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

4
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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(TAC) haplotype present in all six *H. placei* populations across Georgia, Florida and Arkansas. In contrast, there were at least two different F200Y(TAC) haplotypes (up to four) and two different F167Y(TAC) 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.

66 1. Introduction

67 Gastrointestinal nematode parasites are a major cause of disease in grazing ruminants, resulting
68 in billions of US dollars of annual production loss in the livestock industry worldwide (Stromberg
69 and Gasbarre, 2006). Anthelmintic resistance is an ever-increasing threat and understanding the
70 patterns of its emergence is an important goal. *Haemonchus contortus* most commonly infects
71 sheep and goats, causing significant economic losses worldwide, whereas *Haemonchus placei*
72 predominantly infects large ruminants and its economic importance is generally restricted to
73 warmer regions (Hoberg et al., 2004; Lichtenfels et al., 1994; Lichtenfels JR, 1994).
74 Benzimidazole resistance is at an advanced stage in *H. contortus* in many parts of the world and
75 multiple studies have shown regional importance of single nucleotide polymorphisms (SNPs) at
76 codons F167Y(TTC-TAC), E198A(GAA-GCA) and F200Y(TTC-TAC) of the isotype-1 β -
77 tubulin gene (Brasil et al., 2012; Ghisi et al., 2007; Hoglund et al., 2009; Kotze, 2012; Kwa et al.,
78 1994; Redman et al., 2015; Rufener et al., 2009; Silvestre and Cabaret, 2002; Silvestre and
79 Humbert, 2002). Although benzimidazole resistance is now emerging in *H. placei* in cattle, it is
80 generally at a much earlier stage than for *H. contortus* and is much less studied (Ali et al., 2019;
81 Avramenko et al., 2020; Brasil et al., 2012). The F200Y(TAC) isotype-1 β -tubulin resistance
82 mutations have been described in *H. placei* populations in the USA, Pakistan and Brazil (Ali et al.,
83 2019; Avramenko et al., 2020; Brasil et al., 2012) and the F167Y(TAC) mutation has only been
84 recorded in Brazil (Brasil et al., 2012).

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

93

94 2. Materials and Methods

95

96 2.1. Parasite material

97 Parasite material was obtained from three regions of the southern USA, where we anticipated a
98 high prevalence of *Haemonchus*. Adult *Haemonchus* worms were harvested from the abomasa of
99 10 cattle, 2 sheep and 4 goats immediately following their slaughter at three different locations of
100 Arkansas, Florida, and Georgia. Details of the 10 cattle parasite populations have been described
101 in a previous report (Chaudhry et al., 2014). Briefly, three populations were obtained from Georgia
102 (Pop86C, Pop87C, and Pop88C), one population from Florida (Pop85C) and six populations from
103 Arkansas/Northeast Oklahoma (Pop9C, Pop67C, Pop76C, Pop80C, Pop81C, and Pop84C). In the
104 case of Georgia, population Pop86C was collected from an animal pastured on a farm that also
105 raised sheep, population Pop87C was from an animal on a farm where only cattle were pastured
106 and a third population (Pop88C) was collected from an abattoir and so the grazing history was
107 unknown. In the case of Arkansas, population Pop9C was collected from calves that were grazed
108 on a single pasture at the University of Arkansas for 2 months before necropsy. Five populations
109 (Pop67C, Pop76C, Pop80C, Pop81C and Pop84C) were collected from cattle purchased from a
110 sale barn that were derived from different sources in Northwest Arkansas/Northeast Oklahoma and
111 slaughtered immediately after purchase. A final population (Pop85C) was collected from a calf
112 experimentally infected with L₃ derived from several calves in Florida.

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

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

122

123 2.2. *gDNA extraction and pyrosequence genotyping*

124 Adult worms were fixed in 80% ethanol immediately following removal from the host
125 abomasum. The heads of individual worms were dissected and lysed in a single 0.5ul tube
126 containing 40 µl of lysis buffer and stored at -80°C as previously described by Chaudhry et al.
127 (2016). 1 µl of neat single worm lysate was used as a PCR template and identical dilutions of lysis

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

134

135 2.3. Microsatellite genotyping

136 Six previously published microsatellites (Hcms3561, Hcms53265, Hpms43, Hpms52, Hpms53,
137 Hpms102) were selected as potentially useful markers based on our previous data (Chaudhry et
138 al., 2015a; Chaudhry et al., 2016; Santos et al., 2017). These studies produced clear unambiguous
139 genotypes with either a single or double Genescan peaks on single worms, as anticipated for single
140 copy markers in both *H. placei* and *H. contortus*. Individual worm genotyping was performed from
141 6 *H. placei* populations (Pop76C, Pop9C, Pop80C, Pop85C, Pop88C, Pop87C) and 4 *H. contortus*
142 populations (Pop1G, Pop10G, Pop11G, Pop12G) that contained the F200Y(TAC) and
143 F167Y(TAC) resistance-associated SNPs. A summary of primer sequences, allele ranges, PCR
144 amplification, and bioinformatic analysis was described in our previous studies (Chaudhry et al.,
145 2016; Santos et al., 2017).

