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# Drug-efflux and target-site gene expression patterns in *Haemonchus contortus* larvae able to survive increasing concentrations of levamisole *in vitro*

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ABSTRACT

While there is some evidence that changes in nicotinic acetylcholine receptor (nAChR) subunits confer resistance to levamisole in gastrointestinal helminth parasites, the exact nature of the resistance mechanism(s) is unclear. We utilised the presence of a resistant fraction within the Wallangra 2003 isolate of *Haemonchus contortus* larvae in order to subdivide the population into three subpopulations of larvae able to survive increasing concentrations of the drug. We then measured gene expression levels in the subpopulations and the larval population as a whole, focusing on genes encoding the subunit components of levamisole-sensitive receptors, genes encoding ancillary proteins involved in receptor assembly, and P-glycoprotein (P-gp) genes. The subpopulation surviving the lowest levamisole concentration showed increases of 1.5- to 3-fold in a number of P-gp genes (*Hco-pgp-3*, *-4*, *-10*, and *-14*) alongside unchanged receptor genes, compared to the whole Wallangra larval population. On the other hand, the subpopulation surviving the intermediate levamisole concentration showed an increase in only a single P-gp (*Hco-pgp-14*), alongside decreases in some receptor subunit (Hco-unc-63a) and ancillary protein genes (*Hco-unc-50, Hco-ric-3.1* and *3.1*). The subpopulation surviving the highest levamisole concentration showed further decreases in receptor subunit genes (*Hco-unc-63a* and *Hco-unc-29 paralogs*) as well as genes involved in receptor assembly (*Hco-unc-74, Hco-unc-50, Hco-ric-3.1* and *3.1*), alongside no increased P-gp gene levels. This suggests a biphasic pattern of drug resistance in the larvae of this worm isolate, in which a non-specific P-gp-mediated mechanism confers low levels of resistance, while higher level resistance is due to altered receptor subunit composition as a result of changes in both subunit composition and in the levels of proteins involved in receptor assembly.


## 1. Introduction

The use of anthelmintics for the control of intestinal parasites of livestock over the last 50 years has led to the development of resistance to each of the major chemical groups (McKellar and Jackson, 2004; Wolstenholme et al., 2004; Geary et al., 2012). The discovery of new drug compounds to facilitate the management of resistance is a slow and costly process that results in few novel compounds being released (Besier, 2007; Woods and Knauer, 2010). Hence, there is a need to manage the use of existing drugs in such a way as to minimise the impact of resistance, for example through the use of diagnostics to inform on the resistance status of worm populations present on specific properties, and hence inform on which drugs are still likely to be effective. A sensitive molecular test would be valuable for such resistance diagnostic purposes (Prichard et al., 2007). However, before such a test can be designed, an understanding of the mechanism or mechanisms of resistance to a particular anthelmintic or anthelmintic class is required, or at the very least the invariable presence of a genetic marker for that resistance. In addition, elucidating the mechanism/s of resistance may provide a better understanding of the rate at which resistance may emerge, as well as providing a tool to study parasite biology and drug targets (Sangster et al., 2005).

The cholinergenic anthelmintics, levamisole and pyrantel, act as agonists at nicotinic acetylcholine receptors (nAChRs). A subset of these receptors, known as the L-type receptors, are preferentially activated by levamisole and pyrantel (Martin et al., 2012). Activation of this L-type nAChR by the drug leads to sustained neuromuscular depolarization and spastic paralysis (Martin et al., 1997),

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thereby dislodging helminths from the gastrointestinal mucosa and leading to their expulsion from the host. The nAChRs are composed of five subunits forming a ring in the membrane with a central ion channel pore (Levandoski et al., 2005). The subunits are classified as subunits; those bearing two vicinal cysteines (*unc-63, unc-38*, *acr-8*) or β (non-α) subunits that lack the cysteines (*unc-29*, *lev-1*) (Martin et al., 2012). Previous reports on mechanisms of levamisole and pyrantel resistance in *Haemonchus contortus*, *Teladorsagia circumcincta*, *Trichostrongylus colubriformis* and *Ancylostoma caninum* have suggested a role for changes in the expression of a number of nAChR subunit genes: reduced transcription of *unc-63a* (Kopp et al., 2009; Sarai et al., 2013), reduced transcription of *unc-38* (Kopp et al., 2009), reduced transcription of *unc-29* (Kopp et al., 2009; Sarai et al., 2013), the presence of *Hco-acr-8b* (Fauvin et al., 2010; Williamson et al., 2011), and the presence of *Hco-unc-63b* (Neveu et al., 2010). Although not reported to date from studies of parasitic nematodes, it is possible that resistance may also be conferred by changes in ancillary proteins, RIC-3, UNC-74 and UNC-50, that are known to be essential for the proper formation, function and transport of the levamisole receptor, and for which mutations in *Caenorhabditis elegans* are known to confer resistance to levamisole (Gottschalk et al., 2005). Moreover, drug efflux mechanisms such as P-glycoproteins (P-gps) are involved in multidrug resistance in a variety of organisms, including helminths, and have been implicated in resistance to benzimidazoles and macrocyclic lactones (Kerboeuf et al., 2003; Blackhall et al., 2008; Lespine et al., 2012).

