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Comparison of two *in vitro* methods for the detection of ivermectin resistance in *Haemonchus contortus* in sheep

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Summary

Gastrointestinal parasitic nematodes in sheep cause severe economic losses. Anthelmintics are the most commonly used drugs for prophylaxis and therapy against parasitic helminths. The problem of drug resistance has developed for all commercially available anthelmintics in several genera and classes of helminths. *In vitro* and *in vivo* tests are used to detect anthelmintic resistance. Two *in vitro* methods (larval migration inhibition test and micromotility test) for the detection of ivermectin (IVM) resistance were compared using IVM-resistant and IVM-susceptible isolates of *Haemonchus contortus*. The degree of resistance for each test was expressed as a resistance factor (RF). The micromotility test was more sensitive for quantitatively measuring the degree of resistance between susceptible and resistant isolates. The RFs for this test for IVM and eprinomectin ranged from 1.00 to 108.05 and from 3.87 to 32.32, respectively.
Keywords: anthelmintic resistance; *in vitro* detection; *Haemonchus contortus*; Ivermectin

Introduction

Resistance to anthelmintics has become a serious problem in countries with developed sheep and goat industries, especially Australia, New Zealand, South Africa, and South America (Várady *et al*., 2011). Macrocyclic lactones (MLs) are currently the most common group of broad-spectrum anthelmintics for the control of nematode parasites. Reports of resistance to MLs in nematodes of small ruminants, however, have increased over the last decade (Álvarez-Sanchez *et al*., 2006; Diez - Banos *et al*., 2008; Artho *et al*., 2007; Bartley *et al*., 2006; Čerňanská *et al*., 2006). A number of *in vitro* and *in vivo* tests have been developed for the detection of anthelmintic resistance (Taylor *et al*., 2002). Several *in vitro* tests have been described for testing the anthelmintic activity of MLs for the detection of resistance. These tests depend on an assessment of paralysis in larvae (Gill *et al*., 1991; Kotze *et al*., 2006) or of inhibited larval development (Coles *et a*l., 1988; Hubert & Kerboeuf, 1992; Dolinská *et al*., 2013). A micromotility meter has also been developed to evaluate the motility of various larval and adult nematodes as a criterion of paralysis in the absence or presence of anthelmintics, based on a quantitative measurement of motility by photo-detectors (Folz *et al*., 1987a). In this method, infective larvae of *Haemonchus contortus* and *Trichostrongylus colubriformis* are exposed to an anthelmintic for 24 h and then transferred to the micromotility meter (Folz *et al*., 1987b,c; Coles *et al*., 1989).

The present study was designed to evaluate the potential of two *in vitro* methods for the detection of ML resistance in the sheep nematode *H. contortus*. The results of the tests are compared, and the suitability of the methods for the field screening of ML resistance is discussed.

Material and Methods

Parasites isolates

Two susceptible isolates of *H. contortus*, McMaster and ISE (MHco3), and five resistant isolates, White River (WR), CAVR, MOX23, ISE-SL, and ISE-SLI were used in this study. The susceptible ISE isolate was obtained as an inbred isolate of MHCo3 (Roos *et al*., 2004). The McMaster isolate was isolated prior to the introduction of broad-spectrum anthelmintics and is routinely used as a reference susceptible isolate in similar studies (Gill *et al*., 1995). WR was isolated from the field in South Africa and demonstrated resistance to IVM (30 % efficacy at 0.2 mg/kg) and to the benzimidazoles, rafoxanide, and closantel (Van Wyk *et al*., 1988). CAVR is resistant to MLs and moderately resistant to the benzimidazoles (Le Jambre *et al*., 1995). MOX23 has been selected for 23 generations in the laboratory for moxidectin resistance (Ranjan *et al*., 2002). ISE-SL has been selected in the laboratory for IVM resistance (Coles *et al*., 2005). ISE-SLI, derived from ISE-SL, was selected further in our laboratory using 1.5× the recommended dose of IVM.

Trial design

All isolates had been routinely maintained by passage through individually housed, helminth-naive, 5-6 month-old lambs. The lambs were infected orally with 5000 third-stage (L3) larvae of each isolate. Faecal samples were collected 35-50 days after experimental infection, and coprocultures were subsequently prepared by the method described by Henriksen and Korsholm (1983). L3 larvae were isolated from the faecal cultures by standard Baermann filtration and stored at 10 °C in distilled water prior to use. The larvae were tested within one month after collection.

