Evidence of multiple mechanisms of avermectin resistance in haemonchus contortus--comparison of selection protocols

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Keywords

selection, comparison, contortus, haemonchus, resistance, avermectin, protocols, mechanisms, evidence, multiple

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Evidence of multiple mechanisms of avermectin resistance in *Haemonchus contortus*—comparison of selection protocols

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Abstract

Three isolates of *Haemonchus contortus* selected for avermectin resistance in sheep were compared in three in vitro pharmacological tests previously shown to discriminate between field isolates of *H. contortus* resistant and susceptible to the avermectins. Two isolates, F7-A and IVC, were selected for avermectin resistance in the laboratory from a reference susceptible isolate using suboptimal doses of ivermectin (LD_{95}) for 7 and 16 generations, respectively. In these isolates avermectin resistance was not associated with a decreased sensitivity to avermectin inhibition of larval development or L3 motility but was associated with an increased sensitivity to paraherquamide. The third isolate, Warren, was derived from an overwhelmingly avermectin-susceptible, mixed species field isolate in a single generation by propagating the small number of survivors of a 0.2 mg/kg ivermectin treatment (i.e. $10 \times LD_{95}$). This isolate, like previously characterised avermectin-resistant *H. contortus* isolates derived from the field in South Africa and Australia, showed a markedly reduced sensitivity to avermectin inhibition of larval development and L3 motility, as well as an increased sensitivity to paraherquamide. These results suggest that avermectin resistance can manifest itself in different ways and that the two selection protocols used to generate the F7-A, IVC and Warren isolates have resulted in the selection of different resistance phenotypes. © 1998 Australian Society for Parasitology. Published by Elsevier Science Ltd.

Key words: Avermectin; *Haemonchus contortus*; Ivermectin; Nematode; Resistance; Milbemycin

1. Introduction

Anthelmintic resistance poses a threat to the sustainable management of livestock parasites. As new compounds are released there is a need to understand the nature of resistance mechanisms that may arise and their implications for parasite control. Essential to any examination of anthelmintic resistance is the availability of resistant isolates. In the absence of field resistance, isolates are often selected for resistance to an anthelmintic in the laboratory. However, the application of selection pressure on a closed population in the laboratory may not necessarily yield the same resistance mechanisms as selection in the field. It is therefore important to validate results obtained with laboratory selected isolates against the behaviour of resistant isolates from the field.

The avermectins (AVMs) and related milbemycins are broad spectrum anthelmintics with

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potent activity against the common trichostrongylid nematode parasites of sheep [1]. Resistance to these anthelmintics in *Haemonchus contortus* and *Ostertagia* spp. has been reported from the field in many countries [2], while laboratory selection has produced AVM-resistant isolates of *H. contortus* [3] and *Trichostrongylus colubriformis* [4]. Isolates resistant to the AVMs are also resistant to the milbemycins in vivo [5, 6] confirming in vitro evidence that the AVMs and milbemycins share a common mode of action [7].

In vitro techniques using the free-living stages have been developed to characterise the pharmacological effects of the AVM/milbemycins on trichostrongylid nematode parasites and examine the impact of AVM resistance. These include assays that measure effects on larval development [8] and L3 motility [9]. While the motility of L1 larvae is inhibited in the presence of AVM/milbemycins, the concentrations of drug required to induce this effect are significantly higher than those required to inhibit their development to the L3 stage. This suggests that inhibition of larval development results from a different effect of the drug to that which inhibits motility [8]. Field isolates of *H. contortus* resistant to ivermectin (IVM) in vivo have a reduced sensitivity to AVM/milbemycin inhibition of larval development and motility [8, 9].

Paraherquamide, an anthelmintic structurally unrelated to the AVM/milbemycins [10], is also a potent inhibitor of nematode motility [11]. AVM-resistant isolates of *H. contortus* have been found to be up to 10-fold more sensitive to paraherquamide-induced paralysis of L3 larvae than AVM-susceptible isolates [11].

Together, the larval development, L3 paralysis and paraherquamide sensitivity assays provide a basis for characterising AVM resistance in parasitic nematodes. AVM-resistant *H. contortus* isolates derived from the field in South Africa and the Australian CAVR *H. contortus* isolate demonstrate a consistent pattern of responses in these assays [8, 9, 11, 12]. This suggests that a common resistance mechanism has evolved independently in these isolates.

