Contrasting patterns of isotype-1 β-tubulin allelic diversity in Haemonchus contortus and Haemonchus placei in the southern USA are consistent with a model of localised emergence of benzimidazole resistance

Contrasting patterns of isotype-1 β-tubulin allelic diversity in *Haemonchus contortus* and *Haemonchus placei* in the southern USA are consistent with a model of localised emergence of benzimidazole resistance

Umer Chaudhry[a, b, e]*, **E. M. Redman**[b], **Ray Kaplan**[c], **Thomas Yazwinski**[d], **Neil Sargison**[a], **John S. Gilleard**[b]*

a) *University of Edinburgh Royal (Dick) School of Veterinary Studies and Roslin Institute, Scotland, UK*
b) *Department of Comparative Biology and Experimental Medicine, Faculty of Veterinary Medicine, University of Calgary Alberta, Canada*
c) *Department of Infectious Diseases, College of Veterinary Medicine, University of Georgia, Athens, Georgia USA*
d) *Department of Animal Science, University of Arkansas, Fayetteville Arkansas USA*
e) *Department of Veterinary Epidemiology and Public Health, School of Veterinary Medicine, University of Surrey, UK*

*Corresponding authors:
Umer Chaudhry, Department of Veterinary Epidemiology and Public Health, School of Veterinary Medicine, University of Surrey, UK. Email: u.chaudhry@surrey.ac.uk

John Gilleard, Department of Comparative Biology and Experimental Medicine, Host-Parasite Interactions Program, Faculty of Veterinary Medicine, 3330, Hospital Drive, University of Calgary, Calgary, Alberta, T2N 4N1 Canada. Email: jsgillea@ucalgary.ca

Abstract

The benzimidazoles are one of the most important broad-spectrum anthelmintic drug classes for parasitic nematode control in domestic animals and humans. They have been widely used in livestock, particularly in small ruminants for over 40 years. This has resulted in widespread resistance in small ruminant gastrointestinal nematode parasite species, especially *Haemonchus contortus.* Benzimidazole resistance mutations have also been reported in *Haemonchus placei*, but only at low frequencies, suggesting resistance is at a much earlier stage of emergence than is the case for *H. contortus*. Here, we investigate the haplotype diversity of isotype-1 β-tubulin benzimidazole resistance mutations and the population genetic structure of *H. contortus* and *H. placei* populations from sheep and cattle from the southern USA. Microsatellite genotyping revealed a low level of genetic differentiation in six *H.placei* and seven *H. contortus* populations examined. This is consistent with several previous studies from other regions, mainly in *H. contortus*, supporting a model of high gene flow between parasite populations. There was a single F200Y(T**A**C) haplotype present in all six *H. placei* populations across Georgia, Florida and Arkansas. In contrast, there were at least two different F200Y(T**A**C) haplotypes (up to four) and two different F167Y(T**A**C) haplotypes across the seven *H. contortus* populations studied. These results provide further evidence to support a model for benzimidazole resistance in *Haemonchus* spp, in which resistance mutations arise from a single, or the small number of locations, in a region during the early phases of emergence, and subsequently spread due to animal movement.

Keywords: *Haemonchus contortus, Haemonchus placei,* benzimidazole resistance, isotype-1 β-tubulin, resistance emergence and spread.

