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Short Communication

The use of the larval development assay for predicting the *in vivo* efficacy of levamisole against *Haemonchus contortus* and *Trichostrongylus colubriformis*

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ABSTRACT

The larval development assay has been used for many years to measure the sensitivity of the free-living life stages of trichostrongylid nematodes to anthelmintics. The assay has applications in both drug discovery and the diagnosis of drug resistance. We revisited the usefulness of the larval development assay for diagnosis of resistance to levamisole using field-derived isolates of *Haemonchus contortus* and *Trichostrongylus colubriformis* showing varying levels of resistance to this drug *in vivo*. Each of the resistant isolates showed a plateau in their larval development assay dose-response at the highest drug concentrations tested, representing a highly-resistant fraction, amounting to between 6.9 and 55.1% of the populations. The remaining population fractions for the resistant isolates showed IC_{50} values from 1.4- to 17.8-fold higher than their corresponding susceptible isolate of the same species. We used a data set from the DrenchRite® test user manual to derive equations describing the relationship between the IC_{50} values for these low / moderate resistance components of each population and *in vivo* drug efficacy, and then used these equations to estimate the expected *in vivo* efficacy of levamisole against this population component of each isolate. A combination of this expected efficacy, with the known zero efficacy of the drug *in vivo* against the highly-resistant population fractions in each isolate, allowed us to calculate a predicted drug efficacy for the whole population for each isolate. The predicted levamisole efficacies for the three resistant *H. contortus* isolates were 88.8, 84.1 and 43.7%. These compared favourably with the actual efficacy of the drug against these isolates as determined in faecal egg count reduction tests or total worm count studies: 79, 66.3 and 40.6%, respectively. Similarly, for *T. colubriformis*, predicted efficacies of 82.0 and 1.8% compared favourably with the actual efficacies of 65–92 % and 0%, respectively. This study illustrates the usefulness of the larval development assay as a diagnostic tool for predicting *in vivo* efficacy of levamisole against *H. contortus* and *T. colubriformis*.

1. Introduction

Parasitic nematodes have a significant impact on livestock production systems worldwide. The control of these parasites relies on the use of anthelmintics, however they have shown an ability to develop resistance to different chemical groups used for their control over many years (Kaplan and Vidyashankar, 2012; Kotze and Prichard, 2016). One important aspect of controlling parasitic nematodes in the face of increasing levels of drug resistance is an ability to detect resistance in field populations. This allows for drench-use decisions to be made based on some knowledge of the likely efficacy of specific drugs. Ideally, when one detects resistance to a specific drug group, this drug would no longer be used. However, given the limited options available in terms of drugs for which no resistance has been reported, it may not be possible to completely exclude the use of a drug to which some resistance has been detected. Hence, in such cases, it is important to be aware of not only the presence of resistance, but also the likely impact of the resistance on drug efficacy. In this way, drugs whose effectiveness is reduced by only a small degree may still be considered useful, particularly if they are to be used as a component of a combination drench product. Two aspects of the use of such tests are therefore important: firstly, to be able to detect resistance when it is present at only low levels, and secondly to be able to quantify the likely effect of resistance on drench efficacy. The second aspect is often more important than the first for the older anthelmintic drugs as resistance is widespread in the field already, however, its impact on drug efficacy is variable.

There are three general types of anthelmintic resistance tests: firstly, *in vivo* tests (most commonly the faecal egg count reduction test

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(FECRT)), secondly, *in vitro* assays with the free-living life stages (for example, the larval development assay, the egg hatch assay, various migration and motility assays), and thirdly, molecular assays (for example, measurements of beta-tubulin SNP allele frequencies) (Taylor et al., 2002; Coles et al., 2006). The FECRT informs directly on both the presence of resistance and its impact on drug efficacy, however, it is very insensitive, and hence is not suitable for detecting resistance when it is present at only low levels (Martin et al., 1989). *In vitro* assays with free-living stages have shown some promise (Coles et al., 2006), however have not been adopted for use with field isolates largely because they have not been able to effectively quantify resistance across multiple drug classes and worm species. Molecular tests provide the most attractive option in terms of potential sensitivity and cost, however their widespread use in the field is not yet possible, due at least partly to a lack of knowledge on specific resistance mechanisms or useful resistance markers, particularly for the macrocyclic lactone drugs (Kotze et al., 2014).

Given the need for drug resistance diagnostics, the present study aimed to revisit the use of the larval development assay for detection of levamisole resistance in field-derived isolates of *Haemonchus contortus* and *Trichostrongylus colubriformis*. We utilised the relationship between larval development assay data and predicted drug efficacy reported by Lacey et al. (1995) as the basis for the Drenchrite® resistance detection test. We examined the ability of the larval development assay to predict the likely impact of resistance detected in larval life stages on the expected *in vivo* efficacy of levamisole against adult worms from drug-resistant isolates of *H. contortus* and *T. colubriformis*.

