Molecular Genetic Analysis of Benzimidazole Resistance in the Parasitic Nematodes Haemonchus contortus and Haemonchus placei

Chaudhry, Umer

http://hdl.handle.net/11023/2308

Downloaded from PRISM Repository, University of Calgary
Molecular Genetic Analysis of Benzimidazole Resistance in the Parasitic Nematodes

*Haemonchus contortus* and *Haemonchus placei*

by

Umer Naveed Chaudhry

A THESIS
SUBMITTED TO THE FACULTY OF GRADUATE STUDIES
IN PARTIAL FULFILMENT OF THE REQUIREMENTS FOR THE
DEGREE OF DOCTOR OF PHILOSOPHY

GRADUATE PROGRAM IN VETERINARY MEDICAL SCIENCES

CALGARY, ALBERTA

June, 2015

©Umer Naveed Chaudhry 2015
Abstract

The benzimidazoles are one of the most important anthelmintic drug classes used to control parasitic nematodes in domestic animals and humans. They have been intensively used in the livestock sector for over 30 years resulting in widespread resistance in small ruminant parasite species such as *Haemonchus contortus*. The work presented in this thesis investigates the molecular genetics of benzimidazole resistance in *H. contortus* and the closely related species *Haemonchus placei*. The major focus is on India and Pakistan, two regions that were considered to be potentially informative as to how resistance mutations arise and spread due to resistance being at a relatively early stage.

Chapter II, explores parasite populations in small ruminants in the Punjab region of Pakistan showing that co-infections with the two species were relatively common and that interspecies F1 hybrids were present in several populations. This raises the possibility of introgression of anthelmintic resistance genes from one species to another.

Chapter III, presents a study in southern India which provides phylogenetic evidence that the F200Y (T\textsubscript{A}C) mutation has arisen multiple independent times in the region. In contrast, the E198A (G\textsubscript{C}A) mutation appears to have arisen and spread from a single origin. This is the first genetic evidence of the spread of an anthelmintic resistance mutation between locations for any parasitic nematode species to date.

Chapter IV compares the molecular genetics of benzimidazole resistance in *H. contortus* populations under intense drug selection pressure on small ruminant government farms in Pakistan with parasite populations that are under minimal selection pressure in neighbouring rural areas. Overall, the results of this work are consistent with the hypothesis that the F200Y (T\textsubscript{A}C) isotype-1 \( \beta \)-tubulin mutation appears by recurrent mutation during
selection as well as being present in the standing genetic variation present prior to the onset of selection.

Finally, Chapter V, presents evidence that benzimidazole resistance is at an early stage of emergence in *H. placei* from cattle in the southern and mid-west USA. This raises a significant concern that clinical resistance could emerge quite quickly as more benzimidazoles are used in cattle as is the current trend.
Acknowledgements

All praises to “GOD”, the Almighty, most Gracious, the most Merciful and the Sustainer of the worlds, who sent us “MUHAMMAD (PBUH)” as a blessing for whole universe and the best teacher with the ultimate source of wisdom “HOLY QURAN”. I believe it as my utmost pleasure to avail this and express the heartiest gratitude to my respected supervisor Professor Dr. John Gilleard. His skillful guidance, unfailing patience, masterly advice and inspiring attitude made it very easy for me to undertake this work and to write this manuscript. I would also like to thank him for his financial support. I have the honor to express my deep sense of appreciation to the members of supervisory committee (Dr. Dr. Susan Kutz, Dr. Sean Rogers and Dr. Jeroen De Buck) for their generous guidance, expert advice and skillful suggestions during the course of my study. I am extremely thankful to my laboratory colleagues (Dr Libby Redman, Dr Susan Stasiuk, Dr Janneke Wit, Bradley Van Paridon and Andrew Rezansoff) for their great supported in scientific discussions, and all other colleagues who I have not mentioned for their supportive attitude. I find no word to express my appreciation to my wife (AFROZA) and both sons (SAIFULLAH & TALHA) without whom it would have been difficult to manage things during my PhD. I would like to thank my Father, my beloved Mother, my sister Uzma Ali and my friend Shoaib Ashraf, who not only inspired me but also supported to carry myself through the noble ideas of life and their best wishes for my health and success.
# Table of Contents

Abstract .................................................................................................................. II

Acknowledgements ................................................................................................. IV

Table of Contents ..................................................................................................... V

List of Figures .......................................................................................................... X

List of Tables ........................................................................................................... XII

List of Appendices .................................................................................................. XIII

List of Symbols, Abbreviations, Nomenclatures ...................................................... XV

Contributions of Authors ....................................................................................... XVIII

## Chapter I: General Introduction ............................................................................. 1

1.1. Parasitic nematodes and their importance to animal ....................................... 2

1.1.1. Taxonomy and Life cycle of the Trichostongyloidea; the major ............... 2

1.1.2. Molecular genetic identification of parasitic nematodes ............................... 5

1.2. Overview of anthelmintic drugs and their resistance ........................................ 6

1.2.1. Diagnosis of anthelmintic resistance; phenotypic .............................................. 9

1.3. Overview of Haemonchus biology ..................................................................... 9

1.3.1. Relationship between *H. contortus* and *H. placei* ......................................... 11

1.4. *H. contortus* as a model system for anthelmintic drug .................................... 11

1.5. Current understanding of the genetic structure of ............................................. 14

1.6. Mode of action of benzimidazole drugs in *H. contortus* ................................. 15

1.7. Mechanism of benzimidazole resistance in *H. contortus* ............................... 17

1.8. Molecular basis of benzimidazole resistance in *H. contortus* ......................... 21
1.9. Selective sweeps of anthelmintic resistance ................................................................. 22
1.10. Signature of selection anticipated at anthelmintic resistance loci .............................. 25
1.11. Interspecies hybridization in gastro-intestinal parasitic nematodes .......................... 26
1.2. Summary of the objectives and findings of the research presented ............................ 29

Chapter II: Genetic evidence for hybridization between *Haemonchus contortus* ................. 48

Abstract .................................................................................................................................. 49

2.1. Introduction ......................................................................................................................... 51

2.2. Materials and methods ...................................................................................................... 53

2.2.1. Morphologically characterised *H. contortus* and *H. placei* ................................. 53
2.2.2. Field populations of parasites from India and Pakistan .............................................. 54
2.2.3. Genomic DNA extraction ............................................................................................. 55
2.2.4. Pyro sequence species-specific genotyping assay for the position ......................... 55
2.2.5. Microsatellite markers ................................................................................................. 56
2.2.6. rDNA ITS-2 sequencing .............................................................................................. 58
2.2.7. Cloning of *H. placei* isotype-1 β-tubulin gene .......................................................... 58
2.2.8. PCR amplification and sequencing of the β-tubulin and mtND4 markers ............... 59

2.3. Results ............................................................................................................................... 61

2.3.1. Validation of molecular markers using morphologically ......................................... 61
2.3.2. *Haemonchus contortus* but not *H. placei* was identified in small ...................... 63
2.3.3. *Haemonchus contortus* and *H. placei* co-infections are ....................................... 65
2.3.4. Confirmation of *H. contortus/H. placei* F1 hybrids using ......................................... 66
2.3.4. F1 hybrids detected in the field can be the progeny of either ................................... 69

2.4. Discussion ......................................................................................................................... 70
2.5. Acknowledgements

Chapter III: Genetic evidence for the spread of a... 

Abstract...

3.1. Introduction

3.2. Materials and methods

3.2.1. Harvesting of adult Haemonchus worms

3.2.2. Genomic DNA isolation

3.2.3. Pyrosequence genotyping of the rDNA ITS-2 P24 SNP to determine

3.2.4. Pyrosequence genotyping to determine the relative frequencies of

3.2.5. Sequencing of cloned H. contortus isotype-1 β-tubulin

3.2.6. Bioinformatic filtering of H. contortus isotype-1 β-tubulin sequences

3.2.7. Phylogenetic network analysis of H. contortus isotype-1 β-tubulin

3.2.8. Microsatellite genotyping and population genetic analysis

3.3. Results

3.3.1. The F200Y and E198A but not the F167Y benzimidazole

3.3.2. Sequence diversity and phylogenetic relationships of isotype-1

3.3.3. Geographical distribution of F200Y (TAC) and E198A (GCA)

3.3.4. Genetic diversity and population structure of H. contortus

3.4. Discussion

3.5. Acknowledgements

Chapter IV: Comparison of benzimidazole resistance in Haemonchus

Abstract...

4.1. Introduction
<table>
<thead>
<tr>
<th>Section</th>
<th>Title</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>4.2.</td>
<td>Materials and methods</td>
<td>131</td>
</tr>
<tr>
<td>4.2.1.</td>
<td>Parasite collection</td>
<td>131</td>
</tr>
<tr>
<td>4.2.2.</td>
<td>Genomic DNA isolation and species confirmation</td>
<td>132</td>
</tr>
<tr>
<td>4.2.3.</td>
<td>Pyrosequence genotyping to determine the relative frequencies</td>
<td>133</td>
</tr>
<tr>
<td>4.2.4.</td>
<td>Cloning and sequencing of a isotype-1 β-tubulin fragment</td>
<td>134</td>
</tr>
<tr>
<td>4.2.5.</td>
<td>Phylogenetic network analysis of <em>H. contortus</em> isotype-1</td>
<td>135</td>
</tr>
<tr>
<td>4.3.</td>
<td>Results</td>
<td>136</td>
</tr>
<tr>
<td>4.3.1.</td>
<td>The F200Y (TAC) isotype-1 β-tubulin mutation was found</td>
<td>136</td>
</tr>
<tr>
<td>4.3.2.</td>
<td>Sequence diversity and phylogenetic relationships</td>
<td>137</td>
</tr>
<tr>
<td>4.3.3.</td>
<td>Geographical distribution of F200Y (TAC) isotype-1</td>
<td>139</td>
</tr>
<tr>
<td>4.4.</td>
<td>Discussion</td>
<td>142</td>
</tr>
<tr>
<td>4.5.</td>
<td>Acknowledgements</td>
<td>146</td>
</tr>
<tr>
<td>Chapter V:</td>
<td>The presence of benzimidazole resistance mutations in</td>
<td>152</td>
</tr>
<tr>
<td>Abstract</td>
<td></td>
<td>153</td>
</tr>
<tr>
<td>5.1.</td>
<td>Introduction</td>
<td>154</td>
</tr>
<tr>
<td>5.2.</td>
<td>Materials and methods</td>
<td>155</td>
</tr>
<tr>
<td>5.2.1.</td>
<td>Field populations of parasites from USA</td>
<td>155</td>
</tr>
<tr>
<td>5.2.2.</td>
<td>Genomic DNA isolation</td>
<td>156</td>
</tr>
<tr>
<td>5.2.3.</td>
<td>Pyro sequence genotyping of the P24 species-specific SNP</td>
<td>156</td>
</tr>
<tr>
<td>5.2.4.</td>
<td>Sequencing of the <em>H. placei</em> isotype-1 β-tubulin gene</td>
<td>157</td>
</tr>
<tr>
<td>5.2.5.</td>
<td>Pyrosequence genotyping of the isotype-1 β-tubulin P200</td>
<td>158</td>
</tr>
<tr>
<td>5.3.</td>
<td>Results</td>
<td>160</td>
</tr>
<tr>
<td>5.3.1.</td>
<td>Relative prevalence of <em>H. contortus</em> and <em>H. placei</em></td>
<td>160</td>
</tr>
</tbody>
</table>
5.3.2. The frequency of the F167Y, E198A, F200Y polymorphisms............................. 160

5.4. Discussion ............................................................................................................. 162

5.5. Acknowledgements .............................................................................................. 164

Chapter VI: General Discussion ...................................................................................... 169

6.1. New genetic markers for the identification of \textit{H. contortus} and \textit{H. placei} .......... 172

6.2. Differences in the rate of co-infection of small ruminants with \textit{H. contortus} ...... 174

6.3. Genetic analysis of interspecies hybridization between \textit{H. contortus} ............... 176

6.4. Differences in the occurrence of the F200Y, E198A, F167Y ............................... 180

6.5. The occurrence of benzimidazole resistance mutations in ................................... 182

6.6. The nature of the selective sweep associated with benzimidazole .......................... 184

6.7. Contribution of this work to our understanding of how ........................................ 187
List of Figures

Chapter I

Fig. 1.1. Maximum parsimony (MP) analysis of small subunit ribosomal...............................3
Fig. 1.2. Life cycle representing gastrointestinal nematodes ...............................................5
Fig. 1.3. Mode of action of benzimidazole drugs .................................................................16
Fig. 1.4a. Schematic representation of selection of resistance associated mutations ..........23
Fig. 1.4b. Schematic presentation of the origin of resistance associated mutations ..........24
Fig. 1.5. H. contortus and H. placei are distinct species that can hybridize to ..................28

Chapter II

Fig. 2.1. Distribution of Haemonchus and Mecistocirrus spp. identified..............................64
Fig. 2.2. Phylogenetic analysis of isotype-1 β-tubulin haplotypes cloned...............................67
Fig. 2.3. Phylogenetic analysis of mitochondrial NADH dehydrogenase .............................70
Fig. 2.4. The figure represents the allele frequencies of three microsatellite .......................68

Chapter III

Fig. 3.1. Relative frequencies of the F200Y (TAC), F167Y (TAC) .................................103
Fig. 3.2. Split Trees network of the H. contortus isotype-1 β-tubulin.................................105
Fig. 3.3. Median joining network of the H. contortus isotype-1 β-tubulin ............................106
Fig. 3.4. Pairwise FST values based on genotyping 18 individual worms...........................108

Chapter IV

Fig. 4.1. Relative frequencies of the F200Y (TAC), F167Y (TAC) .................................137
Fig. 4.2. Split Trees network of 89 H. contortus isotype-1 β-tubulin.................................138
Fig. 4.3. Histograms showing the frequencies of the resistant and.......................... 140
Fig. 4.4. Median joining network generated in Network 4.6.1................................. 141

Chapter V

Fig. 5.1. Pie charts showing the relative frequency, based on individual..................... 161

Chapter VI

Fig. 6.1. Relative frequencies of the F200Y (T\text{AC}), F167Y (T\text{AC})..........................179
List of Tables

Chapter I
Table 1.1. Percentage of the prevalence of resistance against three broad classes ............. 8
Table 1.2. Published cases of anthelmintic resistance in GI parasitic nematodes ............. 8

Chapter II
Table 2.1. Summary of inter- and intra-species variation ........................................... 66
List of Appendices (Supplementary Figures)

Chapter II

Supplementary Fig. S2.1. Sequence alignment of the 807 bp *Haemonchus placei* .......... 85
Supplementary Fig. S2.2. Alignment of the predicted *Haemonchus placei* ................. 85
Supplementary Fig. S2.3. Comparison of sequence chromatogram of ....................... 86
Supplementary Fig. S2.4. Genescan chromatograms for microsatellite markers...........86
Supplementary Fig. S2.5. Overall allele frequencies of microsatellite markers...........87

Chapter III

Supplementary Fig. S3.1. (A) Comparison of the relative frequency .........................119
Supplementary Fig. S3.2. The distribution of single nucleotide ..............................120
Supplementary Fig. S3.3. Frequency histograms showing resistant .........................121
Supplementary Fig. S3.4. Maximum likelihood tree of ........................................122

Chapter IV

Supplementary Fig. S4.1. The distribution of single nucleotide ..............................150
Supplementary Fig. S4.2. Maximum likelihood tree of ........................................150
List of Appendices (Supplementary Table)

Chapter II
Supplementary Table S2.1. Summary of the morphologically characterized
Supplementary Table S2.2. Summary of the field populations of *Haemonchus*
Supplementary Table S2.3. rDNA internal transcribed spacer 2 (ITS-2)

Chapter III
Supplementary Table S3.1. Allele frequency (%) of SNPs that resulted in an
Supplementary Table S3.2. Panel of microsatellites used for population
Supplementary Table S3.3. Population genetic data for each microsatellite

Chapter IV
Supplementary Table S4.1. Allele frequency (%) of SNPs that resulted in an amino
List of Symbols, Abbreviations, Nomenclatures

RHpl1  Rosario *H. placei* 1
RHpl2  Rosario *H. placei* 2
SpHpl1  Sao Paulo *H. placei* 1
MHpl1  Moredun *H. placei* 1
BZ  Benzimidazole
DAPI  4',6-diamidino-2-phenylindole
E198A  Glutamate to Alanine substitution P198
F167Y  Phenylalanine to Tyrosine substitution P167
F200Y  Phenylalanine to Tyrosine substitution P200
TAC  Thymine-Adenine-Cytosine
TTC  Thymine-Thymine-Cytosine
GCA  Guanine-Cytosine-Adenine
GAA  Guanine-Adenine-Adenine
gDNA  Genomic deoxyribonucleic acid
GI  Gastrointestinal
*H. placei*  *Haemonchus placei*
*H. contortus*  *Haemonchus contortus*
ITS-2  Second internal transcribed spacer
MHco3 (ISE)  Moredun *H. contortus* 3 (Inbred Susceptible Isolate)
MHco4 (WRS)  Moredun *H. contortus* 4 (White River Strain)
MHco10 (CAVR)  Moredun *H. contortus* 10 (Chiswick Avermectin Resistant)
mtDNA  Mitochondrial deoxyribonucleic acid
ND4  NADH dehydrogenase subunit
PCR  Polymerase Chain Reaction
rDNA  Ribosomal deoxyribonucleic acid
RFLP  Restriction Fragment Length Polymorphism
SpHpl1  Sao Paulo *H. placei* 1
SNPs  Single nucleotide polymorphisms
PCR  Polymerase Chain Reaction
DNA  Deoxyribonucleic acid
MD  *Mecistocirrus digitatus*
Hc  *Haemonchus contortus*
Hp  *Haemonchus placei*
Tc  *Teladorsagia circumcinta*
TN  Tamil Nadu
<table>
<thead>
<tr>
<th>Acronym</th>
<th>Description</th>
</tr>
</thead>
<tbody>
<tr>
<td>AP</td>
<td>Andra Predesh</td>
</tr>
<tr>
<td>KE</td>
<td>Kerala</td>
</tr>
<tr>
<td>KA</td>
<td>Karnataka</td>
</tr>
<tr>
<td>Pop</td>
<td>Population</td>
</tr>
<tr>
<td>MP</td>
<td>Maximum Parsimony</td>
</tr>
<tr>
<td>IDT</td>
<td>Integrated DNA technologies</td>
</tr>
<tr>
<td>AMOVA</td>
<td>Analysis of Molecular Variance</td>
</tr>
<tr>
<td>MgSO4</td>
<td>Magnesium Sulfate</td>
</tr>
<tr>
<td>dNTPs</td>
<td>Deoxynucleotide Triphosphates</td>
</tr>
<tr>
<td>G genotype</td>
<td>Guanine</td>
</tr>
<tr>
<td>A genotype</td>
<td>Adenine</td>
</tr>
<tr>
<td>P24</td>
<td>Position 24</td>
</tr>
<tr>
<td>P205</td>
<td>Position 205</td>
</tr>
<tr>
<td>P219</td>
<td>Position 219</td>
</tr>
<tr>
<td>HWE</td>
<td>Hardy-Weinberg Equilibrium</td>
</tr>
<tr>
<td>LD</td>
<td>Linkage Disequilibrium</td>
</tr>
<tr>
<td>ML</td>
<td>Maximum Likelihood</td>
</tr>
<tr>
<td>HKY+G</td>
<td>Hasegawa-Kishino-Yano</td>
</tr>
</tbody>
</table>
Contribution of authors

The experiments were designed and carried out by Umer Naveed Chaudhry under the supervision of Dr. John Gillear. He provided intellectual input, the laboratory space, financial support and resources to conduct the experiments.

For Chapter II (manuscript I) morphologically verified worms were kindly provided by Alessandro Amarante and Monica Amarante (Universidade Estadual Paulista (UNESP), Departamento de Parasitologia, Instituto de Biociências, Sao Paulo, Brazil) and Jacques Cabaret (Annuaire des Labaratoires et des Recherches, INRA, Tours, France). Field samples (adult worms) were collected from the abomasum of ruminants at farm level and abattoirs by Dr. Kamran Ashraf (University of Veterinary and Animal Sciences Lahore, Pakistan) Dr. Muhammad Abbas (Veterinary Research Institute Lahore Pakistan) and Dr. Ramen Muthusamy (Tamil Nadu Veterinary and Animal Sciences University, Chennai, India). Dr. E. M. Redman helped to identify three microsatellite loci to be used as species-specific nuclear genetic markers to distinguish \textit{H. contortus} and \textit{H. placei}.

Chapter III (manuscript II) field samples (adult worms) were collected from the abomasum of ruminants from different abattoirs by Dr. Ramen Muthusamy and colleagues (Tamil Nadu Veterinary and Animal Sciences University, Chennai, India). Dr. E. M. Redman helped to identify nine microsatellite loci that could be used as nuclear genetic markers in order to examine the genetic structure of \textit{H. contortus} from Southern India.
Chapter IV (manuscript III) field samples (adult worms) were collected from the abomasum of ruminants from different abattoirs by myself, Kamran Ashraf, Shoaib Ashraf, Muhammad Zubair Shabbir (University of Veterinary and Animal Sciences, Lahore, Punjab, Pakistan).

Chapter V (manuscript IV) field samples (adult worms) were collected from the abomasum of ruminants at farm level and abattoirs by Dr. Ray Kaplan (University of Georgia, Athens, Georgia USA), Melissa Miller and Dr. Thomas Yazwinski (University of Arkansas, Fayetteville Arkansas USA).

This thesis inclusive of all chapters was written by Umer Naveed Chaudhry with feedback from my supervisor, Dr. John Gilleard.
CHAPTER I

GENERAL INTRODUCTION
1.1. Parasitic nematodes and their importance to animal and human health

Parasitic nematodes are extremely important agents of both human and animal disease (Nieuwhof and Bishop, 2005; Stromberg and Gasbarre, 2006). They are among the most economically important pathogens of ruminants causing serious economic loss and animal welfare problems for the livestock industries worldwide. For example, the annual losses associated with gastrointestinal (GI) parasitic nematodes (superfamily trichostrongyloidea) of small and large ruminants in Australia and USA has been estimated between 1 to 2 billion dollars and are proposed to be billions of dollars worldwide (McLeod, 1995; Stromberg and Gasbarre, 2006). They are also a major contributor to worldwide poverty being ranked amongst the most important animal health problems for livestock producers in the developing world (Perry et al., 2002). Thus, there are major economic gains to be made in agriculture by enhancing the control of key parasitic diseases (McLeod, 1995; Stromberg and Gasbarre, 2006; Gilleard, 2013). It is also estimated that there are 3.5 billion human helminth infection cases worldwide, of which 450 million are individuals who are seriously ill as a result of GI parasitic nematode infections including children and pregnant women (Stepek et al., 2006). Approximately 125,000 deaths occur per year, and these are mainly due to infections with the hookworm infestation (Stepek et al., 2006).

1.1.1. Taxonomy and Life cycle of the Trichostorngyloidea; the major superfamily of gastrointestinal parasitic nematodes of livestock

The phylum Nemathelminthes has six classes, but only one of these, the nematoda, contains parasitic species of significance. The nematodas are commonly called roundworms, from their appearance in cross-section. There are number of further different classification of
nematoda of veterinary importance including order, superfamily, genus and species and a major traditional division was based on bursate and non-bursate groups (Urquhart, 1996). The members of the order Strongylida of nematoda class are mostly of the bursate group, which fall into four well-distinct superfamilies; the trichostrongyloidea (include economically important parasites of ruminants), the strongyloidea (include many important equine parasites), the ancylostomoidea (include hookworms of humans and domestic animals) and the metastrongyloidea (include lungworms species of domestic animals) (Anderson, 2000). A more recent evolutionary framework has been produced based on small subunit ribosomal DNA which divided the nematode into 5 major clades (I-V) (Fig. 1.1) (Blaxter et al., 1998).
**Fig. 1.1.** Maximum parsimony (MP) analysis of small subunit ribosomal (SSU) sequences from 53 nematode taxa. Complete SSU sequences were aligned and processed for MP analysis. 24 shortest trees (including 3,583 nucleotide changes) were found; the tree presented is the strict consensus of these. Branch lengths are strained to be proportional to the number of changes contingent. The ecologies of the taxa are presented by coloured images. No shorter trees were revealed by local searching of subsets of the data. *Priapulus* and *Chordodes* were defined as outgroups (Blaxter et al., 1998).

There are many genera in the superfamily trichostongyloidea (which are within Clade V) that are important GI parasitic nematodes of ruminant livestock (Gilleard, 2013). Most notably, *Haemonchus, Ostertagia, Teladorsagia, Trichostrongylus* and *Cooperia*. Within each of the genera there are several important species. The most important GI parasitic nematode species of small ruminants are *Haemonchus contortus, Teladorsagia circumcincta* and *Trichostrongylus* spp. and of large ruminants are *H. placei, Ostertagia ostertagi* and *Cooperia* spp. (Roeber et al., 2013). The most important species of human GI parasitic nematodes are *Ancylostoma duodenale, Necator americanus, Ascaris lumbricoides* and *Trichuris trichiura* belong to superfamily Ancylostomatoidea (Clade V), Ascaridoidea (Clade III) and Trichuroidea (Blaxter et al., 1998; Gilleard, 2013).

The life cycles of parasitic nematodes are broadly similar (Fig. 1.2). In the case of the GI nematodes, the adult stages are present in the GI tract, where fertilized females produce large numbers of eggs that are passed in the faeces. Trichostrongyloidea eggs usually hatch within 1–2 days within the feces and the hatched L1 stage larvae moults into the L2 and L3 stages in the feces. The infective larval stage (L3) then migrates into the pasture and, once ingested by the host, moults to the L4 stage and then the adult in the GI tract (Zajac, 2006) (Fig. 1.2). After copulation with males, the adult female worms start laying eggs at 18 days post-oral infection with L3 and can be fully gravid by day 33.
Fig. 1.2. Life cycle representing gastrointestinal nematodes (superfamily trichostrongylidea) of ruminants. First, second and third stage larvae (L1, L2 and L3, respectively) are free living in the environment. The fourth stage larvae (L4) and adult parasitic stage is present in the GI of the host. Disease is caused by the L4 and/or adult stages and depends on factors including: species of nematode infecting the host; intensity of the infection; species, age and immunological/health status of the host; host response against the parasite; environment and management aspects.

1.1.2. Molecular genetic identification of parasitic nematodes

In the last few years, there have been major changes occur in the field of parasitology. Those changes have important implications for the development of new approaches for the diagnosis, treatment and control of parasitic diseases, and to study the taxonomy, population genetics, ecology and epidemiology of parasites (Gasser RB, 2006). Parasitic nematodes have been traditionally identified and distinguished on the basis on their morphology using features such as cuticular ridge patterns, sublateral hypodermic chords and morphometrics of the male bursa and spicules (Lichtenfels JR, 1988; Lichtenfels et al., 1994; Lichtenfels JR, 1994; Lichtenfels and Pilitt, 2000). However, these approaches are specialist and time consuming and often insufficient for specific identification. Consequently, various molecular techniques are increasingly used to provide a powerful alternative to overcome the limitations of traditional approaches. These include polymerase chain reaction (PCR), Restriction fragment length polymorphism-polymerase chain reaction (RFLP-PCR), Multiplex PCR, qPCR, Sanger sequencing and Pyrosequence genotyping (Gasser RB, 2006). These have proven to be very
useful in species identification, especially where morphological characters are unreliable to
distinguish between species. Sequence differences in the coding and non-coding regions of the
rDNA cistron have been the most commonly used genetic markers for parasitic nematode
species identification to date. In the case of members of the superfamily trichostrongyloidea,
the internal transcribed spacers of ribosomal DNA cistron (ITS-1 and ITS-2) generally
provide an appropriate level of inter-species variation for reliable species identification
(Stevenson et al., 1995; Hung et al., 1996; Hoste et al., 1998; Zarlenga et al., 1998; Chilton
and Gasser, 1999; Schnieder et al., 1999; Wimmer et al., 2004; Mochizuki et al., 2006;
Roeb et al., 2013).

1.2. Overview of anthelmintic drugs and their resistance in parasitic nematodes of
livestock

Parasitic nematodes are a major cause of disease and productivity loss in grazing livestock
throughout the world. Unlike the situation in humans, where parasitic nematodes are mainly a
problem in developing countries, they are a major problem wherever livestock grazing occurs
(Waller et al., 2004). Contamination of pastures with feces is an inevitable consequence of
livestock grazing and provides an excellent environment for parasite transmission. As a
consequence, modern livestock agriculture has been heavily dependent upon the routine
administration of broad spectrum anthelmintic drugs to control parasitic nematodes. There
have been three broad spectrum anthelmintic classes that have been the mainstay of parasite
control over the last 30 years; the benzimidazoles, the imidazothiazoles and the macrocyclic
lactones (McKellar and Jackson, 2004). The discovery of anthelmintics such as thiabendazole
(first member of the benzimidazole class developed as an anthelmintic) in the 1960s was a major breakthrough in safe effective parasite control (McCracken and Lipkowitz, 1990). The imidazothiazole (levamisole) a nicotinic acetylcholine receptor agonist was discovered in 1966 (Little et al., 2010). In 1980, a another breakthrough in livestock parasite control was made with the introduction of the macrocyclic lactone (ivermectin), a glutamate–gated chloride and GABA channel agonist that had a wide spectrum of activity against not only nematode parasites but many arthropod parasites as well. Two new classes of anthelmintic drugs for parasite control in sheep were introduced in 2008; monepantel, an amino-acetonitrile derivative and derquantel or spiroindole, which both act on novel acetylcholine receptor channels (Kaminsky et al., 2008). However, the benzimidazoles, imidathiozoles and macrocyclic lactones still are the most commonly used drug classes in sheep and cattle.

Anthelmintic resistance has been defined as a reduction in the efficacy of a drug against a population of parasites, which are usually susceptible to this drug by a specific dose or concentration (Prichard et al., 1980) or when a greater concentration of drug is required to reach a certain level of efficacy (Wolstenholme et al., 2004). Consequently, the high level of treatment of livestock with anthelmintic drugs has inevitably led to the development of resistance (Kaplan, 2004; Gillear, 2006; Gillear and Beech, 2007). Resistance has been most rapid to emerge in parasitic nematodes of small ruminants and resistance to all the major classes of anthelmitnics is common and widespread in several parasite species such as 

*Haemonchus contortus* and *Teladorsagia circumcincta*. This is a serious problem and threatens the viability of small ruminant industry in many regions. The situation is summarized in Table 1.1 (Waller, 1997; McKellar and Jackson, 2004).
Table 1.1. Percentage of the prevalence of resistance against three broad classes of anthelmintic drugs in sheep farms. Table adopted from McKellar and Jackson (2004) and Waller (1997).

<table>
<thead>
<tr>
<th>Class of Drug</th>
<th>Australia</th>
<th>South Africa</th>
<th>Brazil</th>
<th>UK</th>
<th>Brazil</th>
<th>Uruguay</th>
<th>Paraguay</th>
</tr>
</thead>
<tbody>
<tr>
<td>Benzimidazoles</td>
<td>90%</td>
<td>79%</td>
<td>90%</td>
<td>60-70%</td>
<td>68%</td>
<td>61%</td>
<td>70%</td>
</tr>
<tr>
<td>Levamisole</td>
<td>80%</td>
<td>73%</td>
<td>84%</td>
<td>30%+</td>
<td>19%</td>
<td>29%</td>
<td>47%</td>
</tr>
<tr>
<td>Macroyclic lactones</td>
<td>60%</td>
<td>73%</td>
<td>13%</td>
<td>30%+</td>
<td>7%</td>
<td>1%</td>
<td>67%</td>
</tr>
</tbody>
</table>

Despite a widespread global concern regarding the development of resistance in parasites of small ruminants, until recently there has been little attention given to the possibility of resistance developing in cattle parasites (Jackson et al., 1987; McKenna, 1996; Coles, 2002). This is mainly due to two main reasons: Firstly, resistance has been slower to develop in cattle than in sheep probably because somewhat less intense anthelmintic use has been applied. Secondly, the consequences of poor parasite control are less obvious in cattle due to the sub-clinical nature of most infections. Nevertheless, anthelmintic resistance in cattle parasites is now emerging and represents a serious challenge to the cattle industry worldwide as summarized in Table 1.2 (Wolstenholme et al., 2004; Gasbarre et al., 2009a; Sutherland and Leathwick, 2011).

Table 1.2. Published cases of anthelmintic resistance in GI parasitic nematodes of cattle. Table adopted from Sutherland and Leathwick (2011).