1. Introduction

Gastrointestinal nematode parasites are a major cause of disease in grazing ruminants, resulting in billions of US dollars of annual production loss in the livestock industry worldwide (Stromberg and Gasbarre, 2006). Anthelmintic resistance is an ever-increasing threat and understanding the patterns of its emergence is an important goal. *Haemonchus contortus* most commonly infects sheep and goats, causing significant economic losses worldwide, whereas *Haemonchus placei* predominantly infects large ruminants and its economic importance is generally restricted to warmer regions (Hoberg et al., 2004; Lichtenfels et al., 1994; Lichtenfels JR, 1994). Benzimidazole resistance is at an advanced stage in *H. contortus* in many parts of the world and multiple studies have shown regional importance of single nucleotide polymorphisms (SNPs) at codons F167Y(T**T**C-T**A**C), E198A(G**A**A-G**C**A) and F200Y(T**T**C-T**A**C) of the isotype-1 β-tubulin gene (Brasil et al., 2012; Ghisi et al., 2007; Hoglund et al., 2009; Kotze, 2012; Kwa et al., 1994; Redman et al., 2015; Rufener et al., 2009; Silvestre and Cabaret, 2002; Silvestre and Humbert, 2002). Although benzimidazole resistance is now emerging in *H. placei* in cattle, it is generally at a much earlier stage than for *H. contortus* and is much less studied (Ali et al., 2019; Avramenko et al., 2020; Brasil et al., 2012). The F200Y(T**A**C) isotype-1 β-tubulin resistance mutations have been described in *H. placei* populations in the USA, Pakistan and Brazil (Ali et al., 2019; Avramenko et al., 2020; Brasil et al., 2012) and the F167Y(T**A**C) mutation has only been recorded in Brazil (Brasil et al., 2012).

In the present study, we have compared the population genetic structure and the isotype-1 β-tubulin haplotype diversity of *H. contortus* and *H. placei* from sheep, goats and cattle sampled from the Arkansans, Florida and Georgia regions of the southern USA. For *H. contortus*, where resistance is at an advanced stage, we find multiple resistance haplotypes across the seven locations sampled. In contrast, for *H. placei,* where resistance is at an early stage of emergence, we find just a single resistance haplotype on all six locations surveyed. These results add to evidence from our previous work suggesting the importance of the spread of resistance from a single, or relatively small number of locations, during the early stages of its emergence.

2. Materials and Methods

2.1. Parasite material

Parasite material was obtained from three regions of the southern USA, where we anticipated a high prevalence of *Haemonchus*. Adult *Haemonchus* worms were harvested from the abomasa of 10 cattle, 2 sheep and 4 goats immediately following their slaughter at three different locations of Arkansas, Florida, and Georgia. Details of the 10 cattle parasite populations have been described in a previous report (Chaudhry et al., 2014). Briefly, three populations were obtained from Georgia (Pop86C, Pop87C, and Pop88C), one population from Florida (Pop85C) and six populations from Arkansas/Northeast Oklahoma (Pop9C, Pop67C, Pop76C, Pop80C, Pop81C, and Pop84C). In the case of Georgia, population Pop86C was collected from an animal pastured on a farm that also raised sheep, population Pop87C was from an animal on a farm where only cattle were pastured and a third population (Pop88C) was collected from an abattoir and so the grazing history was unknown. In the case of Arkansas, population Pop9C was collected from calves that were grazed on a single pasture at the University of Arkansas for 2 months before necropsy. Five populations (Pop67C, Pop76C, Pop80C, Pop81C and Pop84C) were collected from cattle purchased from a sale barn that were derived from different sources in Northwest Arkansas/Northeast Oklahoma and slaughtered immediately after purchase. A final population (Pop85C) was collected from a calf experimentally infected with L_3 derived from several calves in Florida.

Two and three *Haemonchus* populations of sheep and goats, respectively, were collected from Arkansas (Pop1S, Pop2S, Pop10G, Pop11G, Pop12G) and one goat-derived *Haemonchus* population was collected from Georgia (Pop1G). In the case of Arkansas, four populations (Pop2S, Pop10G, Pop11G, and Pop12G) were collected directly from an abattoir, hence the host grazing history was unknown. The Pop1S population was collected from a farm, where sheep had been grazed on a single pasture for 6 months before necropsy. In the case of Georgia, population Pop1G was collected directly from the abattoir, with no grazing history.

Overall, the dataset was composed of 319 individual worms from 10 cattle, 64 individual worms from 2 sheep and 125 individual worms from 4 goats (Supplementary Table S1).

