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Two benzimidazole resistance-associated SNPs in the isotype-1 β-tubulin gene predominate in *Haemonchus contortus* populations from eight regions in China

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ABSTRACT

Haemonchus contortus is one of the most important parasitic nematodes of small ruminants around the world, particularly in tropical and subtropical regions. The control of haemonchosis relies mainly on anthelmintics, but the excessive and prolonged use of anthelmintics is causing serious drug resistance issues in many countries. As benzimidazole (BZ) anthelmintics have been broadly used in China, we hypothesized that resistance is widespread. Given the link between three known single nucleotide polymorphisms (SNPs, designated F167Y, E198A and F200Y) in the isotype-1 β-tubulin gene and BZ resistance, our goal here was to explore the presence of these mutations in *H. contortus* from small ruminants (sheep and goats) from eight provinces in China using PCR-coupled sequencing. In addition, the genetic diversity and genetic relationship of isotype-1 β-tubulin sequence haplotypes were also investigated. Among 192 *H. contortus* adult individuals representing the eight populations, we identified six distinct sequence types, five of which had SNP E198A (GCA) and/or F200Y (TAC). Sequence analysis showed that the frequencies of SNPs E198A and F200Y were 0–70% and 0–31%, respectively. SNP F167Y (TAC) was not detected in any population. In addition, high haplotype diversities (0.455–0.939) and nucleotide diversities (0.018–0.039) were calculated. A network analysis of the isotype-1 β-tubulin gene sequences showed that SNPs E198A and F200Y occurred in multiple distinct groupings, suggesting multiple independent origins of these SNPs. The findings of this first study of SNPs in the isotype-1 β-tubulin gene of *H. contortus* populations suggest that BZ resistance is prevalent in some regions of China, and that any control strategy might focus on monitoring BZ resistance in this country.

1. Introduction

Haemonchus contortus is one of the most economically important trichostrongyloid nematodes affecting small ruminant livestock worldwide. This nematode has a complex three-week life cycle, with a parasitic and a free-living phase. Following the ingestion of infective third-stage larvae (L3s), hosts are affected mainly by the fourth-stage larvae and adults of *H. contortus* primarily due to their blood feeding activity in the abomasum, leading to anaemia and associated complications and often death in heavily infected animals (Waller et al., 2004; Nikolaou and Gasser, 2006; O'Connor et al., 2006).

The control of *H. contortus* and related gastrointestinal nematodes has relied on the treatment with anthelmintic drugs

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including benzimidazoles (BZs). As a consequence of their excessive use, resistance against BZ has developed and spread in *H. contortus* and related strongylid nematodes (Waller, 1997; Jackson and Coop, 2000; Kaplan, 2004). β-tubulin is the target for BZs (Kohler, 2001; Kotze et al., 2014), and, collectively, studies to date recognise three different single nucleotide polymorphisms (SNPs) in the isotype-1 β-tubulin gene at codons 167 (TTC to TAC; F167Y) (Silvestre and Cabaret, 2002), 198 (GAA to GCA; E198A) (Ghisi et al., 2007; Rufener et al., 2009) and 200 (TTC to TAC; F200Y) (Kwa et al., 1994, 1995) to be associated with BZ resistance in *H. contortus*. F200Y has been detected at high prevalence in many countries and appears to be the commonest SNP linked to BZ resistance (Kotze et al., 2014). F167Y is less common than F200Y, with an apparently limited distribution in countries including Argentina, Brazil, Canada, France, the UK and the USA (Silvestre and Cabaret, 2002; Barrere et al., 2012, 2013a,b; Brasil et al., 2012; Chaudhry et al., 2014; dos Santos et al., 2014; Redman et al., 2015). In addition, E198A has been found in three field-derived populations from South Africa and Australia and in another in vitro-selected population from Australia (Ghisi et al., 2007; Rufener et al., 2009; Kotze et al., 2012); recently, this mutation was detected in field populations of *H. contortus* in India (Chaudhry et al., 2015).

BZs and other anthelmintic drugs have been used heavily to control nematode infections in China. Based on some publications from this country (He et al., 1999a,b; Cai et al., 2007a,b; Zhao et al., 2010), we have reason to believe that BZ resistance in *H. contortus* is becoming a major issue. Although SNPs, F167Y, E198A and F200Y have been detected in *H. contortus* in many countries (Kotze et al., 2014), the three studies of isotype-1 β-tubulin gene in China have focused only on the F200Y (Bo and Li, 2005; Hao, 2007; Cai and Bai, 2009); thus, there is no information on the other two SNPs, in spite of the high prevalence of *H. contortus* and the excessive and uncontrolled use of BZs in this country (Shen, 2005).

Understanding the origin and spread of SNPs linked to BZ resistance is important (Skuce et al., 2010), which is why population genetic studies have been conducted recently. For example, Chaudhry et al. (2015) studied 13 *H. contortus* populations from sheep and goats in southern India, and indicated that SNP E198A spread from a single origin to multiple locations in this region, whereas F200Y likely had three independent origins. Similarly, Redman et al. (2015) provided evidence for multiple independent origins of both SNPs F200Y and F167Y in *H. contortus* in the UK. However, there is no such information for China.