In the present paper we compare the in vitro pharmacological profiles of three isolates of *H. contortus* selected in the laboratory for resistance to the AVMs. Two isolates, F7-A and IVC were selected for AVM resistance from a closed parasite population by dosing with suboptimal concentrations of IVM [3, 13]. The third isolate, Warren, was derived by propagating the survivors of a mixed field isolate after selection with IVM at its recommended dose, 0.2 mg/kg. While the pharmacological profile of the Warren isolate was indistinguishable from that of field derived AVM-resistant *H. contortus* isolates, a different AVM resistance phenotype was evident in the F7-A and IVC isolates.

2. Materials and methods

2.1. Chemicals

Avermectin B_1 (AVM B_1), avermectin B_2 (AVM B_2), IVM, IVM-monosaccharide, IVM-aglycone and paraherquamide were gifts from Merck, Sharp and Dohme, USA. Pure samples of levamisole (LVS) and mebendazole (MBZ) were gifts from Smith Kline Beecham, Australia and thiabendazole (TBZ) was a gift from Merck, Sharpe & Dohme, Australia. All other reagents used were of the highest grade commercially available.

2.2. H. contortus *isolates*

The Branchburg (BBH) *H. contortus* isolate was isolated in 1957 and has been not been exposed to anthelmintics since that time [3]. The IV-A F7 (F7-A) isolate was selected from the BBH isolate in the laboratory by suboptimal IVM treatment once per generation and requires a 4-fold greater IVM concentration to kill 95% of the adult population [3]. Selection of this isolate for a further nine generations produced the IVC isolate which requires 10-fold more IVM to kill 95% of the adult population compared to the parent isolate [13]. The McMaster *H. contortus* strain was isolated in Australia prior to the advent of broad spectrum anthelmintics and is routinely used as a reference susceptible strain in resistance studies. Isolation of the Warren *H. contortus* isolate is described below. The AVM-resistant Stellenbosch *H. contortus* isolate was isolated from the field in South Africa [14].

All isolates were routinely maintained by cryopreservation and/or by passage in four to six month old worm-free Merino wethers. Nematode eggs and L3 larvae for in vitro studies were isolated by standard techniques [8, 9].

2.3. Isolation of the Warren H. contortus isolate

A mixed species field isolate was obtained from faeces collected from mixed-aged goats on a property in the Ingleburn area of NSW on which IVM had been used extensively. The faecal samples were collected six weeks after an IVM drench. The initial isolate, comprising 2% *H. contortus* and 98% *T. colubriformis/O. circumcincta* was overwhelmingly AVM-susceptible. A helminth-naive sheep was infested with 10 000 L3s of this isolate then treated with IVM at 0.2 mg/kg once the infection had become patent. Exsheathed L3 larvae collected pre-treatment were found to be 57% *O. circumcincta*, 4% *T. colubriformis* and 39% *H. contortus*. Treatment with IVM at 0.2 mg/kg reduced the egg count of this sheep to < 25 epg, however, approximately 1000 L3s were recovered and used to infect a second sheep. The egg count of the second sheep was increased by reinfecting it with larvae cultured from its own faeces before it was treated with 0.067 mg/kg IVM. This treatment reduced its average egg count from 700 to 300 epg. Speciation of exsheathed L3s pre- and post-treatment found the isolate to be > 90% *H. contortus* with a small number of *O. circumcincta* (< 10%). Pre-treatment, the isolate in the second sheep returned an elevated resistance ratio (*RR*) for AVM B_2 in the larval development assay (*RR* = 4.6), treatment with 1/3 of the recommended dose of IVM increased this ratio (*RR* = 13); further treatment with 0.2 mg/kg did not change the in vitro resistance status of the shed larvae (*RR* = 12). After the 0.2 mg/kg IVM treatment the isolate was 100% *H. contortus* and was designated the first generation of the Warren *H. contortus* isolate. To determine the stability of the AVM-resistance present, the post-0.2 mg/kg IVM Warren *H. contortus* isolate was repassaged. The isolate remained AVM-resistant ($RR_{AVM B2}$ = 13). The presence of AVM-resistant *H. contortus* in the original isolate was confirmed by repeating the above described process starting with a further sample of the original mixed species isolate.