2.2. gDNA extraction and pyrosequence genotyping

Adult worms were fixed in 80% ethanol immediately following removal from the host abomasum. The heads of individual worms were dissected and lysed in a single 0.5ul tube containing 40 µl of lysis buffer and stored at -80°C as previously described by Chaudhry et al. (2016). 1 µl of neat single worm lysate was used as a PCR template and identical dilutions of lysis

buffer, made in parallel, were used as negative controls. To prepare pooled lysates of each population, 1 µl aliquots of individual neat adult worm lysate were pooled, and 1 µl was used as a PCR template. Pyrosequence genotyping of 10 cattle, 2 sheep and 4 goat derived lysates was performed to target the rDNA ITS-2 and codons F167Y (T**A**C), E198A (G**C**A) and F200Y (T**A**C) of isotype-1 β-tubulin of *H. placei* and *H. contortus* was described in our previous studies (Chaudhry et al., 2014; Chaudhry et al., 2015b).

2.3. Microsatellite genotyping

Six previously published microsatellites (Hcms3561, Hcms53265, Hpms43, Hpms52, Hpms53, Hpms102) were selected as potentially useful markers based on our previous data (Chaudhry et al., 2015a; Chaudhry et al., 2016; Santos et al., 2017). These studies produced clear unambiguous genotypes with either a single or double Genescan peaks on single worms, as anticipated for single copy markers in both *H. placei* and *H. contortus*. Individual worm genotyping was performed from 6 *H. placei* populations (Pop76C, Pop9C, Pop80C, Pop85C, Pop88C, Pop87C) and 4 *H. contortus* populations (Pop1G, Pop10G, Pop11G, Pop12G) that contained the F200Y(TAC) and F167Y(TAC) resistance-associated SNPs. A summary of primer sequences, allele ranges, PCR amplification, and bioinformatic analysis was described in our previous studies (Chaudhry et al., 2016; Santos et al., 2017).

2.4. Phylogenetic analysis of the isotype-1 β-tubulin locus

For the isotype-1 β-tubulin gene, a fragment encompassing parts of exons 4 and 5, including codons F167Y(T**T**C-T**A**C), E198A(G**A**A-G**C**A) and F200Y(T**T**C-T**A**C), for *H. placei* (325bp) and *H. contortus* (328bp) were amplified. Pooled lysates were made from 6 *H. placei* populations (Pop9C, Pop76C, Pop80C, Pop87C, Pop88C, Pop85C), in which F200Y (T**A**C) was detected and 7 *H. contortus* populations (Pop1S, Pop2S, Pop10G, Pop11G, Pop12G, Pop1G, Pop86C) in which F200Y(TAC) and F167Y(TAC) were detected. Amplicons were cloned into PJET 1.2/BLUNT vector (Thermo Scientific) and sequenced using standard procedures were described by Chaudhry et al. (2015b). For the phylogenetic analysis, sequences were aligned with *H. placei* and *H. contortus* isotype-1 β-tubulin reference sequences (Acc No KJ598498, Acc. No. X67489) and edited using Geneious Pro 5.4 software (Drummond AJ, 2012). A previously described approach was used to filter the isotype-1 β-tubulin sequences to remove SNPs occurring only once in the

dataset and ensure PCR-induced mutations were not included in the analysis (Chaudhry et al., 2015a; Chaudhry et al., 2016; Redman et al., 2015). The aligned sequences were then imported into the CD-HIT software (Huang et al., 2010) to calculate the number of unique haplotypes present in each population (Table 4). Construction of a network tree of the isotype-1 β-tubulin haplotypes was performed as described in our previous studies (Chaudhry et al., 2015a; Chaudhry et al., 2016).

3. Results

3.1. Confirmation of H. placei and H. contortus species

In our previous study, ITS-2 rDNA pyrosequence genotyping identified *Haemonchus* populations in 7 out of the 10 cattle hosts as comprising of 100% *H. placei* (P24; **G** genotype), one population (Pop86C from Georgia) comprising of 100% *H. contortus* (P24 **A** genotype), one population (Pop9C) comprising 97% *H. contortus* (P24 **A** genotype) and 3% *H. placei* (P24; **G** genotype) and one population (Pop85C) comprising of 100% *H. placei* (P24; **G** genotype) except for a single worm with a heterozygous **A**/**G** at position P24, suggesting that it may be a *H. placei* / *H. contortus* hybrid (Supplementary Table S1 & Fig. 1) (Chaudhry et al., 2014). In the present study, between 29 and 32 individual *Haemonchu*s worms were pyrosequence genotyped for the rDNA ITS-2 P24 SNP (64 worms form sheep and 125 worms from goats) and all worms identified as *H. contortus* (P24 **A** genotype) (Supplementary Table S1 & Fig. 1).

