks in chromatograms characteristic for both species can be due to mixed species infections that may or may not include presence of hybrids. Another relevant consequence of *H. contortus* infection in bovines regards pathogenicity, since calves experimentally infected with *H. contortus* showed severer symptoms and clinical disease compared to *H. placei* infection (Fávero et al., 2016b).

The pyrosequencing assays targeting codons F167Y, E198A and F200Y of the isotype 1 β-tubulin gene in *Haemonchus* spp. revealed that resistance to BZs is in an early stage. However, BZ-resistance is already geographically widespread among beef cattle in Brazil. This was the first extensive study carried out with samples from all geographic regions in Brazil and confirms the results of earlier studies on the phenotypic characterization of trichostrongyloids infecting cattle regarding anthelmintic efficacy in Brazil. These studies demonstrated that BZs remain the most effective drugs and only a few cases of resistance in cattle have been reported (Soutello et al., 2007; Souza et al., 2008, Brasil et al., 2012; Neves et al., 2014, Ramos et al., 2016). Although it is expected that with the continued use of BZ there will be an increase in resistance, as demonstrated by Knapp-Lawitzke et al. (2015), the fact that high frequencies of isotype 1 β-tubulin alleles associated with BZ resistance (>15%) were found in 11.4% of *Haemonchus* spp. populations in this study is worrisome, since this result is much higher than previously described for the species in Brazil (Brasil et al., 2012) and the United States (Chaudhry et al., 2014).

In the present study, we found a high frequency (≥ 15%) of SNPs in the *H. placei* samples to occur in decreasing numbers with respect to codons E198A, F200Y and F167Y. This finding differs from results reported by Chaudhry et al. (2014) and Ali et al. (2019), who found polymorphism only at codon F200Y, whereas no resistance-associated polymorphisms were identified at codons 198 and 167 in *H. placei* in the United States. Brasil et al. (2012) only analyzed two *H. placei* populations from cattle in Brazil and found one carrying F167Y. In contrast to recent findings describing the substitutions E198 L in Brazil, E198 T in Sweden (Baltrušis et al., 2018) as well as E198 V, E198 T, E198R and E198 L in resistant *H. contortus* in Sudan (Mohammedsalih et al., 2020), there was no evidence for such polymorphisms in the data of the present study.

In conclusion, the present study confirmed the presence of *H. conturtus* by molecular analysis and demonstrates that the resistance to BZs is in an early stage but already geographically widespread among *Haemonchus* spp. in beef cattle in Brazil, with the presence of all three β-tubulin isotype 1 polymorphisms associated with BZ resistance (F167Y, E198A and F200Y) in *H. placei*.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Acknowledgments

This study was financed in part by the Brazilian fostering agencies *Coordenação de Aperfeiçoamento de Pessoal de Nível Superior* (CAPES; Finance Code 001), Fundect, (59/300.407/2015 and 71/700.132/2018) and CNPq (47936/2013-6) as well as internal funds from Freie Universität Berlin.

Appendix A. Supplementary data

Supplementary material related to this article can be found, in the online version, at doi:https://doi.org/10.1016/j.prevetmed.2020.105162.

References

Achi, Y.L., Zinsstag, J., Yao, K., Yeo, N., Dorchies, P., Jacquiet, P., 2003. Host specificity of *Haemonchus* spp. for domestic ruminants in the savanna in northern Ivory Coast. Vet. Parasitol. 116, 151–158. https://doi.org/10.1016/s0304-4017(03)00258-9.

Ademola, I.O., Krücken, J., Ramünke, S., Demeler, J., von Samson-Himmelstjerna, G., 2015. Absence of detectable benzimidazole-resistance associated alleles in *Haemonchus placei* in cattle in Nigeria revealed by pyrosequencing of β-tubulin isotype 1. Parasitol. Res. 114, 1997–2001. https://doi.org/10.1007/s00436-015-4406-2.

Ali, Q., Rashid, I., Shabbir, M.Z., Shahzad, K., Ashraf, K., Sargison, N.D., Chaudhry, U., 2018. Population genetics of benzimidazole-resistant *Haemonchus contortus* and *Haemonchus placei* from buffalo and cattle: implications for the emergence and spread of resistance mutations. Parasitol Res. 117, 3575–3583. https://doi.org/10.1007/s00436-018-6055-8.

Ali, Q., Rashid, I., Shabbir, M.Z., Rahman, A.U., Shahzad, K., Ashraf, K., Sargison, N.D., Chaudhry, U., 2019. Emergence and the spread of the F200Y benzimidazole resistance mutation in *Haemonchus contortus* and *Haemonchus placei* from buffalo and cattle. Vet. Parasitol. 265, 48–54. https://doi.org/10.1016/j.vetpar.2018.12.001.

