Short Communication

The presence of benzimidazole resistance mutations in Haemonchus placei from US cattle

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ABSTRACT

Haemonchus populations were collected from cattle from mid-western and eastern Southern US (four and six populations, respectively) to determine the relative prevalence of Haemonchus contortus and Haemonchus placei and the frequency of the three isotype-1 β-tubulin polymorphisms associated with benzimidazole resistance. A minimum of 32 individual adult worms were genotyped at position 24 of the rDNA ITS-2 for each population to determine species identity (296 worms in total). One population from Georgia was identified as 100% H. contortus with the remaining nine populations identified as 100% H. placei. For the H. contortus population, 29 out of 32 worms carried the P200Y (TAG) isotype-1 β-tubulin and 2 out of 32 worms carried the P167Y (TAC) benzimidazole resistance associated polymorphisms respectively. For H. placei, six out of the nine populations contained the P200Y (TAG) isotype-1 β-tubulin benzimidazole resistance associated polymorphism at low frequency (between 1.6% and 9.4%) with no resistance associated polymorphisms being identified at the P198 and P167 codons. This is the first report of the P200Y (TAG) isotype-1 β-tubulin benzimidazole resistance associated polymorphism in H. placei. The presence of this mutation in multiple independent H. placei populations indicates the risk of resistance emerging in this parasite should benzimidazoles be intensively used for parasite control in US cattle.

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1. Introduction

Haemonchus placei and Haemonchus contortus are highly pathogenic and economically important parasites that can infect cattle. H. placei is the species traditionally reported in cattle but H. contortus, which is usually found in small ruminants, has been reported in cattle from several regions of the world (Achi et al., 2003; Akkari et al., 2013; Amarante et al., 1997; d’Alexis et al., 2011; Gasbarre et al., 2009a; Hogg et al., 2010; Jacquet et al., 1998). Benzimidazole resistance is extremely common for H. contortus in small ruminants but there are only a few well-documented reports of resistance to this drug class for Haemonchus spp in cattle. There is one report of benzimidazole resistance for H. contortus from US cattle (Gasbarre et al., 2009a,b) and several reports for H. placei in South American cattle including Brazil (Bricarello et al., 2007; Soutello et al., 2007) and Argentina (Anziani et al., 2004). Fenbendazole use in cattle has been increasing in recent years, including
in N. America, largely in response to concerns caused by avermectin/milbemycin resistant parasites. Consequently, the emergence of benzimidazole resistance in these highly pathogenic parasite species is a major concern.

Three different mutations in the isotype-1 β-tubulin gene have been associated with benzimidazole resistance in *H. contortus* in small ruminants; codon P200 (TTG to TAC; F200Y) (Brasil et al., 2012; Hoglund et al., 2009; Kotze et al., 2012; Niciria et al., 2012; Silvestre and Humbert, 2002; von Samson-Himmelstjerna et al., 2009), codon P167 (TTG to TAC; F167Y) (Brasil et al., 2012; de Lourdes Mottier and Prichard, 2008; Silvestre and Cabaret, 2002) and at codon P198 (GAA to GCA; E198A) (Ghisi et al., 2007; Kotze et al., 2012; Rufener et al., 2009). There have been very few molecular genotyping studies of *H. placei* and only one of the three polymorphisms known to be associated with resistance in *H. contortus* has yet been reported this species; the codon F167Y (TTG to TAC) polymorphism was reported at low frequency in a *H. placei* population from cattle in Brazil (2.5%) (Brasil et al., 2012).

In this short communication, we report the frequency of benzimidazole resistance associated iso-type-1 β-tubulin gene polymorphisms in ten *Haemonchus* spp. populations from cattle in the Midwestern and Eastern portions of the Southern US (nine *H. placei* and one *H. contortus* populations). We identify, for the first time, the presence of the F200Y (TTG to TAC) mutation in *H. placei* from cattle. Its presence at low frequency in multiple independent populations suggests that benzimidazole resistance is beginning to emerge in this highly pathogenic parasite species in US cattle.