2. Materials and methods

2.1. Parasites

Four isolates of *H. contortus* were used for the study:

i) *Hc* Kirby: isolated from the field at the University of New England Kirby Research Farm in 1986; susceptible to all commercial anthelmintics (Albers and Burgess, 1988).
ii) *Hc* Wallangra: isolated from the New England region of Northern New South Wales in 2003; at the time of isolation from the field it was resistant to benzimidazoles, closantel, levamisole, ivermectin and moxidectin (Love et al., 2003). The isolate has been further selected using moxidectin (Cydectin®) over at least five generations. The efficacy of levamisole reported by Love et al. (2003) was 79% (based on a FECRT, n = 10 animals per group).
iii) *Hc* GWBII: a passaged variant of the Wallangra isolate. The isolate has been maintained in donor sheep at the Invetus animal house facility (Armidale, NSW), and further passaged with Triton® drench (containing ivermectin, albendazole and levamisole) treatments. The efficacies of abamectin, albendazole and levamisole were measured as 17.7%, 57.6% and 40.6%, respectively, in 2015 (based on total worm counts, n = 8 animals per group) (trials conducted by Invetus).
iv) *Hc* MPL-R: isolated from a property in southwest Queensland, Australia, in 2014, as described previously (Raza et al., 2016). Zolvix® (active ingredient = monepantel) showed zero efficacy when tested in 2014 (based on total worm counts, n = 4 animals per group). The isolate is also resistant to macrocyclic lactones (ivermectin efficacy 8.8%, based on total worm counts, n = 8 animals per group) as well as albendazole and levamisole (efficacies of 41.8% and 66.3%, respectively, based on FECRTs, n = 10 animals per group) (trials conducted by Invetus).

Three isolates of *T. colubriformis* were used for the study:

i) *Tc* McM: isolated by CSIRO in the 1950's from the Southern Tablelands of NSW; susceptible to all commercial anthelmintics;
ii) *Tc* Mox-R: isolated from a goat farm in the Gold Coast hinterland of QLD (Le Jambre et al., 2005); resistant to macrocyclic lactones (abamectin and moxidectin efficacies of 66% and 58%, respectively); also resistant to levamisole (efficacy 65–92 % in five separate total worm count trials, n = 8 animals per group in each trial) (trials conducted by Invetus).
iii) *Tc* MPL-R: isolated from a farm in the New England region of NSW in 2014. This isolate was recovered from a sheep at the Invetus facility following a positive egg count in an animal treated with Zolvix; levamisole efficacy was zero in a single animal tested (based on faecal egg count; Invetus).

Infected sheep were housed at the Invetus animal house facility in Armidale, NSW, and at the Commonwealth Scientific and Industrial Research Organisation (CSIRO) FD McMaster laboratory near Armidale, NSW. All animal procedures were approved by the University of New England's Animal Ethics Committee (Approval Number 2015-065), or the FD McMaster Animal Ethics Committee, CSIRO (Approval Number AEC 13/23). During the course of the study, faeces were collected into bags attached to the rear of the animals, and sent in sealed plastic bags by overnight courier at ambient temperature to the CSIRO laboratory in Brisbane, Queensland, for recovery of eggs using filtration and sucrose gradient centrifugation as described by Kotze et al. (2009).

2.2. Chemicals

Technical grade levamisole hydrochloride was purchased from Sigma Chemical Co. A stock solution was prepared in DMSO at 10 mg/mL followed by 2-fold serial dilutions in DMSO to generate multiple working solutions.

2.3. Larval development assay

A larval development assay was used to measure the effects of levamisole on the development of *H. contortus* and *T. colubriformis* larvae from eggs to third-stage larvae following the method described previously (Kotze et al., 2009), as modified from the original method of Gill et al. (1995). Levamisole (2 µL of each working solution) was impregnated into 200 µL of 2% agar in 96-well plates. Final levamisole concentrations ranged from 86 µM to 0.042 µM over a series of 2-fold dilutions. Control assays received DMSO alone (final DMSO concentration in levamisole and control wells was 1% v/v). Approximately 80 nematode eggs were added to each well using a repeat dispensing pipette, and plates were incubated overnight at 27 °C. The larvae were fed the next day with growth medium containing live *E. coli* (Kotze et al., 2009). The plates were incubated for another six days, larvae were then killed using Lugol's iodine, and the number of fully grown infective L3 were counted in each well. Each concentration-response experiment consisted of duplicate wells at a range of chemical concentrations, as well as 12 control wells per plate containing DMSO (at 1% v/v) only. Each isolate was examined in replicate experiments.

2.4. Data analysis

The number of fully grown L3 in each well was expressed as a percentage of the mean number of L3 in multiple control (DMSO only) wells. Larval development data from duplicate assay wells in two replicate experiments were combined to provide n = 4 data points at each drug concentration. The data were then analysed using non-linear regression with GraphPad Prism® software (GraphPad Software Inc., USA, version 6.01). The drug resistant isolates showed the presence of a plateau in response at the highest drug concentrations, and hence a non-normalised model with variable slope was used to fit the data. The percentage development at the plateau in the concentration response shown by the resistant isolates at high drug concentrations was used to define the 'highly-resistant fraction' in the larval population. The IC_{50}