<table>
<thead>
<tr>
<th>Anthelmintic class</th>
<th>No of farms</th>
<th>Species</th>
</tr>
</thead>
<tbody>
<tr>
<td>ARG</td>
<td>19</td>
<td><em>H. placei</em>, <em>Cooperia oncophora</em>, <em>Ostertagia ostertagi</em></td>
</tr>
<tr>
<td>AUS</td>
<td>3</td>
<td><em>H. placei</em>, <em>C. oncophora</em>, <em>Trichostrongylus axei</em></td>
</tr>
<tr>
<td>BRA</td>
<td>29</td>
<td><em>H. placei</em>, <em>C. oncophora</em>, <em>C. spatulata</em>, <em>C. pectinata</em>, <em>O. radiatum</em>, <em>Oesophagostomum spp.</em></td>
</tr>
<tr>
<td>UK</td>
<td>1</td>
<td><em>Cooperia spp.</em></td>
</tr>
<tr>
<td>DEU, BEL, SWE</td>
<td>11</td>
<td><em>O. ostertagi</em>, <em>C. oncophora</em></td>
</tr>
<tr>
<td>IND</td>
<td>1</td>
<td><em>H. placei</em></td>
</tr>
<tr>
<td>NZ</td>
<td>80</td>
<td><em>O. ostertagi</em>, <em>C. oncophora</em>, <em>H. placei</em>, <em>H. contortus</em>, <em>Cooperia spp.</em>, <em>Trichostrongylus spp.</em>, <em>T. longispicularis</em></td>
</tr>
<tr>
<td>US</td>
<td>1</td>
<td><em>O. ostertagi</em>, <em>C. oncophora</em></td>
</tr>
</tbody>
</table>

BZ: Benzimidazoles, Lev: Levamisole, ML: Macroyclic lactones

1.2.1. Diagnosis of anthelmintic resistance; phenotypic assays and genetic markers

The growing problem of anthelmintic resistance has led to the increase awareness of the need for reliable and standardized detection methods (Coles, 1999). The most common approaches are the faecal egg count reduction test (FECRT), egg hatch assay (EHAs) and larval development test, which have number of drawbacks either in terms of cost, applicability and interpretation or reproducibility of findings. Moreover, these methodologies are both time consuming and labour intensive. To overcome this drawback, molecular tests for the detection of anthelmintic drug resistance have been developed and employed in a variety of different techniques include SNP-PCR, qPCR, direct sequencing and pyrosequencing genotyping (Beech et al., 2011). The molecular changes occur in isotype-1 β-tubulin, nicotinic acetylcholine gated cation channels, glutamate and GABA gated chloride channels are associated with the development of anthelmintic resistance in parasitic nematodes (Beech et al., 2011), therefore considered to be valuable genetic markers for the identification of anthelmintic resistance in parasitic nematodes.

1.3. Overview of Haemonchus biology

One of the most important genera of the superfamily trichostrongyliidea is Haemonchus, which includes the species H. contortus, H. placei, Haemonchus similis and Haemonchus longistipes. The genus Haemonchus has its evolutionary origins in Africa, where members infect a large number of native antelope hosts with varying degrees of efficiency (Hoberg et al., 2004). The migration of the domesticated animals around the globe by transportation has resulted in the spread of H. contortus, H. placei and H. similis. This has led to H. contortus
and *H. placei* becoming amongst the most important and highly pathogenic GI parasites of small and large ruminants respectively worldwide. *H. contortus* predominantly infects small ruminants, whereas *H. placei* predominantly infects cattle (Lichtenfels et al., 1994). However, these host affiliations are not absolute as will described in further detail later. Although *H. contortus* is originally a tropical parasite, it is now well established in many temperate and even sub-arctic regions with ongoing range expansion possibly associated with global warming (Troell et al., 2003; Waller et al., 2004; Sargison et al., 2007; Barrère et al., 2012; Barrere et al., 2013a; Barrere et al., 2013b). The closely related parasitic nematode *H. placei* is of major economic importance in tropical and sub-tropical regions (Urquhart, 1996). It also occurs sporadically in temperate regions such as western Europe and has also been recently identified as far in north of Sweden and Canada again perhaps suggesting range expansion (Avramenko and Gillear, unpublished data, Johan Hoglund, Susan Kutz, pers comm).

*Haemonchus* species cause severe economic loss in small ruminants due to mortalities, morbidity, poor growth as well as reduced wool and milk production (Coop et al., 1977). These are significant in many regions of the world (Bliss and Todd, 1974). *Haemonchus* species are blood-feeding parasites that live on the mucosal surface of the host abomasum (or stomach) and feed on blood by virtue of their piercing mouthparts (Urquhart, 1996). The blood loss associated with parasite feeding and the haemorrhage from the associated mucosal lesions results in haemorrhagic anemia. The severity of this is related to the worm burden but it is often serious and results in clinical anemia which is often fatal when parasite burdens are high (Urquhart, 1996).
1.3.1. Relationship between H. contortus and H. placei

In late seventies, Gibbons (1979) considered that H. placei and H. contortus belonged to the same species. However, subsequent morphological and genetic work overwhelmingly supports the separate species status (Lichtenfels et al., 1994; Stevenson et al., 1995). There are number of fixed morphological differences including longitudinal ridges on the cuticle surface and morphology of the spicule (Lichtenfels et al., 1994; Jacquiet et al., 1997). There are also differences in karyotype as visualized on DAPI stained metaphase spreads; all the chromosomes of H. contortus including autosomes and the X chromosome are of similar size, whereas in H. placei the X chromosome is larger than the autosomes (Jambre, 1979; Amarante et al., 1997). Additionally, there are fixed nucleotide differences in the second internal transcribed spacer (ITS-2), rDNA non-transcribed spacer (NTS) of ribosomal DNA and mtDNA nd4 gene (Zarlenga et al., 1994; Stevenson et al., 1995; Blouin et al., 1997). In addition, genetic crossing experiments in which F1 hybrids cannot interbreed also support a separate species status as will be reviewed in more detail later (Le Jambre, 1981).

1.4. H. contortus as a model system for anthelmintic drug resistance research

The use of H. contortus as a model system in which to study anthelmintic resistance is based on number of advantages over most other animal parasites. First, this parasite has an extremely high fecundity compared with other trichostongyloid nematodes; a single adult female worm produces up to 4000 eggs per day (Gilleard, 2013). Therefore, millions of eggs can be produced from a single infected host in just in a few days which can be further developed into the L1, L2 and finally L3 infective stage larvae in faeces by harvesting at the
suitable time. This level of production can be sustained from the same infected host over many weeks or even months. Second, the adult worms are comparatively larger than other trichostongyloidea nematode (up to 2.5-3.0 cm in length) allowing certain amounts of material of the adult parasitic stages to be obtained from a single host. Third, *H. contortus* has a short pre-patent period (19-20 days) and burdens of several thousand worms per individual host can be established and maintained for many months from a single oral infection of L3 larvae. Fourth, a useful feature of *H. contortus* is the ability to store large numbers of viable L3 larvae at 15–22 °C for many months. This makes it realistic for a well-equipped laboratory to maintain multiple strains, since each strain only needs to be passage through a single host once or twice per year (Gilleard, 2006, 2013). Finally another important feature of *H. contortus* is its very high propensity to develop resistance to anthelmintics and consequently it is the parasite species which has developed resistance most rapidly.

The value of *H. contortus* as a model system with which to study anthelmintic resistance is heavily dependant on high quality genomic resources. There has been a major effort to sequence the *H.contortus* genome which has been challenging for a number of reasons including a large genome size (currently estimated between 300 to 340 Mb), complex gene structures, a high level of sequence polymorphism and a high level of repetitive sequences (Gilleard, 2013; Laing et al., 2013). Nevertheless a good draft *H. contortus* genome is now available and there are continued efforts for ongoing improvement.

Forward genetic analysis is a potentially extremely powerful approach to identify new candidate genes of anthelmintic resistance. However, genetic crossing of parasitic nematodes is a difficult task because the adult parasites are concealed in the host. Nevertheless, the high fecundity of *H. contortus* and ability to viably transplant adult worms into the abomasum
makes genetic crossing feasible. There have been a number of published examples of successful genetic crossing with *H. contortus* over the years (Jambre, 1979; Le Jambre, 1981; Le Jambre et al., 1999; Hunt et al., 2010; Redman et al., 2015). The most recent application of genetic crossing in this parasite was to undertake a serial backcrossing experiment in order to introgress ivermectin resistance loci into the drug susceptible strain for mapping purposes (Redman et al., 2015). In this particular study, L4 female worms from a drug susceptible strain were transplanted with L4 male worms from a drug resistant strain directly into the abomasum of a recipient ovine host. Eggs recovered in the faeces of the recipient host (F1 progeny) were cultured to the L3 stage *in vitro* and then used to orally infect another host to obtain F1 adult worms. These F1 adult worms could then be surgically recovered from the abomasum on necropsy and used in the next generation cross (Redman et al., 2015). In another recent study, an F2 mapping cross was performed using similar techniques (Hunt et al., 2010). This ability to perform genetic crosses in *H. contortus* makes it one of the very few parasitic nematodes for which there is the potential to genetically map anthelmintic drug resistance loci.

Another important feature of *H. contortus* to highlight is its phylogenetic relationship with *C. elegans* (Blaxter et al., 1998). They are both clade V nematodes and this relatively phylogenetic proximity makes much of the knowledge of drug mode of action and resistance in *C. elegan* of direct relevance to *H. contortus* and related parasites. Furthermore, this relationship has made it possible to undertake functional heterologous expression of *H. contortus* genes in *C. elegans* (Kwa et al., 1995; Delany et al., 1998; Glendinning SK et al., 2011). The ability to use *C. elegans* in this way allows the role of particular *H. contortus*
genes in both drug mode of action and resistance to be functionally studied (Delany et al., 1998).

1.5. Current understanding of the genetic structure of *H. contortus* populations

*H. contortus* populations have a very high level of genetic diversity and this is could be due to two main factors; a large effective population size (approximately 4000 eggs per day) and high mutation rate (Blouin et al., 1995; Prichard, 2001; Gilleard and Beech, 2007). Both factors could contribute to the levels of genetic diversity in parasitic nematodes, although it is difficult to estimate the accurate population size and mutation rate in these organisms. A number of previous studies have suggested that the high level of genetic diversity in *H. contortus* predominantly due to the high population size (Prichard, 2001; Redman et al., 2008; Redman et al., 2015).

A number of studies have investigated how genetic variation is partitioned between *H. contortus* populations (Troell et al., 2003; Redman et al., 2008; Silvestre et al., 2009; Redman et al., 2015). A study of the global population genetic structure of *H. contortus*, using mitochondrial DNA and AFLP markers, revealed a high level of genetic structure between continents with populations from each continent essentially forming monophyletic groups (Troell et al., 2003). Similarly, high levels of genetic divergence were found between laboratory strains derived from different continents by microsatellite genotyping (Redman et al., 2008). These results are not completely surprising because of the limited gene flow that is expected to occur between continents. An early study of *H. contortus*, and a number of related sheep and cattle parasites, in the USA suggested that there was essentially no geographical
sub-structuring in North America due to very large effective population sizes combined with high gene flow due to animal movement (Blouin et al., 1995). However, subsequent work using more discriminatory markers suggest that some population sub-structuring does even occur within a region. Relatively low but significant levels of genetic sub-structuring between *H. contortus* populations within the same country has been described in Sweden, Australia, UK and France (Troell et al., 2003; Hunt et al., 2008; Silvestre et al., 2009; Redman et al., 2015). The results from the UK in particular show that population sub-structuring can still be detected even when high levels of animal movement are known to occur (Redman et al., 2015). It is likely that these findings are due to the complex epidemiology of *H. contortus* in different climatic regions. For example, during the cold winters of Sweden and UK and the dry hot periods in Australia, the vast majority of free-living *H. contortus* larvae in the environment will die. Consequently, parasite survival at these particular times of year is confined to adult worms and inhibited larvae inside the host. Subsequently, there is significant potential for population bottlenecks which may allow sufficient genetic drift to account for measurable genetic sub-structure in these regions.

### 1.6. Mode of action of benzimidazole drugs in *H. contortus*

The mode of action of benzimidazole drugs was originally determined to involve the impairment of microtubule function (Prichard, 2001). Microtubules are an important component of the cytoskeleton serving a variety of functions including movement of chromosomes during cell division, providing a structural skeleton for the cell and a framework for movement of intracellular particles and exocytosis. Microtubules are composed
of two 450 amino acid proteins; one $\alpha$-tubulin and four $\beta$-tubulin subunits. The formation of microtubules is a dynamic process that involves the polymerization of tubulin dimers at one end (the positive pole) and depolymerisation at the other end (the negative pole) (Fig. 1.3).

![Mode of action of benzimidazole (BZ)](image)

**Fig. 1.3.** Mode of action of benzimidazole drugs.

The early studies on the mode-of-action of benzimidazoles in parasitic nematodes were in *Ascaris* where they were shown to disrupt the microtubules of the intestinal cells (Borgers et al., 1975; Van den Bossche et al., 1982). Subsequently, it was shown that microtubules in intestinal cells of a susceptible strain of the parasitic nematode *H. contortus* disappeared after treatment with benzimidazole drugs (Sangster et al., 1985). However, when worms from a resistant strain were treated in the same way the microtubule was still present (Sangster et al., 1985; Jasmer et al., 2007). At the molecular level, it was proposed that benzimidazoles exert their effect by binding to the $\beta$-tubulin protein monomer and preventing the formation of microtubules (Friedman and Platzer, 1978). The tubulin molecules possess three major drug binding sites, the colchicine binding site, the taxol binding site and the vinblastine binding site.
(Wilson, 1970; Lacey, 1988). It is believed that the benzimidazole drugs bind to the colchicine binding site on the β-tubulin monomer in parasitic nematodes forming a tubulin-benzimidazole complex preventing the incorporation of monomers into the positive pole of the polymer thereby inhibiting the formation and maintenance of microtubules (Friedman and Platzer, 1978; Dawson et al., 1984; Sangster et al., 1985; Lacey, 1988) (Fig. 1.4). This is proposed to result in the starvation of nematodes by intestinal disruption (Fig. 1.4) (Martin, 1997; Jasmer et al., 2007). These in-vivo studies are supported by separate work that demonstrated the inhibition of recombinant β-tubulin polymerization by benzimidazole in-vitro (Lubega et al., 1993; Oxberry et al., 2001a; Oxberry et al., 2001b). Recently, a model of β-tubulin was proposed to explain how albendazole fits within the β-tubulin molecule of parasitic nematode *H. contortus* (Prichard, 2001; Robinson et al., 2004; Aguayo-Ortiz R et al., 2013).

1.7. Mechanism of benzimidazole resistance in *H. contortus*

Benzimidazole resistance is common in many organisms ranging from fungi to nematode parasites (Gilleard and Beech, 2007). The elucidation of the role of mutations in the genes encoding β-tubulin in conferring resistance to benzimidazole is the most successful example to date of the candidate gene approach being applied to identify anthelmintic resistance genes in nematodes. In the early 1980s it was first reported that mutations in genes encoding β-tubulin were the likely cause of benzimidazole resistance in fungi (Davidse and Flach, 1977). The mechanism of benzimidazole resistance has been subsequently investigated by molecular genetic means in a number of fungal species including *Aspergillus nidulans, Neurospora*
crassa, Monilinia fructicola and in each case most, or all, of the mutations that confer benzimidazole resistance are in genes that encode β-tubulin (Orbach et al., 1986; Jung et al., 1992; McKay and Cooke, 1997; Park et al., 1997; Ma et al., 2003). Mutations in a number of sites have been shown to give rise to benzimidazole resistance in fungi. These all result in substitution of amino acids in the β-tubulin target at positions P6, P165, P167, P198 and P200.

In late 1980s, Driscoll undertook a mutagenesis in the free-living model nematode C. elegans to identify genetic loci that could give rise to benzimidazole resistance. In all, 28 mutations were identified in C. elegans that confer resistance to benomyl, a benzimidazole drug, and all of these mapped to a single locus, ben-1. Molecular cloning established that ben-1 encodes a β-tubulin (Driscoll et al., 1989).

In early 1990s, the first evidence for the role of a β-tubulin locus in benzimidazole resistance in a parasitic nematode was first shown in H. contortus by probing southern blots with a probe specific for the isotype-1 β-tubulin gene and demonstrating a reduction in the number of hybridizing fragments in resistant compared with susceptible populations (Roos, 1990; Roos et al., 1990; Geary et al., 1992; Kwa et al., 1993; Lubega et al., 1994). In this study, fragments of the cloned β–tubulin isotype-1 gene were used to probe genomic DNA on southern blots to detect restriction fragment length polymorphisms (RFLP) at this locus in H. contortus populations. In the susceptible populations, 2 to 6 different RFLP fragments were detected, depending on the restriction enzyme used, whereas in resistant populations only 1 to 2 fragments were detected. This reduction in polymorphism was interpreted as evidence of selection on the β-tubulin isotype-1 locus in the resistant populations (Roos, 1990; Roos et al., 1990; Kwa et al., 1993). In addition, the purified β-tubulin polypeptide from susceptible H. contortus populations showed high affinity binding for benzimidazole in vitro whereas from
resistant populations it demonstrated low binding affinity characteristics (Lacey and Prichard, 1986; Lubega and Prichard, 1991b, c, a). Furthermore, sequence analysis found that a phenylalanine to tyrosine substitution at the P200 (F200Y) position of β-tubulin isotype-1 polypeptide was the only polymorphism at this locus that was consistently different between susceptible and resistant *H. contortus* populations of sheep (Kwa et al., 1993). In a separate study, a deletion in the isotype-2 β-tubulin was also observed in a resistant *H. contortus* populations isolated directly from small ruminants in the field that had very high levels of benzimidazole resistance (Beech et al., 1994). However, at present, this is the only published example for which an isotype-2 β-tubulin deletion has been described and so its general importance is yet to be determined.

The work described above provides strong circumstantial evidence that the P200 phenylalanine to tyrosine substitution is an important determinant of benzimidazole resistance in *H. contortus*. However, association studies of this type cannot definitively prove that a particular polymorphism is capable of conferring a resistance phenotype since it is possible that a polymorphism in a genetically linked gene could be functionally responsible for resistance. The establishment of a causal relationship between a mutation and the resistance phenotype is a major challenge in the case of parasitic nematodes since there is a lack of reverse genetic and molecular tools to study gene function in these organisms. However, in the case of benzimidazole resistance, this hurdle was overcome by experiments in which the capacity of different alleles of the parasite β-tubulin gene to modulate benzimidazole sensitivity was tested using *C. elegans* as a heterologous expression system (Kwa et al., 1995). Transgenic expression of *H. contortus* isotype-1 β-tubulin alleles that encoded a phenylalanine residue at P200 increased the drug susceptibility of a *C. elegans ben-1* mutant
whereas alleles which encoded a tyrosine residue at P200 did not. In addition, the functional significance of the P200 substitution was directly demonstrated by site-directed mutagenesis experiments. Replacing the phenylalanine residue with a tyrosine reside at the P200 position removed the ability of a β-tubulin allele isolated from a *H. contortus* susceptible strain to revert a *C. elegans ben-1* mutant to a susceptible phenotype (Kwa et al., 1995). Hence, in the case of the *H. contortus* P200 mutation in the isotype-1 β-tubulin locus, persuasive association studies have been backed up with conclusive functional studies.

It is worth noting however, that other mutations in the isotype-1 β-tubulin genes are now thought to also contribute to benzimidazole resistance in *H. contortus*. It was first reported that phenylalanine at position P167 of isotype-1 β-tubulin in *H. contortus* is essential for high affinity binding of benzimidazole drugs to tubulin (Prichard, 2001; Robinson et al., 2004) and polymorphism in this position was subsequently associated with benzimidazole resistance in *H. contortus* (Prichard, 2001). As with codon P200, this polymorphism involves a substitution of phenylalanine with tyrosine. Curiously, substitution at both P167 and P200 have not been observed in the same haplotype of *H. contortus* of the small ruminants and appear to be mutually exclusive F167Y was found only in worms that possessed the wild-type P200 (Silvestre and Cabaret, 2002; de Lourdes Mottier and Prichard, 2008). However recently it was reported that TAC167/TAC200 heterozygous individual was observed in *H. contortus* isolates (Brasil et al., 2012). Recent evidence suggests that a new putative mutation at P198 in β-tubulin isotype-1 can potentially contribute to benzimidazole resistance in *H. contortus*. Ghisi et al. (2007) and Rufener et al. (2009) have identified an adenine to cytosine transversion that leads to a glutamate to alanine substitution at codon P198 in benzimidazole resistance selected populations. The E198A (GCA) polymorphism was found only in
haplotypes that were genotypically wild-type at position F200 (Ghisì et al., 2007; de Lourdes Mottier and Prichard, 2008).

1.8. Molecular basis of benzimidazole resistance in *H. contortus*

The mechanism of benzimidazole resistance (described above) has been investigated by molecular genetic means in *H. contortus* and strong evidence exists that 3 different single amino acid substitutions (i.e., F200Y F167Y and E198A) in the isotype-1 β-tubulin can be responsible for benzimidazole resistance (Kwa et al., 1994; Silvestre and Cabaret, 2002; Silvestre and Humbert, 2002; Ghisi et al., 2007; Hoglund et al., 2009; Rufener et al., 2009; Brasil et al., 2012; Kotze, 2012; Redman et al., 2015). Subsequent work has consistently shown the F200Y (TTC to TAC) SNP occurs in field populations of *H. contortus* suggesting it is of widespread importance worldwide (Silvestre and Humbert, 2002; Hoglund et al., 2009; Barrère et al., 2012; Brasil et al., 2012; Barrere et al., 2013a; Barrere et al., 2013b; Redman et al., 2015). The F167Y (TTC to TAC) SNP has also been detected in a number of countries although is generally considered to be less common than the F200Y (TTC to TAC) mutation (Silvestre and Cabaret, 2002; Brasil et al., 2012; Redman et al., 2015). A SNP at codon E198A (GAA to GCA) resulting in a glutamate to alanine substitution has been detected in just two field-derived laboratory passaged populations of *H. contortus* to date (Ghisì et al., 2007; Rufener et al., 2009; Kotze et al., 2012). It is generally considered that the E198A (GCA) is the rarest of the three benzimidazole mutations globally although it has not been specifically screened for in many of the published studies to date.
1.9. Selective sweeps and the emergence of anthelmintic resistance mutations in *H. contortus*

The application of anthelmintic drugs provides intense positive selection pressure for adaptive mutations in parasite populations. This is expected to result in a “selective sweep” at the loci under selection. There are two different types of selective sweep; “hard” and “soft” (Fig. 1.4a). The classic “hard selective sweep” is characterized by a single resistance haplotype rising to high frequency in parasite populations. This has been described in DDT resistance in *Drosophila* where a transposon insertion in the promoter of the cytochrome P450 gene, is thought to have arisen once and swept through many global populations of *Drosophila melanogaster* (FFrench-Constant et al., 2004). Similarly, hard selective sweeps have been described in mosquitoes (Raymond et al., 1991). In contrast, a classic “soft selective sweep” is characterized by the presence of multiple resistance haplotypes in populations either derived from recurrent mutations appearing on different haplotype backgrounds after the onset of the selection or from pre-existing mutations in the standing genetic variation present before the onset of selection. In recent years, “soft selective sweeps” are being increasingly reported particularly for organisms with large population sizes. For example, resistance to organophosphate insecticides in *Trichobolium Castaneum* (red beetle) involves multiple independent resistance alleles at the acetylcholine esterase and esterase loci respectively (Andreev et al., 1999). Recent work has shown that both “hard selective sweeps” and “soft selective sweeps” have occurred in *H. contortus* and the closely related sheep parasite *T. circumcincta* with differences occurring on different farms (Redman et al., 2015). In that study, the sweeps were generally “softer” for *T. circumcincta* than *H. contortus*. This may
reflect larger effective population size of the former providing both higher levels of genetic diversity and the potential for new mutations.

![Diagram](Fig. 1.4)  
**Fig. 1.4a.** “Schematic representation of selection of resistance associated mutations under hard versus “soft selective sweep” (a) Theoretical phylogeny of susceptible alleles where the different haplotypes are shown in different colors in each population. After selection, one single haplotype (red) become resistance and present in each population. (b) Theoretical phylogeny of susceptible alleles where the different haplotypes are shown in different colors in each population. After selection, multiple different haplotypes (blue, green and orange) become resistance and present in each population”. (Figure and legend from Ffrench-Constant et al. (2004).

Understanding the nature of adaptive changes that occur in response to drug selection may help us to understand how anthelmintic resistance mutations arise and spread (Redman et al., 2015). There are several different ways in which adaptive mutations can arise in populations that are under selection. These processes have recently been reviewed in detail by Messer and Petrov (2013) and will be briefly summarized here in the context of anthelmintic resistance mutations in parasite populations.

(a) A new resistance mutation could arise at a single origin and then spread through parasite populations by migration, likely as a consequence of host movement. As described above, this would result in a classic “hard selective sweep” (Fig. 1.4b). This has been observed for insecticide resistance genes in mosquitoes (Raymond et al., 1991) and Drosophila (Ffrench-Constant et al., 2004).

(b) Anthelmintic resistance mutations could repeatedly arise at multiple independent locations, become fixed by selection and migrate between parasite populations as a result of
host movement (Fig. 1.4b). This would be expected to result in a “soft selective sweep”. This has been recently been proposed for benzimidazole resistance genes in parasitic nematodes (Silvestre and Humbert, 2002; Skuce et al., 2010; Brasil et al., 2012; Redman et al., 2015) and number of cases of insecticide resistance in arthropods such as the red flour beetle, *Tribolium castaneum* (Andreev et al., 1999).

(c) Anthelmintic resistance mutations could be present in the standing genetic variation (so called ancient polymorphisms) present prior to drug use and rise in frequency in each population once selection is applied. This would also be expected to result in a “soft selective sweep” similar to that predicted by recurrent mutation (Fig. 1.4b). This has been a long favored model for anthelmintic resistance mutations in parasitic nematodes by a number of authors, but with little experimental evidence to support it (Roos et al., 1990; Silvestre and Humbert, 2002).

---

*(Figure and legend from Messer and Petrov (2013).*

**Fig. 1.4b.** Schematic presentation of the origin of resistance associated mutations under hard and soft sweeps. (a) In “hard selective sweep” from single mutation, a single resistance haplotype arise from a single origin (shown by x) and coalesce after the onset of positive selection (broken line). (b) In “soft selective sweep” from recurrent mutations, a multiple resistance haplotypes arose from at least two independent origins (shown by x) and lineages coalescence before the onset of positive selection. (c) In a soft sweep from the standing genetic variation, a multiple resistance haplotypes were already present at the onset of positive selection considered to be an ancient origin. The different lineages in a population sample can originate from multiple mutation events. (Figure and legend from Messer and Petrov (2013)).
1.0. Signature of selection anticipated at anthelmintic resistance loci

It is anticipated that selection of adaptive mutations results in changes to the pattern of genetic variation in the genome around the locus under selection. This is often termed the genetic signature of selection. This has been reviewed in detail in general theoretical terms by Pennings and Hermisson (2006) and in the context of anthelmintic resistance by Gilleard and Beech (2007), but the key concepts will be briefly reviewed here. The way in which a resistance mutation originates is an important factor in determining the nature of this signature of selection. Resistance mutations that arise from a single origin and rapidly increase in frequency due to selection will leave little time for recombination to break up the initial haplotype on which the resistance mutation appeared. Therefore, the signature of selection of the resulting “hard selective sweep” is expected to involve a reduction in polymorphism and an increase in linkage disequilibrium over a large genomic region surrounding the locus under selection. Over time, genetic recombination will reduce the extent of the region showing this genetic signature of selection (Gilleard and Beech, 2007).

Therefore length of time the resistance mutations have existed in a population is an important factor in determining the degree to which reduction in polymorphism and linkage disequilibrium extends out from the mutation under selection (Gilleard and Beech, 2007). In the case of trichostrongyloid nematodes such as *H. contortus*, which have large effective population sizes and relatively short generational times, this could potentially occur quite quickly; perhaps over several years. If resistance mutations arise from multiple independent origins or ancient origin, the signature of selection of the resulting “soft selective sweep” would be not expected to include a marked reduction in polymorphism around the locus under
selection. However, linkage disequilibrium would still be predicted and there are methods capable of detecting such signatures (Pennings and Herisson, 2006).

The use of genome-wide approaches to identify the regions under selection are increasingly feasible especially in *H. contortus* due to the recent improvements in the assembly and annotation of the reference genome (Redman et al., 2015). The recent UK study provides some room for optimism in this regard. Both “hard selective sweeps” and “soft selective sweeps” were detected at the isotype-1 β-tubulin locus for *H. contortus* and *T. circumcincta*. In the case of *H. contortus*, a signature of selection could be detected either by a loss of polymorphism or by simple measures of departures from neutrality in 4 out of the 5 farms examined (Redman et al., 2015).

### 1.11. Interspecies hybridization in gastro-intestinal parasitic nematodes of livestock and humans

There have been very few studies to investigate hybridization between different parasitic nematode species of animals or humans. The most detailed work to date has been performed on *Ascaris lubricoides* and *A. suum*, the large roundworms of humans and pigs, respectively, and *Trichuris trichiura* and *T. suis*, parasites of human and pig respectively. In both cases, there is good evidence for interspecies hybridization between the parasites from the two hosts (Criscione et al., 2007; Nissen et al., 2012).

In the case of trichostronglyoid nematode parasites of livestock, the evidence of interspecies hybridization is of particular interest due to its potential to provide a mechanism for the introgression of anthelmintic resistance loci between parasite species. Most of the
work on the superfamily trichostrongyloidea to date has focused on attempts to hybridize closely related species by experimental co-infection followed by detailed morphological analysis to identify potential hybrids (Le Jambre, 1981). The most detailed work has been performed on *H. contortus / H. placei* in sheep, *Cooperia oncophora / Cooperia pectinata* in cattle and *Ostertagia ostertagi / Ostertagia leptospicularis* in sheep; in each case strong evidence for interspecies hybridization has been shown for experimental hybridization between these species pairs (Isenstein, 1971; Le Jambre, 1981; Suarez et al., 1993). Consequently, it is possible that interspecies hybridization occurs in the field for a number of different ruminant trichostrongyloidea nematode species suggesting this could be an important process and mechanism for the spread of anthelmintic resistance mutations.

Although *H. contortus* can infect a wide variety of ruminant species, it is most prevalent in small ruminants (Lichtenfels et al., 1994; Hoberg et al., 2004). The closely related species *H. placei* is predominantly a parasite of cattle (Lichtenfels et al., 1994). Most *Haemonchus* infections in natural field populations are not diagnosed to the species level and so there is a lack of data on true species prevalence and the extent of co-infections. There are a few published reports of apparently pure *H. contortus* infections in cattle (Hogg et al., 2010; Akkari et al., 2013) and *H. placei* infections in small ruminants (Akkari et al., 2013). Further, *H. contortus* and *H. placei* co-infections have been reported in sheep, goats, cattle and buffalo (Amarante et al., 1997; Jacquiet et al., 1998; Achi et al., 2003; Gasbarre et al., 2009a; Gasbarre et al., 2009b; Brasil et al., 2012). Given that *H. contortus* and *H. placei* are sympatric in many regions of the world; such co-infections may be more common than previously reported. The occurrence of co-infection increases the opportunity of interspecies hybridization in parasitic nematodes. Experimental co-transplantation of *H. contortus* and
**H. placei** into the sheep abomasum can result in interspecies hybridization to produce F1 progeny. Although these progeny cannot interbreed, the female F1s are fertile when mated with males of either parental species (Le Jambre, 1981) (Fig. 1.5).

![Experimental Hybridization](image)

**Fig. 1.5.** *H. contortus* and *H. placei* are distinct species that can hybridize to produce viable F1 progeny that can mate with either parental species to produce viable F2 progeny.

Genetic hybridization between *H. contortus* and *H. placei* under field conditions could be a potentially important source of anthelmintic resistance mutations. It could provide a potential mechanism by which resistance genes from populations of *H. contortus* (where they are common) could pass into *H. placei* (where they are presumed to be less common). There are few published reports for presence of intermediate (hybrids) in sheep leading to the suggestion of hybridization (J. R. Lichtenfels, 1986; Lichtenfels JR, 1988). The only genetic evidence for presence of *H. placei* and *H. contortus* hybrids in sheep in the field come from
the identification of two worms from Brazil that were apparently heterozygous at the rDNA ITS-2 locus (Brasil et al., 2012).

1.12. Summary of the objectives and findings of the research presented in this thesis

The overall aim of the research presented in this thesis was to further understand how benzimidazole drug resistance emerges and spreads in parasitic nematode populations. The species chosen for study were *H. contortus* and *H. placei*, two parasitic nematodes of significant economic importance to the sheep and cattle industries respectively. *H. contortus* is the most tractable parasite species in which to study anthelmintic resistance and *H. placei* is a closely related nematode species that has been little studied in this regard and provides an interesting comparative system. I hypothesized that studying these parasites in different countries will provide new insights into the emergence and the spread of benzimidazole resistance mutations. The major focus of this thesis work overall was an investigation of benzimidazole resistance mutations in *Haemonchus* populations of small ruminants in southern India and the Punjab region of Pakistan. These are two regions where we anticipated that co-infections with the two *Haemonchus* species might commonly occur and that resistance may be at an earlier stage of development than most of the countries in North America and Europe that have been examined in detail to date.