Larval migration inhibition test (LMIT)

The LMIT was performed as described by Kotze *et al*. (2006) in 96-well microtiter plates (Millipore, Australia). Stock drug solutions of IVM (10 mg/ml in DMSO) were serially diluted 2-fold, 0.5 µl of each dilution were added to the wells of drug plates, followed by 20 µl of distilled water and 30 µl of a solution containing infective L3 larvae (55 – 60 larvae mixed with amphotericin B – 250 mg/ml). The plates were placed into plastic bags and incubated for 24 h at 27 °C. Rinse plates were prepared at the same time and in the same format as their corresponding drug plates. The culture medium comprised 4.75 µl of each drug dilution and 400 µl of distilled water. These plates were kept at room temperature. Agar/filter plates were also prepared on the same day: 75 µl of agar (0.125 %) were added to the 20-µm filter of each well, and the plates were stored at room temperature. The following day, 325 µl of the solution from the rinse plate were added to the corresponding well of a plate, and the agar/filter plate was lowered into this plate and was incubated several hours at 27 °C. Twenty-four hours after the establishment of the drug plates, the worms from the drug plates were transferred to the agar/filter plates at the corresponding positions. The remaining worms in the drug plates were collected by adding 50 µl of the corresponding solution from the rinse plates to the drug plates, mixed, and then added to the agar/filter plates. The total content of each well consisted of 325 µl of solution from the rinse plates, 75 µl of 0.125 % agar, 50 µl of L3 larvae from the drug plates, and 50 µl of the solution from the rinse plates. The prepared plates were placed into clear plastic bags and incubated under a light at 27 °C for 48 h. The filters were then removed, and all worms were counted.

Micromotility test

The micromotility test was performed as described by Folz *et al*. (1987). Movement of the larvae caused a variation in light rays refracting from the meniscus, and consequently a variation in the electrical signal produced by a photo-detector located at the level of the meniscus. The numerical representation of the modulated signal is termed the motility index. *H. contortus* L3 larvae were mixed with water (500 L3/ml). A stock solution was prepared by dissolving 1 mg of anthelmintic (IVM or eprinomectin) in a mixture of 200 µl of acetone and 50 µl of Tween 20 and then adding 750 µl of distilled water. An identical blank solution but with distilled water replacing the anthelmintic was also prepared. L3 larvae were exposed to anthelmintic in four concentrations: 0.1, 1.0, 10.0, and 100.0 µg/ml. Eprinomectin at the tested concentrations was less effective than IVM against all isolates, so we increased its

Table 1. Origin and status of anthelmintic susceptibility (S) or resistance (R) of ISE, McMaster, ISE-SL, ISE-SLI, MOX-23, WR and CAVR isolates of *Haemonchus contortus*

Isolate	R/S	Reference	Origin
ISE	susceptible	Roos et al. (2004)	Kenya
McMaster	susceptible	CSIRO, Armidale	Australia
ISE-SL	IVM	Coles (2005)	Great Britain
ISE-SLI	IVM	Coles (2005)	Great Britain
MOX-23	IVM	Prichard et al. (2002)	Canada
WR	BZ, CLO, IVM, RAF	Van Wyk and Malan (1988)	South Africa
CAVR	IVM, BZ	LeJambre et al. (1995)	Australia

BZ, benzimidazole; CLO, closantel; IVM, ivermectin; RAF, rafoxanide

Table 2. Arithmetic mean ± SD of LD_{50} and LD_{99} for susceptible and resistant isolates obtained in the larval migration inhibition test with ivermectin

Isolate	LD_{50} (µg/ml) ± SD	LD_{99} (µg/ml) ± SD
McMaster	1.03 ± 0.72	2.80 ± 0.61
ISE	1.25 ± 0.48	3.00 ± 1.24
MOX23	2.13 ± 1.77	34.36 ± 25.14
CAVR	2.80 ± 0.72	20.10 ± 12.12
WR	3.80 ± 0.35	8.77 ± 4.92
ISE-SL	3.65 ± 0.48	48.18 ± 57.59
ISE-SLI	5.05 ± 1.98	22.87 ± 7.45

concentration to 300 µg/ml to obtain reductions in motility. Two susceptible and four resistant isolates of *H. contortus* were tested. For each drug to be tested, 450 µl of the suspension containing *H. contortus* larvae and 50 µl of the acetone/drug solutions at the four concentrations or the blank solution were added to culture tubes (10 × 75 mm). The culture tubes were covered with parafilm to prevent evaporation. The tubes were incubated at 8 – 12 °C for 24 h, and the worms were then processed with the micromotility meter.