3.2. Allele frequencies of the F167Y, E198A, F200Y polymorphisms in the H. placei and H. contortus isotype-1 β-tubulin locus

In our previous study, pyrosequence genotyping was applied to individual worms from the 9 *H. placei* populations to genotype the isotype-1 β-tubulin locus at codon F167Y(T**T**C-T**A**C), E198A(G**A**A-G**C**A) and F200Y(T**T**C-T**A**C). Six of the 9 *H. placei* populations contained the F200Y(T**A**C) benzimidazole resistance-associated SNP at low frequencies between 2-10% (Supplementary Table S2) (Chaudhry et al., 2014). The benzimidazole resistance-associated F167Y(T**A**C) and E198A(G**C**A) SNPs were not detected in any of these cattle populations. In the present study, pyrosequence genotyping was applied to the pooled worms from 7 *H. contortus* populations to genotype the isotype-1 β-tubulin locus at codon F167Y (T**T**C-T**A**C), E198A (G**A**A-

190 G**C**A) and F200Y(T**T**C-T**A**C). Benzimidazole resistance-associated SNPs were found in all 7
191 populations with the F200Y(T**A**C) mutation at high frequencies between 82-100% and 4
192 populations with the F167Y(T**A**C) mutation at low frequencies between 7-24% (Supplementary
193 Table S2). The benzimidazole resistance-associated SNP E198A(G**C**A) was not detected in any
194 of the populations.
195
196 *3.3. Population genetic structure of H. placei and H. contortus*
197 Between 22 and 30 individual worms were successfully genotyped using a panel of six
198 microsatellite markers for each of 6 *H. placei* and 4 *H. contortus* populations. To measure the
199 level of genetic diversity between populations, the diversity index value was estimated. All
200 populations were polymorphic at all loci, with the overall number of alleles per locus (A) ranging
201 from 3 to 16 in *H. placei* and 2 to 10 in *H. contortus* respectively. Several unique alleles (A_U) were
202 observed in each population (Table 1). There was some significant departure from Hardy-
203 Weinberg equilibrium, even after Bonferroni correction, in 4 out of the 36 loci combinations for
204 *H. placei* and 3 out of the 24 loci combinations for *H. contortus*, respectively (Table 1). There
205 were no major departures from linkage equilibrium for any particular combination of loci across
206 all populations indicating that alleles at these loci were randomly associated. *H. placei* and *H.*
207 *contortus* showed a high level of overall genetic diversity in all populations, the mean allele
208 richness (A_C) was 7.750 ± 0.603 and 5.292 ± 0.479 respectively and expected heterozygosity (H_e)
209 was 0.705 (range: 0.042-0.701) and 0.488 (range: 0.048-0.546) respectively (Table 1).
210 To measure the level of genetic difference between populations, the AMOVA and fixation
211 index (F_{ST}) value was estimated. The percentage of variation that partitioned between 6 *H. placei*
212 populations was 0.042% and 4 *H. contortus* populations were 0.015%. This was reflected by levels
213 of pairwise F_{ST} estimates with a maximum of 0.09 for 13 out of 15 possible pairwise comparisons
214 in *H. placei,* and a maximum of 0.02 for 4 out of 6 possible pairwise comparisons in *H. contortus*,
215 showing a low level of genetic differentiation (Table 2).
216
217 *3.4. Haplotype distribution and the network analysis of isotype-1 β-tubulin locus of H. placei and*
218 *H. contortus*
219 A 325bp fragment of the isotype-1 β-tubulin locus was cloned and sequenced from the 6 *H.*
220 *placei* populations containing the F200Y (T**A**C) SNP. The gDNA template was pooled from

between 29 to 36 worms from each population (Supplementary Table S1) and between 6 and 12 clones were sequenced per population (Table 3). A single F200Y(T**A**C) resistance-conferring haplotype (Hr3 F200Y) was present in all six populations (Table 3; Fig. 2A) and five distinct susceptible haplotypes (designated Hs1, Hs2, Hs3, Hs4 and Hs5) were present across the six populations (Table 3, Fig. 2A). All haplotypes, except Hs4, were identified in more than one population supporting their validity (as opposed to PCR or sequencing artefacts). A phylogenetic haplotype network revealed that the single F200Y (T**A**C) resistance haplotype (Hr3 F200Y) was most closely related to the most frequent susceptible haplotype (Hs1) which was also present in all the six cattle populations (Fig. 3A).

