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The soluble proteome phenotypes of ivermectin resistant and ivermectin susceptible *Haemonchus contortus* females compared

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ABSTRACT

Anthelmintics in the absence of vaccines have underpinned a parasite control strategy for over 50 years. However, the continued development of anthelmintic resistance (AR) threatens this control. Measuring early AR is difficult as there many routes that resistance can arise from within multi-nematode populations operating complex metabolism capabilities coupled to different drug management pressures. There is an urgent need to identify and measure early resistance in the field situation. Proteomic profiling of expressed soluble proteins offers a new approach to reveal a drug resistant phenotype within a complex protein pattern. The hypothesis under test was that established differences in drug response phenotypes between nematode isolates can also be measured in their comparative proteomes. As a case study, proteomic differences were measured between an ivermectin resistant and susceptible adult female *Haemonchus contortus*. Adult *H. contortus* females were extracted from the abomasa of six lambs. The nematodes had been maintained in the lambs as monospecific isolates of either ivermectin susceptible or ivermectin resistant worms. Comparative analysis of the soluble proteome was completed along with immunoproteomic analysis using pooled infection sera from the lambs. Following image analysis, spots of interest were excised and analysed by peptide mass fingerprinting and the proteins putatively identified using BLAST. Overall, a relative increase in the expression of proteins involved in the detoxification metabolic area was observed in the resistant isolate. In addition, Western blotting analysis also revealed differences in immuno-reactivity profiles between resistant and susceptible isolates. It can be concluded from this study that proteomic differences can be detectable between ivermectin susceptible and a resistant isolates of *H. contortus*, which could be further explored using other isolates to confirm if proteomic based fingerprinting offers molecular phenotyping or a new panel of resistance biomarkers.

1. Introduction

Parasitic gastroenteritis (PGE) causes serious health and economic problems for domesticated livestock worldwide (Neiuwhoff and Bishop, 2005). The blue-print application of anthelmintic regimes in intensive farming has resulted in increasing and irreversible anthelmintic resistance (AR)

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(Kaplan, 2004; Bartley et al., 2004, 2006; Sargison et al., 2001; Yue et al., 2003; Roos, 1997).

Anthelmintics are currently grouped into four families, the benzimidazoles (BZs), the imidazothiazoles, amino-acetonitrile derivatives (AADs) and the avermectins (Abbott et al., 2004; Kaminsky et al., 2008). Resistance has been reported since the introduction of the BZs in the 1960s (Kaplan, 2004). Resistance to ivermectin (22,23-dihydroavermectin B1) appears to occur via complex mechanisms, with many proposed target and non-target mutations associated with resistant status (Kohler, 2001). For example, reports have suggested that changes in drug transport pathways, detoxification systems and metabolism could also potentially lead to non-specific ivermectin resistance (Rothwell and Sangster, 1997; Xu et al., 1998). To date, ivermectin resistance selection has been reported in β tubulin and in multi-drug resistance proteins (MRP) along with members of the ATP-binding cassette (ABC) transporter families (Pritchard, 2007; Bourguinat et al., 2007; Xu et al., 1998). Current diagnostic methods for AR have disadvantages (Taylor et al., 2002), with regards to time, cost and the requirement that the animal is subjected to parasite challenge (Sayers et al., 2005; Davies et al., 2006) and may often result in false positive or false negative results (Eysker and Ploeger, 2000). Diagnostic methods based on genomic changes have been developed (Sangster and Gill, 1999), although further research is required for application within the field. The identification of genetic markers for AR has been proposed in several studies, namely, SNPs to assess allele frequencies of populations (von Samson-Himmelsjerna, 2006) and internal transcribed spacers (ITS) of ribosomal (r) DNA (Prichard and Tait, 2001) for DNA based assays. PCR methods have also been developed to detect ruminant nematodes in general with some success (von Samson-Himmelstjerna et al., 2002). Although there are disadvantages with regards to sensitivity and cost (Gasser, 2006). Immunological based diagnostics using ELISA for crude worm antigen detection has also been suggested (Eysker and Ploeger, 2000).

Haemonchus contortus is one of the most prevalent and therefore, economically important nematode species of sheep (von Samson-Himmelstjerna et al., 2002). Genetic variation between geographic isolates and drug resistant and susceptible isolates of *H. contortus* has been demonstrated (Redmond and Windham, 2005). Resistant nematode isolates to anthelmintics have been characterised including the *H. contortus* Chiswick avermectin resistant (CAVR) strain (Le jambre et al., 1995) and white river strain (WRS: Carmichael et al., 1987), from Australia and South Africa, respectively. However, the mechanisms underpinning ivermectin resistance remains elusive. Resistance detection arising from a single gene would be relatively simple (Sangster et al., 2005), but it is likely in mixed populations under different selection pressures other downstream processes, such as the modification of gene expression and post translational modifications, may confer the resistant phenotype. Therefore, the determination of protein expression changes in the cellular environment is important (Lilley et al., 2001) as proteins give rise to the phenotype (Barrett et al., 2005). Here we report protein expression differences between two phenotypes via proteomic fingerprinting.

2. Materials and methods

2.1. H. contortus collection and protein preparation

All animals used in this study were cared for under licence in accordance to the Animals (Scientific Procedures) Act 1986. All worm isolates were cultured at the Moredun Research Institute (Edinburgh). Six parasite free whether lambs were randomly selected and allocated to one of two experimental treatments at the Moredun Research Institute. Lambs were infected with either 10,000 ISE (also designated MHco3; Roos et al., 1990) or 10,000 Chiswick avermectin resistant (CAVR also designated MHco10; Le jambre, 1993) infective (L3) *H. contortus* larvae. The ISE (MHco3) strain, which is susceptible to all main classes of anthelmintics and has been accepted as the standard genome strain for *H. contortus* (Redman et al., 2012). The CAVR (MHco10) strain is resistant to avermectin (Le jambre, 1993; Le jambre et al., 1995). Both strains have been passaged through sheep at the Moredun Institute for number of years, the CAVR with ivermectin selection pressure and the CAVR strain is known to have genetic divergence from the susceptible (ISE) strain (Redman et al., 2012). Lambs were individually housed to prevent cross infection. Twenty eight days post infection lambs were humanely killed and exsanguinated prior to abomasal extraction and faecal collection. Abomasal contents were removed by washing with approximately 500 ml warm (39 °C) saline solution. Adult worms were removed from the abomasal wall by manual scraping and the worms transferred into a 50 ml centrifuge tube containing warm saline solution. Approximately 100 adult female worms were manually selected and transferred into a cryovial containing 500 μl of potassium phosphate buffer containing 0.1% Triton-X-100 and protease inhibitors (complete-mini protease inhibitor cocktail tablets, Roche Applied Science, Burgess Hill, West Sussex, UK, according to manufacturer's instructions). Only adult female worms were used during the analysis to prevent protein differences occurring based on sex rather than isolate. Worms were cryogenically frozen and stored at −80 °C prior to subsequent analysis.

2.2. Serum collection and faecal egg counts

Polyclonal anti-*H. contortus* serum was collected from experimentally infected lambs either challenged with ivermectin resistant (CAVR) or ivermectin susceptible (ISE) isolates of *H. contortus.* On days 0 (pre-infection), 14 (patency) and 28 (post-infection) lambs were blood sampled by jugular venepuncture (1 × 7 ml serum tube). Serum was extracted and stored at −30 °C until required. Faecal egg counts were taken throughout the experimental period to confirm infection levels.

2.3. H. contortus cytosolic protein purification

Nematodes were homogenised in 20 mM potassium phosphate buffer pH 7.4 containing 0.1% Triton-X-100