In Chapter II, I present work showing that co-infections with *H. contortus* and *H. placei* are common in Pakistan but not in southern India. Further exploration of the Pakistani populations revealed the presence of F1 hybrids suggesting that interspecies hybridization is relatively common in the region. This provides a potential mechanism for the interspecies
transmission of anthelmintic resistance mutations; a phenomenon that has not been previously considered in the literature.

In Chapter III, I present a molecular genetic study of benzimidazole resistance mutations *H. contortus* in southern India. Since this was a region where resistance was predicted to be less than in examined to date, it was considered that this might provide new insights into the origin and spread of benzimidazole resistance mutations. This study has produced the first clear genetic evidence for the spread of an anthelmintic resistance mutation, E198A (GCA), to multiple different locations from a single origin.

In Chapter IV, I present a study in Pakistan that compares the molecular genetics of benzimidazole resistance mutations between *H. contortus* populations on three government farms in the Punjab region that have been under intense drug selection pressure for many years with *H. contortus* populations from surrounding rural areas that are under little selection pressure. This work provides some interesting insights into the potential origin of the benzimidazole resistance mutations in those populations and provides valuable material for future population genomic studies.

In chapter V, I present an investigation into the presence of benzimidazole resistance mutations in populations of *H. placei* harvested from cattle in several southern US states. This was an important goal as there is little information regarding benzimidazole resistance in this parasite species, or indeed parasitic nematodes of cattle in general. The F200Y (TAC) mutation was found at low frequency in a number of populations. This is the first time this resistance mutation has been reported in this economically important parasite species and suggests resistance is emerging but is still at an early stage. This work provides a foundation
for future studies into the population genetics of benzimidazole resistance in this parasite species.

Finally, in chapter V, I present a general discussion on the overall conclusions from the work in the thesis and the future directions that I believe the work should be taken.

References


Kotze, A.C., Katie; Bagnall, Neil; Hines, Barney; Ruffell, Angela; Coleman, Glen 2012. Relative level of thiabendazole resistance associated with the E198A and F200Y SNPs in larvae of a multi-drug resistant isolate of Haemonchus contortus. International Journal for Parasitology 29 92-97


nematodes of livestock is characterised by multiple independent hard and soft selective sweeps. PLoS Negl Trop Dis. 6;9, :e0003494. doi:


Genetic evidence for hybridization between *Haemonchus contortus* and *Haemonchus placei* in natural field populations and its implications for interspecies transmission of anthelmintic resistance

Umer Chaudhry*, E. M. Redman, Ramen Muthusamy, Muhammad Abbas, Kamran Ashraf, John S. Gillett

Accepted in International Journal for Parasitology, 10.1016/j.ijpara.2014.09.002
Abstract

Genetic hybridization between parasitic nematode species has potentially important consequences. It could lead to the introgression of genes between species including those involved in pathogenicity, host specificity, transmission and drug resistance. It could also complicate diagnosis and control. However, there are few compelling examples of its occurrence in parasites in the field. *Haemonchus contortus* and *Haemonchus placei* are two closely related parasitic nematode species that predominantly infect small ruminants and cattle, respectively. They are capable of experimental hybridisation when adult worms of each species are transplanted into the same individual host. Given that co-infection occurs in both small ruminants and cattle, there is potential for hybridisation in the field. However, this has not been definitively demonstrated and its extent is unknown. We investigated the occurrence of co-infection and interspecies hybridization in *H. contortus* and *H. placei* in field populations from small ruminants from Pakistan and southern India using a number of independent genetic markers. *Haemonchus contortus* and *H. placei* co-infections were common in Pakistan but not in southern India where *H. placei* appeared to be absent in small ruminant hosts. In the former region, a number of worms were identified that were heterozygous for fixed, species-specific rDNA internal transcribed spacer 2 (ITS-2) single nucleotide polymorphisms. Genotyping of these ITS-2 heterozygotes with an additional four nuclear markers conclusively demonstrated them to be F1 interspecies hybrids. Mitochondrial NADH dehydrogenase subunit 4 haplotype analyses demonstrated that four of the hybrid worms had a *H. placei* maternal parent and one had a *H. contortus* maternal parent showing that hybridization could occur in either direction. Interestingly, one of these hybrids contained an *H. contortus* isotype-1 β-tubulin benzimidazole resistance allele, suggesting there is a
potential for interspecies introgression of drug resistance loci. We believe this is the first definitive genetic evidence of hybridization between *H. contortus* and *H. placei* in the field and represents the most comprehensive genetic evidence of F1 hybrids between any human or livestock parasitic nematode species to date. Further, it suggests that interspecies transmission of anthelmintic resistance mutations warrants further investigation.

*Keywords: Haemonchus contortus, Haemonchus placei, Co-infection, Hybridization, Introgression, Anthelmintic resistance, Mecistocirrus*
2.1. Introduction

Members of the genus *Haemonchus* are blood-feeding parasitic nematodes that are amongst the most economically important parasites of grazing ruminants worldwide (Gilleard, 2013). The genus *Haemonchus* has its evolutionary origins in sub-Saharan Africa where there are a variety of species in indigenous artiodacyl hosts (Hoberg et al., 2004). Three of these species - *Haemonchus contortus, Haemonchus placei* and *Haemonchus similis* - are more globally distributed due to anthropogenic translocation by domestic livestock (Gilleard, 2013; Hoberg et al., 2004). *Haemonchus contortus* has an extremely high propensity to develop resistance to anthelmintic drugs and is an established model in which to study the genetics and population dynamics of anthelmintic resistance (Gilleard, 2013).

There was some early debate regarding the relationship between *H. contortus* and *H. placei*. Gibbons (1979) considered *H. contortus* and *H. placei* to be the same species. However, subsequent morphological, molecular and genetic evidence clearly demonstrates their separate species status. There are a number of fixed morphological differences including longitudinal ridges on the cuticle surface (synlophe) and spicule morphology (Jacquiet et al., 1997; Lichtenfels et al., 1994). There are also fixed karyotypic differences as visualized on DAPI stained metaphase spreads; all the chromosomes of *H. contortus*, including the X chromosome, are of similar size, whilst in *H. placei* the X chromosome is larger than the autosomes (Amarante et al., 1997; Le Jambre and Royal, 1980). Furthermore, there are fixed sequence differences in the rDNA second internal transcribed spacer region (ITS-2), rDNA non-transcribed spacer region (NTS) and mitochondrial DNA (mtDNA) NADH dehydrogenase subunit 4 (ND4) gene (Blouin et al., 1997; Stevenson et al., 1995; Zarlenga et
al., 1994a). In addition, experimental crosses between the two species result in F1 hybrids that are only fertile if backcrossed to one of the parental species as described in more detail below (Le Jambre, 1981).

Although *H. contortus* is most commonly found in sheep and goats (Lichtenfels et al., 1994), it can infect a large number of artiodactyl hosts including bovidae (Hoberg et al., 2004). There is less peer-reviewed literature on the host distribution of *H. placei* but it is generally accepted to be most commonly found in cattle (Lichtenfels et al., 1994). However, there are a few published reports of apparently pure *H. contortus* infections in cattle (Akkari et al., 2013; Chaudhry et al., 2014; Hogg et al., 2010) and *H. placei* infections in small ruminants (Akkari et al., 2013). Further, *H. contortus* and *H. placei* co-infections have been reported in sheep, goats, cattle and buffalo (Achi et al., 2003; Amarante et al., 1997; Brasil et al., 2012; Gasbarre et al., 2009b; Gasbarre et al., 2009a; Jacquiet et al., 1998). Given that *H. contortus* and *H. placei* are sympatric in many regions of the world; such co-infections may be more common than previously supposed.

The presence of co-infection raises the possibility of interspecies hybridisation occurring in the field. These two species can hybridize following experimental co-transplantation of adult worms into the abomasum of a recipient sheep (Le Jambre, 1981; Le Jambre and Royal, 1980). Mating of *H. contortus* males with *H. placei* females results in sterile F1 male progeny. However, the F1 female progeny can produce offspring if backcrossed with males of one of the parental species (Le Jambre, 1981; Le Jambre and Royal, 1980). If such hybridization occurs in the field, it could lead to interspecies introgression of genes and provide a mechanism for the transmission of anthelmintic resistance between parasite species. This would be a major concern given the high prevalence
of anthelmintic resistance in *H. contortus*. Hybridization would also have important implications for the role of alternative reservoir hosts where co-infections may be common (Zinsstag et al., 1997).

In this paper we investigate the presence of *H. contortus* and *H. placei* co-infections in *Haemonchus* populations of ruminants from southern India and Pakistan. We demonstrate the presence of *H. contortus/H. placei* F1 hybrids at multiple sites in Pakistan using ITS-2 rDNA, isotype-1 β-tubulin and microsatellite marker loci and demonstrate the presence of an *H. contortus* isotype-1 β-tubulin F200Y resistance allele in a hybrid worm. We believe this is the first genetic confirmation of interspecies hybridization between any parasitic nematodes of ruminant livestock in the field and raises the possibility of interspecies transmission of anthelmintic resistance mutations.

### 2.2. Materials and methods

#### 2.2.1. Morphologically characterised *H. contortus* and *H. placei* populations for genetic marker validation

A number of morphologically characterized populations of adult *H. contortus* and *H. placei* were obtained from small ruminant and bovine hosts from different parts of the world in order to validate the species-specific genetic markers on genetically diverse parasite populations (Supplementary Table S2.1). Four populations of *H. placei* (SpHpl1, RAHpl1, RAHpl2 and MHpl1) were genotyped. SpHpl1 is an experimentally passaged isolate originally derived from a cattle field isolate from Sao Paulo, Brazil and identified as *H. placei* based on the linear discriminating function of male spicule morphometric measurements.
described by Achi et al. (2003) (Dr. Alessandro Amarante and Dr Amarante personal communication). RAHpl1 and RAHpl2 are field isolates derived from post mortem material from two different calves derived from the same farm in Rosario, Argentina and morphological identified as *H. placei* (Dr. J. Cabaret, personal communication [written authorization required]). MHpl1 is a morphologically characterized *H. placei* isolate originally derived from Australia and which has now been passaged by experimental infection and used in various laboratories for many years (Jambre, 1979).

Three morphologically characterized, genetically diverse, strains of *H. contortus* that have been passaged from many years were used; MHco3 (ISE), MHco4 (WRS) and MHco10 (CAVR) (Redman et al., 2008). The geographical origins of the MHco3 (ISE) is not known (Roos et al., 2004) but MHco4(WRS) was derived from the South African White River Strain isolate (van Wyk and Malan, 1988) and MHco10 from the Australian Chiswick Ivermectin-resistant isolate (Le Jambre et al., 1995; Redman et al., 2008).

2.2.2. *Field populations of parasites from India and Pakistan*

Adult worms were harvested on necropsy from the abomasa of ruminant hosts collected from abattoirs in southern India (Tamil Nadu, Andhra Pradesh, Kerala and Karnataka) (27 populations) and from abattoirs and government farms in Pakistan (Punjab) (22 populations) (Fig. 2.1, Supplementary Table S2.2). Following ethanol fixation, worms were examined under a dissecting microscope to determine whether they belonged either to the *Haemonchus* or *Mecistocirrus* genera based on size and gross appearance (Lichtenfels and Pilitt, 2000). *Mecistocirrus* is a genus within the family trichostrongylida which is predominantly reported to infect cattle parasites. Species identity was subsequently confirmed by molecular methods as described in result section 2.3.2.
2.2.3. Genomic DNA extraction

Adult worms were fixed in 70% ethanol immediately following removal from the host abomasum. The heads of individual worms were dissected and lysed in single 0.2 µl tubes containing 50 µl of proteinase K lysis buffer and stored at -80°C as previously described (Redman et al., 2008). One microliter of 1:5 dilution of neat single worm lysate was used as PCR template and identical dilutions of lysate buffer, made in parallel, were used as negative controls. To prepare pooled lysates of each population, 1 µl aliquots of each individual neat adult worm head lysate were pooled. One microliter of a 1:20 dilution of pooled lysates was used as PCR template.

2.2.4. Pyrosequence species-specific genotyping assay for the position 24 (P24) SNP of rDNA ITS-2

The rDNA ITS-2 region was amplified from individual Haemonchus or Mecistocirrus adult worm lysates using a “universal” forward primer complementary to the 5.8S rDNA coding sequence (NC1F: 5’-ACG TCT GGT TCA GGG TTG TT- 3’) and biotin labelled reverse primer complimentary to the 28S rDNA coding sequence (NC2R: 5’-Biotin-TTA GTT TCT TTT CCT CCG CT- 3’) (Stevenson et al., 1995; von Samson-Himmelstjerna et al., 2009). A 321 bp fragment spanning the entire ITS-2 rDNA region was PCR amplified using a 50 µl master mix containing final concentrations of 1X thermopol reaction buffer, 2 mM MgSO₄, 100 µM of each dNTP, 0.1 µM forward and reverse primers and 1.25 U Taq DNA polymerase (New England Biolabs, USA). Thermo-cycling 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 single nucleotide polymorphism (SNP) at position 24 (P24) was determined by pyrosequence genotyping using the PryoMark ID system (Biotage, Sweden). The sequencing primer used was Hsq24 (5’-CATATACTACAATGTGGCTA-3’) and the nucleotide dispensation order was CGAGTCACA. Peak heights were measured using the SNP mode in the PSQ 96 single nucleotide position software. Worms were designated as *H. contortus*, *H. placei* or putative hybrids based on being homozygous A, homozygous G or heterozygous A/G at P24 respectively.

We pyrosequence genotyped *Mecistocirus digitatus* worms using the same PCR protocols, pyrosequencing primers and nucleotide dispensation order as used for *Haemonchus*. In this case, the dispensation order did not precisely match the *M. digitatus* ITS-2 rDNA sequence due to SNPs at positions P25, P28 and P29. Consequently, the pyrograms were read manually to determine the genotypes at these positions. In the case of *M. digitatus*, the expected genotypes are homozygous P25 (A), P28 (A) P29 (T) as opposed to homozygous P25 (T), P28 (T) P29 (A) for *H. contortus* and *H. placei* (accession numbers AB222059 and AB222060) (Mochizuki et al., 2006; Stevenson et al., 1995).

2.2.5. Microsatellite markers

A screen was performed to identify microsatellite loci that could be used as species-specific nuclear genetic markers to distinguish *H. contortus* and *H. placei*. A total of 27 previously published primer pairs that amplify *H. contortus* microsatellite markers were first tested for their ability to amplify loci from *H. placei* (Redman et al., 2008). PCR was performed using a 20 μl master mix containing final concentrations of 1X thermopol reaction
buffer, 2 mM MgSO$_4$, 100 uM of each dNTP, 0.1 uM forward and reverse primers and 1.25 U Taq DNA polymerase (New England Biolabs). Thermo-cycling parameters were 94°C for 2 min followed by 40 cycles of 94°C for 15 s, 54°C for 30 s and 72°C for 1 min with a single final extension cycle of 72°C for 15 min (Redman et al., 2008). A subset of those that could amplify loci from *H. placei* was then tested against several *H. contortus* and *H. placei* populations to compare the allele sizes in each species (data not shown). Three of these markers, Hcms36, Hc53265 and Hc3561, robustly amplified products from both species and had a discrete set of species-specific allelic sizes.

Microsatellite Hcms36 was amplified with primers Hcms36F 5’- HEX-GCA TAG CGG CAA GGA CGT ATG -3’ and Hcms36R 5’- CAT GAC GTA CTC TGG TTG TTC G -3’; Microsatellite Hscm53265 was amplified with primers Hcms53265F 5’- NED-TGT AGC TGG ACT TAC TTT AAA TA -3’ and Hcms53265R: 5’- AGA AGT GGA AAT GCT AGA TG -3’; Microsatellite Hcms3561 amplified with primers Hcms3561F: 5’- HEX-CCT ACA TGT CTC CCA TAT GTC -3’ and Hcms3561R: 5’- TTA GCG AAG TAA TAG CGT GCC 3’. The forward primer of each microsatellite primer pair was 5’ end labelled with either HEX or NED fluorescent dyes (IDT, Canada) and the GeneScan ROX 400 internal size standard was used on the ABI Prism 3100 genetic analyser (Applied Biosystems, USA). Individual chromatograms were analysed using Gene Mapper software to accurately size amplicons and determine genotypes (Applied Biosystems). Exact tests for Hardy-Weinberg equilibrium (HWE) and pairwise linkage disequilibrium (LD) were conducted using Fisher's Exact Test (10,000 runs) using GDA version 1.1. To correct for multiple tests, the *P* value considered significant was adjusted from < 0.05 by sequential Bonferroni correction (Rice, 1989). LD
was calculated by preserving genotypes, to prevent within loci disequilibrium affecting \( P \) value significance (Labate, 2000).

2.2.6. rDNA ITS-2 sequencing

The rDNA ITS-2 region was amplified from pooled Haemonchus adult worm lysates using universal forward and reverse primers described in Section 2.4. rDNA ITS-2 amplicons from pooled worm lysates were cloned into the PCR@ 2.1 TOPO TA vector, and individual clone inserts were sequenced using the reverse primer (NC2R: 5'-TTA GTT TCT TTT CCT CCG CT- 3') on an ABI Prism 377 capillary sequencer. Sequences were edited and aligned with \( H. \) contortus and \( H. \) placei ITS-2 sequences available in GenBank (accession numbers. X78803 and X78812) using Geneious Pro 5.4 software (Drummond AJ, 2012).

2.2.7. Cloning of \( H. \) placei isotype-1 \( \beta \)-tubulin gene

The \( H. \) placei isotype-1 \( \beta \)-tubulin sequence was not available at the start of this project, thus the relevant region was amplified from \( H. \) placei (MHp1) using degenerate primers designed against the \( H. \) contortus isotype-1 \( \beta \)-tubulin sequence (GenBank accession number M76493). The forward degenerate primer was AE16F: 5’-GTIMGIWSIGGICCITAYGGICA-3’ and the reverse degenerate primer was AE25R: 5’-AACATYTGYTGIGTIAGYTCIGC-3’. An 807 bp fragment encompassing four exons (corresponding to exons 3, 4, 5 and 6 in \( H. \) contortus) and the three intervening introns was cloned, sequenced and aligned against the \( H. \) contortus sequence to confirm its identity (accession number KJ598498). Exonic and inronic sequences of the \( H. \) placei isotype-1 \( \beta \)-tubulin fragment had 98.8% and 84% identity overall with that of \( H. \) contortus sequence, respectively (Supplementary Fig. S2.1). 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 (P18, P56 and P68) that have fixed differences between isotype-1 and isotype-2 β-tubulin polypeptides in all trichostrongyloid nematodes sequenced to date (Njue and Prichard, 2003). All three of these residues correspond to those found in the isotype-1 polypeptide (asparagine, serine and alanine, respectively) (Supplementary Fig. S2.2). There was 100% sequence identity between *H. placei* and *H. contortus* over the primer regions used for the *H. contortus* pyrosequencing assay (von Samson-Himmelstjerna et al., 2009), thus the same primers and conditions were used for pyrosequence genotyping of the P167, P198 and P200 mutations for both species (Chaudhry et al., 2014).

### 2.2.8. PCR amplification and sequencing of the isotype-1 β-tubulin and mtND4 markers from *H. contortus* and *H. placei*

For the isotype-1 β-tubulin gene, the same forward (HcPYRF: 5’- GAC GCA TTC ACT TGG AGG AG-3’) and reverse primers (HcPYRR: 5’-CAT AGG TTG GAT TTGTGAGTT-3’) were used to PCR amplify a fragment encompassing parts of exons 4 and 5 and the intervening intron (including codons 167, 198 and 200) for both *H. contortus* (328 bp) and *H. placei* (325 bp) since these primer sites were 100% conserved in both species. In the case of the mtND4 marker, a 466 bp fragment for *H. contortus* and 471 bp fragment for *H. placei* was amplified using forward (HcND4For1-CGACAAACCACCTTGATACTTTATAT) and reverse primers (HcND4Rev-GCTTATTCTTCAGTTACACATATAAGA). In both cases,
PCR conditions consisted of 1X thermopol reaction buffer 2 mM MgSO$_4$, 200 uM dNTPs, 0.2 uM forward and reverse primers and 1 U of Phusion high fidelity DNA polymerase (Finenzyme, Thermo Fisher Scientific, USA) respectively. The thermo-cycling parameters of isotype-1 β-tubulin consisted of an initial 98°C for 30 s followed by 35 cycles of 98°C for 10 s, 59°C for 30 s and 72°C for 1 min with a single final extension cycle of 72°C for 5 min. Thermo-cycling parameters for mtND4 amplification consisted of an initial 98°C for 30 s followed by 35 cycles of 98°C for 10 s, 56°C for 30 s and 72°C for 1 min with a single final extension cycle of 72°C for 5 min. Amplicons were cloned into a PJET 1.2/BLUNT vector (Thermo Fisher Scientific, USA) and sequenced using standard procedures.

Sequences were aligned with *H. contortus* and *H. placei* isotype-1 β-tubulin sequences (accession numbers X67489 and KJ598498) or mtND4 sequences (accession numbers AF070785 and AF070825), respectively, and edited using Geneious Pro 5.4 software (Drummond AJ, 2012). A phylogenetic network tree of the haplotypes was reconstructed using Maximum Likelihood (ML) in MEGA5 (Tamura, 2011) based on the isotype-1 β-tubulin and mtND4 sequence data. The program jModeltest 12.2.0 (Posada, 2008) was used to select the appropriate model of nucleotide substitutions for ML analysis. According to Bayesian information criterion, the best scoring was Hasegawa-Kishino-Yano (HKY+G). The model of substitution was used with parameters estimated from data. Branch supports were obtained by 1000 bootstraps of the data.
2.3. Results

2.3.1. Validation of molecular markers using morphologically characterized H. contortus and H. placei populations

Previous work identified three SNPs in the rDNA ITS-2 (positions 24, 205 and 219) that show consistent sequence-specific differences between H. contortus and H. placei (Stevenson et al., 1995). However this previous study was based on just eight H. contortus and two H. placei worms obtained from sheep and cattle, respectively. Consequently, we first investigated whether these inter-species differences were fixed when larger numbers of individuals from geographically diverse populations were examined. The rDNA ITS-2 sequence was PCR amplified, cloned and sequenced from pooled DNA derived from geographically diverse, morphologically characterized, populations of H. contortus and H. placei. Between four and seven independent clones were sequenced from each of three H. contortus populations (MHco3(ISE), MHco4(WRS), MHco10(CAVR)) populations and each of four H. placei populations (SpHpl1, RAHpl1, RAHpl2, MHpl1)) (Table 2.1, Supplementary Table S2.1). In all cases, the H. contortus sequences contained P24 (A), P205 (G), P219 (A) and the H. placei sequences contained P24 (G), P205 (A), P219 (G) confirming these SNPs are fixed between the two species (Table 2.1). In addition to confirming the three species-specific fixed SNPs, these rDNA ITS-2 sequences identified 21 sites that showed intraspecific variation for H. contortus (positions 18, 19, 21, 22, 27, 58, 59, 63, 79, 83, 114, 115, 123, 130, 141, 174, 179, 196, 202, 215, 218) (Table 2.1) and 11 sites that showed intraspecific variation for H. placei (positions 59, 65, 71, 72, 125, 137, 138, 148, 215, 225, 229) (Table 2.1). This extends the list of previously described rDNA ITS-2 intra-specific
variable sites at positions 4, 10, 18, 19, 21, 58, 59, 78, 117, 123, 174, 179, 196, 202 in *H. contortus* (Gasser et al., 1998; Gharamah et al., 2012; Heise et al., 1999; Stevenson et al., 1995; Troell et al., 2003) and just the one previously reported intra-specific variable site at position 65 in *H. placei* (Stevenson et al., 1995).

The second marker to be validated was the isotype-1 β-tubulin gene. A total of 74 clones were sequenced from three morphologically characterized *H. contortus* populations (MHco3(ISE), MHco4(WRS), MHco10(CAVR)) (approximately 25 clones per population) and 63 clones were sequenced from four morphologically characterized *H. placei* populations (SpHpl1, RAHpl1, RAHpl2, MHpl1) (approximately 15 clones per population) (Fig. 2.2, Supplementary Table S2.1). This identified 18 and seven different haplotypes of *H. contortus* and *H. placei*, respectively, which fell into two distinct phylogenetic clades corresponding to the species of origin (Fig. 2.2).

The third marker to be validated was the gene for mtDNA ND4. A total of 15 clones were sequenced from three *H. contortus* populations (MHco3(ISE), MHco4(WRS), MHco10(CAVR)) (five clones per population) and 15 clones were sequenced from four *H. placei* populations (SpHpl1, RAHpl1, RAHpl2, MHpl1) (approximately four clones per population) (Fig. 2.3, Supplementary Table S2.1). These again formed two distinct phylogenetic clades that correspond to the species of origin (Fig. 2.3).

Three microsatellite loci (Hcms36, Hc53265 and Hc3561) were tested for species specificity by amplification from 123 individual adult worms derived from the three *H. contortus* populations (MHco3(ISE) - 41 worms, MHco4(WRS) - 41 worms and MHco10(CAVR) - 41 worms) and 112 individual adult worms derived from four *H. placei* populations (SpHpl1 - 32 worms, RAHpl1 - 19 worms; RAHpl2 - 20 worms and MHpl1 - 41
worms) (Supplementary Table S2.1). For all three microsatellite markers, the allele sizes were discrete between the two species with no shared alleles detected (Fig. 2.4A). Hence, for these markers, alleles could be unambiguously assigned to each Haemonchus spp. Further, the three markers showed no evidence of LD in the populations, suggesting they are not genetically linked and can be considered to be independent genetic markers (data not shown).

2.3.2. Haemonchus contortus but not H. placei was identified in small ruminants and cattle in southern India

Adult worms were collected from the abomasum of ruminant hosts from abattoirs across southern India (12 sheep, eight goats, six cattle and one buffalo). In all cases, the size and gross morphology of the worms were typical of Haemonchus except for those from four of the cattle which were typical of Mecistocirrus (Md32C, Md7C, Md4C and Md10C). Since we had previously confirmed that the rDNA ITS-2 P24 genotype reliably distinguishes H. contortus and H. placei (see Section 3.3.1), this was used to determine the species of Haemonchus present. Between 15 and 32 worms from each individual host were pyrosequence genotyped for the rDNA ITS-2 P24 SNP (946 worms genotyped in total) (Fig. 2.1, Supplementary Table S2.3). For 12 sheep, eight goats, two cattle, all worms were identified as H. contortus (homozygous A at rDNA ITS-2 P24) (Fig. 2.1). For the remaining four cattle, which contained worms with the physical appearance of Mecistocirrus, the pyrosequence genotypes were consistent with a M. digitatus species identity; homozygous P25 (A), P28 (A) P29 (T) as opposed to P25 (T), P28 (T) P29 (A) for H. contortus and H. placei (Lichtenfels and Pilitt, 2000; Mochizuki et al., 2006). Haemonchus placei (homozygous G at rDNA ITS-2 P24) was
not identified from any of the small or large ruminant hosts examined from southern India (Fig. 2.1, Supplementary Table S2.3).

Fig. 2.1. Distribution of Haemonchus and Mecistocirrus spp. identified in southern India and Pakistan. Geographic locations of abattoirs are indicated with small black circles in four provinces of southern India (B, Tamil Nadu; C, Andra Predesh; D, Karnataka; E, Kerala) and one province of Pakistan (A, Punjab). 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; B, buffalo). Red shading represents worms identified as Haemonchus contortus (Homozygous A at internal transcribed spacer 2 (ITS-2) rDNA position (P24), blue shading represents Haemonchus placei (Homozygous G at ITS-2 rDNA P24), green shading represents putative hybrids (heterozygous A/G at ITS-2 rDNA P24) and yellow shading represents Mecistocirrus digitatus (homozygous A, A and T at ITS-2 rDNA P25, P28 and P29, respectively). For further details of the samples see Supplementary Tables S2 and S3.
2.3.3. *Haemonchus contortus* and *H. placei* co-infections are common in sheep and goats in the Punjab region of Pakistan

Six sheep and 16 goats were autopsied from different regions of the Punjab region of Pakistan. All worms examined from the abomasum had a size and gross morphology typical of *Haemonchus*. Between 15 and 32 individual *Haemonchus* worms from each host were pyrosequence genotyped for the rDNA ITS-2 P24 SNP (644 worms genotyped in total) (Fig. 2.1, Supplementary Table S2.3). For three of the sheep and six of the goats, all worms were identified as *H. contortus* (homozygous A at rDNA ITS-2 P24). The remainder of the sheep and goats examined contained a mixture of *H. contortus* (homozygous A at rDNA ITS-2 P24) and *H. placei* (homozygous G at rDNA ITS-2 P24) indicating co-infection with the two species (Fig. 2.1, Supplementary Table S2.3).

Five of the individual hosts (four goats and one sheep) also contained a single worm with a heterozygous A/G genotype at the rDNA ITS-2 P24 position, suggesting that they may be *H. contortus/H. placei* hybrids. In order to investigate this hypothesis, a 231 bp fragment encompassing the rDNA ITS-2 locus was amplified and directly sequenced from each of these five worms (GenBank accession numbers KJ920741 - KJ920745). All five worms were heterozygous for the three SNPs previously confirmed as having fixed differences between *H. contortus* and *H. placei*: P24 (A/G), P205 (G/A), P219 (A/G) (Table 2.1, Supplementary Fig. S2.3). Furthermore, additional sites showed evidence of heterozygosity in the sequence chromatograms from the five putative hybrid worms: P18, P21, P22, P65, P123, P125, P148 and P196 (Table 2.1). Of these, P18, P21, P22, P123 and P196 have been previously shown only to be polymorphic in *H. contortus* (Table 2.1) and P65, P125 and P148 only to be
polymorphic in *H. placei* (Table 2.1). Hence the pattern of heterozygosity in these additional rDNA ITS-2 sites is consistent with the five worms being *H. contortus/H. placei* hybrids.

**Table 2.1.** Summary of inter- and intra-species variation in the *Haemonchus contortus* and *Haemonchus placei* rDNA internal transcribed spacer 2 (ITS-2) sequences. Positions (P) 24, 205 and 219 (indicated in larger font) have fixed interspecies polymorphisms whereas the other listed positions show intra-specific variation.

| Nucleotide Position | 18 | 19 | 21 | 22 | 24 | 27 | 58 | 59 | 63 | 65 | 71 | 79 | 83 | 111 | 114 | 115 | 123 | 125 | 130 | 137 | 138 | 141 | 148 | 149 | 174 | 179 | 196 | 202 | 205 | 218 | 219 | 225 | 229 |
|---------------------|----|----|----|----|----|----|----|----|----|----|----|----|----|----|----|----|----|----|----|----|----|----|----|----|----|----|----|----|----|----|----|----|----|----|

² rDNA ITS-2 sequence polymorphisms identified from a total of 22 independent clones derived from three morphologically characterised *H. contortus* populations: MHco3 (ISE) - seven clones, MHco4 (WRS) - eight clones, MHco10 (CAVR) - seven clones.

³ rDNA ITS-2 sequence polymorphisms identified from the direct sequences derived from five worms that were determined to be heterozygous at P24 (A/G) by pyrosequence genotyping.

### 2.3.4. Confirmation of *H. contortus/H. placei* F1 hybrids using additional nuclear genetic markers

In order to confirm our hypothesis that the five worms with heterozygous rDNA ITS-2 genotypes are *H. contortus/H. placei* hybrids, we examined four additional nuclear markers - the isotype-1 β-tubulin gene sequence and three microsatellite markers which have discrete allele sizes between the two species.

A fragment encompassing exons 4 and 5 and the intervening intron of the isotype-1 β-tubulin gene were PCR amplified and cloned from each of the five worms. (GenBank accession numbers KJ951066 - KJ951070 and KJ957928 - KJ957932). For each worm, 10 cloned fragments were sequenced to identify both alleles. A total of six isotype-1 β-tubulin haplotypes were identified from the five worms. Four of these clustered with the *H. contortus* sequence clade and the remaining two with the *H. placei* sequence clade (Fig. 2.2). Each
individual hybrid worm was heterozygous, containing one allele of each species type (Fig. 2.2). One hybrid worm (C in Fig. 2.2) contained a *H. contortus* allele (Hc10) with the P200Y (TAC) benzimidazole resistance associated mutation.