2.4. In vitro assays

The sensitivity of the isolates to levamisole, benzimidazoles and a series of AVMs was determined in a larval development assay [8, 15]. Motility assays using L3 larvae were used to determine the resistance status of these isolates with respect to AVMs [9], and their sensitivity to paraherquamide [11]. For the larval development assay the proportion of undeveloped larvae (eggs, L1s and L2s) at each drug concentration was calculated and a log–concentration–logit model [16] fitted to the data to estimate the concentration of drug required to inhibit the development of 50% of the larvae present (LD_{50}), after correction for the mean number of larvae not developed in four control wells (generally 5% to 15%). LP_{50} values for AVM and paraherquamide inhibition of larval motility (i.e. the concentration required to inhibit motility in 50% of the larvae present) were calculated in a similar manner from the proportion of non-motile larvae present at each drug concentration, after correction for the proportion of non-motile larvae in control wells (generally < 5%).

Resistance ratios were calculated as:

$$RR = \frac{LD_{50}(\text{or } LP_{50}) \text{ for the test isolate}}{LD_{50}(\text{or } LP_{50}) \text{ for McMaster } H. \text{ contortus}}$$

3. Results

LD_{50} values for AVM inhibition of larval development in the Branchburg parent isolate and the F7-A and IVC isolates, produced from the Branchburg isolate after seven and 16 generations of IVM selection, respectively, are presented in Table 1. Data for the Australian AVM-susceptible McMaster *H. contortus* isolate and the South African AVM-resistant *H. contortus* isolate, Stellenbosch, are also included in Table 1 for comparison. The AVM-susceptible Branchburg *H. contortus* isolate was similar to the McMaster *H. contortus* isolate in its sensitivity to AVM inhibition of larval development. Unlike the Stellenbosch

Table 1
Sensitivity of the *H. contortus* isolates to a series of avermectins in the larval development assay

Isolate	AVM B_1		AVM B_2		IVM		IVM-monosaccharide		IVM-aglycone	
	LD_{50}[a]	RR[b]	LD_{50}	RR	LD_{50}	RR	LD_{50}	RR	LD_{50}	RR
McMaster[c]	0.51±0.18(17)		1.3±0.4(30)		1.0±0.4(35)		8.1±2.7(26)		4.7±1.0(18)	
BBH	0.61±0.07(2)	1.2	1.5±0.4(2)	1.2	1.1±0.4(5)	1.1	9.0±0.6(2)	1.1	5.5±2.3(2)	1.2
F7-A	0.53±0.07(2)	1.0	1.12±0.07(2)	0.9	1.2±0.6(2)	1.2	7.8±0.9(2)	1.0	4.6±0.6(2)	1.0
IVC	0.60±0.20(3)	1.2	1.42±0.04(3)	1.1	1.0±0.5(8)	1.0	12.2±2.3(3)	1.5	4.9±0.8(3)	1.0
Stellenbosch[c]	1.14±0.15(3)	2.2	11.8±2.8(4)	9.4	2.3±1.1(10)	2.4	31±4(3)	3.8	27±5(3)	5.8
Warren	1.7±0.40(4)	3.2	15±4(8)	12	3.9±2.0(12)	3.9	63±9(4)	7.8	74±19(6)	16

[a] LD_{50} (nM)—mean±S.D. (*n*) where *n* is the number of independent determinations. [b] Resistance ratio relative to the McMaster isolate—see Methods. [c] From Gill et al. [8].

isolate, neither F7-A nor IVC showed a reduced sensitivity to AVM inhibition of larval development.

LP_{50} values for AVM inhibition of L3 motility in the F7-A and IVC isolates obtained after 72 h exposure are presented in Table 2. L3 larvae of the McMaster, Branchburg, F7-A and IVC isolates all showed similar sensitivities to AVM B_1, AVM B_2 and IVM inhibition of L3 motility. Similar trends were observed at 24 and 48 h (data not shown). In contrast, in vivo AVM-resistance in the Stellenbosch isolate was associated with a markedly reduced sensitivity to AVM inhibition of L3 motility.

AVM-resistant isolates of *H. contortus* have been found to have an increased sensitivity to paraherquamide inhibition of L3 motility [11]. The co-selection of increased paraherquamide sensitivity with IVM resistance can be seen by comparing the paraherquamide sensitivity of the Branchburg isolate with that of F7-A, which is 4-fold less sensitive to IVM in vivo after seven generations of IVM selection, and IVC, produced by a further nine generations of selection, which is 10-fold less sensitive to IVM in vivo than the Branchburg isolate (Fig. 1). The IVC isolate was 6-fold more sensitive to paraherquamide than the parent Branchburg isolate.