146

147 2.4. Phylogenetic analysis of the isotype-1 β-tubulin locus

148 For the isotype-1 β-tubulin gene, a fragment encompassing parts of exons 4 and 5, including
149 codons F167Y(TTC-TAC), E198A(GAA-GCA) and F200Y(TTC-TAC), for *H. placei* (325bp)
150 and *H. contortus* (328bp) were amplified. Pooled lysates were made from 6 *H. placei* populations
151 (Pop9C, Pop76C, Pop80C, Pop87C, Pop88C, Pop85C), in which F200Y (TAC) was detected and
152 7 *H. contortus* populations (Pop1S, Pop2S, Pop10G, Pop11G, Pop12G, Pop1G, Pop86C) in which
153 F200Y(TAC) and F167Y(TAC) were detected. Amplicons were cloned into PJET 1.2/BLUNT
154 vector (Thermo Scientific) and sequenced using standard procedures were described by Chaudhry
155 et al. (2015b). For the phylogenetic analysis, sequences were aligned with *H. placei* and *H.*
156 *contortus* isotype-1 β-tubulin reference sequences (Acc No KJ598498, Acc. No. X67489) and
157 edited using Geneious Pro 5.4 software (Drummond AJ, 2012). A previously described approach
158 was used to filter the isotype-1 β-tubulin sequences to remove SNPs occurring only once in the

159 dataset and ensure PCR-induced mutations were not included in the analysis (Chaudhry et al.,
 160 2015a; Chaudhry et al., 2016; Redman et al., 2015). The aligned sequences were then imported
 161 into the CD-HIT software (Huang et al., 2010) to calculate the number of unique haplotypes
 162 present in each population (Table 4). Construction of a network tree of the isotype-1 β -tubulin
 163 haplotypes was performed as described in our previous studies (Chaudhry et al., 2015a; Chaudhry
 164 et al., 2016).

165

166 3. Results

167

168 3.1. Confirmation of *H. placei* and *H. contortus* species

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

179

180 3.2. Allele frequencies of the F167Y, E198A, F200Y polymorphisms in the *H. placei* and *H.* 181 *contortus* isotype-1 β -tubulin locus

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

190 GCA) and F200Y(TTC-TAC). Benzimidazole resistance-associated SNPs were found in all 7
191 populations with the F200Y(TAC) mutation at high frequencies between 82-100% and 4
192 populations with the F167Y(TAC) mutation at low frequencies between 7-24% (Supplementary
193 Table S2). The benzimidazole resistance-associated SNP E198A(GCA) 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 (TAC) SNP. The gDNA template was pooled from

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

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

243

244 **4. Discussion**

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

252 benzimidazole resistance mutations have been reported by Chaudhry et al. (2014) and Avramenko
253 et al. (2020). Indeed, despite the relatively limited use of benzimidazoles in USA beef cattle, the
254 codon F200Y(TAC) mutation appears to be already widespread being detected in 6 out of 9 *H.*
255 *placei* populations examined from Georgia, Arkansas and Florida (Chaudhry et al., 2014) and in
256 15 out of 32 *H. placei* populations examined from Oklahoma, Arkansas and Nebraska (Avramenko
257 et al., 2020). However, this resistance mutation is at low frequencies in these populations (1.6% -
258 9.4% and 0.57 - 27.45%); these levels would not be expected to result in detectable loss of drug
259 efficacy.

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

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

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

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

294

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300

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386

387

388 Figure Legends

389

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

398

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

404

405 **Fig. 3.** Median-joining network of the *H. placei* (panel A) and *H. corturtus* (panel B) isotype-1 β -
 406 tubulin sequences generated in Network 4.6.1. A full median network containing all possible
 407 shortest trees was generated by setting the epsilon parameter equal to the greatest weighted
 408 distance (epsilon = 10). All unnecessary median vectors and links are removed with the MP option
 409 (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(TTC)/ F167Y(TTC)/E198A(GCA) is in black text; F200Y(TAC) resistant
419 haplotype is in blue text; F167Y(TAC) resistant haplotype is in green text.
420