Sarai et al. (2013) showed that larvae of the Wallangra 2003 (WAL) isolate of *H. contortus* exhibited a distinctive plateau in response to levamisole in larval development assays, indicating the presence of a highly resistant fraction accounting for approximately 10–15% of the total population. While the bulk of the population behaved in a similar manner to susceptible worms in the larval development assay (equivalent $IC_{50}$ values) the presence of this resistant fraction resulted in a significant shift in $IC_{95}$ (13-fold higher than the susceptible isolate). The authors suggested that it might be difficult to associate gene transcription patterns with resistance in such heterogeneous isolates as the average transcription levels in a sample of worm larvae derived from the whole population might not be indicative of levels in the resistant fraction within the population. Considering that this resistant fraction, under further drug selection pressure, will most likely over time become the dominant phenotype, ascertaining the differences between the resistant fraction and the bulk of the population might give an indication of the genetic changes occurring in the early stages in the development of resistance. Such changes would therefore be the most critical to monitor in order to detect increasing levels of resistance.

A significant feature of all the parasitic nematode nAChR subunit gene expression studies described above has been the fact that they involved the comparison of genotypes or gene expression patterns between populations showing different levels of resistance, but also differing significantly in genetic background. Field-derived isolates showing resistance to the drug have most often been compared to susceptible isolates collected from different geographical regions, and/or from time periods several decades earlier than the resistant isolate. Hence, the differences seen in gene expression levels could be related to these diverse genetic backgrounds rather than being specifically associated with their level of susceptibility to the drug. The present study aimed to circumvent this issue to some degree by subdividing a single field-derived worm population solely on the basis of the ability of subpopulations to survive exposure to levamisole *in vitro*, and then comparing gene transcription patterns between the whole population and the various subpopulations. The heterogeneity in response to levamisole shown by WAL larvae described above provided an opportunity to subdivide the population based on *in vitro* levamisole sensitivity. Kotze et al. (2012) recently utilised such an experimental design in subdividing the WAL isolate on the basis of susceptibility of larvae to thiabendazole *in vitro*, followed by genotyping of a beta-tubulin gene in the survivors at each drug concentration. This allowed the authors to link specific SNP frequencies to relative drug resistance levels shown by the different subpopulations.

The present study therefore aimed to recover larvae from the levamisole-resistant fraction present within the WAL isolate, at three separate levels of drug exposure, and to compare these subpopulations to each other and to the WAL population as a whole. We measured gene transcription levels for putative subunit constituents of the levamisole sensitive receptor (*Hco-unc-38*, *Hco-unc-29.1–29.4*, *Hco-unc-63a*, *Hco-unc-63b*, *Hco-lev-1*, *Hco-acr-8a* and *Hco-acr-8b*) that have previously been implicated in resistance, as well as two further nAChR subunit genes (*Hco-acr-16* and *Hco-acr-26*). In addition, given their potential role in resistance, we measured transcription levels of four ancillary protein genes (*Hco-unc-50*, *Hco-unc-74*, *Hco-ric-3.1* and *Hco-ric-3.2*) (Martin et al., 2012), and nine P-gp genes.

## 2. Materials and methods

### 2.1. Collection of worms and eggs

The Kirby 1986 and Wallangra 2003 (WAL) isolates of *H. contortus* were maintained in sheep at the McMaster Laboratory, CSIRO Livestock Industries, Armidale, New South Wales (NSW), Australia. All animal procedures were approved by the F.D. McMaster Animal Ethics Committee, CSIRO Animal, Food and Health Sciences (Animal ethics approval number 12/18). The Kirby isolate is susceptible to all commercial anthelmintics (Albers and Burgess, 1988). The WAL isolate was derived from a worm population in the New England region of Northern NSW in 2003 (Love et al., 2003) that was resistant to levamisole (efficacy 79%), as well as benzimidazoles, closantel and short-acting macrocyclic lactones, such as ivermectin. Further selection pressure was applied to this isolate by use of a full dose of Cydectin (active ingredient moxidectin) over at least five subsequent generations. For the present study, sheep were infected with this isolate and were subsequently treated with a full dose of Cydectin 21 days after infection. After a period of at least 6 weeks had elapsed since the Cydectin dose, faeces were collected at various times for recovery of eggs.

Eggs were extracted from faeces by passage through two sieves (250 and 75 μm), followed by centrifugation within a sugar gradient (10% and 25%). Eggs were recovered from the interface between the sucrose layers and washed over a 25 μm sieve with water to remove the sucrose, before being treated with bleach briefly as described by Kotze et al. (2009). The eggs were washed thoroughly to remove the bleach and were then ready for use in larval assays or for *in vitro* drug selection.

### 2.2. Larval development assay

An *in vitro* larval development assay (LDA), as described by Gill et al. (1995) was used to characterize the WAL isolate with respect to levamisole response. Stock drug solution was serially diluted 2-fold in DMSO and 2 μl aliquots were added to a 96-well plate with a final drug concentration ranging from 10 to 0.0049 μg/ml. 200 μl of 2% agar (Davis Gelatine Co., powdered agar Grade J) was dispensed into each well, giving a final DMSO concentration of 1%. Plates were stored at 4 °C for up to a fortnight and re-equilibrated to room temperature before use. 30 μl of egg suspension comprising of ~3500 eggs/mL with amphotericin B (final concentration 25 μl/mL) and tylosin tartare (final