Almeida, F.A., Bassetto, C.C., Amarante, M.R.V., Albuquerque, A.C.A., Starling, R.Z.C., Amarante, A.F.T.D., 2018. Helminth infections and hybridization between *Haemonchus contortus* and *Haemonchus placei* in sheep from Santana do Livramento, Brazil. Rev. Bras. Parasitol. Vet. 27, 208–288. https://doi.org/10.1590/s1984-296120180044.

Altschul, S.F., Gish, W., Miller, W., Myers, E.W., Lipman, D.J., 1990. Basic local alignment search tool. J Mol Biol. 215 (3), 403–410. https://doi.org/10.1016/S0022-2836(05)80360-2.

Amarante, A.F.T., Bagnola, J., Amarante, M.R.V., Barbosa, M.A., 1997. Host specificity of sheep and cattle nematodes in São Paulo state, Brazil. Vet. Parasitol. 73, 89–104. https://doi.org/10.1016/S0304-4017(97)00036-8.

Amarante, M.R.V., Santos, M.C., Bassetto, C.C., Amarante, A.F.T., 2017. PCR primers for straightforward differentiation of *Haemonchus contortus*, Haemonchus placei and their hybrids. J. Helminthol 1–5. https://doi.org/10.1017/S0022149X16000882.

Baltrušis, P., Halvarsson, P., Höglund, J., 2018. Exploring benzimidazole resistance in *Haemonchus contortus* by next generation sequencing and droplet digital PCR. Int. J. Parasitol. Drugs Drug Resist. 8, 411–419. https://doi.org/10.1016/j.ijpddr.2018.09.003.

Borba, M.F.S., 1988. Caracterização de espécies de *Haemonchus* Cobb, 1898 adquiridas por cordeiros traçadores em sistemas de pastoreio misto e simples de ovinos e bovinos. 52p, Dissertação (Mestrado). Faculdade de Medicina Veterinária, Universidade Federal do Rio Grande do Sul, Porto Alegre, Brasil.

Borges, F.A., Almeida, G.D., Heckler, R.P., Lemes, R.T., Onizuka, M.K.V., Borges, D.G.L., 2013. Anthelmintic resistance impact on tropical beef cattle productivity: effect on weight gain of weaned calves. Trop. Anim. Health Prod. 45, 723–727. https://doi.org/10.1007/s11250-012-0280-4.

Borges, F.A., Borges, D.G.L., Heckler, R.P., Neves, J.P.L., Lopes, F.G., Onizuka, M.K.V., 2015. Multispecies resistance of cattle gastrointestinal nematodes to long-acting avermectin formulations in Mato Grosso do Sul. Vet. Parasitol. 212, 299–302. https://doi.org/10.1016/j.vetpar.2015.06.015.

Brasil, B.S.A.F., Nunes, R.L., Bastianetto, E., Drummond, M.G., Carvalho, D.C., Leite, R.C., Molento, M.B., Oliveira, D.A.A., 2012. Genetic diversity patterns of *Haemonchus placei* and *Haemonchus contortus* populations isolated from domestic ruminants in Brazil. Int. J. Parasitol. 42, 469–479. https://doi.org/10.1016/j.ijpara.2012.03.003.

Bremner, K.C., 1955. Cytological studies on the specific distinctness of the ovine and bovine strains of the nematode *Haemonchus contortus* (Rudolphi) Cobb (Nematoda: Trichostrongylidae). Aust. J. Zool. 3, 312–323.

Chaudhry, U., Miller, M., Yazwinski, T., Kaplan, R., Gilleard, J., 2014. The presence of benzimidazole resistance mutations in *Haemonchus placei* from US cattle. Vet. Parasitol. 204, 411–415. https://doi.org/10.1016/j.vetpar.2014.05.025.

Chaudhry, U., Redman, E.M., Abbas, M., Muthusamy, R., Ashraf, K., Gilleard, J.S., 2015. Genetic evidence for hybridisation between *Haemonchus contortus* and *Haemonchus placei* in natural field populations and its implications for interspecies transmission of anthelmintic resistance. Int. J. Parasitol. 45, 149–159. https://doi.org/10.1016/j.ijpara.2014.09.002.

Chaudhry, U., Redman, E.M., Ashraf, K., Shabbir, M.Z., Rashid, M.I., Ashraf, S., Gilleard, J.S., 2016. Microsatellite marker analysis of *Haemonchus contortus* populations from Pakistan suggests that frequent benzimidazole drug treatment does not result in a reduction of overall genetic diversity. Parasit. Vectors. 9, 349. https://doi.org/10.1186/s13071-016-1624-0.