A 328bp fragment of the isotype-1 β-tubulin locus was cloned and sequenced from 7 *H. contortus* populations. The gDNA template was pooled from between 29 to 32 worms from each population (Supplementary Table S1) and between 6 and 15 clones were sequenced per population (Table 3). A total of four *H. contortus* F200Y(T**A**C) resistance haplotypes (Hr12, Hr16, Hr22and Hr23) and two F167Y(T**A**C) resistance haplotypes (Hr20 and Hr29) were were identified in more than one population supporting their validity (Table 3; Fig. 2B), but no susceptible haplotypes were identified among 85 sequences of 7 *H. contortus* populations. A phylogenetic haplotype network was produced to examine the phylogenetic relationship between the six isotype-1 β-tubulin haplotypes (Fig. 3B). Hr12 was by far the most frequent and widely distributed haplotype, being identified in all 7 farms, followed by Hr29 (6 farms), and Hr23(2 farms) (Fig. 3B). Although Hr16, Hr22, and Hr20 haplotypes were at low frequency and only identified on a single farm each, they differed from the other haplotypes by multiple substitutions making them, more likely to be valid haplotypes rather than the result of PCR-induced mutation or sequencing error (Fig. 3B).

4. Discussion

Benzimidazole drugs have been intensively used in small ruminants worldwide for over 40 years leading to the development of resistance in multiple gastrointestinal nematode species including *H. contortus.* In the USA, most *H. contortus* populations in sheep and goats have extremely high levels of benzimidazole resistance (Kaplan and Vidyashankar, 2012). In the case of cattle in the USA, benzimidazoles have not been heavily used due to the predominance of macrocyclic lactone use in parasite control. Although there have been no published studies conclusively demonstrating phenotypic benzimidazole resistance in *H. placei* in North America,

benzimidazole resistance mutations have been reported by Chaudhry et al. (2014) and Avramenko et al. (2020). Indeed, despite the relatively limited use of benzimidazoles in USA beef cattle, the codon F200Y(T**A**C) mutation appears to be already widespread being detected in 6 out of 9 *H. placei* populations examined from Georgia, Arkansas and Florida (Chaudhry et al., 2014) and in 15 out of 32 *H. placei* populations examined from Oklahoma, Arkansas and Nebraska (Avramenko et al., 2020). However, this resistance mutation is at low frequencies in these populations (1.6% - 9.4% and 0.57 - 27.45%); these levels would not be expected to result in detectable loss of drug efficacy.

This situation allows us to explore the patterns of resistance mutations relatively early and late stages of emergence in *H. placei* and *H. contortus* respectively. Our previous work in Pakistan, where there is a similar situation, clearly showed that the resistance was much lower in *H. placei* than in *H. contortus* (Ali et al., 2018). Indeed, the F200Y(T**A**C) mutation in *H. placei* was present on just a single haplotype in the multiple populations sampled, whereas the same mutation in *H. contortus* was present on up to 8 different haplotypes. The presence of just a single F200Y (T**A**C) haplotype in *H. placei* suggested to the spread of a resistance mutation from a single location during the early phases of resistance emergence (Ali et al., 2019). This built on our other previous work on the rarer E198A(G**C**A) mutation in *H. contortus* in India, where a similar pattern of haplotype diversity suggesting a single emergence of this mutation was found in the region (Chaudhry et al., 2015a).