Selection of the Branchburg isolate with IVM did not cause any shift in sensitivity to levamisole or the benzimidazoles (Table 3).

The Warren *H. contortus* isolate was derived from an overwhelmingly susceptible, mixed field isolate in a single generation by propagating the small number of survivors of a 0.2 mg/kg IVM drench. LD_{50} and LP_{50} values obtained for AVMs and paraherquamide against the Warren *H. contortus* isolate are presented in Tables 1 and 2. This

Table 2
Sensitivity of the *H. contortus* isolates to avermectin and paraherquamide inhibition of L3 motility

Isolate	AVM B_1		AVM B_2		IVM		Paraherquamide	
	LP_{50}[a]	RR[b]	LP_{50}[a]	RR	LP_{50}[a]	RR	LP_{50}[c]	RR
McMaster[d]	0.22±0.05(5)		0.84±0.31(5)		0.30±0.11(14)		2.7±0.8(10)	
BBH	0.20±0.01(2)	0.9	0.65±0.12(2)	0.8	0.34±0.11(3)	1.3	5.1±2.5(2)	1.8
F7-A	0.36±0.09(2)	1.6	1.2±0.5(2)	1.4	0.40±0.09(3)	1.6	3.8±1.0(2)	1.4
IVC	0.33±0.06(3)	1.5	0.99±0.02(4)	1.2	0.48±0.10(8)	1.7	0.85±0.21(2)	0.30
Stellenbosch[d]	1.34±0.08(2)	6.1	6.7±1.9(2)	8.0	1.5±0.9(7)	5.0	0.35±0.07(2)	0.13
Warren	1.8	8.1	6.3	7.5	1.4	4.7	0.32	0.12

[a] LP_{50} (μM)±S.D.(*n*)—where *n* is the number of independent determinations. [b] Resistance ratio—see Methods. [c] From Gill et al. [9] except for paraherquamide LP_{50} values from Gill and Lacey [11]. [d] LP_{50} (μg/ml).
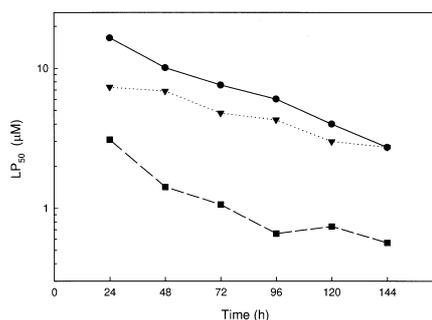
Fig. 1. Paraherquamide inhibition of larval motility in the BBH (●—●), F7-A (▼...▼) and IVC (■— —■) *H. contortus* isolates.

isolate showed a reduced sensitivity to AVM inhibition of larval development and motility compared with the AVM-susceptible McMaster isolate, and an increased sensitivity to paraherquamide. The predominantly *O. circumcincta* field isolate from which the AVM-resistant Warren *H. contortus* isolate was derived was resistant to both the benzimidazoles ($RR = 28$) and levamisole ($RR = 12$). In contrast, the AVM-resistant, Warren *H. contortus* isolate was relatively susceptible to these anthelmintic groups (Table 3).

4. Discussion

The F7-A and IVC *H. contortus* isolates were selected for resistance using doses of IVM chosen to eliminate 95% of the current generation (LD_{95}), then passaging the survivors and redosing with a gradually increasing dose of IVM as resistance

Table 3
Sensitivity[a] of the *H. contortus* isolates to benzimidazoles and levamisole in the larval development assay

Isolate	MBZ	TBZ	LVS
McMaster[b]	$60 \pm 10(36)$	$70 \pm 10(46)$	$1200 \pm 400(41)$
BBH	$65 \pm 13(4)$	$70 \pm 6(4)$	$1040 \pm 180(4)$
F7-A	$62 \pm 9(4)$	$61 \pm 9(3)$	$750 \pm 120(3)$
IVC	$51 \pm 7(4)$	$60 \pm 10(4)$	$750 \pm 190(4)$
Warren	NT[c]	$130 \pm 70(4)$	$1090 \pm 130(3)$