Cobb, N.A., 1898. Extract from MS report on the parasites of stock. Agric. Gaz. N. S. W. 9, 296–321.

Corticelli, B., Lai, M., 1964. La diagnosi di tipo d'infestione nella strongilosi gastrointestinale del bovino in Sardegna con le larve infestive. Vet. Italiana 15, 190–213.

Dean, A.G., Sullivan, K.M., Soe, M.M., 2013. OpenEpi: Open Source Epidemiologic Statistics for Public Health, Versão. Updated 2013/04/06, Acessed 2019/05/13. www.OpenEpi.com.

Demeler, J., Krüger, N., Krücken, J., von der Heyden, V.C., Ramünke, S., Küttler, U., Miltsch, S., López Cepeda, M., Knox, M., Vercruysse, J., Geldhof, P., Harder, A., von Samson-Himmelstjerna, G., 2013. Phylogenetic characterization of β-tubulins and development of pyrosequencing assays for benzimidazole resistance in cattle nematodes. PLoS One 8, e70212. https://doi.org/10.1371/journal.pone.0070212.

Edgar, R.C., 2004. Muscle: multiple sequence alignment with high accuracy and high throughput. Nucleic Acids Res. 32 (5), 1792–1797.

Fávero, F.C., Buzzulini, C., Cruz, B.C., Felippelli, G., Maciel, W.G., Salatta, B., Teixeira, W.F.P., Soares, V.E., Oliveira, G.P., Lopes, W.D.Z., Costa, A.J., 2016a. Experimental infection of calves with *Haemonchus placei* and *Haemonchus contortus*: Assessment of parasitological parameters. Vet. Parasitol 217, 25–28. https://doi.org/10.1016/j.vetpar.2015.12.017.

Fávero, F.C., Buzzulini, C., Cruz, B.C., Felippelli, G., Maciel, W.G., Salatta, B., Siniscalchi, D., Lopes, W.D.Z., Teixeira, W.F.P., Soares, V.E., Oliveira, G.P., Costa, A. J., 2016b. Experimental infection of calves with *Haemonchus placei* or *Haemonchus contortus*: Assessment of clinical, hematological and biochemical parameters and histopathological characteristics of abomasums. Exp. Parasitol 170, 125–134. https://doi.org/10.1016/j.exppara.2016.09.017.

Fortes, F.S., Molento, M.B., 2013. Resistência anti-helmíntica em nematoides gastrintestinais de pequenos ruminantes: avanços e limitações para seu diagnóstico. Pesqui. Vet. Bras. 33, 1391–1402.

Gasbarre, L.C., Smith, L.L., Hoberg, E., Pilitt, P.A., 2009. Further characterization of a cattle nematode population with demonstrated resistance to current anthelmintics. Vet. Parasitol 166, 275–280. https://doi.org/10.1016/j.vetpar.2009.08.019.

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.

Hoberg, E., Lichtenfels, J., Gibbons, L., 2004. Phylogeny for species of *Haemonchus* (Nematoda: Trichostrongyloidea): consideration of their evolutionary history and global biogeography among Camelidae and Pecora (Artiodactyla). J. Parasitol. 90, 1085–1102. https://doi.org/10.1645/GE-3309.

Jacquiet, P., Cabaret, J., Thiam, E., Cheikh, D., 1998. Experimental and natural *Haemonchus* spp. cross infections of domestic ruminants in Sahelian West Africa. Ann. N. Y. Acad. Sci. 849, 465–469. https://doi.org/10.1111/j.1749-6632.1998.tb11098.x.

Knapp-Lawitzke, F., Krücken, J., Ramünke, S., von Samson-Himmelstjerna, G., Demeler, J., 2015. Rapid selection for β-tubulin alleles in codon 200 conferring benzimidazole resistance in an Ostertagia ostertagi isolate on pasture. Vet. Parasitol. 209, 84–92. https://doi.org/10.1016/j.vetpar.2015.02.005.

Keith, R.K., 1953. The differentiation of the infective larvae of some common nematode. Aust. Vet. J. 1, 223–235. https://doi.org/10.1071/ZO9530223.