![Fig. 2.2. Phylogenetic analysis of isotype-1 β-tubulin haplotypes cloned and sequenced from morphologically characterized *Haemonchus* contortus and *Haemonchus placei* populations and putative hybrid worms. Sequences from *H. contortus* and *H. placei* worms are indicated with the prefixes Hc and Hp, respectively. The occurrence of individual haplotypes in each putative hybrid worm is indicated by a different shaped symbol adjacent to the haplotype name. The sequences were aligned by Geneious software and the tree obtained by maximum likelihood analysis using the Hasegawa-Kishino-Yano (HKY+G) model of substitution. Branches with bootstrap support values above 50% (1000 replications) and posterior probability greater than 50, respectively. The phylogeny is rooted with an isotype-1 β-tubulin sequence of *Teladorsagia circumcincta* (Tc) (GenBank accession number FN599034).](image)

The five putative hybrid worms were also genotyped with the three microsatellites previously shown to have discrete allele sizes between the two parasite species - Hcms36, Hc53265 and Hc3561 (Fig. 2.4A). For two of the markers, Hcms36 and Hcms53265, all five worms were heterozygous for one *H. contortus*-specific allele and one *H. placei*-specific allele.
For marker Hcms3561, this was also the case for three of the putative hybrid worms (worms B - D). However, the other two worms (worms A and E) were heterozygous for a *H. contortus*-specific allele (280 bp or 285 bp, respectively) with a second allele that had not been identified in the morphologically characterised *H. contortus* and *H. placei* populations used to validate the markers (302 bp and 328 bp, respectively) (Fig. 2.4, Supplementary Fig. S2.4). We hypothesised that these new alleles may be present in *Haemonchus* populations from Pakistan but absent (or at low frequency) in the morphologically characterised populations examined from other regions of the world. In order to test this, 79 worms that had been identified as *H. placei* from 12 Pakistani populations (H13S, H25S, H24S, H5G, H7G, H6G, H10G, H27G, H28G, H31G, H33G and H19G) and 61 worms that had been identified as *H. contortus* from two Pakistani populations (H3S and H1S) were genotyped for marker Hcms3561. The 302 and 328 bp alleles were present only in the *H. placei* worms, consistent with our hypothesis (Supplementary Fig. S2.5).
Fig. 2.4. The figure represents the allele frequencies of three microsatellite markers (Hcms36, Hc53265, Hc3561). (A) Overall allele frequencies of microsatellite markers Hcms36, Hc53265, Hc3561 in morphologically characterized *Haemonchus contortus* and *Haemonchus placei*. The genotype frequencies are based on 123 individual adult *H. contortus* worms derived from the three populations (MHco3(ISE) - 41 worms, MHco4(WRS) - 41 worms, MHco10(CAVR) - 41 worms) and 112 individual *H. placei* adult worms derived from four populations (SpHpl1 - 32 worms, RAHpl1 - 19 worms, RAHpl2 - 20 worms, MHpl1 - 41 worms). The solid black bars are alleles present in *H. contortus* worms and hatched blocks are alleles present in *H. placei* worms. (B) Schematic representation of the Genescan traces for the three microsatellite markers for the five worms with rDNA internal transcribed spacer 2 (ITS-2) position P24 heterozygous A/G (worms A - E). The black triangles represent the *H. contortus* alleles and the hatched triangles represent the *H. placei* alleles. (The actual Genescan traces are shown in Supplementary Fig. S2.4.)

2.3.4. *F1* hybrids detected in the field can be the progeny of either *H. contortus* or *H. placei* maternal parents

The mtND4 gene was sequenced from the five hybrid worms in order to determine the species identity of their maternal parent (GenBank accession numbers KM259911 - KM259914 and KM 23385). The mtND4 sequence from four of the hybrid worms clustered with the *H. placei* phylogenetic clade, suggesting these were the progeny of matings between a *H. contortus* male and *H. placei* female parent (Fig. 2.3). The mtND4 sequence from the remaining single hybrid worm (Hybrid E) clustered with the *H. contortus* phylogenetic clade, suggesting this was the progeny of a mating between a *H. placei* male and a *H. contortus* female parent (Fig. 2.3).
Fig. 2.3. Phylogenetic analysis of mitochondrial NADH dehydrogenase subunit 4 (mtND4) haplotypes cloned and sequenced from morphologically characterized populations of *Haemonchus contortus* MHco3(ISE), MHco4(WRS), MHco10(CAVR) and *Haemonchus placei* SpHpl1, RAHpl1, RAHpl2, MHpl1 and five putative hybrid worms. Sequences from *H. contortus* and *H. placei* worms are indicated with the prefixes Hc and Hp, respectively. The sequences were aligned by Geneious software and the tree obtained by maximum likelihood analysis using Hasegawa-Kishino-Yano (HKY+G) model of substitution. Branches with bootstrap support values above 50% (1000 replications) and posterior probability greater than 50 respectively. The phylogeny is rooted with a mtND4 sequence of *Teladorsagia circumcincta* (Tc) (GenBank accession number AF383990).

2.4. Discussion

Accurate identification of parasite species is crucial not only for diagnosis, treatment and control but for epidemiological studies and clinical trials. It is well established that *H. contortus* and *H. placei* are separate species based on morphology, molecular markers and genetic crossing studies (Amarante et al., 1997; Blouin et al., 1997; Le Jambre, 1981; Lichtenfels et al., 1994; Stevenson et al., 1995; Zarlenga et al., 1994b). However, they are phylogenetically close and have very similar morphology, making accurate species identification both challenging and time consuming. The most reliable differences between adult worms of the two species are in chromosomal morphology and the patterns of longitudinal cuticular ridges of the synlophe (Bremner, 1955; J. R. Lichtenfels, 1986; Le Jambre and Royal, 1980). However, species identification is more commonly made by morphometric analysis of the male spicules due to its greater speed and ease of application. A discriminating function that combines three different size measurements of the spicules is quite reliable, although misidentification can occur due to overlap in the values for the two species (Amarante, 2011; Jacquiet et al., 1997). Furthermore, it is not possible to definitively identify hybrid worms by morphological examination alone.
Molecular techniques offer some advantages over morphology-based identification. They are more objective, more scalable and easier to implement in non-specialist laboratories. However, reliable molecular identification needs appropriate, well-validated markers for which both intra- and inter- species genetic variation are well defined (Gasser, 1999, 2006; McKeand, 1998). In this paper, we have used a number of well characterized *H. contortus* and *H. placei* strains and isolates to provide more information on the genetic variation of the ITS-2 rDNA locus and further validate its use as a species-specific marker. The SNPs at positions P24, P205 and P219 all showed invariant fixed differences between the two species, even when the isolates were from diverse geographical origins. This allowed us to use the P24 position to screen a large number of samples for evidence of co-infection and for potential hybrids. We also identified a larger number of intra-specific variable positions than previously reported, with 21 out of 231 and 11 out of 231 variable positions in *H. contortus* and *H. placei*, respectively. Intraspecific variation was mainly at different positions in the two species with only positions 59 and 215 being variable in both (Table 2.1). This distinct pattern of intraspecific ITS-2 rDNA sequence variation between the two species provided additional evidence of heterozygosity of the ITS-2 rDNA locus in putative hybrid worms (Table 2.1).

We also demonstrated that haplotype diversity for both the isotype-1 β- tubulin and the mtDNA ND4 loci was completely congruent with the species identity determined by morphology and ITS-2 rDNA sequence analysis (Figs. 3, 4). Finally, three microsatellite markers were identified that show discrete allele sizes between the two species. Hence, this work has significantly expanded the set of validated molecular markers available to distinguish *H. contortus* and *H. placei* and identify hybrid worms.
Although *H. contortus* and *H. placei* are generally considered to predominantly infect small ruminant and cattle hosts, respectively, *H. contortus* is known to be capable of infecting a large number of different ruminant hosts (Lichtenfels et al., 1994). Although *H. placei* appears to be somewhat less promiscuous, it can infect small ruminants as well as cattle (Achi et al., 2003; Amarante et al., 1997; Chaudhry et al., 2014; Gasbarre et al., 2009a; Jacquiet et al., 1998; Lichtenfels et al., 1994). Most *Haemonchus* infections in the field are not diagnosed to the species level, thus there is a lack of data on true species prevalence and the extent of co-infections. From the relatively small number of available studies, it is clear that the situation varies from region to region. For example, in the United Kingdom, *H. placei* appears to be absent and clinical haemonchosis in cattle and small ruminants is solely due to *H. contortus* (Hogg et al., 2010). In many other regions of the world, the two species are sympatric and co-infection is common (Achi et al., 2003; Amarante et al., 1997; Gasbarre et al., 2009a; Jacquiet et al., 1998; Lichtenfels et al., 1994).

Our study has revealed a striking difference in the prevalence of *H. placei*, and of co-infection with *H. contortus*, between small ruminants from southern India and Pakistan. In southern India, *H. placei* was not found in any of the 20 small ruminant hosts sampled from a variety of locations in the region. In contrast, *H. placei* was widespread in small ruminants from the Punjab region of Pakistan with co-infections being more common than single *H. contortus* infections. There are several possible reasons for this difference between the regions.

(i) Anthelmintic use. The greater use of anthelmintics in small ruminants in rural southern India than in the Punjab region could play a role in the species balance. Anthelmintic resistance is generally less common in *H. placei* than in *H. contortus*, thus intensive
anthelmintic use could lead to a predominance of *H. contortus* single species infections. Consistent with this hypothesis, we found no *H. placei* in small ruminants from three government farms in Pakistan that used intensive anthelmintic treatment regimes (H1S, H2G, H3S) in contrast to the rest of the samples from rural areas where *H. placei* was common (Fig. 2.1).

(ii) Differences in climate and transmission. Following repeated experimental infections, small ruminants mount a more effective immune response to *H. placei* than to *H. contortus* (Santos et al., 2014). Hence, in southern India, an area with year round high humidity and high transmission, repeated exposure could result in immunity to *H. placei* and a predominance of *H. contortus* single species infections. In contrast, *H. placei* may be able to better establish infections in small ruminants in the Punjab region, where there may be less frequent challenge and lower immunity due to a shorter transmission season and drier climate. However, for this to be important, there would need to be a high level of *H. placei* challenge to small ruminants in southern India since the immunity induced by larval challenge is largely species-specific (Santos et al., 2014). At present there is no evidence for this and, as explained below, the reverse may actually be true.

(iii) Perhaps the most likely explanation is the role played by *M. digitatus* in southern Indian cattle. In 28 out of 31 cattle examined on autopsy from a variety of regions in southern India, *M. digitatus* was the predominant abomasal parasite on the basis of gross morphological appearance (unpublished data). Hence, the absence of *H. placei* in small ruminants in southern India might be due to the presence of only a small reservoir of *H. placei* in cattle. More information on the prevalence of *H. placei* in large ruminants in the two regions (both cattle and buffalo) would help test this hypothesis.
The ability of *H. contortus* and *H. placei* to hybridize in experimental crosses and the presence of co-infections raises the possibility of interspecies hybridization between these two species in the field. An early study reported six individual worms recovered from sheep in Georgia, United States of America, with spicule measurements that were intermediate between *H. placei* and *H. contortus*, leading to the suggestion of hybridization (J. R. Lichtenfels, 1986; Lichtenfels JR, 1988). The only genetic evidence for hybridisation to date was the recovery of two worms from sheep in Brazil that were heterozygous for *H. contortus* and *H. placei* rDNA ITS-2 sequences (Brasil et al., 2012). However, no detailed genetic analysis of these individuals was performed. In the study presented here, we have identified five individual worms, each from different small ruminant hosts from Pakistan, that were heterozygous for species-specific alleles of five independent genetic markers from the nuclear genome (ITS-2 rDNA, isotype-1 β tubulin and three unlinked microsatellite markers). This represents the first definitive genetic evidence of hybridization between these two species in the field. The fact that each of these worms was heterozygous for all five nuclear markers strongly suggests they are F1 hybrids (as opposed to F2 or subsequent generations). Mitochondrial ND4 sequence analysis further suggests that hybrids can be derived from interspecies mating of either *H. contortus* or *H. placei* female worms.

The relatively low number of hybrids found overall is unsurprising since interspecies crosses are typically less efficient than those within each species, even for very closely related sympatric species (Anderson et al., 1998; Isenstein, 1971; Nissen et al., 2012; Suarez et al., 1993). In the absence of selection, small numbers of hybrids are likely be lost a population and not necessarily be of major biological significance. However, if selection pressure favors hybrids and their backcrossed progeny, then the presence of even low numbers of F1 hybrids
could be very important. For example, if F1 progeny inherited anthelmintic resistance mutations from either parent, then drug treatments would provide positive selection for the survival and reproduction of such individuals and their progeny. This is a distinct possibility given the high frequency of anthelmintic resistance mutations in *H. contortus* populations and the intensive anthelmintic treatment regimes used in ruminants. Indeed, one of the five hybrid worms we detected was a female worm which was heterozygous for an *H. contortus* isotype-1 β tubulin allele containing the P200Y benzimidazole resistance polymorphism. If such an individual were to backcross with a *H. placei* male to produce fertile offspring, which we know is biologically possible (Le Jambre, 1981; Le Jambre and Royal, 1980), it could result in interspecies introgression of anthelmintic resistance alleles into the *H. placei* genetic background. Such alleles could then increase in frequency under the influence of positive selection by anthelmintic treatments. Future work is needed to test this hypothesis such as the screening of large numbers of *H. placei* worms (homozygous G at rDNA-ITS-2 P24) for evidence of introgression of the isotype-1 β tubulin from *H. contortus*.

It is increasingly apparent that hybridization between closely related animal species occurs more commonly in nature than previously believed (Mallet, 2005). However, there have been relatively few genetic studies to investigate this in parasitic nematodes of animals or humans. The most detailed work to date has been performed on *Ascaris lubricoides* and *Ascaris suum*, the large roundworms of humans and pigs, respectively. Although their species status and the extent of cross infection has been subject to much debate, there is strong evidence for genetic hybridization between the parasites from the two hosts (Criscione et al., 2007). Recently, hybridisation between the human and pig nematode parasites *Trichuris trichiura* and *Tricuris suis* has been suggested (Nissen et al., 2012). This was based the
analysis of worms from humans, one of which was heterozygous at the ITS-2 rDNA locus and several others that contained either ITS-2 rDNA or β-tubulin sequences that clustered with *T. suis* sequence clades. In the case of trichostronglyid species of livestock, the issue of hybridization is of particular interest due to its potential to provide a mechanism for the introgression of anthelmintic resistance loci between parasite species. Also, there are a large number of closely related species which commonly co-infect the same individual hosts, providing the opportunity for interspecies hybridization in the field. Most of the work to date involves older studies that focused on attempts to hybridize closely related species by experimental co-infection followed by detailed morphological analysis to identify potential hybrids. Isenstein (1971) demonstrated that *Cooperia oncophora* and *Cooperia pectinata* hybridise under experimental conditions in cattle and hybrids have also been experimentally produced between *Ostertagia ostertagia* and *Ostertagia leptospicularis* in sheep (Suarez et al., 1993). Consequently, it is possible that interspecies hybridization occurs in the field for a number of different ruminant trichostrongyloid nematode species. Given the increasing prevalence of anthelmintic resistance in this group of parasites and the implications for interspecies transmission of resistance genes, further investigation of interspecies hybridization between strongylid nematode species is warranted.

2.5. Acknowledgements

We are grateful to Alessandro Amarante and Monica Amarante (Universidade Estadual Paulista (UNESP), Departamento de Parasitologia, Instituto de Biociências, Sao Paulo, Brazil), for kindly supplying morphologically characterized SpHpl1 *H. placei* worms; also to
Jacques Cabaret (Annuaire des Labaratoires et des Recherches, INRA, Tours, France) for the morphologically characterized RAHpl1 and RAHpl2 *H. placei* from Rosario, Argentina. We would like to thank the Natural Sciences and Engineering Research Council of Canada (NSERC) for funding support (Grant number RGPIN/371529-2209) as well as the NSERC-CREATE Host Pathogen Interactions (HPI) graduate training program at the University of Calgary, Canada.

**References**


Appendices: Supplementary Figure Legends

**Supplementary Fig. S2.1.** Sequence alignment of the 807 bp *Haemonchus placei* isotype-1 β-tubulin amplicon with the equivalent region of the *Haemonchus contortus* isotype-1 β-tubulin amplicon (accession number KJ598498) encompassing four exons (corresponding to exons 3, 4, 5 and 6) and the three intervening introns.

**Supplementary Fig. S2.2.** Alignment of the predicted *Haemonchus placei* β-tubulin polypeptide with *Haemonchus contortus* isotype-1 (accession number M76493) and *H. contortus* isotype-2 (accession number M76491) polypeptide sequences. The three amino acid positions 167, 198, 200 associated with benzimidazole resistance mutations are highlighted by red boxes. The amino acids positions showing fixed differences between isotype-1 and isotype-2 β-tubulin polypeptides from all trichostrongyloid nematodes examined to date are indicated with black stars (Njue and Prichard, 2003). The color scheme of amino acids represents the traditional amino acid properties.
Supplementary Fig. S2.3. Comparison of sequence chromatogram of *H. contortus* and *H. placei* with five putative hybrid worms (A - E) showing heterozygosity at the fixed species-specific positions (P24, P205, P219) of the rDNA internal transcribed spacer 2 (ITS-2) sequence.

Supplementary Fig. S2.4. Genescan chromatograms for microsatellite markers Hcms36, Hc53265 and Hc3561 for each of the five putative hybrid worms (A - E).
Supplementary Fig. S2.5. Overall allele frequencies of microsatellite markers Hc3561 in *Haemonchus contortus* and *Haemonchus placei* field populations in the Punjab region of Pakistan. The genotype frequencies are based on 61 individual adult *H. contortus* worms derived from two populations (H3S and H1S) and 79 individual *H. placei* adult worms derived from 12 populations (H13S, H25S, H24S, H5G, H7G, H6G, H10G, H27G, H28G, H31G, H33G and H19G). The solid black bars are alleles present in *H. contortus* worms and hatched blocks are alleles present in *H. placei* worms.

Appendices: Supplementary Table Legends

Supplementary Table S2.1. Summary of the morphologically characterized *Haemonchus contortus* and *Haemonchus placei* populations from different geographical regions used for genetic marker validation.

<table>
<thead>
<tr>
<th>Morphologically characterised populations</th>
<th>Host</th>
<th>Worm number</th>
<th>Parasite species</th>
<th>Isolate type</th>
<th>Geographical origin</th>
</tr>
</thead>
<tbody>
<tr>
<td>SpHpl1</td>
<td>Cattle</td>
<td>32</td>
<td><em>H. placei</em></td>
<td>Laboratory isolate</td>
<td>Sao Paulo, Brazil</td>
</tr>
<tr>
<td>RAHpl1</td>
<td>Cattle</td>
<td>19</td>
<td><em>H. placei</em></td>
<td>Field isolate</td>
<td>Rosario, Argentina</td>
</tr>
<tr>
<td>RAHpl2</td>
<td>Cattle</td>
<td>20</td>
<td><em>H. placei</em></td>
<td>Field isolate</td>
<td>Rosario, Argentina</td>
</tr>
<tr>
<td>MHpl1</td>
<td>Cattle</td>
<td>41</td>
<td><em>H. placei</em></td>
<td>Laboratory isolate</td>
<td>Australia</td>
</tr>
<tr>
<td>MHco3(1SE)</td>
<td>Sheep</td>
<td>41</td>
<td><em>H. contortus</em></td>
<td>Laboratory isolate</td>
<td>Unknown</td>
</tr>
<tr>
<td>MHco4(WRS)</td>
<td>Sheep</td>
<td>41</td>
<td><em>H. contortus</em></td>
<td>Laboratory isolate</td>
<td>South Africa</td>
</tr>
<tr>
<td>MHco10(CAVR)</td>
<td>Sheep</td>
<td>41</td>
<td><em>H. contortus</em></td>
<td>Laboratory isolate</td>
<td>Australia</td>
</tr>
</tbody>
</table>
Supplementary Table S2.2. Summary of the field populations of *Haemonchus* spp. and *Mecistocirrus* spp. collected from sheep, goats, cattle and buffalo hosts from Tamil Nadu (TN), Andhra Predesh (AP), Kerala (KE) and Karnataka (KA) regions of southern India and the Punjab (PN) region of Pakistan.

<table>
<thead>
<tr>
<th>Indian field populations</th>
<th>Host</th>
<th>Worm number</th>
<th>Origin</th>
<th>Location, country</th>
<th>Pakistani field populations</th>
<th>Host</th>
<th>Worm number</th>
<th>Origin</th>
<th>Location, country</th>
</tr>
</thead>
<tbody>
<tr>
<td>H2S Sheep</td>
<td>44</td>
<td>Abattoir</td>
<td>Chennai (TN), India</td>
<td>H16S Sheep</td>
<td>32</td>
<td>Abattoir</td>
<td>Lahore (PN), Pakistan</td>
<td></td>
<td></td>
</tr>
<tr>
<td>H1S Sheep</td>
<td>44</td>
<td>Abattoir</td>
<td>Salem (TN), India</td>
<td>H13S Sheep</td>
<td>32</td>
<td>Abattoir</td>
<td>Lahore (PN), Pakistan</td>
<td></td>
<td></td>
</tr>
<tr>
<td>H51S Sheep</td>
<td>41</td>
<td>Abattoir</td>
<td>Salem (TN), India</td>
<td>H25S Sheep</td>
<td>32</td>
<td>Abattoir</td>
<td>Sargodha (PN), Pakistan</td>
<td></td>
<td></td>
</tr>
<tr>
<td>H52S Sheep</td>
<td>36</td>
<td>Abattoir</td>
<td>Salem (TN), India</td>
<td>H24S Sheep</td>
<td>30</td>
<td>Abattoir</td>
<td>Sargodha (PN), Pakistan</td>
<td></td>
<td></td>
</tr>
<tr>
<td>H16S Sheep</td>
<td>44</td>
<td>Abattoir</td>
<td>Tiruchirapalli (TN), India</td>
<td>H3S Sheep</td>
<td>32</td>
<td>Farm</td>
<td>Lahore (PN), Pakistan</td>
<td></td>
<td></td>
</tr>
<tr>
<td>H41S Sheep</td>
<td>43</td>
<td>Abattoir</td>
<td>Madurai (TN), India</td>
<td>H1S Sheep</td>
<td>29</td>
<td>Farm</td>
<td>Lahore (PN), Pakistan</td>
<td></td>
<td></td>
</tr>
<tr>
<td>H42S Sheep</td>
<td>36</td>
<td>Abattoir</td>
<td>Madurai (TN), India</td>
<td>H2G Goat</td>
<td>32</td>
<td>Farm</td>
<td>Lahore (PN), Pakistan</td>
<td></td>
<td></td>
</tr>
<tr>
<td>H71S Sheep</td>
<td>42</td>
<td>Abattoir</td>
<td>Vijayawada (AP), India</td>
<td>H5G Goat</td>
<td>18</td>
<td>Abattoir</td>
<td>Lahore (PN), Pakistan</td>
<td></td>
<td></td>
</tr>
<tr>
<td>H72S Sheep</td>
<td>36</td>
<td>Abattoir</td>
<td>Vijayawada (AP), India</td>
<td>H7G Goat</td>
<td>32</td>
<td>Abattoir</td>
<td>Lahore (PN), Pakistan</td>
<td></td>
<td></td>
</tr>
<tr>
<td>H27S Sheep</td>
<td>36</td>
<td>Abattoir</td>
<td>Tirupathi (AP), India</td>
<td>H6G Goat</td>
<td>24</td>
<td>Abattoir</td>
<td>Lahore (PN), Pakistan</td>
<td></td>
<td></td>
</tr>
<tr>
<td>H28S Sheep</td>
<td>44</td>
<td>Abattoir</td>
<td>Tirupathi (AP), India</td>
<td>H4G Goat</td>
<td>22</td>
<td>Abattoir</td>
<td>Lahore (PN), Pakistan</td>
<td></td>
<td></td>
</tr>
<tr>
<td>H26S Sheep</td>
<td>45</td>
<td>Abattoir</td>
<td>Tirupathi (AP), India</td>
<td>H8G Goat</td>
<td>15</td>
<td>Abattoir</td>
<td>Lahore (PN), Pakistan</td>
<td></td>
<td></td>
</tr>
<tr>
<td>H11G Goat</td>
<td>32</td>
<td>Abattoir</td>
<td>Dharmapuri (TN), India</td>
<td>H10G Goat</td>
<td>32</td>
<td>Abattoir</td>
<td>Lahore (PN), Pakistan</td>
<td></td>
<td></td>
</tr>
<tr>
<td>H12G Goat</td>
<td>36</td>
<td>Abattoir</td>
<td>Dharmapuri (TN), India</td>
<td>H27G Goat</td>
<td>32</td>
<td>Abattoir</td>
<td>Okara (PN), Pakistan</td>
<td></td>
<td></td>
</tr>
<tr>
<td>H21G Goat</td>
<td>36</td>
<td>Abattoir</td>
<td>Trichy (TN), India</td>
<td>H28G Goat</td>
<td>32</td>
<td>Abattoir</td>
<td>Okara (PN), Pakistan</td>
<td></td>
<td></td>
</tr>
<tr>
<td>H22G Goat</td>
<td>36</td>
<td>Abattoir</td>
<td>Trichy (TN), India</td>
<td>H29G Goat</td>
<td>32</td>
<td>Abattoir</td>
<td>Okara (PN), Pakistan</td>
<td></td>
<td></td>
</tr>
<tr>
<td>H32G Goat</td>
<td>36</td>
<td>Abattoir</td>
<td>Cochin (KE), India</td>
<td>H31G Goat</td>
<td>32</td>
<td>Abattoir</td>
<td>Sahiwal (PN), Pakistan</td>
<td></td>
<td></td>
</tr>
<tr>
<td>H31G Goat</td>
<td>32</td>
<td>Abattoir</td>
<td>Cochin (KE), India</td>
<td>H33G Goat</td>
<td>32</td>
<td>Abattoir</td>
<td>Sahiwal (PN), Pakistan</td>
<td></td>
<td></td>
</tr>
<tr>
<td>H61G Goat</td>
<td>36</td>
<td>Abattoir</td>
<td>Mangalore (KA), India</td>
<td>H71G Goat</td>
<td>32</td>
<td>Abattoir</td>
<td>Gujranwala (PN), Pakistan</td>
<td></td>
<td></td>
</tr>
<tr>
<td>H62G Goat</td>
<td>36</td>
<td>Abattoir</td>
<td>Mangalore (KA), India</td>
<td>H91G Goat</td>
<td>32</td>
<td>Abattoir</td>
<td>Gujranwala (PN), Pakistan</td>
<td></td>
<td></td>
</tr>
<tr>
<td>H2C Cattle</td>
<td>32</td>
<td>Abattoir</td>
<td>Vijayawada (AP), India</td>
<td>H29G Goat</td>
<td>32</td>
<td>Abattoir</td>
<td>Gujranwala (PN), Pakistan</td>
<td></td>
<td></td>
</tr>
<tr>
<td>H3C Cattle</td>
<td>18</td>
<td>Abattoir</td>
<td>Cochin (KE), India</td>
<td>H21G Goat</td>
<td>26</td>
<td>Abattoir</td>
<td>Gujranwala (PN), Pakistan</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Md4C Buffalo</td>
<td>32</td>
<td>Abattoir</td>
<td>Cochin (KE), India</td>
<td>Md4C Buffalo</td>
<td>32</td>
<td>Abattoir</td>
<td>Cochin (KE), India</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Md7C Buffalo</td>
<td>32</td>
<td>Abattoir</td>
<td>Cochin (KE), India</td>
<td>Md10C Buffalo</td>
<td>17</td>
<td>Abattoir</td>
<td>Chennai (TN), India</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Md32C Buffalo</td>
<td>15</td>
<td>Abattoir</td>
<td>Cochin (KE), India</td>
<td>Hc41B Buffalo</td>
<td>29</td>
<td>Abattoir</td>
<td>Chennai (TN), India</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>
**Supplementary Table S2.3.** rDNA internal transcribed spacer 2 (ITS-2) species-specific single nucleotide polymorphism (SNP) of position 24, (P24) genotypes identified by pyrosequencing of *Haemonchus contortus, Haemonchus placei* and *Mecistocirrus digitatus* worms from India and Pakistan.

<table>
<thead>
<tr>
<th>Indian field populations</th>
<th>Host</th>
<th>No. of <em>H. contortus</em> worms</th>
<th>No. of <em>M. digitatus</em> worms</th>
<th>Pakistani field populations</th>
<th>Host</th>
<th>No. of <em>H. contortus</em> worms</th>
<th>No. of <em>H. placei</em> worms</th>
<th>No. of heterozygous worms</th>
</tr>
</thead>
<tbody>
<tr>
<td>H2S Sheep</td>
<td>44</td>
<td></td>
<td></td>
<td>H16S Sheep</td>
<td>32</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>H15 Sheep</td>
<td>44</td>
<td></td>
<td></td>
<td>H13S Sheep</td>
<td>29</td>
<td>2</td>
<td></td>
<td>1</td>
</tr>
<tr>
<td>H35 Sheep</td>
<td>41</td>
<td></td>
<td></td>
<td>H25S Sheep</td>
<td>2</td>
<td>30</td>
<td></td>
<td></td>
</tr>
<tr>
<td>H52S Sheep</td>
<td>36</td>
<td></td>
<td></td>
<td>H34S Sheep</td>
<td>27</td>
<td>3</td>
<td></td>
<td></td>
</tr>
<tr>
<td>H16S Sheep</td>
<td>44</td>
<td></td>
<td></td>
<td>H3S Sheep</td>
<td>32</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>H41S Sheep</td>
<td>43</td>
<td></td>
<td></td>
<td>H1S Sheep</td>
<td>29</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>H42S Sheep</td>
<td>36</td>
<td></td>
<td></td>
<td>H2G Goat</td>
<td>31</td>
<td>1</td>
<td></td>
<td></td>
</tr>
<tr>
<td>H71S Sheep</td>
<td>42</td>
<td></td>
<td></td>
<td>H5G Goat</td>
<td>14</td>
<td>3</td>
<td>1</td>
<td></td>
</tr>
<tr>
<td>H72S Sheep</td>
<td>36</td>
<td></td>
<td></td>
<td>H7G Goat</td>
<td>27</td>
<td>5</td>
<td></td>
<td></td>
</tr>
<tr>
<td>H27S Sheep</td>
<td>36</td>
<td></td>
<td></td>
<td>H6G Goat</td>
<td>14</td>
<td>9</td>
<td>1</td>
<td></td>
</tr>
<tr>
<td>H28S Sheep</td>
<td>44</td>
<td></td>
<td></td>
<td>H4G Goat</td>
<td>22</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>H26S Sheep</td>
<td>45</td>
<td></td>
<td></td>
<td>H8G Goat</td>
<td>15</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>H11G Goat</td>
<td>32</td>
<td></td>
<td></td>
<td>H10G Goat</td>
<td>29</td>
<td>3</td>
<td></td>
<td></td>
</tr>
<tr>
<td>H12G Goat</td>
<td>36</td>
<td></td>
<td></td>
<td>H27G Goat</td>
<td>26</td>
<td>6</td>
<td></td>
<td></td>
</tr>
<tr>
<td>H21G Goat</td>
<td>36</td>
<td></td>
<td></td>
<td>H28G Goat</td>
<td>30</td>
<td>1</td>
<td>1</td>
<td></td>
</tr>
<tr>
<td>H22G Goat</td>
<td>36</td>
<td></td>
<td></td>
<td>H29G Goat</td>
<td>32</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>H32G Goat</td>
<td>36</td>
<td></td>
<td></td>
<td>H31G Goat</td>
<td>20</td>
<td>12</td>
<td></td>
<td></td>
</tr>
<tr>
<td>H31G Goat</td>
<td>32</td>
<td></td>
<td></td>
<td>H33G Goat</td>
<td>28</td>
<td>4</td>
<td></td>
<td></td>
</tr>
<tr>
<td>H61G Goat</td>
<td>36</td>
<td></td>
<td></td>
<td>H17G Goat</td>
<td>32</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>H62G Goat</td>
<td>36</td>
<td></td>
<td></td>
<td>H19G Goat</td>
<td>31</td>
<td>1</td>
<td></td>
<td></td>
</tr>
<tr>
<td>H2C Cattle</td>
<td>32</td>
<td></td>
<td></td>
<td>H20G Goat</td>
<td>32</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>H3C Cattle</td>
<td>18</td>
<td></td>
<td></td>
<td>H21G Goat</td>
<td>26</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Md4C Cattle</td>
<td>29</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Md7C Cattle</td>
<td>32</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Md10C Cattle</td>
<td>32</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Md32C Cattle</td>
<td>17</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Hc41B Buffalo</td>
<td>15</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>Total 27</strong></td>
<td><strong>850</strong></td>
<td></td>
<td></td>
<td><strong>Total 22</strong></td>
<td><strong>560</strong></td>
<td></td>
<td><strong>79</strong></td>
<td><strong>5</strong></td>
</tr>
</tbody>
</table>
CHAPTER III

MANUSCRIPT II

Genetic evidence for the spread of a benzimidazole resistance mutation across southern India from a single origin in the parasitic nematode *Haemonchus contortus*

Umer Chaudhry a, E. M. Redman a, Muthusamy Raman b, John S. Gilleard a **

Accepted in International Journal for Parasitology, IJPARA-S-15-00127-2
Abstract

It is important to understand how anthelmintic drug resistance mutations arise and spread in order to determine appropriate mitigation strategies. We hypothesized that a molecular genetic study of *Haemonchus contortus* in southern India, a region where resistance may be less advanced than in Western Europe and North America, might provide some important insights into the origin and spread of anthelmintic resistance. The F200Y (TAC) isotype-1 β-tubulin benzimidazole resistance mutation is common in *H. contortus* throughout the world and the F167Y (TAC) and E198A (GCA) mutations, although less common, have been reported in a number of different countries. We have investigated the haplotypic diversity and phylogenetic relationship of isotype-1 β-tubulin benzimidazole resistance alleles for twenty three *H. contortus* populations from small ruminants across southern India. The F200Y (TAC) mutation was most common, being detected in 18/23 populations at frequencies between 9 to 84% and the E198A (GCA) mutation was also detected in 8/23 populations at frequencies between 8 and 18%. The F167Y (TAC) mutation was not detected in any of the 23 populations. Phylogenetic haplotype network analysis suggested that the F200Y (TAC) mutation has arisen multiple independent times in the region with at least three independent origins of resistance alleles across the populations surveyed. In contrast, the E198A (GCA) was present on a single haplotype which, given the high level of haplotypic diversity of the susceptible alleles in the region, suggests this particular mutation has spread from a single origin; likely by anthropogenic animal movement. Population genetic analysis of twelve of the *H. contortus* populations using a panel of eight microsatellite markers revealed extremely low genetic differentiation between populations consistent with the hypothesis of high gene flow among sites. Additionally, there was no significant genetic differentiation between *H.*
contortus taken from sheep and goats consistent with H. contortus populations being freely shared between these two different hosts. Overall, these results provide the first clear genetic evidence for the spread of an anthelmintic resistance mutation to multiple different locations from a single origin.

