Parasitol Res (2012) 111:2201–2204
DOI 10.1007/s00436-012-2944-4

SHORT COMMUNICATION

Is the micro-agar larval development test reliable enough to detect ivermectin resistance?

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Received: 27 February 2012 / Accepted: 23 April 2012 / Published online: 12 May 2012

Abstract To aid the diagnosis of anthelmintic resistance, a range of in vivo and in vitro techniques have been developed. Amongst in vitro techniques, the larval development test is the most widely employed. Six lambs were infected with susceptible (three) and ivermectin-resistant (three) isolates of *Haemonchus contortus*. The micro-agar larval development test (MALDT) was able to easily distinguish between susceptible and resistant isolates. Different proportions of resistant and susceptible eggs were subsequently incubated, i.e. development to the third larval stage occurred only in the resistant isolate. The percentage of resistant eggs ranged from 2 to 20.0 % of all eggs in the wells. In all cases, the MALDT was able to detect the presence of a minimum of 10 % of resistant worms amongst a susceptible background population. The probability was approximately 87 % of positively diagnosing a proportion of resistant worms of only 2–4 % within the population.

Introduction

The intensive use of anthelmintics for the control of helminthic infections has resulted in the development of resistance, which has become a major practical problem in many countries (Várady et al. 2011). The situation in Europe is serious mainly for resistance to benzimidazoles and levamisoles, but reports of resistance to macrocyclic lactones in nematodes parasitic in small ruminants have increased over the last decade (Bartley et al. 2003; Čerňanská et al. 2006; Borgsteede et al. 2007; Traversa et al. 2007). A rapid emergence of resistant worms in the coming years may thus well occur, as it has in Australia, New Zealand and many countries in Latin America.

To detect anthelmintic resistance (AR), in vivo and in vitro methods may be used (reviewed by Johansen 1989; Taylor et al. 2002). Most of these methods, though, have drawbacks in cost, applicability, interpretation or reproducibility. Larval development tests are the most widely used in vitro methods for the detection of AR in ovine nematodes. Coles et al. (1988) first reported a larval development test that could detect resistance to benzimidazoles and levamisoles. A number of versions of larval development tests that can detect resistance to all classes of anthelmintics have since been developed (Taylor 1990; Hubert and Kerboeuf 1992). Two variations, based on either liquid or agar, are being used (Várady et al. 2009). Both types of test rely on the development of eggs to the third larval stage (L3). Gill et al. (1995) developed an assay to detect resistance to avermectins and milbemycins that led to the development of a commercially available assay, the DrenchRite® assay (Microbial Screening Technologies, Kemps Creek, NSW, Australia). Similarly, Coles et al. (2006) described the micro-agar larval development test (MALDT) that uses 96-well microtitre plates containing drug-impregnated agar. By using the MALDT, we have been able to readily distinguish ivermectin-resistant isolates from susceptible isolates, particularly when using ivermectin aglycone (Várady et al. unpublished). The aim of this study was to evaluate the potential of the MALDT to assess low levels of ivermectin resistance in isolates of *Haemonchus contortus*, a parasitic nematode of sheep.

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Table 1 LD_{50}, LD_{99} and RF of the two isolates of *H. contortus* in the MALDT

Ivermectin aglycone	LD_{50} (ng/ml)± SD	CV (%)	Resistance factor	LD_{99} (ng/ml)± SD	CV (%)	Resistance factor
ISE	1.59±0.34	21.7	15.1	5.01±1.16	23.2	54.7
ISE-SI	24.02±8.96	37.3		274.40±249.9	91.0	

Materials and methods

Two isolates of *H. contortus* were used, one susceptible and one resistant to macrocyclic lactones. The susceptible isolate, ISE, was obtained as an inbred isolate of MHCo3 (Roos et al. 2004). A derived isolate, ISE-SI, was selected for resistance to ivermectin in the laboratory (Coles et al. 2005). Additionally, the isolate was selected by exposure to 1.5× the recommended dose. Both isolates have been routinely maintained by passage through worm-free merino lambs (three animals for each isolate) that were 5–6 months old and housed separately. Lambs were infected orally with 5,000–6,000 L3 larvae of each isolate. Faecal samples for the MALDT were collected on three separate days after inoculation. The number of replicates in the 'sensitivity test' varied from 17–28.