Kotze, A.C., Hunt, P.W., Skuce, P., von Samson-Himmelstjerna, G., Martin, R.J., Sager, H., Krücken, J., Hodgkinson, J., Lespine, A., Jex, A.R., Gilleard, J.S., Beech, R. N., Wolstenholme, A.J., Demeler, J., Robertson, A.P., Charvet, C.L., Neveu, C., Kaminsky, R., Rufener, L., Alberich, M., Menez, C., Prichard, R.K., 2014. Recent advances in candidate-gene and whole-genome approaches to the discovery of anthelmintic resistance markers and the description of drug/receptor interactions. Int. J. Parasitol. 4 (3), 164–184. https://doi.org/10.1016/j.ijpddr.2014.07.007.

Kwa, M.S.G., Veenstra, J.G., Van Dijk, M., Roos, M.H., 1995. β-tubulin genes from the parasitic nematode *Haemonchus contortus* modulate drug resistance in *Caenorhabditis elegans*. J. Mol. Biol. 246, 500–510. https://doi.org/10.1006/jmbi.1994.0102.

Lichtenfels, J.R., Pilitt, P.A., Hoberg, E.P., 1994. New morphological characters for identifying individual specimens of *Haemonchus* sp. (Nematoda: Trichostrongyloidea) and a key to species in ruminants of North America. J. Parasitol 80, 107–119. https://doi.org/10.2307/3283353.

Martin, P.J., Anderson, N., Jarrett, R.G., 1989. Detecting benzimidazole resistance with faecal egg count reduction tests and in vitro assays. Aust. Vet. J. 66 (8), 236–240. https://doi.org/10.1111/j.1751-0813.1989.tb13578.x.

Mohammedsalih, K.M., Krücken, J., Khalafalla, A., Bashar, A., Juma, F.R., Abakar, A., Abdalmalaik, A.A.H., Coles, G., von Samson-Himmelstjerna, G., 2020. New codon 198 β-tubulin polymorphisms in highly benzimidazole resistant *Haemonchus contortus* from goats in three different states in Sudan. Parasit. Vectors 13, 114. https://goi.org/10.1186/s13071-020-3978-6.

Neves, J.H., Carvalho, N., Rinaldi, L., Cringoli, G., Amarante, A.F.T., 2014. Diagnosis of anthelmintic resistance in cattle in Brazil: a comparison of different methodologies. Vet. Parasitol 206, 216–226. https://doi.org/10.1016/j.vetpar.2014.10.015.

Niec, R., 1968. Cultivo e identificación de larvas infectantes de nematodes gastrointestinales del bovino y ovino. INTA, Argentina.

Place, F.E., 1893. Anaemic diarrhoea in young cattle. Vet. Rec. 5, 589.

Ramos, F., Portella, L.P., Rodrigues, F.S., Reginato, C.Z., Pötter, L., Cezar, A.S., Sangioni, L.A., Vogel, F.S.F., 2016. Anthelmintic resistance in gastrointestinal nematodes of beef cattle in the state of Rio Grande do Sul, Brazil. Int. J. Parasitol. Drugs Drug Resist. 6, 93–101. https://doi.org/10.1016/j.ijpddr.2016.02.002.

Ramünke, S., Melville, L., Rinaldi, L., Hertzberg, H., de Waal, T., von Samson-Himmelstjerna, G., Cringoli, G., Mavrot, F., Skuce, P., Krücken, J., Demeler, J., 2016. Benzimidazole resistance survey for *Haemonchus*, *Teladorsagia* and *Trichostrongylus* in three European countries using pyrosequencing including the development of new assays for Trichostrongylus. Int. J. Parasitol. Drugs Drug Resist. 6, 230–240. https://doi.org/10.1016/j.ijpddr.2016.10.002.

Ramünke, S., Borges, F.A., von Son-de Fernex, E., von Samson-Himmelstjerna, G., Krücken, J., 2018. Molecular marker sequences of cattle Cooperia species identify Cooperia spatulata as a morphotype of Cooperia punctata. PLoS One 13, e0200390. https://doi.org/10.1371/journal.pone.0200390.

Redman, E., Whitelaw, F., Tait, A., Burgess, C., Bartley, Y., Skuce, P.J., Jackson, F., Gilleard, J.S., 2015. The emergence of resistance to the benzimidazole anthlemintics in parasitic nematodes of Livestock is characterised by multiple independent hard and soft selective sweeps. PLoS Negl. Trop. Dis. 9, 1–24. https://doi.org/10.1371/journal.pntd.0003494.

Riggs, N.L., 2001. Experimental cross-infections of *Haemonchus placei* (Place, 1893) in sheep and cattle. Vet. Parasitol. 94, 191–197. https://doi.org/10.1016/s0304-4017(00)00381-2.