**Keywords:** Haemonchus contortus, isotype-1 β-tubulin, anthelmintic drug resistance, benzimidazoles resistance, sheep.
3.1. Introduction

Anthelmintic resistance in parasitic nematodes is a threat to sustainable livestock production worldwide and an understanding of how resistance mutations arise and spread is important to the development of effective mitigation strategies. However, we still have a limited understanding of these processes at the population level, even for the parasites in which anthelmintic resistance has been most intensively studied such as the gastro-intestinal parasites of small ruminants. Benzimidazole resistance in *H. contortus* is an excellent system in which to study the population genetics of anthelmintic resistance because of the available molecular tools and our increasing knowledge of its genetics and population biology (Silvestre and Humbert, 2002; Redman et al., 2008; Laing et al., 2013; Redman et al., 2015).

Three resistance-associated non-synonymous single nucleotide polymorphisms (SNPs) in the isotype-1 β-tubulin gene have been associated with benzimidazole resistance in *H. contortus*. A SNP in codon F200Y (TTC to TAC), resulting in a phenylalanine to tyrosine substitution, is widespread and often at high frequency in many countries (Silvestre and Humbert, 2002; Hoglund et al., 2009; Brasil et al., 2012; Redman et al., 2015). A SNP at codon F167Y (TTC to TAC), resulting in a phenylalanine to tyrosine substitution, has also been detected in a number of countries although it is generally considered to be less common than the F200Y (TTC to TAC) mutation (Silvestre and Cabaret, 2002; de Lourdes Mottier and Prichard, 2008; Barrère V, 2012; Brasil et al., 2012; Chaudhry and Gilleard, 2015; Redman et al., 2015). However, it is noteworthy that the F167Y (TAC) was present in six out of seven *H. contortus* populations examined in a recent UK study and its frequency across these six farms was similar to that of the F200Y (TAC) mutation overall (Redman et al., 2015). Indeed, in one
population where the F200Y (TAC) was detected at very low frequency, the F167Y (TAC) was present almost at fixation (>95% frequency). Hence, the F167Y (TAC) mutation may be of similar importance as the F200Y (TAC) mutation for benzimidazole resistance of *H. contortus* in the UK. A third SNP at codon E198A (GAA to GCA) resulting in a glutamate to alanine substitution has been detected in just two field-derived laboratory passaged populations of *H. contortus* to date; one from South Africa and one from Australia (Ghisi et al., 2007; Rufener et al., 2009; Kotze et al., 2012). It appears the E198A (GCA) is the rarest of the three benzimidazole mutations globally, although it has not been focus of any published studies to date.

In our recent population genetic study of *H. contortus* on seven UK farms, we presented evidence that both the F200Y (TAC) and the F167Y (TAC) mutations present on those farms had multiple independent origins (Redman et al., 2015). This suggests that benzimidazole resistance mutations have repeatedly arisen in the UK, although we still have a poor understanding of how often this has occurred and of the relative importance of recurrent versus pre-existing mutation (Redman et al., 2015). We are investigating this question in a number of different countries and southern India was chosen on the basis that resistance might be at an earlier stage than many of the regions studied to date. Anthelmintic treatment of livestock is generally less regimented in rural southern India than it is in Western Europe and North America and generic drug formulations of unknown efficacy are often used. In addition, the large in refugia population of free-living larvae in a warm, humid tropical climate is expected to reduce the rate at which resistance develops. There are only a few published studies on anthelmintic resistance from India but benzimidazole resistance has been demonstrated by fecal egg count reduction tests and the F200Y (TAC) mutation has been
detected by allele-specific PCR in Northern India (Tiwari et al., 2006; Chandra et al., 2014). However, the overall situation and how it compares to other countries is unclear.

In this paper, we present evidence for the spread of the E198A (GCA) mutation from a single origin to multiple locations in southern India. In contrast, the F200Y (TAC) mutation appears to have multiple independent origins consistent with its more frequent occurrence. The results presented here provide the first genetic evidence of the spread of an anthelmintic resistance mutation from a single source presumably due to the large amount of anthropogenic livestock movement in the region.

### 3.2. Materials and Methods

#### 3.2.1. Harvesting of adult Haemonchus worms

Adult *Haemonchus* worms were harvested from the abomasum of twenty-three individual ruminant hosts that were slaughtered at ten different abattoirs located across four different provinces of southern India in 2011 (Tamil Nadu, Andhra Pradesh, Kerala and Karnataka) (Fig. 3.1, Supplementary Table S3.1). Each animal that was necropsied in each abattoir was known to originate from a different pasture/village in the region, although the precise location was not available. Hence, each of the twenty-three animals harboured geographically distinct parasite populations distributed across the four provinces. Twelve of the *H. contortus* populations were collected from sheep; two from the Chennai abattoir (Pop2S and Pop1S), two from the Salem abattoir (Pop51S and Pop52S), one from the Tirunelveli abattoir (Pop16S), two from the Madurai abattoir (Pop41S and Pop42S), two from the Vijayawada abattoir (Pop71S and Pop72S) and three from the Tirupathia abattoir (Pop27S, Pop28S and
Pop26S). Eight of the *H. contortus* populations were collected from goats; two from the Dharmapuri abattoir (Pop11G and Pop12G), two from the Trichy abattoir (Pop21G and Pop22G), two from the Cochin abattoir (Pop32G and Pop31G) and two from the Mangalore abattoir (Pop61G and Pop62G). Two of the *H. contortus* populations were collected from cattle, one each from the Vijaywada (Pop2C) and Cochin abattoirs (Pop3C) and a single *H. contortus* population was collected from a buffalo at the Chennai abattoir (Pop41B) (Fig. 3.1, Supplementary Table S3.1).

3.2.2. Genomic DNA isolation

Adult worms were fixed in 70% ethanol immediately following removal from the host abomasum. The heads of individual male and female worms were dissected and lysed in single 0.2 µl tubes containing 50 µl of proteinase K lysis buffer and stored at -80°C as previously described (Redman et al., 2008; Chaudhry et al., 2015). One µl of a 1:5 dilution of neat single worm lysate was used as PCR template and identical dilutions of lysate buffer, made in parallel, were used as negative controls. To prepare pooled lysates of each population, 1 µl aliquots of each individual neat adult worm head lysate were pooled. One µl of a 1:20 dilution of pooled lysate was used as template for subsequent PCRs.

3.2.3. Pyrosequence genotyping of the rDNA ITS-2 P24 SNP to determine Haemonchus species identity

A 321bp fragment encompassing the rDNA ITS-2 region was PCR amplified from individual *H. contortus* adult worm lysates using a “universal” forward primer complementary to 5.8S rDNA coding sequence (NC1F: 5’-ACG TCT GGT TCA GGG TT GTT- 3’) and a
biotin-labelled reverse primer complementary to the 28S rDNA coding sequence (NC2R: 5’-Biotin-TTA GTT TCT TTT CCT CCG CT- 3’) (Stevenson et al., 1995; Chaudhry et al., 2015). The SNP at position 24 of the ITS-2 (which discriminates between *H. contortus* and *H. placei*) was determined by a previously described pyrosequence genotyping assay using sequencing primer 5’-CATATACTACAATGTGGCTA-3’ (Chaudhry et al., 2015). A minimum of 18 and a maximum of 45 individual adult worms (850 worms in total) were genotyped per population (23 populations in total) (Supplementary Table S1). All the worms genotyped in the 23 populations described above were confirmed as *H. contortus* (P24; A genotype)(Chaudhry et al., 2015).

3.2.4. Pyrosequence genotyping to determine the relative frequencies of the isotype-1 β-tubulin P200, P198 and P167 benzimidazole resistance-associated SNPs in *H. contortus* populations

A 328bp fragment, spanning exons 4 and 5 and intervening intron of the isotype-1 β-tubulin gene was PCR amplified from pooled DNA lysates for each of the 23 *H. contortus* populations (Supplementary Table S3.1). Final PCR conditions were 1X thermopol reaction buffer, 2mM MgSO₄, 100μM dNTPs, 0.1μM forward and reverse primers and 1.25U Taq DNA polymerase (New England Biolabs) and a previously published primer pair was used; forward primer (HcPYRF: 5’-GAC GCA TTC ACT TGG AGG AG-3’) and reverse primer (HcPYRR: 5’-Biotin-CAT AGG TTG GAT TTGTGA GTT-3’) (von Samson-Himmelstjerna et al., 2009). The thermo-cycling parameters consisted of an initial 95°C for 5 min followed by 35 cycles of 95°C for 1 min, 53°C for 1 min and 72°C for 1 min and a single final extension cycle of 72°C for 5 min.
Following PCR amplification, the relative frequency of the F167Y (T\text{AC}), E198A (G\text{CA}) and F200Y (T\text{AC}) isotype-1 β-tubulin SNPs in the amplicons derived from each parasite population were determined by pyrosequence genotyping using the PryoMark ID system using the allele quantification (AQ) mode (Biotage, Sweden). Previously published sequencing primers were used for the F167Y (T\text{AC}) and F200Y (T\text{AC}) mutations; Hcsq167: 5’-ATA GAA TTA TGG CTT CGT-3’ and Hcsq200: 5’- TAG AGA ACA CCG ATG AAA CAT-3’ respectively (Hoglund et al., 2009; von Samson-Himmelstjerna et al., 2009). A new sequencing primer, Hcsq198: 5’-ACT GGT AGA GAA CAC CG-3’, was used for the E198A (G\text{CA}) mutation. The base dispensation was set to GATGCTCGT for codon 167, GATGCAGCA for codon 198 and GATGCTGTA for codon 200. Peak heights were measured using the AQ mode of the PSQ 96 single nucleotide position software. In order to validate the use of peak heights to estimate relative SNP frequencies in amplicons derived from pooled DNA, between 32 and 44 individual worms from nine of the populations were also individually genotyped at the three SNPs. The frequency of the F200Y (T\text{AC}) SNP based on pooled data correlated well with that derived from the individual worm genotyping for these nine populations (see Supplementary Fig. 3.1). The E198A (G\text{CA}) was only present at low frequency in the populations and so the correlation was less precise. Nevertheless the pooled data still provided a sufficient estimate of the allele frequency appropriate for the work undertaken in this study (see Supplementary Fig. 3.1).

3.2.5. Sequencing of cloned \textit{H. contortus} isotype-1 β-tubulin

The same 328bp isotype-1 β-tubulin fragment described above was also amplified, cloned and sequenced from the pooled lysates from thirteen of the populations (Pop28S,
Pop1S, Pop2S, Pop16S, Pop3C, Pop26S, Pop27S, Pop11G, Pop12G, Pop51S, Pop31G and Pop32G). Primers HcPYRF: 5’- GAC GCA TTC ACT TGG AGG AG-3’ and HcPYRR: 5’-CAT AGG TTG GAT TTGTGA GTT-3’ were again used but with the following PCR reaction conditions; 5X Phusion HF reaction buffer, 2mM MgSO4, 200uM dNTPs, 0.2uM forward and reverse primers and 1U of Phusion high fidelity DNA polymerase (Finenzyme inc.). The thermo-cycling parameters of isotype-1 β-tubulin consisted of an initial 98°C for 30 sec followed by 35 cycles of 98°C for 10 sec, 59°C for 30 sec and 72°C for 1 min with a single final extension cycle of 72°C for 5 min. Amplicons were cloned into PJET 1.2/BLUNT vector (Thermo Scientific) and sequenced using previously described procedures (Chaudhry et al., 2014). Sequences were aligned with H. contortus isotype-1 β-tubulin sequences (Acc No X67489) and edited using Geneious Pro 5.4 software (Drummond AJ, 2012).

3.2.6. Bioinformatic filtering of H. contortus isotype-1 β-tubulin sequences to remove PCR induced mutations

Polymorphisms appearing more than once in the sequence data set are expected to be real, whereas polymorphisms that only occur once are possible artefacts due to polymerase induced errors. We used a previously described method to test this for our data (Redman et al., 2015). The distribution of the SNPs, when plotted along the isotype-1 β-tubulin gene model, strongly supports the hypothesis that the majority of unique SNPs were PCR-induced mutations whereas those appearing more once were genuine polymorphisms (Supplementary Fig. S3.2). Specifically, SNPs appearing more than once were either clustered within the introns or were synonymous changes in the exons (except for those occurring at the resistance associated positions P198 and P200). This is the pattern expected for genuine polymorphisms.
In contrast, the SNPs that occurred only once in the entire dataset were evenly distributed across intron and exons with no bias for synonymous mutations in the exons. Consequently, we only considered SNPs occurring more than once in the dataset in order to take a conservative approach and ensure only real polymorphisms were considered. This resulted in 21 different *H. contortus* haplotypes in the final filtered dataset which were used for all following analyses. For completeness, we also constructed trees and haplotype networks with the full unfiltered dataset and, other than increasing the total number of haplotypes, this did not change any of the phylogenetic relationships or overall conclusions (data not shown).

3.2.7. Phylogenetic network analysis of *H. contortus* isotype-1 β-tubulin sequence data

Networks based on genetic distance were computed using the neighbour-net method employed in SplitsTrees4 (Huson and Bryant, 2006) to produce circular (equal angle) split networks. Median joining networks were generated in Network 4.6.1 software (Fluxus Technology Ltd.). 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 were removed with the Maximum Parsimony (MP) option (Polzin and Daneschmand, 2003). A phylogenetic network tree of the isotype-1 β-tubulin haplotypes was reconstructed using maximum Likelihood (ML) in MEGA5 (Tamura, 2011). The program jModeltest 12.2.0 (Posada, 2008) was used to select the appropriate model of nucleotide substitutions for ML analysis. According to Bayesian information criterion, the best scoring was Hasegawa-Kishino-Yano (HKY+G). The model of substitution was used with parameters estimated from the data. Branch supports were obtained by 1000 bootstraps of the data. The most probable ancestral node was determined by rooting the
networks to a closely related outgroup and *Teladorsagia circumcincta* sequence was used to root the *H. contortus* network.

### 3.2.8. Microsatellite genotyping and population genetic analysis

Previously described *H. contortus* microsatellite markers (Otsen et al., 2000; Redman et al., 2008; Redman et al., 2015) were screened to produce a panel of eight markers that consistently amplified loci from the Indian populations under study (data not shown). A summary of primer sequences and allele ranges are given in Supplementary Table S3.2 and the PCR conditions for each marker were as previously described by Redman et al. (2008). The forward primer of each microsatellite primer pair was 5’ end labelled with fluorescent dye (IDT, Canada) and the GeneScan ROX 400 internal size standard was used on the ABI Prism 3100 genetic analyser (Applied Biosystems, USA). Individual chromatograms were analysed using Gene Mapper software version 4.0 to accurately size the amplicons and determine genotypes (Applied Biosystems, USA).

From the multi-locus microsatellite genotype data, heterozygosity ($H_e$ and $H_o$), allele richness and estimates of $F_{IS}$ for each locus were calculated using Arlequin 3.1 (Excoffier et al., 2005). Guo & Thompson’s (1992) exact test was used to statistically evaluate deviations from Hardy-Weinberg equilibrium for all populations (Guo and Thompson, 1992). Significance levels were adjusted using the sequential method of Bonferroni for multiple comparisons in the same dataset (Rice, 1989). There was a significant departure from Hardy-Weinberg equilibrium, even after Bonferroni correction, in addition to relatively high $F_{IS}$ values for 10 out of the 108 loci combinations for *H. contortus* (Supplementary Tables S3.3). The presence of null alleles for microsatellite loci has been previously reported and is the
likely reason for these departures from Hardy-Weinberg Equilibrium (Grillo et al., 2007; Hunt et al., 2008; Redman et al., 2008; Silvestre et al., 2009). Linkage disequilibrium analysis was carried out by GENEPOP version 3.3 (Rousset and Raymond, 1995) using a log likelihood ratio test statistic (G-test). Partition of microsatellite diversity between and within populations were estimated through an analysis of molecular variance AMOVA (Excoffier et al., 1992). Data were defined as ‘standard’ rather than ‘microsatellite’, as loci did not necessarily adhere to the stepwise mutation model. Pairwise $F_{ST}$ values were calculated and significance testing was undertaken by random permutation in Arlequin 3.11.

3.3. Results

3.3.1. The F200Y and E198A but not the F167Y benzimidazole resistance-associated isotype-1 $\beta$-tubulin polymorphisms were commonly found in H. contortus populations across southern India

A 328bp fragment of the isotype-1 $\beta$-tubulin gene was PCR amplified from twenty three H. contortus populations across southern India and the relative frequencies of the three currently known benzimidazole resistance-associated SNPs F167Y (T$\rightarrow$A$\rightarrow$C), E198A (G$\rightarrow$C$\rightarrow$A) and F200Y (T$\rightarrow$A$\rightarrow$C) was determined by pyrosequence genotyping (using pools of 18 to 44 worms per population; see Supplementary Table S3.1). Benzimidazole resistance-associated SNPs were found in eighteen of the twenty three populations with the F200Y (T$\rightarrow$A$\rightarrow$C) and E198A (G$\rightarrow$C$\rightarrow$A) SNPs being identified in multiple populations. The F200Y (T$\rightarrow$A$\rightarrow$C) and the E198A (G$\rightarrow$C$\rightarrow$A) SNPs were detected in 18 and 8 populations at frequencies between 9-84% and 8-18% respectively (Fig. 3.1 and Supplementary Table S3.1). The benzimidazole resistance associated SNP F167Y (T$\rightarrow$A$\rightarrow$C) was not detected in any of the populations.
Fig. 3.1. Relative frequencies of the F200Y (TAC), F167Y (TAC) and E198A (GCA) isotype-1 β-tubulin benzimidazole resistance associated mutations in *H. contortus* populations from southern India. Each *H. contortus* population is represented by three pie charts one for each resistance mutation; F200Y (TTC/TAC), F167Y (TTC/TAC) and E198A (GAA/GCA). Each of these pie charts shows the relative frequency of the resistant versus susceptible SNP based on allele quantification by pyrosequence genotyping of pooled DNA from between 18-44 worms per population (supplementary table 3.1). The resistance-associated SNP genotype frequency - F200Y (TAC) or E198A (GCA) - is shown in light grey hatch and “susceptible” SNP genotype frequency - F200Y (TTC), F167Y (TTC) and E198A (GAA) - is shown in black. Geographic locations of abattoirs are indicated with small circles on the map and the abattoir names from which the samples were obtained are shown above the labelling lines. The four provinces are indicated on the map (A) Tamil Nadu (B) Andhra Pradesh (C) Karnataka (D) Kerala.

3.3.2. Sequence diversity and phylogenetic relationships of isotype-1 β-tubulin alleles in *H. contortus* from southern India

The 328bp isotype-1 β-tubulin fragment was PCR amplified, cloned and sequenced from pooled DNA from each of thirteen populations. For each population, template DNA was pooled from between 18 and 44 worms (Supplementary Table S3.1) and the inserts of at least 10 clones were sequenced per population. Following bioinformatics filtering (see materials and methods), a total of twenty one different *H. contortus* isotype-1 β-tubulin haplotypes (GenBank accession numbers KP792514-KP792534) were identified in the 159 sequences
generated from the thirteen populations. Seven out of the twenty one haplotypes encoded the F200Y (TAC) resistance polymorphism and one encoded the E198A (GCA) resistance polymorphism. A SplitsTrees network was constructed with all twenty one isotype-1 β-tubulin haplotypes to examine their phylogenetic relationships (Fig. 3.2). The F200Y (TAC) resistance polymorphism was present on haplotypes located in three distinct parts of the network, each of which also contained at least one susceptible haplotype. In contrast, the E198A (GCA) was present on a single haplotype in the network. A Maximum Likelihood (ML) tree was also constructed and showed the same phylogenetic relationships (Supplementary Fig. S3.4).

3.3.3 Geographical distribution of F200Y (TAC) and E198A (GCA) isotype-1 β-tubulin benzimidazole resistance haplotypes in southern India

Frequency histograms of both the susceptible and resistance haplotypes showed that most of the populations contained multiple different resistance haplotypes (Supplementary Fig. 3.3). The only exceptions were populations Pop31G, Pop32G and Pop1S in which the F200Y (TAC) resistance associated polymorphism was detected on a single resistance haplotype (Supplementary Fig. 3.3). The Median-Joining network was constructed to integrate the geographical distribution and phylogenetic relationships of the different isotype-1 β-tubulin haplotypes (Fig. 3.3). The Median-Joining network was congruent with the SplitTrees network (Fig. 3.2) and Maximum Likelihood tree (Supplementary Fig. S3.4) analysis. The only difference was in the relative position of haplotype Hr25 in the network. The two F200Y (TAC) resistance-associated haplotypes that are present at the highest frequency in the dataset (Hr23 and Hr34) were present in a large number of different parasite populations across
southern India (11 and 7 different populations respectively). The only F200Y (TAC) resistance-associated haplotypes that were present in just a single population were those present at low frequency in the data set overall. In contrast, the E198A (GCA) resistance-associated mutation was present on only a single haplotype in the network (Hr24) for all six of the populations in which it was sequenced (Fig. 3.3).

Fig. 3.2. Split Trees network of the *H. contortus* isotype-1 β-tubulin sequences generated with the neighbour-net method of SplitsTrees4 (Huson and Bryant, 2006). The circles in network represent the different haplotypes and the size of the circles is proportional to the frequency in the population. The haplotypes containing the different mutations are shaded as follows: susceptible haplotypes containing F200Y (TTC)/F167Y (TTC)/E198A (GAA) are black; P200Y resistant haplotypes containing F200Y (TAC)/F167Y (TTC)/E198A (GAA) are light hatch; the P198A resistant haplotype (HR24) containing F200Y (TTC)/F167Y (TTC)/E198A (GCA) is shown by vertical line shading.
Fig. 3.3. Median joining network of the *H. contortus* isotype-1 β-tubulin sequences generated in Network 4.6.1 (Fluxus Technology Ltd.). 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 circle representing each haplotype is proportional to its frequency in the dataset and the colours in the circles reflect the frequency distribution in each population as indicated on the colour key on the inset map. Small red dots represent median vectors. The number of mutations
separating adjacent sequence nodes or median vectors is indicated along connecting branches and the length of the lines connecting the haplotypes is proportional to the number of nucleotide changes. The most probable ancestral node is determined by rooting the network to a closely related outgroup T. circumcincta (Tc) (GenBank accession number FN599034). The text providing the name of each haplotype is colour coded as follows; susceptible haplotype F200Y (TTC)/ F167Y (TTC)/E198A (GAA) is in black text; P200Y resistant haplotype F200Y (TAC)/ F167Y (TTC)/E198A (GAA) is in blue text; P198A resistant haplotype F200Y (TTC)/ F167Y (TTC)/E198A (GCA) is in pink text.

3.3.4. Genetic diversity and population structure of H. contortus in southern India

A panel of eight microsatellites were used to genotype eighteen individual worms in each of twelve populations in order to investigate the basic population genetic structure of H. contortus in southern India. Six of the populations (Pop26S, Pop27S, Pop51S, Pop52S, Pop71S and Pop22S) were from sheep and six from goats (Pop11G, Pop12G, Pop31G, Pop32G, Pop61G and Pop62G). Eight of the populations contained both the F200Y (TAC) and E198A (GCA) resistance associated SNPs (Pop11G, Pop12G, Pop26S, Pop27S, Pop32G, Pop52S and Pop62G), two populations just the F200Y (TAC) resistance associated SNP (Pop31G and Pop51S) and two populations did not contain any resistance associated SNPs (Pop61G and Pop71S). There were no major departures from linkage equilibrium for any particular combination of loci across all populations, indicating that alleles at these loci were randomly associating and not genetically linked (data not shown). All populations were polymorphic at all loci, with the number of alleles per locus ranging from 2 to 13 (Supplementary Table S3.3). H. contortus showed a high level of overall genetic diversity in all populations; the mean allele richness was 6.565 ±0.371 alleles per locus with an expected heterozygosity (He) of 0.6449 (range: 0.651-0.6900). There was very little difference in overall genetic diversity between the twelve populations. AMOVA analysis estimated that the percentage of variation that partitioned between twelve populations was 0.006% suggesting extremely low genetic sub-structuring of H. contortus in Southern India. This was reflected by
the very low pairwise $F_{ST}$ estimates calculated between each of the twelve populations. Only 5 out of 66 possible pairwise comparisons showed significant genetic differentiation (Fig. 3.4) based on $F_{ST}$ values and, even in these cases, the $F_{ST}$ values were very low ranging from 0.0244 (between Pop31G and Pop52S) to 0.0351 (between Pop61G and Pop22S).

<table>
<thead>
<tr>
<th></th>
<th>Pop11G</th>
<th>Pop12G</th>
<th>Pop26S</th>
<th>Pop27S</th>
<th>Pop31G</th>
<th>Pop32G</th>
<th>Pop51S</th>
<th>Pop52S</th>
<th>Pop61G</th>
<th>Pop62G</th>
<th>Pop71S</th>
</tr>
</thead>
<tbody>
<tr>
<td>Pop12G</td>
<td>-0.0101</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Pop26S</td>
<td>0.0030</td>
<td>0.0041</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Pop27S</td>
<td>-0.0010</td>
<td>0.0034</td>
<td>0.0132</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Pop31G</td>
<td>-0.0095</td>
<td>-0.006</td>
<td>-0.0035</td>
<td>0.0059</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Pop32G</td>
<td>-0.0020</td>
<td>-0.0086</td>
<td>-0.0057</td>
<td>0.0123</td>
<td>0.0046</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Pop51S</td>
<td>-0.0035</td>
<td>0.0003</td>
<td>-0.0041</td>
<td>0.0201</td>
<td>-0.0099</td>
<td>0.0089</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Pop52S</td>
<td>-0.0040</td>
<td>0.0040</td>
<td><strong>0.0248</strong></td>
<td><strong>0.0268</strong></td>
<td><strong>0.0244</strong></td>
<td>0.0062</td>
<td>0.0211</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Pop61G</td>
<td>-0.0174</td>
<td>0.0021</td>
<td>0.0123</td>
<td>0.0110</td>
<td>0.0019</td>
<td>0.0009</td>
<td>0.0061</td>
<td>0.0012</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Pop62G</td>
<td>-0.0075</td>
<td>0.0059</td>
<td>0.0038</td>
<td>0.0079</td>
<td>-0.0052</td>
<td>0.0031</td>
<td>0.0045</td>
<td>0.0137</td>
<td>0.0072</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Pop71S</td>
<td>-0.0051</td>
<td>-0.0069</td>
<td>-0.0052</td>
<td>0.0253</td>
<td>-0.0070</td>
<td>-0.0002</td>
<td>-0.0109</td>
<td>0.0087</td>
<td>0.0029</td>
<td>0.0056</td>
<td></td>
</tr>
<tr>
<td>Pop22S</td>
<td>0.0157</td>
<td>0.0053</td>
<td>0.0008</td>
<td>0.0299</td>
<td>0.0055</td>
<td>0.0102</td>
<td>0.0118</td>
<td><strong>0.0347</strong></td>
<td><strong>0.0351</strong></td>
<td>0.0164</td>
<td>0.0002</td>
</tr>
</tbody>
</table>

**Fig. 3.4.** Pairwise $F_{ST}$ values based on genotyping 18 individual worms from each of 12 *H. contortus* populations with eight microsatellite markers. Pairwise comparisons with statistically significant genetic differentiation, ($p<0.002$) are highlighted in bold and underlined.

### 3.4. Discussion

The frequency at which anthelmintic drug resistance arises and the extent to which it spreads are important considerations for its prevention and management (Silvestre and Humbert, 2002; Skuce et al., 2010; Redman et al., 2015). Previous work characterizing benzimidazole resistance mutations in *H. contortus* allows us to study the origin and spread of resistance in a much more targeted way than for other drug classes and parasite species. In this paper, we present a molecular genetic study of benzimidazole resistance in *H. contortus* in southern India. We chose this region for two main reasons. Firstly, small ruminants in rural areas of southern India are treated in a sporadic manner often with generic drugs of unknown
quality. Also, the warm, humid climate is expected to allow a relatively large in refugia population of free-living larvae on pasture for most of the year. These factors are expected to lead to less intense selection pressure overall and so led us to hypothesize that benzimidazole resistance might at an earlier stage in southern India than in many of the countries examined to date. Secondly, there is a large amount of unregulated animal movement in the region providing significant potential for the spread of mutations between locations. We believed that studying benzimidazole resistance in such a region should provide some interesting insights into the how resistance emerges and spreads and how this can be examined using molecular genetic approaches.

Although benzimidazole resistance is already widespread in southern India, our results suggest it is indeed less advanced than in those European and North American countries examined to date (Silvestre and Cabaret, 2002; de Lourdes Mottier and Prichard, 2008; Barrère et al., 2012; Brasil et al., 2012; Barrere et al., 2013a; Barrere et al., 2013b; Chaudhry and Gilleard, 2015; Redman et al., 2015). Although, the isotype-1 β-tubulin P200Y (TAC) mutation was present in 18 out of 23 populations, it was still at a relatively low frequency (<25%) in over half of these (13/23). Similarly, although the E198A (GCA) mutation was present in 8/23 populations, it was at low frequency in all cases (maximum frequency of 18%). It is interesting that the E198A (GCA) mutation was identified in so many populations given it has only been previously reported in just two H. contortus field derived isolates (from Australia and South Africa) and was not detected in surveys of benzimidazole resistant H. contortus from the UK, Canada and USA (Ghisi et al., 2007; Rufener et al., 2009; Barrère et al., 2012; Kotze et al., 2012; Barrere et al., 2013a; Barrere et al., 2013b; Chaudhry and Gilleard, 2015). Hence the E198A (GCA) appears to be much more common in southern
India than in the other countries examined to date, even though benzimidazole resistance appears less advanced based on the P200Y (TAC) distribution and frequency overall (Höglund et al., 2009; Barrère et al., 2012; Barrere et al., 2013a; Barrere et al., 2013b; Chaudhry and Gilleard, 2015; Redman et al., 2015). In contrast, the opposite applies to the F167Y (TAC) mutation. We did not identify this mutation in any of the 23 southern Indian populations, in spite of this mutation being found in most other countries examined to date, (Silvestre and Cabaret, 2002; de Lourdes Mottier and Prichard, 2008; Barrère et al., 2012; Brasil et al., 2012; Barrere et al., 2013a; Barrere et al., 2013b). Indeed, in our recent UK study, the F167Y (TAC) was found in *H. contortus* on 6 out of 7 farms examined and was at a similar overall frequency to the P200Y (TAC) mutation. The contrast between southern India and the UK is striking; the F167Y (TAC) mutation was commonly found in the UK but the E198A (GCA) mutation not identified but the exact opposite situation was the case for southern India. Hence, although the P200Y (TAC) mutation seems to be inevitably present in *H. contortus* populations throughout the world, there appears to be marked regional variation in the relative occurrence of the F167Y (TAC) and E198A (GCA) mutations. The most parsimonious explanation of these observed patterns of occurrence is that the F167Y (TAC) and E198A (GCA) mutations appear rarely in a region but then become widespread by migration. In this model, it is unsurprising that a particular mutation would be widespread in some countries or regions but absent in others.

