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Benzimidazole resistance allele haplotype diversity in United Kingdom isolates of *Teladorsagia circumcincta* supports a hypothesis of multiple origins of resistance by recurrent mutation ☆

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ABSTRACT

Polymorphisms in the isotype I β-tubulin gene are important genetic determinants of benzimidazole (BZ) resistance in a number of parasitic nematode species including *Teladorsagia circumcincta*, a major gastrointestinal nematode of sheep. This study investigates the genetic diversity at this locus in a BZ-resistant isolate of *T. circumcincta* (MTci5) derived from a sheep farm in the United Kingdom (UK) that was open to animal, and therefore parasite, migration. Pyrosequencing was used to determine the frequency of single nucleotide polymorphisms (SNPs) known to be associated with BZ resistance. This was followed by a combination of single strand conformation polymorphism (SSCP) analysis and nucleotide sequencing to sample allelic diversity in a 276 bp fragment immediately surrounding the isotype I β-tubulin F200Y mutation. The genetic diversity at this locus was extremely high, with seven different haplotypes found to contain the resistant F200Y polymorphism in this single resistant isolate. Genotyping by SSCP interfaced with pyrosequencing demonstrated that the $P200^{Y}$ mutation is also present on multiple haplotypes in two other BZ-resistant *T. circumcincta* isolates from the UK. This contrasts with much lower levels of haplotype diversity in BZ-resistant alleles present in *T. circumcincta* isolates from French goat farms that are closed to parasite migration. Taken together with our knowledge of *T. circumcincta* population genetic structure, these results are most consistent with multiple independent origins of resistance and mixing of alleles due to the large amount of livestock movement in the UK.

1. Introduction

Parasitic nematodes represent a major burden to the livestock industry worldwide; their control is now seriously compromised by the increasing prevalence of anthelmintic resistance. An understanding of how resistant mutations arise and spread in parasite populations is an important goal in devising control strategies that minimise the development of resistance. A mutation leading to a F200Y substitution in the isotype I β-tubulin gene is strongly linked to benzimidazole (BZ) resistance in several trichostrongylid nematodes of sheep, including the abomasal nematode, *Teladorsagia circumcincta* (for reviews see von Samson-Himmelstjerna et al., 2007; Gilleard, 2006). Other mutations in isotype I β-tubulin, most notably F167Y and E198A, have also been reported in BZ-resistant trichostrongylids, but F200Y appears to be the most commonly associated genetic determinant (Silvestre and Cabaret, 2002; Ghisi et al., 2007). Since the role of the F200Y mutation in BZ resistance is well established and understood, it provides an excellent opportunity to study how anthelmintic resistance may have arisen in the field.

In this context, we are undertaking population genetic studies of *T. circumcincta* in United Kingdom (UK) sheep in order to investigate a number of important questions. For example, do BZ-resistant alleles have many or just a few independent origins? To what extent do resistant alleles spread between farms and over what scale? These questions are more challenging to address for diploid obligate sexually reproducing organisms than for haploid organisms with limited genetic exchange, such as bacteria or protozoa. Nevertheless, they are still amenable to population genetics approaches that have been applied to other diploid organisms with large population sizes such as insects (Ffrench-Constant et al., 2004).

We have undertaken an investigation of a multi-drug resistant *T. circumcincta* strain (MTci5), isolated from a UK farm that is open

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to animal movement and, consequently, to parasite gene flow. The main purposes of this study were to investigate haplotype diversity and phylogenetic relationships of resistant alleles within this isolate, and to compare results with those previously obtained on farms closed to animal movement (Silvestre and Humbert, 2002; Silvestre et al., 2009). The overall aim was to provide an insight into the origin of resistant alleles and the implications of animal movement in their spread.

2. Materials and methods

2.1. Parasite isolates

MTci1 is BZ-susceptible and was isolated in 1979 from lambs grazing on pastures at Moredun Research Institute, UK. MTci2 was obtained from the Central Veterinary Laboratories (Weybridge, UK) in 2000 and is known to be susceptible to ivermectin and levamisole, however, low-level BZ resistance is suspected from previous egg hatch test results (D. Bartley, personal communication). MTci3 is BZ-resistant and was originally isolated from sheep on Firth Mains farm, Central Scotland, in 1983. MTci4 is a caprine-derived BZ- and ivermectin-resistant isolate previously characterised by Jackson et al. (1992a,b,c). The origin of the multi-drug resistant MTci5 isolate is described in detail by Sargison et al. (2001) and initial phenotypic characterisation of this population was carried out by Bartley et al. (2004). This isolate has been passaged two to three times per year at Moredun Research Institute since being brought into the laboratory in 2002.

2.2. DNA extraction

Genomic DNA was extracted from single exsheathed L_3 of the MTci1, MTci2, MTci3 and MTci4 isolates, essentially as described in Silvestre and Humbert (2002). DNA isolation from adult MTci5 worms was performed using a miniprep DNA purification kit (QiaQuick DNA mini kit, Qiagen) eluted in 100 μl volumes. All lysates were stored at −20 °C.