Data analysis

Motility indexes for the larval micromotility test were transformed to percent reductions of motility by the formula: [(vehicle control index - treatment index)/(vehicle control index - background index)]×100. The results are presented as a resistance factor: the ratio of the LD_{50} or LD_{99} for the resistant strain to the LD_{50} or LD_{99} for the susceptible strain. The LD_{50} and LD_{99} values were determined by a logistic regression model (Dobson *et al*., 1987).

Results

Larval migration inhibition test

The results of the LMIT for IVM resistance are shown in Table 2. The LD_{50} values of the resistant strains differed from those of the susceptible strains by an RF from 1.7 to 4.9, and the LD_{99} values differed by an RF from 2.9 to 17.2 (Table 3). The LD_{99} values were more variable, producing a higher coefficient of variation. The mean RFs for the LMIA are shown in Table 3. The LD_{99} data were better able to distinguish between the susceptible and resistant isolates.

Micromotility test

The mean reduction in motility and the LD_{50} values for the susceptible and resistant isolates of *H. contortus* after treatment with IVM and eprinomectin are presented in Tables 4 and 5. An IVM concentration of 10 µg/ml (Table 4) significantly reduced the larval motility of the susceptible *H. contortus* isolates, but only the treatment with 100 µg/ml IVM substantially reduced motility in the resistant isolates. The sensitive isolates displayed higher susceptibilities to the drugs tested, as indicated by the LD_{50} values.

Discussion

The values of RFs in LMIT in our study were as high as 12.2 for MOX-23 and 7.17 for CAVR. Kotze *et al*. (2006) with identical test obtained RFs for CAVR and MOX-23 of 1.7 and 5.7, respectively. LMIT utilize the ability of larvae to migrate through a filter mesh.

Table 3. Resistance factors for ivermectin (IVM) in the larval migration inhibition test

Strains	IVM RF_{50}	IVM RF_{99}
WR/McM	3.68	3.13
CAVR/McM	2.71	7.17
MOX23/McM	2.06	12.27
ISE-SL/McM	3.54	17.20
ISE-SLI/McM	4.90	8.16
WR/ISE	3.04	2.92
CAVR/ISE	2.24	6.70
MOX23/ISE	1.70	11.45
ISE-SL/ISE	2.92	16.06
ISE-SLI/ISE	4.04	7.62

Table 4. Mean reduction (%) in motility of L3 larvae of resistant and susceptible strains of *H. contortus* after incubation in different concentrations of ivermectin (IVM)

	Concentration of IVM (µg/ml)				
Isolate	**0.1**	**1**	**10**	**100**	**LD_{50}**
WR	0	0	26.2	49.7	90.76
CAVR	0	55.2	52.8	76.1	4.2
McMaster	0	39.7	88.9	80.9	1.57
ISE	0	59.3	97.8	91.4	0.84
MOX23	13.7	41.2	78.9	96.4	1.57
ISE SL	11.2	32	6.7	80.5	78.33

A layer of 0.125 % agar is applied to the filter mesh to create an additional barrier for the migrating larvae. The test was able to detect a level of resistance of 10 % in a population. Kotze *et al*. (2006) detected resistance to MLs in *H. contortus* but not in *T. colubriformis* and *Ostertagia circumcincta*. Despite the potential of LMITs, the tests present some difficulties. Several factors may contribute to poor sensitivity. In mixed field parasitic populations, IVM may have different potencies against different species of gastro-intestinal parasites. The LMIT is suitable only for *H. contortus*, which seriously limits the utility of this test for monitoring resistance in field surveys. The RFs obtained by LMITs are significantly lower than those obtained by larval development tests (Dolinská *et al*., 2012, 2013), which may indicate a lower sensitivity of the LMIT for the detection of IVM-resistant parasites. Additionally the cost of the microtiter plates (Millipore), in which the LMIT is performed, is considerable. From a practical point of view, the test cannot thus be used for the detection of IVM resistance in mixed populations of trichostrongylids.

Calculation of LD_{99} values in the in vitro tests can significantly increase test sensitivity and identify resistance when only a small proportion of the worm population is resistant (Várady *et al*., 2007). Based on our data from LMIT the RF99 values obtained from CAVR, MOX23, ISE-SL and ISE-SLI isolates were 1.6 – 6.7 times higher compare to RF50. Only LD_{99} values from WR isolate were almost identical with LD_{50} values. The possible reason for this could be the different proportion of resistant individuals in the isolates. While CAVR, MOX23, ISE-SL and ISE-SLI isolates showed high resistance in vivo, WR isolated from the field in South Africa demonstrated moderate resistance to IVM.