In the case of the E198A (GCA) mutation in southern India, the phylogenetic analysis of the resistance haplotypes also supports the hypothesis that it arises rarely. Indeed, in this case, it appears that this mutation has spread to multiple different locations in southern India from a single origin. The mutation is present on a single haplotype in all six of the *H.*
contortus populations from which it was sequenced (Fig. 3.2 and 3.3). In contrast, there was a large amount of sequence diversity of susceptible haplotypes even within the short fragment that was sequenced (13 different susceptible haplotypes in the dataset). Hence, given this high level of susceptible haplotype diversity in southern India, it would be extremely unlikely that the E198A (G\text{CA}) repeatedly arose only on the same haplotype (Hr24). Hence, the evidence strongly suggests that the E198A (G\text{CA}) mutation in the populations examined is derived from a single origin. This represents the first clear genetic evidence of the spread of an anthelmintic resistance mutation across multiple locations in a country.

In contrast, the P200Y (TAC) mutation was present on multiple, phylogenetically diverse haplotypes and their phylogenetic relationship suggests there are at least three independent origins of the P200Y (T\text{AC}) mutation in the populations examined (Fig. 3.2 and 3.3). Within the haplotype networks, each of three groups of resistance haplotypes (Hr23/Hr27/Hr36; Hr22/Hr35; Hr25/Hr34) were more closely related to one or more susceptible haplotypes in the network than they were to any of the other resistant haplotypes (Fig. 3.2 and 3.3). This provides strong evidence that these are independently derived alleles. Hence, the phylogenetic evidence supports the view that the P200Y (T\text{AC}) mutation originates much more commonly in parasite populations than the E198A (G\text{CA}) mutation. This helps to explain why P200Y (T\text{AC}) mutation is seen more commonly and consistently across the world than the E198A (G\text{CA}) whose distribution is much more highly variable as discussed above.

We examined the population structure of twelve H. contortus populations from southern India using a panel of eight highly polymorphic microsatellite markers. The diversity within each population was very high as has been previously reported for this parasite (Blouin
et al., 1995; Silvestre and Humbert, 2002; Redman et al., 2015). However, the genetic differentiation between populations was extremely low with only 0.006% of the genetic variation being partitioned between populations overall and only 5/66 pairwise Fst comparisons showing statistically significant differentiation (highest Fst value being 0.0351). This is consistent with previous studies showing genetic differentiation between *H. contortus* populations to be relatively low within a number of different countries (including the UK, France and Sweden) (Silvestre et al., 2009; Redman et al., 2015). This has been suggested to be due to a combination of large effective population sizes and high gene flow by migration due to anthropogenic movement of livestock hosts (Blouin et al., 1995; Silvestre et al., 2009; Redman et al., 2015). The genetic differentiation in the populations examined here is lower than previously reported (Redman et al., 2015). For example, in our UK study using 7 out of 8 of the same markers used here, AMOVA analysis of microsatellite data revealed 2.84% of the genetic variation was partitioned between populations overall (compared with 0.006% here). Also in that study 10 out of 21 Fst comparisons showed statistically significant differentiation between *H. contortus* populations (highest Fst was 0.0757) compared with just 5 out of 66 here (highest Fst was 0.0351). The lower level of genetic differentiation between *H. contortus* populations in southern India could be due to higher gene flow consistent with the large amount of unregulated anthropogenic animal movement that occurs in the region. This would predispose to the rapid spread of anthelmintic resistance mutations across the region. One caveat to this interpretation though is that the low genetic differentiation observed could also be at least partly due to the high levels of pasture larval survival in the year-round warm humid climate which would minimize population bottlenecks compared to more temperate regions. A final point worth noting is that there was no significant genetic differentiation
detected between the *H. contortus* populations taken from sheep and goats (Fig. 3.4). Indeed, the Fst values were lower between many of the pairwise comparisons between sheep and goat populations than between pairwise comparisons of these populations in the same host. This supports the view that *H. contortus* is freely shared between the two hosts with little or no host species barrier.

### 3.5. Acknowledgements

We are grateful to the Natural Sciences and Engineering Research Council of Canada (NSERC) (Grant number RGPIN/371529-2209), NSERC-CREATE Host Pathogen Interactions (HPI) graduate training program at the University of Calgary as well as the BBSRC for funding support (Grant number BB/E018505/1).

### Reference


Chaudhry, U., Redman, E., Abbas, M., Muthusamy, R., Ashraf, K., Gilleard, J., 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.


Polzin, T., Daneschmand, S.V., 2003. On Steiner trees and minimum spanning trees


Appendices: Supplementary Figure Legends

Supplementary Fig. S3.1. (A) Comparison of the relative frequency of resistant and susceptible SNPs - F200Y (TTC/TAC), E198A (GAA/GCA) and F167Y (TTC/TAC) – as determined by individual worm genotyping (upper chart) and allele quantification from pooled worm lysates (lower chart) for nine field populations of *H. contortus* in southern India (32 - 44 worms per population). Susceptible genotypes are represented by dark shading; purple, red and green for F200Y (TTC), E198A (GAA) and F167Y (TTC) respectively whereas resistant genotypes are indicated by light shading; purple red and green shading for P200Y(TAC), E198A (GCA) and F167Y (TAC) respectively. (B) Regression analysis comparing the F200Y (TAC) and E198A (GCA) resistance SNP frequencies as determined by individual worm genotyping (upper chart) versus allele quantification from pooled worm lysates (lower chart) for nine populations of *H. contortus*. 
Supplementary Fig. S3.2. The distribution of single nucleotide polymorphisms among 159 *H. contortus* sequences across the 382bp PCR amplicon of the isotype-1 β-tubulin gene spanning exons 4 and 5 and intervening intron. The position of the SNPs along with the 328bp sequences for *H. contortus* is shown along the X-axis and the number of sequences that contain each SNP is plotted on the Y-axis.
Supplementary Fig. S3.3. Frequency histograms showing resistant and susceptible isotype-1 β-tubulin haplotypes identified from the thirteen populations. F200Y (TTC)/F167Y (TTC)/E198A (GAA) susceptible haplotypes are shown in black, P200Y (TAC) resistant haplotypes light grey; P198A (GCA) resistant dark grey.)
Supplementary Fig. S3.4. Maximum likelihood tree of isotype-1 β-tubulin haplotypes cloned and sequenced from thirteen *H. contortus* populations from four provinces of southern India [(A) Tamil Nadu (B) Andra Predesh (C) Karnataka (D) Kerala]. Susceptible haplotypes - F200Y (TTT)/ F167Y (TTT)/ E198A (GAA) - are indicated with a yellow circle; P200Y resistant haplotypes - F200Y (TAC)/ F167Y (TTT)/E198A (GAA) - are indicated with a blue circle and the E198A resistant haplotype - F200Y (TTT)/ F167Y (TTT)/E198A (GCA) – are indicated with a pink circle. The coloured rectangles indicate the populations in which each haplotype was identified as indicated by the colour key on the inset map. The sequences were aligned by Geneious software and the tree obtained by maximum likelihood analysis using the Hasegawa-Kishino-Yano (HKY+G) model of substitution (Drummond AJ, 2012). Branches with bootstrap support values above 50% (1000 replications) and posterior probability greater than 50, respectively. The phylogeny is rooted with an isotype-1 β-tubulin sequence of *Teladorsagia circumcincta* (*Tc*) (GenBank accession number **FN599034**).
Appendices: Supplementary Table Legends

**Supplementary Table S3.1.** Allele frequency (%) of SNPs that resulted in an amino acid changes at codon F200Y (TTC/TAC), F167Y (TTC/TAC) and E198A (GAA/GCA) in isotype-1 β-tubulin obtained from twenty three *H. contortus* populations from southern India.

<table>
<thead>
<tr>
<th>Indian field Populations</th>
<th>Host</th>
<th>No of worms in each pool</th>
<th>P167</th>
<th>P198</th>
<th>P200</th>
<th>Abattoirs Location (Province)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Pop2S</td>
<td>Sheep</td>
<td>44</td>
<td>100</td>
<td>0</td>
<td>100</td>
<td>0</td>
</tr>
<tr>
<td>Pop3S</td>
<td>Sheep</td>
<td>44</td>
<td>100</td>
<td>0</td>
<td>100</td>
<td>0</td>
</tr>
<tr>
<td>Pop5S</td>
<td>Sheep</td>
<td>41</td>
<td>100</td>
<td>0</td>
<td>100</td>
<td>0</td>
</tr>
<tr>
<td>Pop52S</td>
<td>Sheep</td>
<td>36</td>
<td>100</td>
<td>0</td>
<td>92</td>
<td>8</td>
</tr>
<tr>
<td>Pop16S</td>
<td>Sheep</td>
<td>44</td>
<td>100</td>
<td>0</td>
<td>100</td>
<td>0</td>
</tr>
<tr>
<td>Pop41S</td>
<td>Sheep</td>
<td>43</td>
<td>100</td>
<td>0</td>
<td>100</td>
<td>0</td>
</tr>
<tr>
<td>Pop42S</td>
<td>Sheep</td>
<td>36</td>
<td>100</td>
<td>0</td>
<td>100</td>
<td>0</td>
</tr>
<tr>
<td>Pop71S</td>
<td>Sheep</td>
<td>42</td>
<td>100</td>
<td>0</td>
<td>100</td>
<td>0</td>
</tr>
<tr>
<td>Pop72S</td>
<td>Sheep</td>
<td>36</td>
<td>100</td>
<td>0</td>
<td>100</td>
<td>0</td>
</tr>
<tr>
<td>Pop27S</td>
<td>Sheep</td>
<td>36</td>
<td>100</td>
<td>0</td>
<td>85</td>
<td>15</td>
</tr>
<tr>
<td>Pop28S</td>
<td>Sheep</td>
<td>44</td>
<td>100</td>
<td>0</td>
<td>100</td>
<td>0</td>
</tr>
<tr>
<td>Pop26S</td>
<td>Sheep</td>
<td>45</td>
<td>100</td>
<td>0</td>
<td>84</td>
<td>16</td>
</tr>
<tr>
<td>Pop11G</td>
<td>Goat</td>
<td>32</td>
<td>100</td>
<td>0</td>
<td>89</td>
<td>11</td>
</tr>
<tr>
<td>Pop12G</td>
<td>Goat</td>
<td>36</td>
<td>100</td>
<td>0</td>
<td>82</td>
<td>18</td>
</tr>
<tr>
<td>Pop21G</td>
<td>Goat</td>
<td>36</td>
<td>100</td>
<td>0</td>
<td>100</td>
<td>0</td>
</tr>
<tr>
<td>Pop22G</td>
<td>Goat</td>
<td>36</td>
<td>100</td>
<td>0</td>
<td>100</td>
<td>0</td>
</tr>
<tr>
<td>Pop12G</td>
<td>Goat</td>
<td>36</td>
<td>100</td>
<td>0</td>
<td>92</td>
<td>8</td>
</tr>
<tr>
<td>Pop31G</td>
<td>Goat</td>
<td>45</td>
<td>100</td>
<td>0</td>
<td>100</td>
<td>0</td>
</tr>
<tr>
<td>Pop61G</td>
<td>Goat</td>
<td>36</td>
<td>100</td>
<td>0</td>
<td>100</td>
<td>0</td>
</tr>
<tr>
<td>Pop62G</td>
<td>Goat</td>
<td>36</td>
<td>100</td>
<td>0</td>
<td>87</td>
<td>13</td>
</tr>
<tr>
<td>Pop2C</td>
<td>Cattle</td>
<td>32</td>
<td>100</td>
<td>0</td>
<td>100</td>
<td>0</td>
</tr>
<tr>
<td>Pop3C</td>
<td>Cattle</td>
<td>18</td>
<td>100</td>
<td>0</td>
<td>100</td>
<td>0</td>
</tr>
<tr>
<td>Pop41B</td>
<td>Buffalo</td>
<td>29</td>
<td>100</td>
<td>0</td>
<td>100</td>
<td>0</td>
</tr>
</tbody>
</table>

**Supplementary Table S3.2.** Panel of microsatellites used for population genetics analysis of *H. contortus*

<table>
<thead>
<tr>
<th>Microsatellite</th>
<th>Primer sequences (5' → 3')</th>
<th>Allele size range (bp)</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>Hc36</td>
<td>F: (HEX) GCATAcCGGCAAGGGACGTATG R: CGAGGTACTCTGGTGTTCGTG</td>
<td>138-158</td>
<td>Redman, 2015</td>
</tr>
<tr>
<td>Hc22193</td>
<td>F: (NED) ATCCACTTTCACCTCTATCA R: GTGTGGGATCCTGTGTG</td>
<td>198-225</td>
<td>Redman, 2015</td>
</tr>
<tr>
<td>Hc3086</td>
<td>F: (FAM) AAGCCCAACAAAAGACAAAT R: CACATAAGACACTCTCTT</td>
<td>250-400</td>
<td>Redman, 2015</td>
</tr>
<tr>
<td>Hc53265</td>
<td>F: (FAM) GTAGGCTGACTCTTTAAAGA R: AGAAGTGAAAAGCTGTAGTG</td>
<td>155-248</td>
<td>Redman, 2015</td>
</tr>
<tr>
<td>Hc25</td>
<td>F: (FAM) AAGGGAAGGTAGAAGTTCCGG R: GCCATTGTGTAAGGCTCCC</td>
<td>170-312</td>
<td>Otzen et al., 2000</td>
</tr>
<tr>
<td>Hc33</td>
<td>F: (HEX) ATGGGGTTGTCGGAGGGATTTCC R: CCGCCTCAATTAAGGGCCTAGA</td>
<td>180-240</td>
<td>Otzen et al., 2000</td>
</tr>
<tr>
<td>Hc8a20</td>
<td>F: (FAM) CAAACTTGCACCCAGACCTCTC R: AGGGGGTGTCGACAAAAACATT</td>
<td>170-300</td>
<td>Redman et al., 2008</td>
</tr>
<tr>
<td>Hc3561</td>
<td>F: (HEX) CTTACAGTGTCTCTCCATATGTC R: TTAGCGAAGTAFAAGGCTGCC</td>
<td>262-288</td>
<td>Unpublished primer</td>
</tr>
</tbody>
</table>
**Supplementary Table S3.3.** Population genetic data for each microsatellite marker from twelve populations of *H. contortus* based on panel of 8 microsatellite loci.

<table>
<thead>
<tr>
<th>Population</th>
<th>He53265 (15')</th>
<th>He3086 (20')</th>
<th>He22193 (11')</th>
<th>Hems36 (8')</th>
<th>He33 (11')</th>
<th>He8a20 (16')</th>
<th>He2561 (11')</th>
<th>Hems25 (20')</th>
<th>All loci</th>
</tr>
</thead>
<tbody>
<tr>
<td>H11 (18°)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>N_e</td>
<td>1</td>
<td>2</td>
<td>2</td>
<td>3</td>
<td>1</td>
<td>3</td>
<td>3</td>
<td>2</td>
<td></td>
</tr>
<tr>
<td>H_e</td>
<td>0.7011</td>
<td>0.2333</td>
<td>0.8805</td>
<td>0.8810</td>
<td>0.6712</td>
<td>0.5098</td>
<td>0.6758</td>
<td>0.8608</td>
<td>0.6152</td>
</tr>
<tr>
<td>H_o</td>
<td>0.9333</td>
<td>0.8235</td>
<td>0.7500</td>
<td>0.6666</td>
<td>0.5229</td>
<td>0.6000</td>
<td>0.8125</td>
<td>0.5822</td>
<td></td>
</tr>
<tr>
<td>p-value</td>
<td>0.9574</td>
<td>0.0676</td>
<td>0.8502</td>
<td>0.2208</td>
<td>0.3166</td>
<td>1.0000</td>
<td>0.0448</td>
<td>0.3786</td>
<td></td>
</tr>
<tr>
<td>F_is</td>
<td>-0.3470</td>
<td>1.0000</td>
<td>0.0666</td>
<td>0.1529</td>
<td>0.0070</td>
<td>-0.0397</td>
<td>0.1157</td>
<td>0.0579</td>
<td></td>
</tr>
<tr>
<td>A</td>
<td>9</td>
<td>2</td>
<td>10</td>
<td>10</td>
<td>5</td>
<td>5</td>
<td>5</td>
<td>9</td>
<td>6.4♀</td>
</tr>
<tr>
<td>H12 (18°)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>N_e</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>1</td>
<td>0</td>
<td></td>
<td></td>
</tr>
<tr>
<td>H_e</td>
<td>0.5349</td>
<td>0.3938</td>
<td>0.8476</td>
<td>0.8793</td>
<td>0.7396</td>
<td>0.5174</td>
<td>0.6934</td>
<td>0.8301</td>
<td>0.6276</td>
</tr>
<tr>
<td>H_o</td>
<td>0.6666</td>
<td>0.6666</td>
<td>0.8888</td>
<td>0.8888</td>
<td>0.5000</td>
<td>0.7058</td>
<td>0.7777</td>
<td>0.5907</td>
<td></td>
</tr>
<tr>
<td>p-value</td>
<td>0.9341</td>
<td><strong>0.0002</strong></td>
<td>0.3578</td>
<td>0.4897</td>
<td>0.3097</td>
<td>0.7587</td>
<td>0.6111</td>
<td>0.4862</td>
<td></td>
</tr>
<tr>
<td>F_is</td>
<td>-0.2553</td>
<td>1.0000</td>
<td>0.2183</td>
<td>-0.0111</td>
<td>-0.2088</td>
<td>0.0347</td>
<td>-0.0185</td>
<td>0.0648</td>
<td></td>
</tr>
<tr>
<td>A</td>
<td>6</td>
<td>3</td>
<td>8</td>
<td>13</td>
<td>5</td>
<td>6</td>
<td>5</td>
<td>12</td>
<td>7.0♂</td>
</tr>
<tr>
<td>H26 (18°)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>N_e</td>
<td>0</td>
<td>1</td>
<td>3</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>2</td>
<td>1</td>
<td></td>
</tr>
<tr>
<td>H_e</td>
<td>0.6793</td>
<td>0.1894</td>
<td>0.8920</td>
<td>0.7771</td>
<td>0.7873</td>
<td>0.6619</td>
<td>0.6875</td>
<td>0.8484</td>
<td>0.6525</td>
</tr>
<tr>
<td>H_o</td>
<td>0.8888</td>
<td>0.8888</td>
<td>0.7647</td>
<td>0.6111</td>
<td>0.6666</td>
<td>0.6875</td>
<td>0.6470</td>
<td>0.5727</td>
<td></td>
</tr>
<tr>
<td>p-value</td>
<td>0.1861</td>
<td>0.0524</td>
<td>0.5800</td>
<td>0.0529</td>
<td>0.2663</td>
<td>0.7187</td>
<td>0.2459</td>
<td>0.0254</td>
<td></td>
</tr>
<tr>
<td>F_is</td>
<td>-0.3203</td>
<td>1.0000</td>
<td>0.0036</td>
<td>0.0165</td>
<td>0.2288</td>
<td>-0.0074</td>
<td>0</td>
<td>0.243</td>
<td></td>
</tr>
<tr>
<td>A</td>
<td>7</td>
<td>2</td>
<td>10</td>
<td>9</td>
<td>6</td>
<td>7</td>
<td>4</td>
<td>11</td>
<td>6.5♀</td>
</tr>
<tr>
<td>H27 (18°)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>N_e</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>2</td>
<td>0</td>
<td>1</td>
<td>0</td>
<td>0</td>
<td></td>
</tr>
<tr>
<td>H_e</td>
<td>0.7076</td>
<td>0.6153</td>
<td>0.8269</td>
<td>0.9238</td>
<td>0.7681</td>
<td>0.5730</td>
<td>0.7650</td>
<td>0.8698</td>
<td>0.6900</td>
</tr>
<tr>
<td>H_o</td>
<td>0.9411</td>
<td>0.8888</td>
<td>0.8333</td>
<td>0.8125</td>
<td>0.4444</td>
<td>0.5555</td>
<td>0.7222</td>
<td>0.5960</td>
<td></td>
</tr>
<tr>
<td>p-value</td>
<td>0.9737</td>
<td><strong>0.0009</strong></td>
<td>0.4811</td>
<td>0.1417</td>
<td>0.5007</td>
<td>0.4754</td>
<td>0.0153</td>
<td>0.4204</td>
<td></td>
</tr>
<tr>
<td>F_is</td>
<td>-0.3438</td>
<td>1.0000</td>
<td>-0.0772</td>
<td>0.1065</td>
<td>-0.0597</td>
<td>0.2294</td>
<td>0.2796</td>
<td>0.1738</td>
<td></td>
</tr>
<tr>
<td>A</td>
<td>10</td>
<td>3</td>
<td>7</td>
<td>13</td>
<td>5</td>
<td>8</td>
<td>10</td>
<td>7.1♂</td>
<td></td>
</tr>
<tr>
<td>H31 (18°)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>N_e</td>
<td>1</td>
<td>1</td>
<td>2</td>
<td>2</td>
<td>1</td>
<td>0</td>
<td>1</td>
<td>0</td>
<td></td>
</tr>
<tr>
<td>H_e</td>
<td>0.6381</td>
<td>0.2898</td>
<td>0.8984</td>
<td>0.8395</td>
<td>0.7575</td>
<td>0.5615</td>
<td>0.6773</td>
<td>0.8746</td>
<td>0.6221</td>
</tr>
<tr>
<td>H_o</td>
<td>0.8333</td>
<td>0</td>
<td>0.8823</td>
<td>0.8823</td>
<td>0.7058</td>
<td>0.5294</td>
<td>0.7058</td>
<td>0.6666</td>
<td>0.5853</td>
</tr>
<tr>
<td>p-value</td>
<td>0.9279</td>
<td><strong>0.0001</strong></td>
<td>0.2158</td>
<td>0.4387</td>
<td>0.4023</td>
<td>0.6180</td>
<td>0.7161</td>
<td>0.0007</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Ns</td>
<td>1</td>
<td>2</td>
<td>3</td>
<td>4</td>
<td>5</td>
<td>6</td>
<td>7</td>
<td>8</td>
</tr>
<tr>
<td>------------------</td>
<td>-------------</td>
<td>---</td>
<td>---</td>
<td>---</td>
<td>---</td>
<td>---</td>
<td>---</td>
<td>---</td>
<td>---</td>
</tr>
<tr>
<td></td>
<td>Hs</td>
<td>0.6825</td>
<td>0.2333</td>
<td>0.8714</td>
<td>0.9019</td>
<td>0.6952</td>
<td>0.6047</td>
<td>0.6793</td>
<td>0.8127</td>
</tr>
<tr>
<td></td>
<td></td>
<td>0.8333</td>
<td>0.9444</td>
<td>0.7647</td>
<td>0.6111</td>
<td>0.5555</td>
<td>0.8888</td>
<td>0.7777</td>
<td>0.6181</td>
</tr>
<tr>
<td></td>
<td>p-value</td>
<td>0.2259</td>
<td>0.0667</td>
<td>0.8930</td>
<td>0.5355</td>
<td>0.5743</td>
<td>0.9325</td>
<td>0.5600</td>
<td>0.8901</td>
</tr>
<tr>
<td></td>
<td>Fis</td>
<td>-0.2289</td>
<td>1.0000</td>
<td>-0.0864</td>
<td>0.1561</td>
<td>0.1241</td>
<td>0.0835</td>
<td>-0.3203</td>
<td>0.0441</td>
</tr>
<tr>
<td></td>
<td></td>
<td>6.7^*</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>6.7^*</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>6.7^*</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>6.7^*</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>6.7^*</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>6.7^*</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>6.7^*</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>6.7^*</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>6.7^*</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>6.7^*</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>6.7^*</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>6.7^*</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>6.7^*</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>6.7^*</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>6.7^*</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>6.7^*</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>6.7^*</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>6.7^*</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>6.7^*</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>6.7^*</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>6.7^*</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>6.7^*</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>6.7^*</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>6.7^*</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>6.7^*</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>6.7^*</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>6.7^*</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>6.7^*</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>6.7^*</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

N_s apparent null homozygotes, i.e. number of worms in the population which failed to give an amplification product for a particular marker; H_s expected heterozygosity; Fis, inbreeding coefficient. P-values indicate a significant deviation from Hardy–Weinberg equilibrium following bonferroni correction; A number of alleles; A_U, number of alleles unique to that population; N_f, estimated null allele frequency.

^* Total number of individuals genotyped for each population is given in parenthesis under the population name.

^+ Total number of alleles for each marker across all populations is given in parenthesis below each marker name.

^4 Mean number of alleles in each population for eight markers.
Comparison of benzimidazole resistance in *Haemonchus contortus* on government and rural farms in the Punjab Pakistan provides further insights into the origin of resistance alleles

Umer Chaudhry, Muhammad Zubair Shabbir, Kamran Ashraf, Shoaib Ashraf, John S. Gilleard

Manuscript in-preparation for publication in Parasitology and Vector
Abstract

We have compared the molecular genetics of benzimidazole resistance alleles in *H. contortus* populations under intense drug selection pressure on small ruminant government farms that are closed to animal movement with parasite populations that are under minimal selection pressure in the surrounding rural areas of the Punjab region of Pakistan. The F200Y (TAC) isotype-1 β-tubulin benzimidazole resistance mutation was present at very high frequency in all three government farms examined whereas it was either absent, or present at very low frequency, in eighteen parasite populations from surrounding rural areas. Phylogenetic analysis suggests that the F200Y (TAC) mutation has multiple independent origins in the region consistent with several previous studies from other countries. The F200Y (TAC) mutation was present on multiple haplotypes on each of the three government farms demonstrating that a soft selective sweep has occurred on these closed herds even though parasite migration is essentially absent. Although the F200Y (TAC) appears to have repeatedly arisen, the F167Y (TAC) and E198A (GCA) mutations were absent from all populations, even on the government farms with over 30 years of frequent benzimidazole use. This is consistent with the view that these two mutations arise more rarely than the F200Y (TAC). One of the F200Y (TAC) haplotypes (Hr22) present on the three government herds, that were originally founded from locally sourced breeding stock, was also present in a contemporary rural parasite population suggesting it could have been present in the parasite populations prior to the formation of the government herds over 30 years ago. However, there were also two resistance haplotypes (Hr31, Hr23) present on the government farms that were not found in any of rural populations suggesting that they may have appeared after the formation of the herds. Overall, this work provides further evidence to support the hypothesis
that the F200Y (TAC) mutation isotype-1 \( \beta \)-tubulin mutation can repeatedly appear by recurrent mutation although its presence in the standing genetic variation prior to the onset of selection may also occur.

**Keywords:** *Haemonchus contortus*, isotype-1 \( \beta \)-tubulin, anthelmintic drug, benzimidazole, soft selective sweep.
4.1. Introduction

Anthelmintic resistance in parasitic nematodes is a threat to sustainable livestock production worldwide (Kaplan, 2004). However, we still have a limited understanding of how resistance emerges at the population level. *Haemonchus contortus* is one of the most economically important helminth parasites of small ruminants worldwide and is an important model for anthelmintic resistance research (Gilleard 2013). This parasite has developed resistance to all the major anthelmintic drug classes and resistance to multiple drug classes occurs, often at high frequency, in many parts of the world (Waller, 1997; McKellar and Jackson, 2004).

Benzimidazole drugs have been used to control livestock parasites for over 40 years and resistance to this drug class is at an advanced stage in many parts of the world (Chaudhry and Gilleard, 2015; Redman et al., 2015). We have sufficient knowledge of the molecular basis of resistance for this drug class in order to study the emergence and spread of anthelmintic resistance mutations in field populations. Three single nucleotide polymorphisms (SNPs) in the isotype-1 β-tubulin gene have been associated with benzimidazole resistance in *H. contortus*. The F200Y (TTC to TAC) mutation has been found in every country examined to date and is often present at high frequency (Silvestre and Humbert, 2002; Hoglund et al., 2009; Brasil et al., 2012; Chaudhry and Gilleard, 2015; Redman et al., 2015). We have recently presented genetic evidence that this mutation independently arises multiple times in a region and spreads between sites by migration (Redman 2015, Chaudhry 2015). The SNPs at codons F167Y (TTC to TAC) and E198A (GAA to GCA) have also been reported in multiple countries but have a more variable occurrence and are generally present at lower frequency
than the F200Y (TTC to TAC) mutation (Barrère et al., 2012; Brasil et al., 2012; Chaudhry and Gilleard, 2015; Redman et al., 2015). We have recently provided genetic evidence to show that E198A (GCA) mutation present in multiple populations in southern India was derived from a single origin (Chaudhry et al., 2015a).

We are investigating the population genetics of benzimidazole resistance in *H. contortus* in a variety of different countries (Chaudhry et al., 2014; Chaudhry et al., 2015a; Chaudhry and Gilleard, 2015; Redman et al., 2015). Benzimidazole resistance is at an advanced stage in most countries that we, and others, have examined to date due to the widespread use of this drug class over many years. In places such as the UK, Western Europe, Australia and North America most *H. contortus* populations have moderate to high levels of benzimidazole resistance (Brasil et al., 2012; Chaudhry and Gilleard, 2015; Redman et al., 2015). In these countries, most parasite populations have at least one of the resistance-associated mutations at high frequency. We have been seeking locations where we can investigate benzimidazole resistance at an earlier stage of its emergence and Pakistan is a potentially interesting country in this respect. Economic constraints mean that most farmers do not use specific anthelmintic treatment regimes, anthelmintics are often diluted before administration and generic drugs of unknown quality are often used. Consequently, we anticipated that benzimidazole resistance might be at a relatively early stage of emergence in animals from small rural farms or owned for subsistence purposes. In contrast, government farms tend to use frequent treatments with benzimidazole drugs providing a potentially interesting contrast in the same geographical region. There are only a few published studies on benzimidazole resistance in Pakistan but there is information to suggest resistance may not yet be widespread (Saeed et al 2010, Hussain 2014). Fecal egg count reduction tests performed on Beetal goats on eighteen farms
in the Faisalabad district of the Punjab showed oxfendazole efficacies of between 80-100% (over 95% fecal egg count reduction on eleven on the farms) (Saeed et al., 2010). The sequencing of an isotype-1 β-tubulin gene fragment from 95 adult *H. contortus* worms collected from abattoirs in the Punjab and North West Frontier provinces identified revealed just a 7.86% frequency of the F200Y (TAC) mutation (Hussain et al., 2014).

In the present paper, we show that the F200Y (TAC) benzimidazole resistance mutation is present only at very low frequencies in eighteen *H. contortus* populations sourced from sheep and goats from rural locations in the Punjab Province of Pakistan. In contrast this mutation is present at very high frequency in three government farms which use frequent and regular anthelmintic treatments. The distribution and phylogenetic relationships between resistance and susceptible haplotypes suggest that the F200Y (TAC) mutation has arisen multiple independent times in the region and provides further evidence of the importance of recurrent mutation in the emergence of benzimidazole resistance in this parasite species.

### 4.2. Materials and Methods

#### 4.2.1. Parasite collection

Adult *Haemonchus* worms were harvested from the abomasas of eighteen individual ruminant hosts necropsied at local abattoirs across the Punjab province of Pakistan in 2012 that were known to originate from different pastures/villages in the region (Fig. 4.1, Supplementary Table S4.1). Adult *Haemonchus* worms, were also obtained from animals from three government farms following transportation and necropsy at the Veterinary Research Institute Lahore. Five of the *H. contortus* populations were collected from sheep;
two from the Lahore abattoir (Pop16S and Pop13S), one from the Sargodha abattoir (Pop24S), one from the Jahangirabad government farm (Pop3S) and one from the Okara government farm (Pop1S). Sixteen of the *H. contortus* populations were collected from goats; six from the Lahore abattoir (Pop5G, Pop7G, Pop6G, Pop4G, Pop8G, Pop10G), three from the Okara abattoir (Pop27G, Pop28G, Pop29G), two from the Sahiwal abattoir (Pop31G, Pop33G), four from the Gujranwala abattoir (Pop17G, Pop19G, Pop20G, Pop21G) and one from the Layyah government farm (Pop2G) (Fig. 4.1, Supplementary Table S4.1).

The Layyah government farm (pop2G) was established in 1980 from angora goats sourced from Turkey which were crossed with locally sourced goats of the “hairy breed”. This herd has been treated with anthelmintics (levamisole HCL and oxfendazole) approximately every 3 months since its establishment and has been closed to animal movement since that time. The Okara (Pop1S) and Jahangirabad (Pop3S) government farms were established using local sheep breeds (lohi and kajli) in 1985 and 1989 respectively. Both these herds have been treated alternately with albendazole and oxfendazole approximately every 3 months since their establishment. There has been some historical movement of sheep between the Okara and Jahangirabad farms but these herds have otherwise been closed to animal movement since they were established.