2.3. Single strand conformation analysis (SSCP)

A small region of the isotype I β-tubulin gene (a 276 bp fragment from codons 151 to 216 containing a 120 bp intron) was chosen for PCR amplification. This region was known to be polymorphic and included putative BZ resistance-associated single nucleotide polymorphism (SNP) sites at P167 (Silvestre and Cabaret, 2002), P198 (Ghisi et al., 2007) and P200 (Silvestre and Humbert, 2002), that have been reported in *T. circumcincta* and other nematode species. The primer sequences used for SSCP analyses are listed in Table 1. PCR products spanning this polymorphic region of the β-tubulin gene were mixed with SSCP loading buffer (95% deionised formamide (Sigma), 10 mM sodium hydroxide (Sigma), 0.25% Bromophenol blue (Sigma), 0.25% Xylene cyanol (Sigma)) at a ratio of 1:2 and denatured at 95 °C for 5 min on a hotplate. Samples were then quenched and stored on ice whilst the gels were loaded with 10 μl of each denatured product. A 20% (37.5:1) polyacrylamide:bisacrylamide (Severn Biotech, UK) gel mixture in TBE buffer (0.5*x* = 0.045 M tris–borate; 0.001 M EDTA, pH 8.0) was found to give optimal resolution. Gels were subjected to vertical electrophoresis at a constant voltage of 120 V for 18 h. Following electrophoresis, gels were soaked in GelRed solution (Biotium, Inc., Hayward, CA, USA) in preparation for scanning under UV light in a BioRad FX Molecular Imager scanner.

SSCP was used as an initial screen to investigate the level of polymorphism of the isotype I β-tubulin gene and, subsequently, to determine single worm genotypes in terms of SSCP allele class. In order to provide definitive nucleotide sequence for the respective alleles, PCR products from 28 single MTci5 worms were cloned into the pGEM-T Easy vector (Promega). The 276 bp isotype I β-tubulin fragment was PCR amplified from six colonies from each cloning experiment and these were run on SSCP alongside the PCR product amplified from the corresponding single worm. This allowed the genotypes of the 28 worms to be determined in terms of four polymorphic allele classes. The remaining 44 worms could then be genotyped using the validated SSCP profiles. Cloned alleles run as reference standards on SSCP gels were used to determine single worm genotypes. Allele and genotype frequencies were calculated in Microsoft Excel. Data were also analysed for deviation from Hardy Weinberg Equilibrium (HWE) using GenAlEx add-in software (Peakall and Smouse, 2006) for Microsoft Excel.

2.4. Pyrosequencing assay to determine F200Y, E198A and F167Y genotypes

Pyrosequencing assays were designed, using the Biotage proprietary assay design software, to specifically target the F200Y, A198E and F167Y SNPs in the parasite's isotype I β-tubulin gene. The putative E198A SNP (GAA to GCA) was within six bases of the F200Y SNP and so could be accurately called in the same pyrosequencing reaction. The primer sequences and any modifications are summarised in Table 1. Briefly, biotinylated PCR products were generated using a 5′ Biotin-labelled primer and an unmodified primer. Each reaction contained 1/10th reaction volume of worm lysate as template, 0.67 μM Biotin-forward primer and 2 μM reverse primer (MWG Biotech, Germany), 1× Platinum *Taq* buffer (10×, Invitrogen), 0.32 mM dNTP mix (10 mM, Roche), 1.5 mM $MgCl_2$ (50 mM, Invitrogen) plus 0.04 U Platinum *Taq* DNA polymerase (5 U/μl, Invitrogen) made up in molecular grade water (Fisher Scientific). PCR cycling was carried out on an ABI 2700 thermocycler (Applied Biosystems) with one cycle of 94 °C for 5 min; 45 cycles of 55 °C for 30 s (to exhaust the biotinylated primer), 72 °C for 30 s and 92 °C for 1 min; with a final extension step of 10 min at 72 °C. For the sample preparation step, 3 μl streptavidin-coated Sepharose beads (Biotage) and 37 μl binding buffer (Biotage) were added to each 40 μl PCR product in a 96 well plate and agitated for 5 min at room temperature to allow binding of the biotin-labelled DNA to the beads. The beads were then processed using the customised sample preparation tool and reagents (Biotage) and dispensed into the assay plate (Biotage) containing 40 μl of 0.4 μM sequencing primer per well. The respective P200 and P167 pyrosequencing primers were annealed at 80 °C on an ABI 2700 thermocycler (Applied Biosystems) for 3 min. Samples were then cooled to room temperature before the pyrosequencing run was initiated. The target sequences to genotype were, for F200Y: 5′-AGWAYGTTTCA-3′ and for F167Y: 5′-ATTCATYCTC-3′. A number of controls were included in each assay: a positive heterozygote control, at least three negative PCR controls (reaction mix, no template) and another negative control containing sequencing

Table 1
PCR primers used for single strand conformation polymorphism and subsequent pyrosequencing assays for F200Y and F167Y single nucleotide polymorphisms, respectively.

Primer name	Sequence 5′–3′	Modifications
BTUB_FOR	CCAAAATTCGCGAGGAGTA	–
BTUB_REV	TTTCAAGGTGCGGAAGCAGA	–
F200Y_FOR	ACCTTACAATGCCACTCTTTCTG	5′ Biotin
F200Y_REV	GCGGAAGCAGATATCGTACAG	–
F200Y_SEQ	RGAGCYTCATTATCGATR	Degeneracy
F167Y_FOR	GCATTCTTTGGGAGGAGGTA	–
F167Y_REV	TGCACCTCGAGAACCTGTACATA	5′ Biotin
F167Y_SEQ	CGGATAGAATCATGGCT	–