Roberts, F.H.S., Bremmer, K.C., 1955. The susceptibility of cattle to natural infestation of nematode *Haemonchus contortus* (Rudolphi) Cobb 1898. Aust. Vet. J. 31, 133–134.

Roberts, F.H.S., ÓSullivan, P.J., 1950. Methods for egg counts and larvas cultures for strongyles infecting tract of cattle. Aust. J. Agric. Res. 1, 99–192.

Rudolphi, C.A., 1803. Neue beobachtungen über die eingeweidewürmer. Arch. Zool. Zoot. 3, 1–32.

Santiago, M.A.M., 1968. *Haemonchus* Cobb, 1898 (Nematoda: Trichostrongyloidea) contribuição ao estudo da morfologia, biologia e distribuição geográfica das espécies parasitas de ovinos e bovinos, no Rio Grande do Sul. Thesis. Faculdade de Veterinária, Universidade Federal de Santa Maria, Santa Maria.

Santos, J.M.L., Monteiro, J.P., Ribeiro, W.L.C., Macedo, I.T.F., Camurça-Vasconcelos, A. L.F., Vieira, L.S., Bevilaqua, C.M.L., 2014a. Identification and quantification of benzimidazole resistance polymorphisms in *Haemonchus contortus* isolated in Northeastern Brazil. Vet. Parasitol 199, 160–164. https://doi.org/10.1016/j.vetpar.2013.11.006.

Santos, M.C., Amarante, M.R.V., Silva, M.R.L., Amarante, A.F.T., 2014b. Differentiation of *Haemonchus placei* from *Haemonchus contortus* by PCR and by morphometrics of adult parasites and third stage larvae. Rev. Bras. Parasitol. Vet 23, 495–500. https://doi.org/10.1590/S1984-29612014085.

Santos, T.R., Lopes, W.D.Z., Buzulini, C., Borges, F.A., Sakamoto, C.A.M., Lima, R.C.A., Oliveira, G.P., Costa, A.J., 2010. Helminth fauna of bovines from the Central Western, Minas Gerais, Brazil. Cienc. Rural. 40, 934–938. https://doi.org/10.1590/S0103-84782010005000040.

Silva, M.R.L., 2014. Diagnóstico morfológico e molecular de Haemonchus spp. em bovinos e ovinos. Dissertação. Instituto de Biociência, Universidade Estadual Paulista, Campus de Botucatu.

Silvestre, A., Cabaret, J., 2002. Mutation in position 167 of isotype 1 beta-tubulin gene of trichostrongylid nematodes: role in benzimidazole resistance? Mol. Biochem. Parasitol. 120, 297–300.

Soutello, R.G.V., Seno, M.C.Z., Amarante, A.F.T., 2007. Anthelmintic resistance in northwestern São Paulo state. Braz. Vet. Parasitol. 148, 360–364.

Souza, A.P., Ramos, C.I., Bellato, V., Sartor, A.P., Schelbauer, C.A., 2008. Resistência de helmintos gastrintestinais de bovinos a anti-helmínticos no Planalto Catarinense. Cienc. Rural 38, 1363–1367. https://doi.org/10.1590/S0103-84782008000500026.

Tamura, K., Stecher, G., Peterson, D., Filipski, A., Kumar, S., 2013. MEGA6: Molecular Evolutionary Genetics Analysis version 6.0. Mol. Biol. Evol 30, 2725–2729.

Travassos, L.P., 1914. Trichostrongylinae brasileiras. Bras. Med. 28.

Van Wyk, J.A., Cabaret, J., Michael, L.M., 2004. Morphological identification of nematode larvae of small ruminants and cattle simplified. Vet. Parasitol. 119, 277–306. https://doi.org/10.1016/j.vetpar.2003.11.012.

von Samson-Himmelstjerna, G., 2006. Molecular diagnosis of anthelmintic resistance. Vet. Parasitol. 136 (2), 99–107. https://doi.org/10.1016/j.vetpar.2005.12.005.

von Samson-Himmelstjerna, G., Blackhall, W., Mccarthy, J.S., Skuce, P.J., 2007. Single nucleotide polymorphism (SNP) markers for benzimidazole resistance in veterinary nematodes. Parasitology 134, 1077–1086. https://doi.org/10.1017/S0031182007000054.

von Samson-Himmelstjerna, G., Walsh, T.K., Donnan, A.A., Carrière, S., Jackson, F., Skuce, P.J., Rohn, K., Wolstenholme, A.J., 2009. Molecular detection of benzimidazole resistance in *Haemonchus contortus* using real-time PCR and pyrosequencing. Parasitology 136, 349–358. https://doi.org/10.1017/S003118200800543X.