4.2.2. Genomic DNA isolation and species confirmation

Adult worms were fixed in 70% ethanol immediately following removal from the host abomasum. The heads of individual worms were dissected and lysed in single 0.2 μl tubes containing 50 μl of proteinase K lysis buffer and stored at -80°C as previously described by Redman et al. (2015) and Chaudhry et al. (2015b). One microliter of 1:5 dilution of neat
single worm lysate was used as PCR template and identical dilutions of lysate buffer, made in parallel, were used as negative controls. To prepare pooled lysates of each population, 1 µl aliquots of each individual neat adult worm head lysate were pooled. One microliter of a 1:20 dilution of pooled lysates was used as template for subsequent PCRs.

A combination of gross morphological identification and genotyping of the SNP at position 24 of the ITS-2 rDNA was used to identify *H. contortus* in these populations as previously reported by Chaudhry et al. (2015b). A minimum of 14 and a maximum of 32 individual adult *H. contortus* worms were identified per population (560 *H. contortus* worms in total). Individual *H. contortus* DNA lysates were pooled for each population for isotype-1 β-tubulin genotyping.

4.2.3. Pyrosequence genotyping to determine the relative frequencies of the isotype-1 β-tubulin F200Y (TAC), E198A (GCA) and F167Y (TAC) benzimidazole resistance-associated SNPs in *H. contortus* populations

A 328bp fragment, spanning exons 4 and 5 and intervening intron of the isotype-1 β-tubulin gene was PCR amplified from pooled DNA lysates for each of the 21 *H. contortus* populations (Supplementary Table S4.1). Final PCR conditions were 1X thermopol reaction buffer, 2mM MgSO₄, 100µM dNTPs, 0.1µM forward and reverse primers and 1.25U Taq DNA polymerase (New England Biolabs) and a previously published primer pair was used; forward primer (HcPYRF: 5’- GAC GCA TTC ACT TGG AGG AG-3’) and reverse primer (HcPYRR: 5’-Biotin-CAT AGG TTG GAT TTGTGA GTT-3’) (von Samson-Himmelstjerna et al., 2009). The thermo-cycling parameters consisted of an initial 95°C for 5 min followed
by 35 cycles of 95°C for 1 min, 53°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, the relative frequency of the F167Y (TAC), E198A (GCA) and F200Y (TAC) isotype-1 β-tubulin SNPs in the amplicons derived from each parasite population were determined by pyrosequence genotyping using the PryoMark ID system using the allele quantification (AQ) mode (Biotage, Sweden). Previously published sequencing primers were used for the F167Y (TAC), E198A (GCA) and F200Y (TAC) mutations; Hcsq167: 5'-ATA GAA TTA TGG CTT CGT-3', Hcsq198: 5'-ACT GGT AGA GAA CAC CG-3' and Hcsq200: 5'- TAG AGA ACA CCG ATG AAA CAT-3' respectively (Hoglund et al., 2009; von Samson-Himmelstjerna et al., 2009) (Chaudhry et al., 2015a). The base dispensation was set to GATGCTCGT for codon 167, GATGCAGCA for codon 198, and GATGCTGTA for codon 200. Peak heights were measured using the AQ mode of the PSQ 96 single nucleotide position software. SNPs. This approach to resistance allele quantification on DNA pools has been previously validated and shown to detect F200Y (TAC) resistance mutations at a minimum threshold of ~16% in each population and E198A (GCA) resistance mutations at a minimum threshold of ~8% in each population (Chaudhry et al., 2015a).

4.2.4. Cloning and sequencing of a isotype-1 β-tubulin fragment encompassing the F200Y (TAC), E198A (GCA) and F167Y (TAC) mutations

The same 328bp isotype-1 β-tubulin fragment described above was also amplified, cloned and sequenced from the pooled lysates from the seven H. contortus populations in which the F200Y (TAC) was detected (Pop24S, Pop1S, Pop27G, Pop2G, Pop3S, Pop5G and
Primers HcPYRF: 5’- GAC GCA TTC ACT TGG AGG AG-3’ and HcPYRR: 5’-CAT AGG TTG GAT TTGTGA GTT-3’ were again used but with the following PCR reaction conditions: 5X Phusion HF reaction buffer, 2mM MgSO4, 200uM dNTPs, 0.2uM forward and reverse primers and 1U of Phusion high fidelity DNA polymerase (Finenzyme Inc.). The thermo-cycling parameters of isotype-1 β-tubulin consisted of an initial 98°C for 30 sec followed by 35 cycles of 98°C for 10 sec, 59°C for 30 sec and 72°C for 1 min with a single final extension cycle of 72°C for 5 min. Amplicons were cloned into PJET 1.2/BLUNT vector (Thermo Scientific) and sequenced using standard procedures previously described by (Redman et al., 2015). Sequences were aligned with H. contortus isotype-1 β-tubulin sequences (Acc No X67489) and edited using Geneious Pro 5.4 software (Drummond AJ, 2012). The isotype-1 β-tubulin sequences were bioinformatically filtered to remove PCR induced mutations using a previously described approach (Chaudhry et al., 2015a; Redman et al., 2015) (Supplementary Fig. S4.1).

4.2.5. Phylogenetic network analysis of H. contortus isotype-1 β-tubulin sequence data set

Networks based on genetic distance were computed using the neighbour-net method employed in SplitsTrees4 (Huson and Bryant, 2006) to produce circular (equal angle) split networks. Median joining networks were generated in Network 4.6.1 software (Fluxus Technology Ltd.). 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 were removed with the MP (Maximum Parsimony) option (Polzin and Daneschmand, 2003). A phylogenetic network tree of the isotype-1 β-tubulin haplotypes was reconstructed using maximum Likelihood (ML) in MEGA5 (Tamura,
The program jModeltest 12.2.0 (Posada, 2008) was used to select the appropriate model of nucleotide substitutions for ML analysis. According to Bayesian information criterion the best scoring was Hasegawa-Kishino-Yano (HKY+G). The model of substitution was used with parameters estimated from data. Branch supports were obtained by 1000 bootstraps of the data. The most probable ancestral node was determined by rooting the networks to a closely related outgroup and Teladorsagia circumcincta sequence was used to root the \textit{H. contortus} network.

4.3. Results

4.3.1. The $F200Y$ (\texttt{TAC}) isotype-1 $\beta$-tubulin mutation was found at high frequency in \textit{H. contortus} on government farms but only rarely from animals from rural locations.

A 328bp fragment of the isotype-1 $\beta$-tubulin gene was PCR amplified from twenty one \textit{H. contortus} populations (3 populations from government farms and 18 populations from rural farms) and the relative frequencies of the three currently known benzimidazole resistance-associated SNPs $F167Y$ (\texttt{TAC}), $E198A$ (\texttt{GCA}) and $F200Y$ (\texttt{TAC}) was determined by pyrosequence genotyping (using pools of 14 to 32 worms per population; see Supplementary Table S4.1). The benzimidazole resistance-associated $F200Y$ (\texttt{TAC}) SNP was found at high frequency (81-100\%) in all 3 parasite populations from government farms. In contrast, it was only detected at low frequency (2-6\%) in four out of eighteen parasite populations from animals sourced from rural locations (Fig. 4.1 and Supplementary Table S4.1). The $F167Y$ (\texttt{TAC}) and $E198A$ (\texttt{GCA}) SNPs were not detected in any of the populations.
Fig. 4.1. Relative frequencies of the F200Y (TAC), F167Y (TAC) and E198A (GCA) isotype-1 β-tubulin benzimidazole resistance mutations in H. contortus populations from the Punjab region of Pakistan. Each H. contortus population is represented by three pie charts; one for each resistance mutation; F200Y (TTC/TAC), F167Y (TTC/TAC) and E198A (GAA/GCA). Each of these pie charts shows the relative frequency of the resistant versus susceptible SNP, based on allele quantification by pyrosequence genotyping of pooled DNA from between 14-32 worms per population (supplementary Table S4.1). The resistance-associated SNP genotype frequency - F200Y (TAC) - is shown in white and the “susceptible” SNP genotype frequencies - F200Y (TTC), F167Y (TTC) and E198A (GAA) – are shown in black. Geographic locations of abattoirs are indicated with small circles on the map and the abattoir names from which the samples were obtained are shown above the labelling lines. The province is indicated on the map (A) Punjab.

4.3.2. Sequence diversity and phylogenetic relationships of isotype-1 β-tubulin alleles identified in H. contortus on government and rural farms

The 328bp isotype-1 β-tubulin fragment was PCR amplified, cloned and sequenced from pooled DNA from each of the seven parasite populations in which the F200Y (TAC) resistance mutation was detected (three populations from government farms and four populations from rural locations). At least ten clones per population were sequenced (Supplementary Table S4.1). Following bioinformatic filtering (see materials and methods), a
total of twenty one different isotype-1 β-tubulin haplotypes (GenBank accession numbers KR269920-KR269940) were identified in the 89 sequences generated from the seven populations. Six out of the twenty one haplotypes encoded the F200Y (T→C) resistance polymorphism. A SplitsTrees network was constructed with all twenty one isotype-1 β-tubulin haplotypes to examine their phylogenetic relationships (Fig. 4.2). There was a higher degree of haplotypic diversity for the susceptible haplotypes compared to the resistance haplotypes consistent with the F200Y (T→C) SNP being under purifying selection. Nevertheless the F200Y (T→C) resistance polymorphism was present on haplotypes located in four different parts of the network. Each of these resistance haplotypes were more closely related to one or more susceptible haplotypes than to any of the other resistance haplotypes suggesting they were independently derived. A Maximum Likelihood (ML) tree was also constructed and showed the same phylogenetic relationships (Supplementary Fig. S4.2).
**Fig. 4.2.** Split Trees network of 89 *H. contortus* isotype-1 β-tubulin sequences from seven populations generated with the neighbour-net method of SplitsTrees4 (Huson and Bryant, 2006). The circles in network represent the different haplotypes and the size of the circles is proportional to the frequency in the overall dataset. The haplotypes containing the different mutations are shaded as follows: susceptible haplotypes containing F200Y (TTC)/F167Y (TTC)/E198A (GAA) are black; P200Y resistant haplotypes containing F200Y (TAC)/F167Y (TTC)/E198A (GAA) are white.

4.3.3. *Geographical distribution of F200Y (TAC) isotype-1 β-tubulin benzimidazole resistance haplotypes*

The three parasite populations from government farms (Pop1S, Pop3S and Pop2G) and one of the parasite populations (Pop27G) from a rural location each contained multiple resistance haplotypes. The three remaining parasite populations from rural locations (Pop24S, Pop5G, and Pop13S) contained just a single resistance haplotype (Fig. 4.3). All four parasite populations from rural locations (Pop24S, Pop27S, Pop5S and Pop13S) showed a high degree of susceptible haplotype diversity whereas the three parasite populations from government farms (Pop1S, Pop3S and Pop2G) did not (Fig. 4.3).
Fig. 4.3. Histograms showing the frequencies of the resistant and susceptible isotype-1 β-tubulin haplotypes identified from the seven *H. contortus* parasite populations in which resistance alleles were detected (3 government farms and four rural populations). F200Y (TTC)/F167Y (TTC)/E198A (GAA) susceptible haplotypes are shown in black; P200Y (TAC) resistance haplotypes light hatch.
A median-Joining network was constructed to integrate the phylogenetic relationships and frequencies of the different isotype-1 β-tubulin haplotypes with geographical information (Fig. 4.4). The Median-Joining network was largely congruent with the SplitTrees network and Maximum Likelihood tree with only minor differences (Fig. 4.2, Supplementary Fig. S4.2). The three most common resistance haplotypes (Hr22, Hr23 and Hr12) were present on multiple farms. The resistant haplotype that was present at the highest frequency overall (HR22) was present on all three government farms (Pop1S, Pop3S, and Pop2G) as well as in one rural parasite population (Pop24S). The resistance haplotype with the next highest frequency (Hr12) was present on just one government farm (Pop2G) and also in several rural parasite populations (Pop13S, Pop27G and Pop5G). Resistance haplotypes Hr23 and HR 31 were both present on two different government farms (Pop2G /Pop3S and Pop1S/Pop3S respectively). The two remaining resistance haplotypes (Hr16 and Hr3) were present at very low frequency in just a single rural farm population (Pop27G) (Fig. 4.4).
Fig. 4.4. Median joining network generated in Network 4.6.1 (Fluxus Technology Ltd.) from multi-aligned sequences for *H. contortus*. 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 circle representing each haplotype is proportional its frequency in the overall dataset and the colours in the circles reflect the frequency distribution in each geographical population as indicated on the colour key on the inset map. Small red dots represent median vectors. The number of mutations separating adjacent sequence nodes or median vectors is indicated along connecting branches and the length of the lines connecting haplotypes is proportional to the number of nucleotide changes. The most probable ancestral node is determined by rooting the network to a closely related outgroup *T. circumcincta* (*Tc*) (GenBank accession number [FN599034](https://www.ncbi.nlm.nih.gov/nuccore/FN599034)). The text providing the name of each haplotype is colour coded as follows; susceptible haplotype F200Y (TTC)/ F167Y (TTC)/E198A (GAA) is in black text; P200Y resistant haplotype F200Y (TAC)/ F167Y (TTC)/E198A (GAA) is in blue text.

4.4. Discussion

In most regions of the world examined to date, benzimidazole resistance is at an advanced stage with one, or more, of the known resistance mutations being at a high frequency in most populations (Brasil et al., 2012; Chaudhry and Gilleard, 2015; Redman et al., 2015). In parasite populations where resistance alleles have reached, or have nearly reached, fixation it is potentially difficult to disentangle the population genetics of resistance and draw conclusions about its origins and spread. In contrast, small ruminants in rural regions of Punjab, Pakistan are rarely treated with effective anthelmintic drugs due to economic and logistical constraints. However, small ruminants on government farms are treated with anthelmintic drugs very frequently providing an interesting contrast. The nature of the three government herds examined in this study provided an interesting opportunity to explore the origin of resistance alleles in the region. These three government herds were founded from locally derived breeding stock over 30 years ago and have been subsequently closed to animal movement. In the case of the Okara (Pop1S) and Jahangirabad (Pop3S) herds, they were derived exclusively from local breeding stock and, although there has been
some limited movement of stock between these two farms, they have been otherwise closed to animal movement since their foundation. Further, the sheep on these farms have been treated with benzimidazole drugs approximately every three months over the last 30 years with no history of any other drug class being used. The third government farm in Layyah (Pop2G) was originally established from angora goats imported for Turkey crossed with locally derived goats and has also been closed to animal movement since its foundation. We reasoned that comparison of isotype-1 β-tubulin resistance alleles in *H. contortus* from the government farms with resistant and susceptible haplotypes in the surrounding rural populations might provide some insights into the origin of resistance alleles.

Consistent with the known patterns of drug use, we found that the F200Y (TA C) resistance mutation was present at very high frequency on all three government farms we examined but was absent, or present a very low frequency, on all the rural farms sampled. The F200Y (TA C) mutation was present on multiple haplotypes on each of the government farms; Hr12/Hr22/Hr23 for Layyah (Pop2G), Hr22/Hr31/Hr23 for Jahangirabad (Pop3S) and HR22/Hr31 for Okara (Pop1S). These resistance haplotypes are phylogenetically distant to each other in the haplotype network of resistance and susceptible alleles in the region and so likely to be independently derived (with the possible exception of haplotypes Hr22 and Hr31 which are more closely related) (Fig. 4.4). The occurrence of the F200Y (TA C) mutations on multiple divergent haplotypes shows that a soft selective sweep has occurred at the isotype-1 β-tubulin locus has occurred on the government farms (Fig. 4.4). Previous work has demonstrated that soft selective sweeps can occur at the isotype-1 β-tubulin locus in both *H. contortus* and *T.circumcincta* (Chaudhry et al., 2015a; Redman et al., 2015). However, this previous study was on sheep farms in the UK with high levels of animal movement and so the
resistance haplotype diversity found on some UK sheep farms could have been due, at least in part, to the migration of resistance haplotypes between farms (Redman et al., 2015). However, in this case, the government farms have been closed to animal movement for over 30 years demonstrating that soft selective sweeps can occur in the absence of significant migration of resistance alleles between farms. In the absence of migration, soft selective sweeps can result from either selection acting on resistance mutations already in the standing genetic variation or pre-existing mutations present before the onset of selection or recurrent mutations appearing on different susceptible haplotype backgrounds after the onset of the selection.

It is very difficult, if not impossible, to definitively distinguish between pre-existing and recurrent mutations purely on the basis of the nature of the selective sweep (Messer and Petrov, 2013). However, examination of parasite populations from rural locations of the Punjab, that have been subject to minimal drug selection pressure, provides an indication of the likely isotype-1 β-tubulin haplotype diversity in parasite populations before they were subject to the intense selection pressure that has been applied on government farms. The Hr22 resistance haplotype is the most frequent benzimidazole resistance haplotype on both Jahangirabad (Pop3S) and Okara (Pop1S) government farms. This haplotype could have been shared by migration since animal movement is known to have historically occurred between these two farms. However, this resistance haplotype is also present on the Layyah (Pop 2G) government farm even though it has been closed to animal movement since its foundation with no movement of livestock with the other two government farms. Given that the Hr22 resistance haplotype was also found in one of the rural populations (Pop24S), as well as on all three government farm, it seems likely that it existed in the parasite populations prior to formation of the government herds.
In contrast, the Hr23 resistant haplotype is present in parasite populations present in the Jahangirabad (Pop3S) and Layyah (Pop 2G) government farms but is absent from the Okara (Pop1S) government farm. There are two possible explanations for this observation. First, it could have pre-existed mutation in parasite populations in the animals that founded the government farms and been subsequently lost by population bottlenecking of the Okara (Pop1S) population. Alternatively, it could have arisen by recurrent mutations on the same susceptible haplotype background on two of the farms under intense selection pressure. Given this Hr23 resistance haplotype was not detected in any of the 18 populations rural parasite populations examined, it must either be absent or be present at very low frequency the standing genetic variation of the overall parasite population. These results are similar to those of previous work on benzimidazole resistance of *H. contortus* and *T. circumcincta* on French goat farms that were closed to animal movement since their formation in the 1980s (Silvestre and Humbert, 2002). In that case, as we found on the government farms in the Punjab, some resistance haplotypes were shared and some were not. The most parsimonious explanation of the data from both the French and Pakistan studies is that both pre-existing and recurrent mutations contribute to the supply of F200Y (TA\textit{C}) resistant mutations present in contemporary parasite populations. This is arguably an unsurprising hypothesis if the mutation confers no (or little) fitness cost but provides a significant adaptive advantage under the influence of benzimidazole drug selection in very large parasite populations.

It is noteworthy that the F167Y (TA\textit{C}) and E198A (G\textit{C}A) mutations were not detected in any of the government or rural *H. contortus* populations even after intense benzimidazole selection pressure over 30 years. This is consistent with previous studies that consistently show that the F200Y (TA\textit{C}) mutation is found globally, whereas the other two mutations
occur much more sporadically. For example, in our recent work in southern India the F167Y (TAC) was apparently absent and the E198A (GCA), although present at multiple locations, appears to have spread from a single origin in the region. In summary, the work here provides further support to the hypothesis that the F200Y (TAC) mutations arise commonly and repeatedly whereas the F167Y (TAC) and E198A (GCA) are much rarer which may reflect differences in fitness costs between these different mutations.

4.5. Acknowledgements

We would like to thank Natural Sciences and Engineering Research Council of Canada (NSERC) (Grant number RGPIN/371529-2209), NSERC-CREATE Host Pathogen Interactions (HPI) graduate training program at the University of Calgary as well as the BBSRC for funding support (Grant number BB/E018505/1). We are also grateful to the Vice Chancellor (Prof. Dr. Talat Naseer Pasha) of the University of Veterinary and Animal Science Lahore Pakistan for his great support to collect samples from government and rural farms.

Reference


from the faecal egg count reduction test and resistant allele frequencies of the beta-
tubulin gene. Vet Parasitol 161, 60-68.


Sympatric species distribution, genetic diversity and population structure of Haemonchus isolates from domestic ruminants in Pakistan. Veterinary parasitology 206, 188-199.

Trends Parasitol 20, 477-481.


Polzin, T., Daneschmand, S.V., 2003. On Steiner trees and minimum spanning trees 

1256.

Redman, E., Whitelaw, F., Tait, A., Burgess, C., Bartley, Y., Skuce, P., Jackson, F., Gilleard, 
J., 2015. The emergence of resistance to the benzimidazole anthelmintics in parasitic 
nematodes of livestock is characterised by multiple independent hard and soft selective 
sweeps. PLoS Negl Trop Dis. 6;9, :e0003494. doi:.


**Supplementary Fig. S4.1.** The distribution of single nucleotide polymorphisms among 89 *H. contortus* sequences across the 382bp PCR amplicon of the isotype-1 β-tubulin gene spanning exons 4 and 5 and intervening intron. The position of the SNPs along with the 328bp sequences for *H. contortus* is shown along the X-axis and the number of sequences that contain each SNP is plotted on the Y-axis.
Supplementary Fig. S4.2. Maximum likelihood tree of isotype-1 β-tubulin haplotypes cloned and sequenced from the seven *H. contortus* populations from the Punjab provinces of Pakistan in which resistance alleles were detected. Susceptible haplotypes - F200Y (TTC)/ F167Y (TTC)/ E198A (GAA) are indicated with a yellow circle and P200Y resistant haplotypes - F200Y (TAC)/ F167Y (TTC)/E198A (GAA) are indicated with a blue circle. The colored rectangles indicate the populations in which each haplotype was identified as indicated by the color key on the inset map. The sequences were aligned by Geneious software and the tree obtained by maximum likelihood analysis using the Hasegawa-Kishino-Yano (HKY+G) model of substitution (Drummond AJ, 2012). Branches with bootstrap support values above 50% (1000 replications) and posterior probability greater than 50, respectively. The phylogeny is rooted with an isotype-1 β-tubulin sequence of *Teladorsagia circumcincta* (Tc) (GenBank accession number [FN599034](https://www.ncbi.nlm.nih.gov/nuccore/FN599034)).

Appendices: Supplementary Table Legends

**Supplementary Table S4.1:** Allele frequency (%) of SNPs that resulted in an amino acid changes at codons F200Y (TTCTAC), F167Y (TTCTAC) and E198A (GAA/GCA) in isotype-1 β-tubulin obtained from twenty one *H. contortus* populations across the Punjab province in Pakistan.

<table>
<thead>
<tr>
<th>Pakistani field populations</th>
<th>Host</th>
<th>No of worms in each pool</th>
<th>P167</th>
<th>P198</th>
<th>P200</th>
<th>Abattoir / Farms location</th>
<th>Origin</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td>TC</td>
<td>TA</td>
<td>GAA</td>
<td>GCA</td>
<td>TC</td>
</tr>
<tr>
<td>Pop1SS Sheep</td>
<td>32</td>
<td>100</td>
<td>0</td>
<td>100</td>
<td>0</td>
<td>100</td>
<td>0</td>
</tr>
<tr>
<td>Pop13S Sheep</td>
<td>29</td>
<td>100</td>
<td>0</td>
<td>100</td>
<td>98</td>
<td>2</td>
<td>Lahore</td>
</tr>
<tr>
<td>Pop24S Sheep</td>
<td>27</td>
<td>100</td>
<td>0</td>
<td>100</td>
<td>95</td>
<td>5</td>
<td>Sargodha</td>
</tr>
<tr>
<td>Pop38 Sheep</td>
<td>32</td>
<td>100</td>
<td>0</td>
<td>100</td>
<td>0</td>
<td>100</td>
<td>P200Y</td>
</tr>
<tr>
<td>Pop1S Sheep</td>
<td>29</td>
<td>100</td>
<td>0</td>
<td>100</td>
<td>21</td>
<td>81</td>
<td>Okara</td>
</tr>
<tr>
<td>Pop2G Goat</td>
<td>31</td>
<td>100</td>
<td>0</td>
<td>100</td>
<td>0</td>
<td>100</td>
<td>Layyah</td>
</tr>
<tr>
<td>Pop5G Goat</td>
<td>14</td>
<td>100</td>
<td>0</td>
<td>100</td>
<td>95</td>
<td>5</td>
<td>Lahore</td>
</tr>
<tr>
<td>Pop7G Goat</td>
<td>27</td>
<td>100</td>
<td>0</td>
<td>100</td>
<td>100</td>
<td>0</td>
<td>Lahore</td>
</tr>
<tr>
<td>Pop9G Goat</td>
<td>14</td>
<td>100</td>
<td>0</td>
<td>100</td>
<td>100</td>
<td>0</td>
<td>Lahore</td>
</tr>
<tr>
<td>Pop4G Goat</td>
<td>22</td>
<td>100</td>
<td>0</td>
<td>100</td>
<td>100</td>
<td>0</td>
<td>Lahore</td>
</tr>
<tr>
<td>Pop8G Goat</td>
<td>15</td>
<td>100</td>
<td>0</td>
<td>100</td>
<td>100</td>
<td>0</td>
<td>Lahore</td>
</tr>
<tr>
<td>Pop10G Goat</td>
<td>29</td>
<td>100</td>
<td>0</td>
<td>100</td>
<td>100</td>
<td>0</td>
<td>Lahore</td>
</tr>
<tr>
<td>Pop27G Goat</td>
<td>26</td>
<td>100</td>
<td>0</td>
<td>100</td>
<td>94</td>
<td>6</td>
<td>Okara</td>
</tr>
<tr>
<td>Pop28G Goat</td>
<td>30</td>
<td>100</td>
<td>0</td>
<td>100</td>
<td>100</td>
<td>0</td>
<td>Okara</td>
</tr>
<tr>
<td>Pop29G Goat</td>
<td>32</td>
<td>100</td>
<td>0</td>
<td>100</td>
<td>100</td>
<td>0</td>
<td>Okara</td>
</tr>
<tr>
<td>Pop31G Goat</td>
<td>20</td>
<td>100</td>
<td>0</td>
<td>100</td>
<td>100</td>
<td>0</td>
<td>Sahival</td>
</tr>
<tr>
<td>Pop33G Goat</td>
<td>28</td>
<td>100</td>
<td>0</td>
<td>100</td>
<td>100</td>
<td>0</td>
<td>Sahival</td>
</tr>
<tr>
<td>Pop17G Goat</td>
<td>32</td>
<td>100</td>
<td>0</td>
<td>100</td>
<td>100</td>
<td>0</td>
<td>Sahival</td>
</tr>
<tr>
<td>Pop19G Goat</td>
<td>31</td>
<td>100</td>
<td>0</td>
<td>100</td>
<td>100</td>
<td>0</td>
<td>Gujranwala</td>
</tr>
<tr>
<td>Pop20G Goat</td>
<td>32</td>
<td>100</td>
<td>0</td>
<td>100</td>
<td>100</td>
<td>0</td>
<td>Gujranwala</td>
</tr>
<tr>
<td>Pop21G Goat</td>
<td>26</td>
<td>100</td>
<td>0</td>
<td>100</td>
<td>100</td>
<td>0</td>
<td>Gujranwala</td>
</tr>
</tbody>
</table>
CHAPTER V

MANUSCRIPT IV

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

Umer Chaudhry*, Melissa Miller, Thomas Yazwinski, Ray Kaplan, John Gilleard

Accepted in Journal of Veterinary Parasitology, S0304-4017(14)00304-5
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, twenty nine out of thirty two worms carried the P200Y (TAC) isotype-1 β-tubulin and two out of thirty two worms carried the P167Y (TAC) benzimidazole resistance associated polymorphisms respectively. For *H. placei*, six out of the nine populations contained the P200Y (TAC) 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 (TAC) 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 benzimidazole be intensively used for parasite control in US cattle.

**Keywords:** *Haemonchus contortus*, *Haemonchus placei*, Anthelmintic resistance, Cattle parasite, Benzimidazole.
5.1. 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 (Amarante et al., 1997; Jacquiet et al., 1998; Achi et al., 2003; Gasbarre et al., 2009a; Hogg et al., 2010; d'Alexis S, 2011; Akkari et al., 2013). 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; Gasbarre et al., 2009b) 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 (TTC to TAC; F200Y) (Silvestre and Humbert, 2002; Hoglund et al., 2009; von Samson-Himmelstjerna et al., 2009; Brasil et al., 2012; Kotze, 2012; Niciura et al., 2012), codon P167 (TTC to TAC; F167Y) (Silvestre and Cabaret, 2002; de Lourdes Mottier and Prichard, 2008; Brasil et al., 2012) and at codon P198 (GAA to GCA; E198A) (Ghisi et al., 2007; Rufener et al., 2009; Kotze, 2012). 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 (TTC 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 isotype-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 (TTC 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.

### 5.2. Materials and Methods

#### 5.2.1. 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 abomasae 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. 5.1). 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 barn that were derived from different sources in Northwest Arkansas / Northeast Oklahoma and slaughtered immediately after purchase. A final population (H85) was collected from a calf experimentally infected with L3 derived from a number of calves in Florida. Overall, the dataset was composed of 294 individual specimens of adult worms from the genus *Haemonchus* distributed among ten different populations from individual cattle hosts.

5.2.2. Genomic DNA isolation

Adult worms were fixed in 70% ethanol immediately following removal from the cattle abomasa. 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 0.2μl tube containing 50μl of proteinase K lysis buffer and stored at -80°C as previously described (Redman et al., 2008). 1μl 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.

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

A 321bp fragment spanning the entire ITS-2 rDNA region was PCR amplified from individual adult worm lysates using a “universal” forward primer complementary to the 5.8S rDNA coding sequence (DITSF: 5’-ACG TCT GGT TCA GGG TTG TT- 3’) and biotin
labelled reverse primer complimentary to the 28S rDNA coding sequence (DITSR: 5’-Biotin-
TTA GTT TCT TTT CCT CCG CT- 3’) (Stevenson et al., 1995). Reaction mixtures consisted of 50μl master mix containing final concentrations of 1X thermopol reaction buffer (New England BioLabs), 2mM MgSO₄, 0.5μl of 100μM dNTPs, 0.5μl of each 0.1μM 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’-CATATACTACAATGTGGCTA-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 CGAGTCACA. 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).

5.2.4. 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’-GTIMGIWSIGCITAYGGICA-3’, AE25F: 5’-CARCTITTYMGICCIAYTA-3’, AE34F: 5’-GARGGIGCIGARCTITIGA-3’) and reverse degenerate primers (AE16R: 5’-TGIGTIAGYTCIGCICIGTISW-3’, AE25R: 5’-
A 385bp 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 GAT TTGTGA GTT-3”). A 50μl master mix was used containing final concentrations of 1X thermopol reaction buffer (New England BioLabs), 2mM MgSO₄, 0.5μl of 100μM dNTPs, 0.5μl of each 0.1μM 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, 53°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 PryoMark ID system, (Biotage, Sweden) essentially using the method described by (von Samson-Himmelstjerna et al., 2009). The sequencing primers used were Hcsq167: 5’-ATA GAA TTA TGG CTT CGT-3’ and Hcsq200: 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-sequencing™ AB) with the base dispensation set to codon 167: GATGCTCGT, 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:

P167 (homozygous susceptible for a TTC genotype, homozygous resistant for an TAC genotype and heterozygous for a TT/AC genotype). P198 (homozygous susceptible for an GA genotype, homozygous resistant for a GCA genotype and heterozygous for an GA/CA
genotype). P200 (homozygous susceptible for a TTC genotype, homozygous resistant for an TAC genotype and heterozygous for a TT/AC genotype).

5.3. Results

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

Over the 231bp 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 characterized populations (Stevenson et al., 1995; Chaudhry et al., 2015). 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 the 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).

5.3.2. 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 (TAC) 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. 5.1). In total, out of the 264 individual worms genotyped across the nine populations, ten were identified as P200Y
(TT/AC) heterozygous resistant, one was identified as P200Y (TAC) homozygous resistant and the remaining 253 were identified as P200Y (TT/AC) homozygous susceptible.

The one *H. contortus* population (H86) contained the P200Y (TAC) polymorphism at high frequency 38/64 alleles (59.4%) and also the P167Y (TAC) at lower frequency 2/62 alleles (3.1%) (Fig. 5.1). In this population at position P200, nine worms were homozygous resistant (TAC), three worms were homozygous susceptible (TT/AC) and twenty worms were heterozygous (TT/AC). At position P167, two worms were heterozygous resistance (TT/AC) and thirty were identified as homozygous susceptible (TT/AC). All thirty two individual worms were identified as homozygous susceptible (GCA) at P198.

![Fig. 5.1.](Image)

**Fig. 5.1.** 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* and one population of *H. contortus* from cattle. Parasite species identity was determined by the SNP genotype at position 24 of the rDNA ITS-2 sequence (where *H. placei* is G and *H. contortus* is A). In the pie chart for position P200, blue represents the “resistance SNP” (TAC) and yellow represents the “susceptible SNP” (TT/AC). In the pie chart for position P167, the green colour represents “resistance SNP” TAC and the yellow represents the “susceptible SNP” (TT/AC). 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.