We have used the MALDT described by Coles et al. (2006). Tests were performed on 96-well microtitre plates. Stock drug solutions of ivermectin aglycone were serially diluted 1:2 with dimethyl sulphoxide (DMSO) to produce 12 final concentrations ranging from 0.084 to 173.6 μg/ml. Subsequently, 12 μl of the stock solutions were mixed with 150 μl of 2 % Bacto agar. After solidification of the agar, 10 μl of eggs in a 0.3 mg/ml solution of amphotericin B (final number of eggs per well was 50–80) were mixed with 10 μl of yeast extract and then added to the agar. For control wells, no drug and only DMSO (1.6 %) was used. Yeast extract was prepared as described by Hubert and Kerboeuf (1984) (e.g. 1 g of yeast extract in 90 ml of 0.85 % NaCl was autoclaved for 20 min, and then 27 ml of this solution were mixed with 3 ml of 10× concentrated Earle's solution). The plates were incubated for 7 days at 25°C. Larvae were then killed with Lugol's iodine solution, and all eggs and first-, second- and third-stage larvae in each well were counted under an inverted stereomicroscope.

Results of the MALDT are presented as estimates of the LD_{50} and LD_{99}, which are defined as the concentrations of ivermectin aglycone that inhibit development to the L3 stage by 50 and 99 %, respectively. The data were analysed by a logistic regression model to determine the LD_{50} and LD_{99} (Dobson et al. 1987). The degree of AR was expressed as the resistance factor (RF), calculated as the estimates of the LD_{50} or LD_{99} in the MALDT of the resistant isolate divided by the respective estimates of the susceptible isolate.

Based on the results obtained from the MALDT, different proportions of resistant and susceptible eggs were incubated in two concentrations (5.4 and 10.9 ng/ml) of ivermectin aglycone. Both concentrations represent 'resistant wells', i.e. development to the L3 stage occurred only in the resistant isolate (ISE-SI). The percentage of resistant eggs ranged from approximately 2 to 20 % of all eggs in the wells. After 7 days of cultivation, the number of L3 larvae at each concentration was determined.

Table 2 Detection by MALDTs of different proportions of the resistant ISE-SI isolate mixed with the susceptible isolate (ISE) of *H. contortus* using two different concentrations of ivermectin aglycone

Concentration[a] (ng/ml) IA	Percent of resistant eggs[b]	Number[c]	Positivity[d]	Percent[e]
5.4	2–4	20	17	85
10.9	2–4	17	15	88
5.4	5–10	23	21	91
10.9	5–10	21	18	85
5.4	10–20	27	27	100
10.9	10–20	28	28	100

IA ivermectin aglycone

[a] Concentrations of drug indicating resistance

[b] Percentage of resistant eggs (ISE-SI) in the microtitre well; total number of eggs (ISE-SI + ISE) is approximately 50–80

[c] Number of replicates

[d] Positivity (at least one L3 larva found in the well after termination of the test)

[e] Percentage of positivity (the chance of positively diagnosing the resistant segment of the population)

Results and discussion

The RFs and mean values of the LD_{50} and LD_{99} for the MALDTs are shown in Table 1. The degree of distinction between the ISE and ISE-SI isolates was much greater using the LD_{99} (RF=54.7) as the threshold value. The sensitivity of the MALDT for the detection of ivermectin resistance in *H. contortus* was assessed by mixing susceptible (ISE) and resistant (ISE-SI) eggs at different proportions. The results of these tests are shown in Table 2. We chose the concentrations of 5.4 and 10.9 ng/ml of ivermectin aglycone because no development of the ISE isolate occurred at these drug concentrations. A slight suppression (max. 5 %) of development occurred in the ISE-SI isolate at the 10.9 ng/ml concentration. The test was able to clearly indicate the presence of approximately 10 % of resistant worms amongst a susceptible background population. The probability was approximately 87 % of positively diagnosing a proportion of resistant worms of only 2–4 % within the population.

