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Gene expression changes in a P-glycoprotein (*Tci-pgp-9*) putatively associated with ivermectin resistance in *Teladorsagia circumcincta* ☆

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ABSTRACT

Anthelmintic resistance in parasitic nematodes of small ruminants is widespread and, in some parts of the world, threatens the sustainability of sheep production. The genetic changes underlying resistance to anthelmintics, particularly ivermectin (IVM), remain to be determined. The majority of studies to date have investigated target site mutations; relatively little attention has been paid to the role of changes in gene expression. In this study, we investigated the expression of putative drug transporter molecules, P-glycoproteins (Pgps), in *Teladorsagia circumcincta*, the predominant parasitic nematode species of sheep in the UK and the major anthelmintic resistant species. Utilising a degenerate PCR approach, 11 partial Pgp sequences were identified. Constitutive differences in gene expression between an IVM-susceptible (MTci2) and a multidrug-resistant (MTci5) isolate were determined for 10 of the Pgps using the ΔΔCt TaqMan® real-time PCR method. Gene expression differences were particularly marked in one of these genes, namely *Tci-pgp-9*. In the MTci5 isolate, statistically significant increases in *Tci-pgp-9* expression, at the mRNA level, were observed across all life-cycle stages and most notably in eggs (55-fold increase). Comparison of the partial *Tci-pgp-9* nucleotide sequences from MTci2 and MTci5 also identified high levels of polymorphism. This work has shown that constitutively increased expression in *Tci-pgp-9*, coupled with increased sequence polymorphism, could play a role in allowing multidrug-resistant *T. circumcincta* to survive IVM exposure. The genetic changes underpinning these gene expression changes remain to be elucidated and need to be investigated in other isolates. These changes could form the basis of an IVM resistance marker to monitor the spread of resistance and to evaluate management practices aimed at delaying its spread.

1. Introduction

Infection of ruminants with gastrointestinal nematode parasites such as *Haemonchus contortus* and *Teladorsagia circumcincta* causes parasitic gastroenteritis (PGE), leading to reduced liveweight gain, anaemia and, in severe cases, death. PGE is associated with a range of costs for the farmers and their animals, such as loss of efficient meat, milk and wool production and compromised animal welfare (Boyne et al., 2006; Broughan and Wall, 2007). Parasitism is seen as the single most important cause of production losses in small ruminants worldwide (McLeod, 1995; Molento, 2009). The cost of gastrointestinal nematode infection in the UK sheep industry was estimated to be £84 million per annum, calculated as the cost to the industry due to reduced lamb growth rates plus treatment and prevention measures (Nieuwhof and Bishop, 2005). The predominant nematode species infecting sheep in temperate regions such as the UK, and the focus of this research, is *T. circumcincta*, which is also the predominant anthelmintic-resistant species (Jackson and Coop, 2000; Bartley et al., 2003).

The principal method of controlling parasitic nematodes in livestock is the regular strategic treatment of susceptible hosts with anthelmintics (Prichard et al., 2007), however the sustainability of this approach is threatened by increasing levels of drug resistance, an inevitable consequence of continued anthelmintic use (James et al., 2009). Anthelmintic resistance has developed rapidly and is a major threat to livestock production in many parts of the world. It is a global phenomenon but is more prevalent in the Southern Hemisphere (Bartley et al., 2004; Kaplan, 2004; Sutherland et al., 2010). In some severe cases in the UK, the inability to control parasitic nematodes with anthelmintics has led to the abandonment of sheep farming enterprises (Sargison et al., 2005). The true extent of anthelmintic resistance is not known, as few farmers routinely check the efficacy of the anthelmintics they use and no routine surveillance for resistance is carried out (Sargison et al., 2001, 2007). In addition, current in vitro and

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in vivo diagnostic tests for anthelmintic resistance are limited in their use, as not all drugs can be tested using each assay, they are time-consuming, expensive and labour intensive and, in some cases, require the use of test animals (Coles et al., 1992). Since resistance has a genetic component, there has been much interest in the development of molecular genetic tests to determine the resistance status of individual parasites or populations but, with the exception of the benzimidazole (BZ) anthelmintics, such tests are still lacking.

The macrocyclic lactone anthelmintic, IVM was released onto the market in 1981 and, when fully effective, has an efficacy of almost 100%. As such, it has become the biggest selling anti-parasitic drug for livestock (Omura, 2008; Van Zeveren, 2009. Ivermectin resistance in the bovine nematode *Ostertagia ostertagi*. Doctor of Philosophy. Ghent University). The mode of action of IVM is relatively well understood; it binds irreversibly to γ-aminobutyric acid- (GABA) and glutamate-gated chloride (GluCl) channels, causing the hyperpolarisation and flaccid paralysis of pharyngeal and somatic muscle cells, leading to starvation and expulsion of the worms (Martin et al., 1998; Blackhall et al., 1998a, 2003). In contrast, the mechanisms by which parasites exhibit IVM resistance have not been determined (Geary, 2005). The majority of studies have focused on the identification of gene mutations associated with resistance, particularly in IVM target genes (Wolstenholme et al., 2004; von Samson-Himmelstjerna et al., 2007). Changes in allele frequencies of Glu- and GABA-gated Cl channel subunits in *H. contortus* have been observed, but no single allele has been associated with resistance between different IVM-resistant populations (Blackhall et al., 1998a, 2003). In the free-living model nematode, *Caenorhabditis elegans*, concurrent mutations in three genes encoding the GluCl α-type subunits, *avr*-14, *avr*-15 and *glc*-1 are required to confer high levels of IVM resistance. Mutation of any two of these three genes only confers low level or no resistance (Dent et al., 2000; Cook et al., 2006; McCavera et al., 2007). Parasitic nematodes have a different set of GluCl subunit genes compared with *C. elegans* but do share some, such as *avr-14*, an orthologue of which, in the cattle parasite, *Cooperia oncophora*, has been shown to carry a polymorphism making the subunit less sensitive to IVM (McCavera et al., 2007).