In our study the micromotility meter was used to evaluate the effect of two ML anthelmintics on the motility of *H. contortus* L3 larvae. Paralysis tests described previously by Martin and Le Jambre (1979), Barton (1983), and Geerts *et al*. (1989) were based on visual evaluations of larval paralysis, which is considered to be subjective and not sufficiently reproducible. By using a micromotility meter, the degree of subjectivity (assessing whether a larva is in motion) can be minimized. The micromotility meter has been described as a sensitive tool for measuring the *in vitro* motility of larval and adult *H. contortus* and *T. colubriformis* (Bennett and Pax, 1986; Folz *et al*., 1987b, c). Some studies, however, have demonstrated a lower ability to distinguish between susceptible and resistant isolates of *H. contortus* (Coles *et al*., 1989; Várady & Čorba, 1998).

In the current study, the RFs ranged from 1.00 to 108.05 for IVM and from 3.87 to 32.32 for eprinomectin. Similarly high RFs (maximum 88.51-345.6) were obtained by Demeler (2005) in a comparison of the motilities of susceptible and resistant adult *T. colubriformis* and *O. circumcincta*. The use of adult parasites, however, requires the sacrifice of animals and so is not financially practical for field surveys. Our micromotility testing provided relatively high RFs, indicating a good ability to differentiate between IVM-resistant and -susceptible strains of *H. contortus*. The ability to measure the motility of larvae in mixed infections of gastrointestinal parasites, however, is questionable, because different species of gastrointestinal nematodes have different motilities, e.g. the motility of *H. contortus* is much higher initially but is markedly lower after about 10 minutes (Gill *et al*., 1995). The present version of the micromotility test is thus unsuitable for measuring levels of resistance under field conditions.

Table 5. Mean reduction (%) in motility of L3 larvae of resistant and susceptible strains of *H. contortus* after incubation in different concentrations of eprinomectin (EPM)

	Concentration of EPM (µg/ml)						
Isolate	0.1	1	10	100	200	300	LD_{50}
WR		43.4	43.4	53.3	72.2	71.7	41.04
CAVR	35		49.48	29.3	65.3	64.7	49.77
McM	0	37.1	76.4	63.9	100	100	3.98
ISE	34.6	53.23	65.2	69.3	100	100	1.54
MOX23	15.8	27.7	40.3	70.7	100	100	15.4
ISE SL	14.26	11.65	68.5	50.16	45.1	66.9	47.27

Table 6. Resistance factors for ivermectin (IVM) and eprinomectin (EPM) in the micromotility test

Strains	IVM RF_{50}	EPM RF_{50}
WR/McM	57.81	10.31
CAVR/McM	2.67	12.50
MOX23/McM	1.00	3.87
ISE-SL/McM	49.89	11.88
WR/ISE	108.05	26.65
CAVR/ISE	5.00	32.32
MOX23/ISE	1.87	10.00
ISE-SL/ISE	93.25	30.69

As it was demonstrated in the previous studies, the use of avermectin analogs (eprinomectin, ivermectin aglycone) significantly increased the ability of the in vitro tests to differentiate between IVM-resistant and -susceptible isolates (Dolinská *et al*., 2013). These analogues produce 2 – 3 times higher RR compared to IVM, which was not a case of our study in MMT. The reason for this is unclear and could be related to the motility cycles of the larvae. After incubation in the dark at 25 °C, the L3 larvae of *H. contortus* were stimulated to move in a rapid sinusoidal motion by exposure to light. They then remain active for at least 10 minutes and maximum activity was reached after a short lag time of l-2 min. Thus LD_{50} values were more dependent on exposure of L3 larvae to the light and not the length of incubation.

The lowest RR obtained for MOX23 isolate in both tests suggested different or additional genetic mechanism in MOX resistance compared to IVM resistance. IVM is >130-fold more potent than moxidectin at inhibiting pharyngeal pumping (and thus blocking feeding) in *Caenorhabditis elegans*, and IVM initially stimulates motility before paralyzing this nematode. Moxidectin appears to only cause paralysis (Ardelli *et al*., 2009). Additionally slightly higher RR (especially for LD_{99}) obtained in LMIT suggest that LMIT may be a superior tool to monitor resistance to MOX.

The potential of the two tests for use in monitoring IVM resistance is questionable and additional experimental work is require optimizing the test in field condition.

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