The ISE-SI isolate of *H. contortus* resistant to the normal therapeutic dose (0.2 mg/kg) was produced after three rounds of infection, resulting in egg counts reduction of 69 % (Coles et al. 2005). We have continued to select this isolate for resistance in our laboratory by using 1.5× the recommended dose of ivermectin, which lowered the egg counts by a further 40 %, approximately. All eggs recovered from these sheep and cultured to L3 were, thus, most likely to be resistant to ivermectin.

The sensitivity and standardisation of the test is of great importance, because the ability to detect low levels of resistant worms in a population is crucial for the early detection of AR (von Samson-Himmelstjerna et al. 2009). For this purpose, we mixed eggs from the susceptible and resistant isolates in different proportions and performed the MALDT. By using this approach, we were previously able to diagnose benzimidazole-resistant individuals at a level of 4 % within the tested population (Várady et al. 2007). Our results demonstrate the potential of the MALDT to also detect a low proportion of ivermectin-resistant individuals, which would be useful for monitoring resistance in field studies. As documented in a previous study, the use of ivermectin aglycone in MALDTs significantly increases the sensitivity of the tests (Várady unpublished data). The current study indicates that ivermectin aglycone at concentrations of 5.4 and 10.9 ng/ml could be considered as threshold discriminating doses, or minimum inhibitory concentration values that prevent development to the L3 stage for *H. contortus*.

Despite MALDT's great potential, performing the test presents some difficulties. Two factors may contribute to poor sensitivity. Firstly, in mixed parasitic populations in the field, ivermectin aglycone may have different potencies against different species of gastro-intestinal parasites. Species of *Haemonchus*, *Ostertagia* and *Trichostrongylus* are generally the most common parasites of small ruminants in which resistance to macrocyclic lactones occurs. The lowest values of the LD_{50} in different analogues of ivermectin have been demonstrated in *H. contortus* (Lacey et al. 1991; Demeler 2005). These values were 2–4 times lower than those in *Ostertagia circumcincta* and *Trichostrongylus colubriformis*. The discriminating doses of 5.4 and 10.9 ng/ml of ivermectin aglycone, found in this study with *H. contortus*, might thus be higher for species of *Ostertagia* and *Trichostrongylus*. For monitoring ivermectin resistance in field studies, increasing the discriminating dose to 21.6 ng/ml of ivermectin aglycone may be a reasonable strategy to avoid the misdiagnosis of susceptible *Ostertagia* and *Trichostrongylus* as resistant. Secondly, the correlation between the in vitro MALDT and the in vivo faecal egg count reduction test is questionable. Kotze et al. (2002) subjected several ivermectin-resistant isolates of *H. contortus*, unaffected by the recommended dose of ivermectin, to a larval development test. The in vitro and in vivo data did not fully correlate in one of three isolates when the LD_{50} criterion was used. When a discriminating dose based on the LD_{99} was used, however, the misdiagnosed isolate was readily identifiable as resistant, showing the presence of a small proportion (<20 %) of highly resistant worms.

In conclusion, the MALDT showed comparable and reliable results for the detection of ivermectin resistance in *H. contortus*. Additionally, the test was able to reveal a relatively small proportion of resistant worms in the population, a sensitivity that should have potential in determining resistance in field tests.

Acknowledgments The study was supported by VEGA grant agency, grant no. 2/0135/10 of the Scientific Agency of the Slovak Academy of Sciences and by Slovak Research and Development Agency project no. LPP-0186-07.

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