## 2. Materials and methods

### 2.1. Field populations of parasites from USA

We chose to study several different US regions where we anticipated *Haemonchus* spp. to be prevalent. Adult worms that were harvested from the abomasa of cattle immediately following slaughter were grossly identified as belonging to the *Haemonchus* genus. Three populations were obtained from Georgia (H86, H87 and H88), one population from Florida (H85) and six populations from Arkansas/Northeast Oklahoma (H9, H67, H76, H80, H81, and H84) (Fig. 1). In the case of Georgia, samples were collected from individual hosts from three distinct locations in northeast Georgia. One population (H86) was collected from an animal pastured on a farm that also raised sheep, another population (H87) was from an animal from a farm where only cattle were pastured and a third population (H88) was collected from an abattoir and so the grazing history was unknown. In the case of Arkansas, one population (H9) was collected from calves that were grazed on a single pasture at the University of Arkansas for 2 months prior to necropsy. Five populations (H67, H76, H80, H81 and H84) were collected from cattle purchased from a sale

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**Fig. 1.** Pie charts showing the relative frequency, based on individual worm pyrosequence genotyping, of SNPs that result in amino acid changes at the P200, P198 and P167 positions of isotype-1 β-tubulin in nine populations of *H. placei* (H9, H67, H76, H80, H81, H84, H85, H87 and H88) and one population of *H. contortus* from cattle (H86). Parasite species identity was determined by the SNP genotype at position 24 of the rDNA ITS-2 sequence (where *H. placei* is C and *H. contortus* is A). In the pie chart for position P200, blue represents the “resistance SNP” (TAC) and yellow represents the “susceptible SNP” (TTG). In the pie chart for position P167, the green colour represents “resistance SNP” TAC and the yellow represents the “susceptible SNP” (TTG). For position P198, the yellow represents the “susceptible SNP” (GAA) (no resistance-associated SNPs were detected at this position). The figures under the pie charts indicate the number of “resistance SNPs” detected over the total number of alleles that were genotyped in the population. These figures are only given in those instances where “resistance SNPs” were detected in the population. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

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2.2. Genomic DNA isolation

Adult worms were fixed in 70% ethanol immediately following removal from the cattle abomasum. Subsets of fixed male and female worms were taken for each population and the mid-body and posterior region removed by sharp dissection and the heads used to make DNA template. Individual adult worm heads were lysed in single 0.2 μl tube containing 50 μl of proteinase K lysis buffer and stored at −80 °C as previously described (Redman et al., 2008). 1 μl of 1:5 dilution of single worm lysate was used as PCR template and identical dilutions of lysate buffer, made in parallel, were used as negative controls.

2.3. Pyrosequence genotyping of the P24 species-specific SNP in the rDNA ITS-2

A 321 bp fragment spanning the entire ITS-2 rDNA region was PCR amplified from individual adult worm lysates using a "universal" forward primer complimentary to the 5.8S rDNA coding sequence (DITSF: 5'-AGC TCT TGG TCA GGG TTG TT-3') and biotin labelled reverse primer complimentary to the 28S rDNA coding sequence (DITSR: 5'-Biotin-TTA GTT TTC CCT CCG CT-3') (Stevenson et al., 1995). Reaction mixtures consisted of 50 μl master mix containing final concentrations of 1× thermopol reaction buffer (New England Biolabs), 2 mM MgSO4, 0.5 μl of 100 μM dNTPs, 0.5 μl of each 0.1 μM forward and reverse primers and 1.25U Taq DNA polymerase (New England Biolabs). Thermocycling parameters were 95 °C for 5 min followed by 35 cycles of 95 °C for 1 min, 57 °C for 1 min and 72 °C for 1 min with a single final extension cycle of 72 °C for 5 min.

Following PCR amplification of rDNA ITS-2, the SNP at position 24 (P24) was determined by pyrosequence genotyping using the PyroMark ID system, (Biotage, Sweden) essentially using the method described by (Hoglund et al., 2009). The sequencing primer used was Hsq24 (5'-CATATACATAATGCGCTA-3'). The SNP at position P24 was determined using a PSQ 96 system with single nucleotide position software (Pyrosequencing™ AB) with the base dispensation set to CGACTACA. Peak heights were measured using the SNP mode in the PSQ 96 single nucleotide position software. Worms were identified as *H. contortus* (homozygous for an A nucleotide at position 24), *H. placei* (homozygous for an G nucleotide at position 24).