Changes in alternative, non-target, drug-handling molecules could also allow an IVM-resistant phenotype to develop, either through increased drug efflux or metabolism (Wolstenholme et al., 2004). One such family of molecules, which have not been studied to date in *T. circumcincta*, and for which IVM is a known substrate, are the p-glycoproteins (Pgps) (Pouliot et al., 1997; Lespine et al., 2008). These are large transmembrane proteins which are products of the ATP binding cassette (ABC) superfamily of genes, found throughout *Archaea*, *Eubacteria* and *Eukarya*. These molecules are involved in the active transport of endogenous and exogenous hydrophobic molecules (Sangster et al., 1999; Kerboeuf et al., 2003; Jones and George, 2005). Over-expression of Pgps has been linked to drug resistance in cancerous tumours, malaria parasites and HIV (Beugnet et al., 1997; Zhang et al., 1998; Loo and Clarke, 1999). As such, although not definitive targets of IVM, changes in the expression levels of these genes (and their gene products) could enable parasites to survive IVM exposure. A number of experiments have suggested that Pgps are implicated in IVM resistance in parasitic nematodes. For example, the use of Pgp inhibitors has been shown to cause a regression towards an IVM-susceptible phenotype in an IVM-resistant *T. circumcincta* isolate using the larval feeding inhibition test (Bartley et al., 2009). Also, changes in the expression levels or allelic frequencies of Pgps have been described in IVM-selected *H. contortus*, *Onchocerca volvulus* and *C. elegans* (Blackhall et al., 1998b; Xu et al., 1998; James and Davey, 2009).

In this study, the role of changes in Pgp expression in the IVM resistance phenotype in *T. circumcincta* was investigated by real-time PCR using the relative quantification (or ΔΔCt) method to compare the expression of Pgps between an IVM-susceptible and IVM-resistant isolate.

2. Materials and methods

2.1. Parasite isolates

Two *T. circumcincta* isolates were investigated; the anthelmintic-susceptible MTci2 isolate and the anthelmintic-resistant MTci5 isolate, the 'M' denoting an isolate identified by and maintained at Moredun Research Institute, Scotland. The MTci2 isolate was isolated prior to the use of levamisole and IVM and, in an egg hatch test for BZ resistance, was shown to have an ED_{50} (the effective dose at which 50% of eggs hatch) of 0.09 μg/mL where the cut-off for resistance is 0.1 μg/mL, indicating that it is also BZ-susceptible (D. Bartley, personal communication). MTci5 is a multidrug-resistant field isolate with efficacies for fenbendazole, levamisole and IVM, revealed through controlled efficacy and faecal egg count reduction tests of 59%, 88% and 60%, respectively (Bartley et al., 2004). Eggs, L_1, exsheathed L_3 (xL_3), L_4 and adults of each isolate were obtained from donor sheep infected following standard protocols. Infection of donor animals was subject to formal institutional ethical approval and animals were cared for, in accordance with UK Home Office guidelines, by individuals holding a Personal License permitting them to do so. Pools of parasites were stored in PBS under liquid nitrogen until required.

2.2. RNA extraction and cDNA synthesis

Parasites were homogenised using a pestle and mortar under liquid nitrogen and RNA extracted using TRIzol® reagent (Invitrogen), following the manufacturer's protocol. The RNA was resuspended in 75 μL nuclease free water and stored at −80 °C. Confirmation of RNA quality was obtained by running 5 μL on a 1% agarose gel with GelRed™ (Biotium, USA) to identify the 18S band and by determining the RNA concentration using the NanoDrop® ND-1000 spectrophotometer (Thermo Fisher Scientific Inc., UK).

MTci2 L_4 and xL_3 cDNA synthesised using the SMART™ cDNA synthesis kit (Clontech) and single-stranded cDNA derived from adult worms were used for gene identification. cDNA for use in the real-time PCR experiments was synthesised from 1.5 μg total RNA using the SuperScript™ III Reverse Transcriptase kit (Invitrogen) and Random Primers (Promega) for first strand synthesis, following the manufacturer's protocol for each isolate and life-cycle stage. The cDNA concentration was determined using the NanoDrop® ND-1000 spectrophotometer. The cDNA was diluted to 50 ng/μL and aliquots made prior to storage at −20 °C.

2.3. Identification of T. circumcincta Pgps

Pgps were identified from MTci2 L4 and xL3 cDNA using degenerate PCR, followed by cloning and sequencing of the resulting PCR products. Degenerate primers designed to amplify the internucleotide binding domain (IBD) of *H. contortus* Pgps were as described previously by Sangster et al. (1999). PCR was carried out by combining 12.75 μL dH_2O, 2.5 μL 10× PCR buffer, 0.75 μL 50 mM $MgCl_2$, 1.0 μL dNTP (Roche), 0.5 μL Platinum® *Taq* DNA Polymerase (Invitrogen) and 2.5 μL each of the forward and reverse primers and template. All PCR reactions were run on an Applied Biosystems 2720 thermal cycler as follows: 5 min at 94 °C for a denaturing step followed by 40 cycles of 94 °C for 30 s, 55 °C for 30 s and 72 °C for 30 s, followed by 10 min at 72 °C and then a hold at 4 °C. PCR products were visualised on a 1% agarose gel with GelRed™ (Biotium,