[a] LD_{50} (nM) $\pm$ S.D. (*n*) where *n* is the number of independent determinations. [b] From Gill et al. [8]. [c] NT—not tested.

developed. In vivo resistance to IVM in these isolates was not associated with any decrease in sensitivity to AVM inhibition of larval motility or development. The Warren *H. contortus* isolate was selected from a susceptible, mixed field isolate in a single generation by treatment at the recommended dose of IVM, i.e. a dose 10-fold greater than the LD_{95} for this drug against susceptible *H. contortus* in sheep [3]. For this isolate, as for AVM-resistant *H. contortus* isolates derived from the field in South Africa [8, 9] and Australia [12], in vivo IVM resistance is associated with a decreased sensitivity to AVM inhibition of larval motility and development. These differences in behaviour in in vitro tests suggest that selection with suboptimal doses has produced a different mechanism of AVM resistance to that produced by selection with the recommended dose.

McKenzie [17] has argued that selection protocols that require drenching with suboptimal doses of an anthelmintic are more likely to select for polygenic resistances, whereas field exposure to concentrations of the drug far in excess of its LD_{95} will select for monogenic resistances. AVM resistance in CAVR, another Australian isolate of *H. contortus*, is known to be inherited as a dominant trait, probably controlled by a single gene [18, 19]. The isolation of the CAVR strain has parallels to the isolation of the Warren *H. contortus* isolate in that both involved the propagation of small numbers of survivors of a field isolate after treatment with 0.2 mg/kg IVM [12]. The ease with which the AVM-resistant Warren *H. contortus* isolate was selected from the original mixed species isolate suggests that the AVM resistance present may also be a dominant trait. This has been confirmed in studies of the inheritance of AVM resistance which indicate that for the Warren isolate AVM resistance is also a dominant, monogenic trait (Gill, Le Jambre and Lenane, unpublished data). The nature of the inheritance of AVM resistance in the IVC isolate is unknown.

AVM/milbemycins are known to affect both motility and pharyngeal pumping in parasitic nematodes. The rapidity of expulsion of *H. contortus* from sheep following IVM treatment suggests that in this species the effect on motility is more critical (Gill, Bhardwaj and Sangster, unpub-

lished data). It was therefore surprising that the F7-A and IVC isolates showed no decreased sensitivity to AVM inhibition of larval motility. One explanation for this could be that resistance is developmentally regulated in these isolates. Alternatively AVM resistance in these isolates may be achieved by a mechanism that allows only a transient relief from the effects of the drug which is masked by the extended periods of drug exposure required for the L3 motility assay used in this study. In the L3 motility assay the larvae are exposed to the drug in the dark for at least 24 h and comparative dose response data is usually accumulated after three cycles of light and dark, i.e. after 72 h of exposure [9]. Concentrations of IVM sufficient to inhibit motility in adult *H. contortus* are only present in the gut for a relatively short time (1–2 h) following oral anthelmintic treatment.

Like other isolates of *H. contortus* resistant to the AVMs in vivo, both the IVC and Warren isolates had an increased sensitivity to paraherquamide inhibition of L3 motility compared with AVM-susceptible isolates. The evidence for an association between paraherquamide sensitivity and AVM resistance is made more compelling by the apparent co-selection of these traits in the selection of the F7-A and the IVC isolates from the original Branchburg strain although the pharmacological basis of such an association remains unclear.

In the early years of an anthelmintic's use isolates selected for resistance by exposure to the anthelmintic in the field are not available. To overcome this researchers have often used model organisms, such as the free-living nematode, *Caenorhabditis elegans*, which are more easily manipulated than obligate parasites, or have selected parasite populations for anthelmintic resistance in the laboratory, often with suboptimal doses. While these studies have provided insights into the genetic and pharmacological potential for resistance in target species neither is guaranteed to predict the kinds of resistances that will emerge in the field situation. Isolation and propagation of resistant individuals present at very low levels (<1%) in otherwise susceptible field populations is a way to provide resistant isolates for study which are more likely to reflect the nature of the resistances that will be encountered in the field. While highlighting the need for

caution when extrapolating results obtained with laboratory selected resistant isolates to the field situation, the pharmacological differences between the F7-A and IVC isolates and field-derived AVM-resistant *H. contortus* also indicate that AVM resistance can be achieved by more than one mechanism in this nematode parasite of sheep.

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