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The role of polymorphisms at β tubulin isotype 1 codons 167 and 200 in benzimidazole resistance in cyathostomins ☆

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Abstract

Cyathostomins are recognised as the primary parasitic pathogens of horses. Despite the use of benzimidazole (BZ) anthelmintics in horses for more than 40 years and widespread drug resistance in the field, the mechanisms of resistance to this drug class in cyathostomins are not fully understood. The results presented here constitute a detailed comparison of β tubulin gene mutations and mRNA transcript levels in populations of BZ-susceptible (BZ-S) and -resistant (BZ-R) cyathostomins. Full-length cDNA sequences were generated from individual parasites of four ($n = 24$) and two ($n = 19$) cyathostomin species for isotypes 1 and 2, respectively. Levels of intra- and inter-specific nucleotide sequence variation were comparable with previous findings and single amino acid substitutions were observed at several locations. On comparison of BZ-S and BZ-R parasites, differences were consistently observed at only two sites, codons 167 and 200 of the β tubulin isotype 1 gene. Four populations of parasites were genotyped at these two loci by pyrosequencing; one that was fenbendazole (FBZ)-sensitive (FBZ-rS), two that were FBZ-resistant (FBZ-R1 and -R2) and one that was oxibendazole-resistant (OBZ-R), as previously assessed by faecal egg count reduction tests. This analysis revealed statistically significant differences between FBZ-rS and FBZ-R populations at both loci and this was highly significant for codon 167. For the OBZ-R population, the only significant difference compared with the FBZ-rS population was observed at codon 200. These observations suggest that mutations at codons 167 and 200 are important in BZ resistance and raise the possibility that selection at different loci may occur in FBZ- and OBZ-resistant parasites. Multiple parasites ($n = 158$) were genotyped for both codons 167 and 200, the majority of which showed homozygous 'resistant' mutations at one locus only and none showed homozygous 'resistant' genotypes at both loci. No significant differences in mRNA levels of β tubulin isotypes 1 and 2 were observed between the FBZ-rS and FBZ-R1 populations.

Keywords: Cyathostomins; β Tubulin isotypes 1 and 2; Codons 167 and 200 mutations; mRNA transcript; Benzimidazole resistance

1. Introduction

Almost all grazing horses carry a cyathostomin burden at some stage of, if not throughout, their lives (Lyons et al., 1999; Proudman and Matthews, 2000). Up to 52 cyathostomin species have been described (Lichtenfels et al., 1998, 2002; Matthee et al., 2002), although it is generally accepted that 90% of the burden in an individual horse consists of 5–10 common species (Reinemeyer et al., 1984; Lyons et al., 1999; Lichtenfels et al., 2002; Chapman et al., 2002). Heavily-infected horses can harbour hundreds of thousands of nematodes, a large proportion of which are present as larvae in the large intestinal wall (Dowdall et al., 2002; Lichtenfels et al., 2002). Such infections induce a protein-losing enteropathy leading to weight loss and inappetance (Murphy and Love, 1997) or colic (Uhlinger, 1990; Mair and Pearson, 1995; Murphy et al., 1997; Mair

☆ *Note:* Nucleotide sequence data reported in this paper are available in the GenBank, EMBL and DDBJ databases under Accession Nos.: EU251433–EU251475.

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et al., 2000). The emergence of large numbers of previously encysted larvae from the mucosa/submucosa can cause larval cyathostominosis, a severe inflammatory enteropathy associated with acute weight loss and diarrhoea and which has a case fatality rate of up to 50% (Giles et al., 1985; Love and McKeand, 1997; Love et al., 1999).

Benzimidazole (BZ) drugs have been used to control cyathostomin infections for more than 40 years. Unfortunately, extensive, frequent use of these anthelmintics has led to widespread resistance (Kaplan et al., 2004). Benzimidazoles are considered to target β tubulin proteins (Roos et al., 1995) and resistance is thought to involve a structural change in the β tubulin molecule, leading to a loss of high-affinity receptors resulting in reduced drug binding (Lubega and Prichard, 1990; Lacey and Gill, 1994). Research into the mechanisms of BZ resistance in nematodes has focused on β tubulin genes, specifically point mutations observed at two loci in β tubulin isotype 1, 167 (Silvestre and Cabaret, 2002) and 200 (Kwa et al., 1995).