2.4. Sequencing of the *H. placei* isotype-1 β-tubulin gene

In order to obtain the *H. placei* isotype-1 β-tubulin genomic sequence encompassing the region of interest, gDNA template from *H. placei* (MHp1) was used as template for PCR using degenerate primers designed (AE16F: 5'-GTGIMGWGGCCITAYGGIC-3'; AE25F: 5'-CARCITTYMGICCGAYAAYT-3'; AE34F: 5'-GARGGGGCG-ARCTTIGAYA-3') and reverse degenerate primers (AE16R: 5'-TGITIAAYTGDIAGCITISW-3'; AE25R: 5'-AACATYTGGITGATGCIG-3'; AE34R: 5'-TGTAYGTGRTAYTCI-SWRAT-3') against the conserved regions of isotype-1 β-tubulin sequence from *H. contortus*. An 807 bp fragment was cloned, sequenced and aligned against the *H. contortus* sequence to confirm its identity (Accession number KJ598498). Exonic and intronic sequence of the *H. placei* isotype-1 β-tubulin fragment had 98.8% and 84% identity overall with *H. contortus* sequence, respectively. We are confident that this gene fragment is the *H. placei* isotype-1 orthologue since, at the polypeptide level, there is 100% amino acid identity with *H. contortus* isotype-1 but only 98.4% amino acid identity with the next most closely related *H. contortus* polypeptide; the isotype-2 β-tubulin (Saunders et al., 2013). Furthermore, the translated polypeptide of the *H. placei* isotype-1 β-tubulin gene fragment encompasses three residues that have fixed differences with isotype-2 β-tubulin polypeptides in all trichostrongylid nematodes sequenced to date (Njue and Prichard, 2003). These are at positions P18, P56, and P68 and all of these have the amino acid characteristic of the isotype-1 polypeptide; asparagine, serine and alanine, respectively (data not shown). There was 100% sequence identity between *H. placei* and *H. contortus* over the primer regions used in the *H. contortus* pyrosequencing assay and so the same primers and conditions were used for pyrosequence genotyping of the P167, P198 and P200 mutations for both species.

2.5. Pyrosequence genotyping of the isotype-1 β-tubulin P200, P198 and P167 SNPs

A 385 bp fragment, spanning exon 5, exon 6 and intervening intron 6 of the isotype-1 β-tubulin gene, was amplified from *H. contortus* or *H. placei* respectively using previously published primers (von Samson-Himmelstjerna et al., 2009); forward primer (HcPYRF: 5'-GAC GCA TTC ACT TGG AGG AG-3') together with reverse primer (HcPYRR: 5'-Biotin-CAT AGG TTG CAG TGT GTA-3'); A 50 μl master mix was used containing final concentrations of 1× thermopol reaction buffer (New England Biolabs), 2 mM MgSO4, 0.5 μl of 100 μM dNTPs, 0.5 μl of each 0.1 μM forward and reverse primers and 1.25U Taq DNA polymerase (New England Biolabs). Thermocycling parameters were 95 °C for 5 min followed by 35 cycles of 95 °C for 1 min, 57 °C for 1 min and 72 °C for 1 min with a single final extension cycle of 72 °C for 5 min.

Following PCR amplification of isotype-1 β-tubulin from either species, the SNP at position P167, P198 and P200 was determined by pyrosequence genotyping using the PyroMark ID system, (Biotage, Sweden) essentially using the method described by (von Samson-Himmelstjerna et al., 2009). The sequencing primers used were Hcqs167: 5'-ATA GAA TTA TGG CTT CGT-3' and Hcqs200: 5'-TAG AGA ACA CCG ATG AAA CAT-3' for the P167 and P200 mutations respectively (Hoglund et al., 2009; von Samson-Himmelstjerna et al., 2009). Subsequent
analysis of the isotype-1 β-tubulin at codons 198, the new sequencing primers Hcsq198: 5’-ACT GGT AGA GAA CAC CG-3’ was used. The SNP at position P167, P198 and P200 was determined using a PSQ 96 system with single-nucleotide position software (Pyro-seqencing™ AB) with the base dispensation set to codon 167: GATGCCTGT, codon 198: GATGCAGCA and codon 200: GATGCTGTA. Peak heights were measured using the SNP mode in the PSQ 96 single nucleotide position software. Worms were identified as follows.


3. Results

3.1. Relative prevalence of H. contortus and H. placei

Over the 231 bp of ITS-2 sequence, there are three sites at positions 24, 205 and 219 have previously been shown to have fixed single nucleotide polymorphisms (SNPs) between H. contortus and H. placei in multiple geographically diverse morphologically characterised populations (Stevenson et al., 1995; Brasil et al., 2012; Chaudhry and Gilleard, unpublished data). Here we used the P24 SNP genotype to distinguish the two species. A minimum of 32 individual adult worms were genotyped per population (294 worms in total). Nine out of ten populations were identified as 100% H. placei (P24; G genotype) and one population (H86 from Georgia) was identified as 100% H. contortus (P24 A genotype) (H86).