Therefore, for the first time in China, we explored genetic variation and the three known BZ-associated SNPs (F167Y, E198A and F200Y) in the isotype-1 β-tubulin gene in populations of *H. contortus* from sheep and goats from eight provinces using PCR-coupled sequencing, to begin to understand the status of BZ resistance and the origin of these SNPs.

2. Materials and methods

2.1. Parasite populations and genomic DNA isolation

Animal ethics approval was granted (permit SYXK2015-0084) by the Science and Technology Department of Hubei province, China. Adult *H. contortus* populations were collected from eight different geographical regions from provinces in subtropical to tropical climatic zones of China (Fig. 1). Samples from Hubei, Yunnan, Shaanxi and Guangxi were from goats, whereas others were from sheep. The history of use of anthelmintics, including BZs or other drugs, was not clear in many cases. After collection from the abomasum, worms were washed in physiological saline, fixed in 75% ethanol and submitted to the College of Veterinary Medicine, Huazhong Agricultural University, Wuhan. Upon arrival, individual adult male worms were morphologically identified (Lichtenfels et al., 1994). Then, following the rehydration of worms, total genomic DNA was extracted from individual adults using sodium dodecyl-sulfate/proteinase K treatment, followed by spin-column purification (Wizard DNA Clean-Up, Promega) (Gasser et al., 2006). Genomic DNA samples were stored at −20 °C until use. For the identification of BZ-associated SNPs, genomic DNA from individual worms was used. For the analysis of genetic diversity within and among populations, 'pooled' DNA was subjected to PCR, cloned and then sequenced. To prepare DNA template representing individual populations, 2 μl aliquots of the genomic DNAs from each of the 24 individual worms from each population were pooled.

2.2. Specific identification of individual H. contortus

The specific identity of each worm as *H. contortus* was confirmed by molecular means. A 265 bp region of the second internal transcribed spacer (ITS-2) of nuclear ribosomal DNA gene was specifically amplified by conventional PCR from individual genomic DNA samples using the primer pair HAE (5′-CAAATGGCATTTGTCTTTTAG-3′) + NC2 (5′-TTAGTTTCTTTTCCTCCGCT-3′) (Bott et al., 2009). In brief, PCR was performed in 25 μl of 10 mM Tris-HCl, pH 8.3, 50 mM KCl, 4 mM $MgCl_2$, 250 μM each of dNTP, 100 pmol of each primer and 1 U *Taq* polymerase (TaKaRa) in thermal cycler (Eastwin Life Sciences, Inc., China) using the following protocol: 94 °C/5 min, followed by 35 cycles of 94 °C/30 s, 55 °C/30 s and 72 °C/30 s, with a final extension at 72 °C/7 min. Known positive and a negative (no-template) control samples were included in each PCR run. All amplicons were examined on 1.5% agarose gels, and detected upon ultraviolet transillumination using an electronic documentation system (Alphamager Mini) to confirm a single band of the appropriate size. Subsequently, the amplicons were column-purified (Wizard PCR-Preps, Promega) and then directly sequenced (in separate reactions) with both primer HAE and NC2 using the Big-Dye Terminator v.3.1 cycle sequencing kit (Applied Biosystems) in an automated sequencer (PRISM3730, ABI). The identity of each sequence was established based on the comparison with reference sequences of *H. contortus* ITS-2 available in the GenBank database (GenBank accession nos. X78803 and EU084691.1). Eventually, 24 individual adult male worms from each of the eight populations were identified as *H. contortus* (Table 1).

2.3. PCR amplification and direct sequencing of the isotype-1 β-tubulin gene fragment of H. contortus

A 385 bp region (including exons 4 and 5 and their intervening intron) of the isotype-1 β-tubulin gene was amplified from individual *H. contortus* genomic DNA samples using primer pair HcPy2PCR-F (5′-GACGCATTCACTTGGAGGAG-3′) and HcPy2PCR-R (5′-CATAGGTTGGATTTGTGAGTT-3′) (von Samson-Himmelstjerna et al., 2009) by PCR (as described in Subsection 2.2) using the following cycling protocol: 94 °C/3 min, followed by 40 cycles at 94 °C/40 s, 53 °C/40 s and 68 °C/40 s, with a final extension at 68 °C/7 min. Amplicons were subjected to agarose gel (1.5%) electrophoresis to confirm that each represented a single band, followed by individual PCR product purification and direct sequencing (as described in Subsection 2.2) with primers HcPy2PCR-F and HcPy2PCR-R (in both directions).

2.4. Determination of the frequencies of the isotype-1 β-tubulin F167Y, E198A and F200Y BZ resistance-associated SNPs in H. contortus populations

Sequence traces were examined using Chromas v.2.4.1 software, focusing on the SNPs F167Y, E198A and F200Y. As described by