The major genetic mechanism conferring BZ resistance in field populations of trichostrongylids is considered to be a TTC → TAC point mutation at codon 200 of the β tubulin isotype 1 gene (F200Y, Silvestre and Cabaret, 2002). This mutation has been associated with BZ resistance in other organisms such as *Caenorhabditis elegans* (Driscoll et al., 1989), *Aspergillus nidulans* and *Penicillium* spp. (Kwa et al., 1994). The same polymorphism, at codon 167 (F167Y), is thought to play a role in BZ resistance in *Neurospora crassa* (Orbach et al., 1986) and *Saccharomyces cerevisiae* (Li et al., 1996) as well as in trichostrongylid nematodes (Prichard, 2001; Silvestre and Cabaret, 2002). In the latter species, the codon 167 mutation is found in the absence of the codon 200 mutation, but has been observed to occur only rarely in the field (Silvestre and Cabaret, 2002). Selection at the same codons in the β tubulin isotype 2 gene is also thought to affect BZ sensitivity (Kwa et al., 1993a; Beech et al., 1994; Prichard, 2001), whilst deletion of this gene has been observed in highly-resistant *Haemonchus contortus* individuals (Kwa et al., 1993b).

Alterations in levels of β tubulin mRNA could also play a role in BZ resistance (Burkhart et al., 2001). Few data are available with regard to this phenomenon in parasitic nematodes however, in vertebrates, β tubulin isotype expression levels have been observed to change in cell lines selected for resistance to antimitotic agents such as Taxol (Burkhart et al., 2001).

To date, DNA sequences encoding two β tubulin isotypes (1 and 2) have been isolated from cyathostomins (Pape et al., 1999; Clark et al., 2005). Thus far, the isotype 1 codon 200 mutation has been the focus of research into BZ resistance in cyathostomins (von Samson-Himmelstjerna et al., 2002a,b; Pape et al., 2003), although other polymorphisms along this gene have been identified in small numbers of parasites from BZ-susceptible and -resistant populations (Drogemuller et al., 2004). Here, we believe we present the first description of sequence variation along full-length cDNAs of both β tubulin isotypes from several species obtained from two populations of known fenbendazole (FBZ) sensitivity. We also report a genotyping analysis of parasites derived from FBZ-sensitive (FBZ-rS) and -resistant (FBZ-R1, -R2) populations and from a further population known to be resistant to oxibendazole (OBZ-R). Finally, we report β tubulin isotype 1 and isotype 2 mRNA transcript levels observed in BZ-resistant versus -susceptible cyathostomins. Statistically significant differences were revealed amongst FBZ-rS, FBZ-R and OBZ-R populations, at both loci, suggesting that these loci are involved in BZ resistance. The analysis of β tubulin mRNA transcript levels did not support a role for isotype 2 deletion in BZ resistance in cyathostomins. Genotyping multiple parasites at both codons 167 and 200 raise important questions about the mechanisms of BZ resistance in these parasites and highlight the need to explore the heritability of BZ resistance in these important equine parasites.

2. Materials and methods

2.1. Parasite populations

Two populations of adult Cyathostominae were provided courtesy of Professor Tom Klei (Louisiana State University (LSU), Baton Rouge, LA, USA). Species identification was performed by Dr. Melanie Chapman (LSU) following the key of Lichtenfels et al. (1998) and was confirmed for a subset of these parasites by PCR–ELISA (Hodgkinson et al., 2003). Parasite population 1, designated as FBZ-rS, was recovered at necropsy from 2- to 3-year-old animals derived from a herd of ponies which had not been exposed to anthelmintic treatment for more than 18 years. A faecal egg count reduction (FECR) test was performed on foals that were siblings of the necropsied ponies and showed that, at 14 days post-treatment with FBZ, the egg output was reduced by >80%, suggesting that the majority of the parasites present in this herd were relatively sensitive to FBZ. A second parasite population, FBZ-R1, was harvested at necropsy from a 6-month-old male pony from a breeding herd with a history of BZ treatment. The pony received FBZ 28 and 14 days before necropsy and the FECR test performed after the latter treatment showed a reduction in faecal egg output of only 10.3%, suggesting that the majority of worms derived from this animal were FBZ-resistant. A third population of adult parasites (FBZ-R2) was harvested in 2005, at the necropsy of a female pony from within a breeding herd maintained at the University of Liverpool. This breeding herd had been on permanent pastures for more than 20 years during which time a number of anthelmintic treatments were administered, including FBZ. This herd was subjected to several FECR tests between 1 October 1999 and 1 January 2002, including two tests carried out 14 and 21 days, respectively, after the administration of five daily treatments with FBZ and another test 14 days after a single FBZ treatment. The FECR test results were 63% ($n = 8$,