3.2. The frequency of the F167Y, E198A, F200Y polymorphisms in the H. placei and H. contortus populations

Pyrosequence genotyping was applied to the individual worms from the nine H. placei and single H. contortus populations in order to genotype the isotype-1 β-tubulin gene at positions P167, P198, P200. Six of the nine H. placei populations contained the P200Y (TA/C) benzimidazole resistance associated SNP at low frequency; 1/60 allele (1.7%), 1/60 allele (1.7%), 1/50 allele (2%), 2/62 alleles (3.2%), 6/64 alleles (9.4%) and 1/64 allele (1.6%) in populations H9, H76, H80, H85, H87, and H88, respectively (Fig. 1). In total, out of the 264 individual worms genotyped across the nine populations, 10 were identified as P200Y (TT/AC) heterozygous resistant, 1 was identified as P200Y (TA/C) homozygous resistant and the remaining 253 were identified as P200Y (TT/C) homozygous susceptible.

The one H. contortus population (H86) contained the P200Y (TA/C) polymorphism at high frequency 38/64 alleles (59.4%) and also the P167Y (TA/C) at lower frequency 2/62 alleles (3.1%) (Fig. 1). In this population at position P200, nine worms were homozygous resistant (TA/C), three worms were homozygous susceptible (TT/C) and 20 worms were heterozygous (TT/AC). At position P167, two worms were heterozygous resistant (TT/AC) and 30 were identified as homozygous susceptible (TT/C). All 32 individual worms were identified as homozygous susceptible (GCA) at P198.

4. Discussion

Haemonchus spp. are important, highly pathogenic blood feeding parasites of cattle in many parts of the world, particularly in warmer regions with high humidity, such as South America and the southern USA. H. placei is the most common species traditionally reported from cattle but H. contortus infections also occur (Gasbarre et al., 2009a,b). Of the 10 populations examined (6 from Arkansas/East Oklahoma, 1 from Florida and 3 from Georgia), 9 were 100% H. placei suggesting that this is still the predominant Haemonchus species infecting the cattle in the southern USA. Only 1 of the 10 populations was H. contortus (farm H86 from Georgia). Interestingly, this farm rotationally grazed cattle and sheep and clinical Haemonchosis has been previously diagnosed in lambs on this farm by one of the authors (RMK). Thus, it is likely that the H. contortus in the cattle were derived from pastures contaminated by infected sheep. Such co-grazing is not generally common in the US which may explain why H. placei is the predominant Haemonchus species found in this study. It is noteworthy that of the ten Haemonchus populations in this study, the only one with a high frequency of benzimidazole resistance-associated polymorphisms was H. contortus population (H86). Given the high prevalence of benzimidazole resistance in H. contortus in small ruminants in the southern USA (Howell et al., 2008; Mortensen et al., 2003), it seems likely that the benzimidazole resistance in this H. contortus population from cattle was originally selected in sheep with resistant parasites subsequently infecting cattle.

The most important finding of this study is the presence of the P200Y (TA/C) isotype-1 β-tubulin polymorphism in H. placei. To our knowledge this is the first report of this resistance-associated SNP in H. placei. This polymorphism is the predominant benzimidazole associated polymorphism in numerous trichostrongylid parasite species of sheep and has been more recently reported in two cattle parasites, Ostertagia ostertagi and Cooperia oncophora (Njue and Prichard, 2003; Winterrowd et al., 2003). We detected this polymorphism in three out of six independent H. placei populations from Arkansas, one from Florida and two from Georgia. Since, these nine H. placei isolates were obtained from calves originating from different locations, this result suggests the P200Y (TA/C) resistance mutation is likely to be widespread in H. placei populations in US cattle. The low frequency of the mutation in each of the populations suggests benzimidazole drugs would still have high efficacy in these cases and resistance would not be detected clinically or by in vivo or in vitro phenotypic assays. Nevertheless, the presence of this polymorphism in multiple populations suggests that there is significant risk of benzimidazole resistance emerging in this parasite species in the US cattle herd. This is a major concern given the high pathogenicity of this parasite and recent observations suggesting that
resistance to avermectin/milbemycin anthelmintics is quite common in this species (Yazwinski et al., 2013). Furthermore, the current trend of increased use of benzimidazole anthelmintics for cattle parasite control in N. America, due to concerns with avermectin/milbemycin resistant parasites, is likely to result in greater selection pressure for resistance and speed the further development of resistance in cattle parasites. This study illustrates the value of molecular genotyping techniques to detect the emergence of resistance mutations at an early stage, well before resistance would be detectable with phenotypic assays.

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