The work presented in this paper was performed to further test the hypothesis that resistance spreads from a single, or a small number of locations, during the early phases of its emergence. We have found that for *H. placei*, where resistance is at a relatively early stage, there is just a single F200Y (T**A**C) haplotype (Hr3) in all 6 of the *H. placei* populations studied. The dominance of the Hs1 susceptible haplotype in the *H. placei* populations means there is insufficient susceptible allelic diversity to allow us to strongly conclude that the Hr3 haplotype is likely to have arisen just once in the region. However, the results are consistent with our previous work and provide further evidence for the genetic model that resistance mutations spread from a single, or a small number of locations in a region during the early phases (Ali et al., 2019; Chaudhry et al., 2015a). The *H. placei* results contrast with those of *H. contortus*, where resistance is more advanced since we identified at least two different F200Y(T**A**C) (likely four) and two different F167Y(T**A**C) haplotypes across the 7 *H. contortus* populations sampled. The early spread of resistance from one

or a small number of locations in a region emphasis the importance of livestock movement in the spread of benzimidazole resistance mutations in ruminants (Chaudhry et al., 2016).

There have been several studies on the population genetics of *H. contortus* but much less is known for *H. placei* (Chaudhry et al., 2015a; Chaudhry et al., 2016; Hunt et al., 2008; Redman et al., 2015; Silvestre et al., 2009). Microsatellite genotyping revealed a high level of genetic diversity among *H. placei* (allele richness 7.750 ± 0.603, expected heterozygosity 0.705) and *H. contortus* (allele richness 5.292 ± 0.47, expected heterozygosity 0.488) populations and a low level of genetic differentiation between the populations; *H. placei* (F_{st} estimates a maximum of 0.09) and *H. contortus* (F_{st} estimates a maximum of 0.02). This population genetic structure is consistent with that expected when high levels of gene flow occur between parasite populations and further supports the likelihood of the spread of resistance alleles in the southern USA.

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Figure Legends

Fig. 1. Distribution of *Haemonchus* spp. identified in from several locations in the southern USA. Geographic locations of abattoirs/farms are indicated with small black circles in three states (A) Arkansas (B) Georgia (C) Florida. Each pie chart represents a single parasite population taken from an individual host. The final letter of the parasite population name indicates the host species of origin (S, sheep; G, goat; C, cattle). Black shading represents worms identified as *H. placei* (Homozygous **G** at ITS-2 rDNA P24), vertical line shading represents worms identified as *H. contortus* (Homozygous **A** at ITS-2 rDNA position P24) and the light dot represents worms identified as putative hybrids (heterozygous **A/G** at ITS-2 rDNA P24).

Fig. 2. Frequency histograms showing resistant and susceptible isotype-1 β-tubulin haplotypes identified from six *H. placei* populations in panel A and seven *H. contortus* populations in (panel B). F200Y(TTC)/ F167Y(TTC)/E198A(GAA) susceptible haplotypes are shown in blue, F200Y (T**A**C) resistant haplotypes in red colour and F167Y(T**A**C) resistant haplotypes in green colour. The number of clones sequenced corresponding to each haplotype is shown above each bar (n).

Fig. 3. Median-joining network of the *H. placei* (panel A) and *H. corturtus* (panel B) isotype-1 β-tubulin sequences generated in Network 4.6.1. A full median network containing all possible shortest trees was generated by setting the epsilon parameter equal to the greatest weighted distance (epsilon = 10). All unnecessary median vectors and links are removed with the MP option (Polzin and Daneschmand, 2003). The size of the circle representing each haplotype is proportional

410 to its frequency in the dataset and the colours in the circles reflect the spread of this haplotype in
411 each population as indicated on the colour key on the inset map. The number of mutations
412 separating adjacent sequence nodes or median vectors is indicated along connecting branches and
413 the length of the lines connecting the haplotypes is proportional to the number of nucleotide
414 changes. The most probable ancestral node is determined by rooting the network to a closely
415 related outgroup *H. contortus* (Hc) against *H. placei* network (GenBank accession number
416 **X67489**) and outgroup *H. placei* (Hp) against *H. contortus* network (GenBank accession number
417 **KJ598498**). The text providing the name of each haplotype is colour coded as follows; susceptible
418 haplotypes F200Y(T**T**C)/ F167Y(T**T**C)/E198A(G**C**A) is in black text; F200Y(T**A**C) resistant
419 haplotype is in blue text; F167Y(T**A**C) resistant haplotype is in green text.
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