5.4. Discussion

*Haemonchus* spp. are important, highly pathogenic blood feeding parasites of cattle in many parts of the world, particular 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; Gasbarre et al., 2009b). Of the ten populations examined (six from Arkansas/East Oklahoma, one from Florida and three from Georgia), nine were 100% *H. placei* suggesting this is still the predominant *Haemonchus* species infecting cattle in the southern USA. Only one of the ten 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 (Mortensen et al., 2003; Howell et al., 2008), 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 (TAC) 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 (TAC) 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 TA1, 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.
5.5. Acknowledgments

We would like to thank Natural Sciences and Engineering Research Council of Canada (NSERC) for funding support (Grant number RGPIN/371529-2209) as well as the NSERC-CREATE Host Pathogen Interactions (HPI) graduate training program at the University of Calgary.

Reference


Kotze, A.C., Katie; Bagnall, Neil; Hines, Barney; Ruffell, Angela; Coleman, Glen 2012. Relative level of thiabendazole resistance associated with the E198A and F200Y SNPs in larvae of a multi-drug resistant isolate of Haemonchus contortus. International journal for parasitology 2 92-97


CHAPTER VI

GENERAL DISCUSSION
The benzimidazoles are one of the most important broad spectrum anthelmintic drug classes available for the control of parasitic nematodes of domestic animals and humans (Waller, 1997). They have been intensively used in the livestock sector, particularly in small ruminants, for over 30 years. This has led to the widespread emergence of resistance in a number of small ruminant parasite species including, and most extensively, in *H. contortus*. In many counties - including Australia, New Zealand, USA, Canada, UK and France - benzimidazole resistance has become so common in this parasite that the use of this drug class for control is severely compromised (McKellar and Jackson, 2004). Indeed, in such countries studies suggest that the majority of *H. contortus* populations are now resistant. Consequently, there has been an increasing reliance on other drug classes such as the macrocyclic lactones to which resistance in *H. contortus* has now also become very common. This has led the pharmaceutical industry to develop new drug classes such as the AADs (monepantel) and the spironolactones (derquantel) that have been licensed for use in a number of countries in the last few years. Resistance in *H. contortus* has already been reported to these drugs in those countries where they are being used (Baker et al., 2012; Scott et al., 2013; Mederos et al., 2014). Hence it is extremely important that we understand how resistance mutations arise and spread in parasite populations and develop tools and approaches to detect resistance at an early stage of its development.

Benzimidazole resistance in *H. contortus* is currently the best system in which to study the genetics of anthelmintic resistance at the population level because there is already a good understanding of the molecular basis of resistance (Gillear, 2013). In spite of this, we still have a relatively poor understanding of how resistance emerges and spreads at the population level. Although, there has been a lot of work on the occurrence of the known resistance
mutations - F200Y, E198A and F167Y - in many developed countries, there has been relatively little research in less developed countries. A major focus of the work in this thesis was to investigate the occurrence and the molecular genetics of the known benzimidazole resistance mutations in two such regions; Pakistan and southern India. It was anticipated that benzimidazole resistance would be at an earlier stage of development in such regions due to less intense selection pressure because of lower drug use. Consequently, investigating the molecular genetics of anthelmintic resistance in Pakistan and India would not only address a major practical knowledge gap but might also provide new insights into the origin and spread resistance mutations in parasite populations. Additionally, benzimidazole resistance has been little studied in the closely related parasite of cattle *H. placei* and so it is unclear what the current situation is in this parasite. However, it was considered likely that resistance would be at an earlier stage of development in *H. placei* than in *H. contortus* due to the lower amount of benzimidazole use in cattle than in sheep and the lack of clinical reports of poor treatment efficacy. Consequently, another aspect of this thesis work was to investigate the relationship between *H. contortus* and *H. placei* and to study the occurrence of candidate benzimidazole resistance mutations in this latter species.

In this general discussion I will highlight some of the major findings of my thesis work and undertake a more speculative discussion than in the results chapters including the future directions that I believe the research should take.
6.1. New genetic markers for the identification of *H. contortus* and *H. placei*

For the effective diagnosis, treatment, and control of parasitic nematode infections, it is essential that individual species can be accurately identified (McKeand, 1998). It was previously recognized that *H. contortus* and *H. placei* are separate species based on morphology and molecular markers (Le Jambre, 1981; Lichtenfels et al., 1994; Zarlenga et al., 1994b; Stevenson et al., 1995; Amarante et al., 1997; Blouin et al., 1997). Although *H. contortus* and *H. placei* are closely related species with similar morphology, they can be differentiated from each other based on the patterns of longitudinal cuticular ridges of the synlophe and the morphometrics of the male spicules (Bremner, 1955; Le Jambre and Royal, 1980; J. R. Lichtenfels, 1986). However, misidentification can occur due to number of overlapping features between the two species (Jacquiet et al., 1997; Amarante, 2011). Furthermore, it is not possible to definitively identify hybrid worms by morphological examination alone or to reliably differentiate the eggs or larvae recovered from the feces of live animals.

DNA based methods circumvent many of the problems of morphology based identification especially where morphological characters are unreliable to distinguish between the two species (Duncan et al., 1992). In their previous studies, Zarlenga et al. (1994a) identified sequence differences in the external transcribed spacer (NTS) region of rDNA between *H. contortus* and *H. placei*. However only single isolates of each species was compared. This left the question open as to whether there is geographical variation in the ribosomal rDNA (NTS) region in both species. The use of the first and second internal transcribed spacers (ITS-1, ITS-2) region of rDNA has been reported to accurately delineate
between closely related species in a range of parasite groups (Porter and Collins, 1991; Adlard et al., 1993; Wachira TM, 1993). The previous study of Stevenson et al. (1995) identified *H. contortus* and *H. placei* species based on the sequence of the rDNA ITS-2 in which three sites at positions 24, 205 and 219 have been reported to show fixed single nucleotide polymorphisms (SNPs) between the two species. However, only eight *H. contortus* and two *H. placei* worms were compared in that study. Hence, it was unclear as to whether there was geographical variation in the ribosomal rDNA ITS-2 sequence in these species that might compromise the use of these SNPs as reliable diagnostic markers of species identity.

In Chapter II, sequence analysis of the rDNA ITS-2 region from geographically diverse and morphologically verified populations of *H. placei* and *H. contortus* was used to verify the three SNPs at positions 24, 205 and 219 as species-specific markers. The results showed that there were fixed differences at these three positions between the two species even when the isolates were obtained from different continents. This allowed us to apply the P24 SNP marker using a pyrosequence genotyping assay, to screen a large number of parasite populations to look for the evidence of co-infection and potential interspecies hybridization between *H. contortus* and *H. placei*. We also demonstrated that haplotype diversity in both the isotype-1 β-tubulin and the mtDNA ND4 loci was completely congruent with the species identity determined by rDNA ITS-2 region (Figs. 2.2, 2.3 CHAPTER II) and identified three microsatellite markers with discrete allele sizes between the two species. Hence, this work has significantly expanded the set of validated molecular markers available to discriminate *H. contortus* and *H. placei* and to identify hybrid worms (Fig. 2.4 CHAPTER II).
6.2. Differences in the rate of co-infection of small ruminants with *H. contortus* and *H. placei* in field populations from the Punjab region of Pakistan and southern India

Parasites that are geographically sympatric are often either separated by their distributions in different host species or by having distinct locations in the same host. Hence, two phylogenetically closely related parasite species with a sympatric geographic distribution might never come together for mating. However, this is not the situation for *H. placei* and *H. contortus*, which both inhabit the abomasum of their ruminant hosts. Although *H. contortus* is most common in sheep and goats, it can infect a large number of artiodactyl hosts (Hoberg et al., 2004) including cattle (Hogg et al., 2010). There is less peer-reviewed literature on the host distribution of *H. placei* but it is most commonly found in cattle and, although it can infect some other ruminant hosts including sheep, its host distribution appears to be more limited than *H. contortus* (Akkari et al., 2013). *H. placei* and *H. contortus* co-infections have been reported in small and large ruminants although it is unclear how commonly this occurs in different regions (Amarante et al., 1997; Jacquiet et al., 1998; Achi et al., 2003; Gasbarre et al., 2009a; Gasbarre et al., 2009b).

*Haemonchus* infections in the field are not usually diagnosed to the species level and so there is a lack of data on true species prevalence and the extent of co-infections. In many regions of the world, the two species are sympatric and co-infection appears to be quite common (Lichtenfels et al., 1994; Amarante et al., 1997; Jacquiet et al., 1998; Achi et al., 2003; Gasbarre et al., 2009a). However, from the small number of available studies, it is clear that the situation differs from region to region. For example, in Brazil, *H. contortus* and *H. placei* co-infection was found in cattle and sheep (Amarante et al., 1997; Brasil et al., 2012).
and in Tunisia, *H. contortus* and *H. placei* co-infection was encountered as the most predominant form of infection in cattle (Akkari et al., 2013). In contrast, *H. placei* appears to be absent for the UK with *H. contortus* being the only *Haemonchus* species reported to infect sheep and cattle (Hogg et al., 2010).

In Chapter II, we investigated the extent to which co-infections with these two species occur in a number of different host species (sheep, goats, cattle and buffalo) in southern India and Pakistan. In southern India, all worms were identified as *H. contortus* in 12 sheep, 8 goats, 2 cattle and one buffalo. However in Pakistan, 3 sheep and 10 goats contained a mixture of *H. contortus* and *H. placei* indicating co-infection within the two species are common. Therefore our study revealed a striking difference in the prevalence of *H. contortus* and *H. placei* co-infections between sheep and goats from southern India and Pakistan. This difference was initially a surprise since cattle and sheep co-graze in both regions and there have been previous anecdotal and published reports of *H. placei* in southern India. However the reliability of the species identification in those reports or studies is not clear. One possible reason for the difference in the occurrence of *H. placei* in sheep between Pakistan and southern India might relate to differences in the cattle parasite faunas. *Mecistocirrus digitatus* appears to be the predominant abomasal parasite of cattle in southern India. This is a blood feeding trichostrongyloid parasite from a closely related genus to *Haemonchus* that thrives in a tropical climate. It is significantly larger and so easy to differentiate on gross morphological appearance (Lichtenfels and Pilitt, 2000). I visually examined 31 adult parasite populations, each consisting of several hundred worms, obtained from cattle from abattoirs across the region and 28 of which were apparently solely *M. digitatus* based on gross morphology. Four of these populations were confirmed as *M. digitatus* using ITS-2 rDNA pyrosequence
genotyping. The remaining three populations were *H. contortus* based on both visual inspection and pyrosequence genotyping. Hence it is possible that *H. placei* is absent or rare in cattle (its primary host) in southern India due to competition from *M. digitatus* that may be better suited to the year round hot humid climate. This might account for the absence in *H. placei* in small ruminants in the region. There are no reports of *M. digitatus* from Pakistan which has a significantly different climate to southern India and so it may be that *H. placei* is the predominant abomasal parasite in cattle there. However, there is a lack of peer review data to support this hypothesis and future studies focused on this question would be worthwhile. This is a potentially interesting example of how competition between parasite species and differences in species assemblages in hosts in different regions can have practical implications; in this case with respect to anthelmintic resistance.

6.3. Genetic analysis of interspecies hybridization between *H. contortus* and *H. placei* field populations and implications for the potential interspecies transmission of anthelmintic resistance

The presence of co-infections of *H. contortus* and *H. placei* raises the possibility of interspecies hybridization between these two phylogenetically close species. If this occurs, it could potentially provide a mechanism for the interspecies introgression of genetic loci between these parasite species. Adaptive mutations can potentially be introgressed between species under the influence of selection even if hybridisation events are relatively rare. One can hypothesize that this may indeed be the case for benzimidazole resistance mutations as a result of the selection pressure applied by the intensive use of benzimidazole drugs.
Interspecies transmission of anthelmintic resistance loci has not been considered as a potential source of resistance mutations to date. However, this could be a major concern with respect to *H. placei* given the currently high prevalence of anthelmintic resistance in *H. contortus*.

*Haemonchus contortus* and *H. placei* have been shown to be capable of hybridization following experimental co-transplantation of adult worms into the abomasum of a recipient sheep (Jambre, 1979; Le Jambre, 1981). Mating of *H. contortus X H. placei* resulted in F1 and F2 female progeny that were fertile. However, cytological studies of the testis from the F1 and F2 males showed a failure of chromosome pairing and some spermatocytes with extra chromosomes. In all observed cases, degeneration of the chromosomes occurred before anaphase I and was followed by a subsequent degeneration of the spermatocyte. However, the F1 females of both crosses (*H. contortus* male X *H. placei* female; *H. contortus* female X *H. placei* male) could produce offspring if backcrossed with males of one of the parental species (Jambre, 1979; Le Jambre, 1981). Hence, it is possible that such interspecies matings could occur in the field if co-infections occur. It was first reported that six individual worms recovered from sheep in Georgia USA with spicule measurements that were intermediate between *H. placei* and *H. contortus* (J. R. Lichtenfels, 1986; Lichtenfels JR, 1988). However, it was not clear from these studies whether these were hybrids, “morphotypes” or members of a cryptic species. The only evidence, previous to this thesis work, that hybridization might occur in the field was a recent report of two individual worms from sheep in Brazil that appeared heterozygous for several putative species-specific SNPs on sequence chromatograms generated by direct sequencing of rDNA ITS-2 amplicons (Brasil et al., 2012).

In Chapter II, we used a molecular genetic approach to investigate whether interspecies hybridization occurred between the two *Haemonchus* species in the Punjab region of Pakistan.
where co-infections were found to be common. Five individual hosts (four goats and one sheep) contained individual worms that were heterozygous for the P24 ITS-2 rDNA species specific SNP. These five worms were examined with five independent genetic markers from the nuclear genome; ITS-2 rDNA, isotype-1 β tubulin and three unlinked microsatellite markers with discrete allele sizes between the two species. All five worms were heterozygous for species–specific alleles for all the five nuclear markers providing strong evidence that they were F1 hybrids. In addition, analysis of mitochondrial ND4 sequence suggested that hybrids can be derived from interspecies mating of either H. contortus or H. placei female worms with males of the alternate species. Interestingly, one of the five hybrid worms carried a H. contortus isotype-1 β-tubulin allele with a P200Y (TAC) resistance mutation. If such an individual were to backcross with a H. placei to produce fertile offspring, which we know is biologically possible (Le Jambre and Royal, 1980; Le Jambre, 1981), it could result in interspecies transmission of anthelmintic resistance alleles into the H. placei genetic background. Such alleles could then potentially rise in frequency under the influence of positive selection by anthelmintic treatments. The risk of interspecies introgression of benzimidazole resistance alleles is probably not high in Pakistan at present due to the low frequency of resistance mutations in H. contortus populations except on government farms. However, I have recently helped to supervise a summer student (Ephraim Hui, Bachelor of Health Sciences Program, University of Calgary) to undertake a similar, albeit it smaller, study in large ruminants in the USA. Screening just nine populations of H. placei from calves identified an H. contortus / H. placei F1 hybrid containing a H. contortus isotype-1 β-tubulin allele with a P200Y (TAC) resistance mutation that was isolated from a calf in Florida (Fig. 5.1, CHAPTER V) (Chaudhry and Ephraim Unpublished data). Given the high frequency of
benzimidazole resistance mutations in *H. contortus* and the intensive use of benzimidazole drugs in the US, it seems that the possibility of introgression of *H. contortus* benzimidazole resistance mutations into *H. placei* should be explored (Fig. 6.1). This could be investigated by screening large numbers of *Haemonchus* worms from cattle across the US searching for individual worms that are homozygous for a *H. placei* P24 ITS-2 rDNA marker but contain a *H. contortus* isotype-1 β-tubulin resistance allele(s). The genetic background of such worms could be then interrogated in more detail either using microsatellite marker panels or more discriminatory genome-wide approaches such as RAD sequencing.

**Fig. 6.1.** Relative frequencies of the F200Y (TAC), F167Y (TAC) and E198A (GCA) isotype-1 β-tubulin benzimidazole resistance associated mutations in *H. contortus* populations from sheep in the southern USA (Chaudhry and Gilleard, unpublished data). Each *H. contortus* population is represented by three pie charts; one for each resistance mutation; F200Y (TTC/TAC), F167Y (TTC/TAC) and E198A (GAA/GCA). Each of these pie charts shows the relative frequency of the resistant versus susceptible SNP, based on allele quantification by pyrosequence genotyping of pooled DNA from 32 worms per population (supplementary table 6.1). The resistance-associated SNP genotype frequency is shown in white - F200Y (TAC) or F167Y (TAC) and susceptible associated resistance SNP is shown in black - F200Y (TTC), F167Y (TTC) and E198A (GAA). Geographic locations of abattoirs are indicated with small circles on the map and the abattoir names from which the samples were obtained are shown above the labelling lines. The two states are indicated on the map [(A) Arkansas (B) Georgia].
6.4. Differences in the occurrence of the F200Y, E198A, F167Y benzimidazole resistance mutations in *H. contortus* populations in different regions and the implications for the origins of resistance

The F200Y (TTC to TAC) benzimidazole resistance conferring SNP is widespread and often present at high frequency in many developed countries including Australia, New Zealand, France, Sweden, Brazil and UK (Silvestre and Humbert, 2002; Hoglund et al., 2009; Brasil et al., 2012; Kotze et al., 2012; Bisset SA et al., 2014; Redman et al., 2015). In this thesis work, I found that although benzimidazole resistance is less progressed, the F200Y (TTC to TAC) is also the predominant resistance mutation in *H. contortus* populations in southern India and the Punjab region of Pakistan. This mutation is quite widespread in southern India and is the sole benzimazole resistance mutation identified in Pakistan (although much less common in this region). This is consistent with the view that this SNP could have been present as an ancient polymorphism and/or arises repeatedly by recurrent mutation in *H. contortus* populations under the influence of selection. In either case, this suggests that this polymorphism has little fitness cost, at least in the presence of drug selection. This will be discussed further in discussion section 6.7 below.

In contrast, the other two known benzimidazole resistance mutations - F167Y (TTC to TAC) and E198A (GCA) - are more variable in their occurrence in different regions. Although the F167Y (TTC to TAC) been detected in quite a number of countries including USA, Canada, UK, France and Brazil, it is generally less widespread and at much lower frequencies than the F200Y (TTC to TAC) mutation (Silvestre and Cabaret, 2002; Barrère et al., 2012; Brasil et al., 2012; Barrere et al., 2013a; Barrere et al., 2013b). One notable
exception to this appears to be in a study of seven UK sheep farms where the F167Y (T\textsubscript{A}C) mutation was present at almost as high a frequency as the F200Y (T\textsubscript{A}C) mutation in \textit{H. contortus} populations (Redman et al., 2015). In this thesis work, the F167Y (T\textsubscript{A}C) mutation was not found in any of the populations from Pakistan or southern India. Overall, these results suggest that the F167Y (T\textsubscript{A}C) is not likely to be present as a pre-existing “ancient polymorphism” since, even in those counties where it occurs, it often absent from farms under intense selection pressure and appears to be completely absent in some regions of the world such as southern India and the Punjab, Pakistan. This may suggest that this polymorphism has a higher fitness cost than the F200Y (T\textsubscript{A}C) mutation. In that case, although it is predicted to arise many times in populations by recurrent mutation (by virtue of the huge populations sizes of \textit{H. contortus}) it would only become fixed in the face of intense drug selection (although the exact conditions for this to occur or poorly defined). A similar argument applies to the E198A (G\textsubscript{A}A to G\textsubscript{C}A) mutation which is even rarer than the F167Y (T\textsubscript{A}C) mutation. It has been detected in just two field-derived populations of \textit{H. contortus} to date; one from South Africa and one from Australia and in one laboratory population following in vitro selection of parasite populations during serial passage in experimentally infected animals (Ghisi et al., 2007; Rufener et al., 2009; Kotze et al., 2012). To my knowledge there have been no other reports of the E198A (G\textsubscript{C}A) resistance mutation in \textit{H. contortus} natural field populations. In this thesis study, the E198A (G\textsubscript{C}A) mutation was absent from the Pakistani populations examined and although it was moderately widespread in southern India it was much less so than the F200Y (T\textsubscript{A}C) mutation. More importantly, the phylogenetic analysis of resistance alleles suggested that E198A (G\textsubscript{C}A) mutation originated from a single origin whereas the
F200Y (TAC) mutation appears to have originated multiple times. This will be discussed further in the discussion section 6.7 below.

One final relevant point comes from the results of the three government farms in Pakistan. In each case, there has been intense selection pressure by the benzimidazole drugs with there being at least 4 treatments a year for approximately thirty years. Moreover, these farms are closed to animal movement. In these farms the F200Y (TAC) was present at high frequency and was present on several haplotype backgrounds on the same farm. In contrast, the F167Y (TTC to TAC) and E198A (GCA) mutations were absent on these farms in spite of many years of intense selection. This again, is consistent with the view that the mutations are not present in the pre-existing standing genetic variation of the parasite populations and also are likely to incur some fitness cost. One might ask why these mutations have not yet emerged under such strong selection pressure applied over many years. It might be that once the F200Y (TAC) reaches high frequency on these farms, additional resistance mutations are not of sufficient adaptive advantage to become fixed and increase in frequency.

6.5. The occurrence of benzimidazole resistance mutations in H. placei populations collected from cattle in the southern USA

Resistance to benzimidazole drugs has been reported in H. placei but there is relatively little information on its prevalence (Anziani et al., 2004; Soutello et al., 2007). Haemonchus placei resistant to benzimidazoles in large ruminants has been recorded in Brazil (Bricarello et al., 2007; Soutello et al., 2007), Argentina (Anziani et al., 2004) and USA (Gasbarre et al., 2009a; Gasbarre et al., 2009b) and recently north India (Yadav and Verma, 1997). All
documented reports, above indicate the presence of *H. placei* resistant to benzimidazole by using parasitological methods at the field level such as the fecal egg count reduction test. These methods give an estimate of the benzimidazole resistance or susceptibility of a parasite community as a whole, but cannot be used to assess the proportion of benzimidazole resistance worms in a particular population. (Jacquiet et al., 1998; Achi et al., 2003). Although there have been relatively few investigations into the molecular genetics of benzimidazole resistance in cattle parasites, there are some indications that same genetic determinants are involved in some cases. Winterrowd et al. (2003) amplified partial genomic sequences from *Cooperia oncophora* and demonstrated that benzimidazole resistance in field isolates of *C. oncophora* was associated with the F200Y mutation. Njue and Prichard (2003) subsequently cloned and sequenced full-length cDNAs representing both isotypes of β-tubulin from *C. oncophora* and found a small proportion of individuals that carried F200Y resistant isotype-1 allele (Demeler et al., 2013). Also There have been very few molecular genetic studies of *H. placei* and only one of the three polymorphisms known to be associated with resistance in *H. placei* has yet been reported this species; the codon F167Y (TAC) polymorphism was reported at low frequency in a *H. placei* population from cattle in Brazil (2.5%) (Brasil et al., 2012). However, there have been no previous reports of the presence of F200Y (TAC) and E198A (GCA) resistance mutations in *H. placei* under natural field conditions.

In Chapter V, we demonstrated the frequency of known benzimidazole resistance–conferring mutations F167Y (TAC), E198A (GCA) and F200Y (TAC) in *H. placei* populations of cattle from several regions of the southern USA. We detected P200Y (TAC) mutation in 6 out of 9 *H. placei* populations at frequencies between 1.7-9.4% (Fig. 5.1, CHAPTER V). Although this is a relatively small study, these results suggested that P200Y
(TAC) resistance mutation is likely to be present in many *H. placei* populations in southern US cattle. The presence of this mutation at such a low frequency would not be expected to currently result in a detectable loss of efficacy of benzimidazole drugs. However, this is an important finding since it suggests that benzimidazole resistance is likely to emerge in this parasite species. In cattle, there has been relatively little benzimidazole use over the last 30 years because producers have relied mainly on macrocyclic lactone preparations applied as pour-ons for parasite control. However, in recent years the awareness of ivermectin resistance in cattle parasites has led to producers now starting to use more benzimidazole drugs more often. Hence, increased selection together with the presence of resistance mutations at low frequencies in many parasite populations makes resistance very likely to emerge; possibly relatively quickly. *H. placei* is an extremely pathogenic parasite and so the emergence of resistance to multiple drug classes would be a major concern. This study is a good illustration of 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 or be clinically apparent (Fig. 5.1 CHAPTER V).

**6.6. The nature of the selective sweep associated with benzimidazole resistance in *H. contortus***

Understanding the genetic signature of selection is not only important for understanding how resistance mutations arise and spread but also to provide the background information needed to search for evidence of selection in parasite genomes. The genomic resources are rapidly improving for *H. contortus* and there is increasing potential to apply
genome-wide scans in order to identify novel resistance mutations. However, there is currently, little understanding of the nature of the signature of selection associated with the emergence of anthelmintic resistance in parasitic nematodes. Analysis of the signature of selection was not a major focus of my thesis work but I will briefly discuss some points of relevance that will be important to consider in future research. A study of previous UK sheep farms found that for T. circumcincta, numerous resistance haplotypes were detected on each of six farms demonstrating a “soft selective sweep” in each case (Redman et al., 2015). For H. contortus, the overall diversity of resistance haplotypes was somewhat less but “soft selective sweeps” with multiple resistance haplotypes were still found on four farms. On one farm there was a single resistance haplotype at high frequency more typical of a classic “hard selective sweep” (Redman et al., 2015). Hence, although overall pattern of selection associated with benzimidazole resistance in H. contortus on UK sheep farms was that of a “soft selective sweep”, the details varied between individual farms (Redman et al., 2015). We have little understanding yet of why this is the case, although it was hypothesized that it could, at least in part, be due to population bottlenecking of H. contortus that might occur during UK winters leading to the loss of resistance alleles in some situations. Similar results were also obtained in studies on goat farms in France that were closed to animal movement (Silvestre and Humbert, 2002; Silvestre et al., 2009). In those studies, in the case of H. contortus two resistance haplotypes were identified on two of the farms whereas only a single haplotype was present on the remaining five farms (Silvestre and Humbert, 2002; Silvestre et al., 2009). However, only very few worms were examined on most of the farms and so the true resistance haplotype diversity on each farm was not clear.
Overall, the results presented in this thesis from India and Pakistan are consistent with these previous studies showing an overall pattern of a “soft selective sweep”. There was a lot of resistance haplotype diversity overall. In the southern Indian study, the majority of the parasite populations contained multiple different resistance haplotypes with distant phylogenetic relationships. Indeed the only populations where a single resistance haplotype was present were those where resistance was just beginning to emerge and had a very low frequency of the resistance mutation. Hence there is a very clear pattern of “soft selective sweeps” being present. The absence of any populations with a “hard selective sweep”, like the one described in the UK, is consistent with a lack of population bottlenecking in southern India due to the year round warm humid climate. Similarly in Pakistan, the only three populations with a high frequency of benzimidazole resistance mutations were three government farms and again multiple phylogenetically divergent resistance haplotypes for the F200Y (TAC) was present in each case. (Supplementary Fig. S3.3 and Fig. 4.3 CHAPTER III & IV).

The populations from Pakistan in particular will be potentially very useful to undertake genome-wide scans to investigate signatures of selection and to investigate whether there are loci additional the isotype-1 β-tubulin locus that contribute to benzimidazole resistance. There is a very high frequency of the F200Y (TAC) resistance mutations on the three government farms due to many years of intense drug selection. Indeed for two of the farms (Pop 1 and Pop 3), it is likely that only benzimidazole drugs have been used making these extremely useful as most populations available for such studies have been subject to selection with multiple drug classes. The breeding stock used to found the herds on these government farms were sourced from local animals (exclusively from local animals in the case of two farms; Pop 1 and Pop
3). Hence, comparison with the contemporary parasite populations in rural sheep from the surrounding areas which have been subject to little selection and have a very low frequency of the F200Y (TAC) mutation should be extremely informative. It will be interesting to undertake genome-wide scans using reduced representation approaches such as RAD sequencing, or even whole genome sequencing, to compare the genomes of parasites on these government farms with those from the neighboring rural populations. This will be a potentially powerful approach to characterize the signature of selection at the isotype-1 β-tubulin locus in drug resistance populations and determine the extent to which it extends from the causal resistance mutation. This information could then help guide the analysis of the data from whole genome scans seeking to identify additional genomic regions that harbor additional loci contributing to the benzimidazole resistance phenotype. The results from the work in this thesis not only provides well characterized material for such studies but also provides an initial indication of the types of analysis that may need to be undertaken. Analytical approaches that can identify regions of linkage disequilibrium that are typically seen in “soft selective sweeps” are likely to be more appropriate than those that detect reductions in polymorphism that are more characteristic of “hard selective sweeps” (Messer and Petrov, 2013).

6.7. Contribution of this work to our understanding of how benzimidazole resistance mutations originate and spread in *H. contortus* populations

There is currently a poor understanding how anthelmintic resistance mutations emerge and spread in parasite populations and providing new insights into this question was an
important goal of this thesis. Early work suggested that anthelmintic resistance mutations typically arose from the standing genetic variation that pre-existed the use of anthelmintic (Roos et al., 1990; Silvestre and Humbert, 2002). More recently several authors have suggested that mutations recurrently appear during the period of selection may also be important, perhaps even more important (Silvestre and Humbert, 2002; Skuce et al., 2010; Brasil et al., 2012; Redman et al., 2015). It is impossible to draw definitive conclusions on this issue from the currently available data but there is an increasing amount of circumstantial support for this latter model. As discussed earlier in the section 6.5, examination of the occurrence of the benzimidazole resistance-associated mutations in different countries and regions itself provides some important insights. It is clear that the widespread (and consistent) occurrence of the P200Y (TAC) mutation is consistent with both pre-existing and recurrent mutation models. In contrast, as discussed earlier in section 6.5, the F167Y (TAC) and E198A (GCA) mutations are much more variable in their occurrence and distribution such that it seems unlikely they were part of the standing genetic variation that pre-existed drug selection. Their distribution is more consistent with them appearing and become fixed during the period of drug selection and the phylogenetic relationships of the resistance alleles in this study are consistent this view. The P200Y (TAC) mutation is present on multiple independent haplotypes in both southern India and Pakistan suggesting multiple independent origins. This is very consistent with the previous studies in both France and UK (Silvestre and Humbert, 2002; Redman et al., 2015). The situation in Pakistan was of particular interest in this regard as there were three government farms that founded from local breeding stock (and largely closed to subsequent animal movement). These government farms had been under intense selection pressure with benzimidazole resistant drugs for over 30 years whereas the parasite
populations in the surrounding rurally located sheep and goats were not. As discussed in more
detail in Chapter IV, there were multiple divergent resistance haplotypes on each government
farm, some of which were detected at low frequency in rural populations and some of which
were not. This is consistent with results of Silvestre and Humbert (2002) on closed goat farms
in France and provides additional evidence that resistance mutations originate both from the
pre-existing mutations and from more recent recurrent mutations.

A particularly important finding from this thesis work was the phylogenetic evidence
that the E198A (GCA) resistance mutation that was present in multiple geographically
separate H. contortus populations was derived from a single origin. This is the first time there
has been unequivocal genetic evidence of the spread of any anthelmintic resistance allele
between locations. Knowledge of parasite epidemiology and farming practices in different
countries makes it very likely that the migration of resistance alleles is an important element
in the emergence of resistance in many situations. However, this is difficult to experimentally
investigate and demonstrate. In most countries where benzimidazole resistance is well
advanced, the diversity of the resistance haplotypes, and the complexity of their relationships,
makes it difficult to use genetic analysis to definitively demonstrate that a particular resistance
allele has spread from one location to another. However, studying a region in which resistance
is at an earlier stage provides a simpler situation from which to draw conclusions. Even here
though, the widespread occurrence of the P200Y (TAC) and the presence of multiple
haplotypes prevents us from directly inferring spread of this particular mutation (although it is
clearly likely). However the detection of the rarer E198A (GCA) at multiple sites but only on
a single haplotype provides persuasive evidence that it originated just once in the region. It is
effectively in the early stage of a “hard selective sweep”. The fact that this mutation has
become so widespread from a single origin is a dramatic illustration of the role of migration in the spread of resistance alleles. This emphasizes the critical importance of biosecurity measures and quarantine dosing in managing the emergence of resistance in any country where there is significant animal movement. It also implies that the migration of resistance mutations between locations plays an extremely important role in producing the complex patterns of resistance haplotypes seen at the later stages of resistance development in countries such as France and the UK. It is interesting to speculate that, although the more common P200Y (TAC) mutation clearly has multiple independent origins, it may be that there are a smaller number than might be concluded from an initial analysis of the complex resistance haplotype networks observed.

Reference


Baker, K.E., George, S.D., Stein, P.A., Seewald, W., Rolfe, P.F., Hosking, B.C., 2012. Efficacy of monepantel and anthelmintic combinations against multiple-resistant Haemonchus contortus in sheep, including characterisation of the nematode isolate. Veterinary parasitology 186, 513-517.


nematodes of livestock is characterised by multiple independent hard and soft selective sweeps. PLoS Negl Trop Dis. 6;9, :e0003494. doi:


