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An In Vitro Test for Drug Resistance in *Haemonchus contortus*

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ABSTRACT: A cambendazole-resistant (CR) strain of *Haemonchus contortus,* the large stomach nematode of sheep, had similar in vitro growth characteristics compared to the parent strain (BPL). When the in vitro cultivation system was modified to detect drug resistance: (1) exsheathed infective larvae of the BPL strain did not develop in a culture medium with a concentration of 2.5 μg/ml thiabendazole (TBZ), but the CR strain developed to the mid-4th larval stage in 6 days at the same concentration of TBZ; and, (2) larvae of both strains of *H. contortus* were killed prior to or in the 3rd molt in a culture medium with 2.5 μg/ml levamisole.

KEY WORDS: Nematoda, Trichostrongyloidea, *Haemonchus contortus,* bioassay, in vitro cultivation, drug resistance, levamisole, thiabendazole.

The most objective criteria for defining a drug-resistant population of a species is its ability to grow and develop in the presence of a drug concentration that either kills a susceptible population or inhibits its growth and development. Some methods previously used for detecting drug resistance in parasitic nematodes include: drug efficacy studies, egg hatching, larval motility assays, tubulin binding, and aggregation assays. These methods are discussed later in this paper.

Recently, Stringfellow (1984, 1986) cultured *Haemonchus contortus* to egg-laying and sperm-producing adults in vitro. The use of in vitro methods to test the effects of anthelmintics on *Haemonchus contortus,* the large stomach nematode of sheep, goats, and other ruminants, is not new. Stringfellow and Fetterer (unpubl. data) used these in vitro culture methods to study development of drug resistance in this parasite. Douvres et al. (1980) used in vitro methods to study the effects of drugs on *Ostertagia ostertagi* grown in vitro, and Rew et al. (1982) studied the effects of drugs on adult *H. contortus* maintained in vitro. However, studies of the effects of drugs on the developmental stages of susceptible and drug-resistant strains of *Haemonchus contortus* are rare. The cambendazole-resistant strain (CR) used in the present study was selected from the susceptible strain (BPL) by Kates et al. (1973). The objective of the present study was to determine if in vitro methods could be adapted for use as an assay for detecting thiabendazole resistance in *H. contortus.*

Materials and Methods

Experimental animals and nematodes

Neutered Polled Dorset sheep, raised helminth free except for minimal infection with *Strongyloides papillosus,* were used as hosts for the nematodes. They were maintained in individual concrete-floored pens and fed a pelleted feed (mixture of alfalfa meal, barley, bran, corn, meal, oats, and salt).

Susceptible (BPL) and the cambendazole-resistant (CR) strains of *H. contortus* were maintained in the sheep. The cambendazole-resistant strain was originally isolated from the susceptible parent strain by Kates et al. (1973). Infective larvae were recovered from fecal cultures, freed of debris, cleaned with sterile 0.85% saline, and exsheathed by treatment with 1.25% sodium hypochlorite in saline. They were then washed with sterile saline and Earle's balanced salt solution containing antibiotics (penicillin: 5 million units; streptomycin: 5 g; fungazone: 5 mg/5.1 liters) (Douvres, 1983).

Culture procedures

The 1st series of experiments compared the growth and development of the BPL and the CR strains of *H. contortus* in vitro. Techniques for the cultivation of this worm from infective larvae to advanced stages have been previously described (Stringfellow, 1984, 1986). Preparation of medium API-1 was described previously by Douvres and Malakatis (1977) and Fildes' reagent (a peptic digest of defibrinated bovine blood) was described by Douvres (1983). The ovine gastric contents (OGC) was prepared fresh. It was first cleared of ingesta by straining it through cheese cloth, then centrifuged at 3,900 *g* and 2,400 *g* on a Damon Cu5000 and Sorvall RC5C for 30 min each. It was then filtered through an AP25 clarifying pad at 40 psi. After clarifying the OGC, it was Millipore filtered and separated by Dacron separators. The top filter was the AP25 clarifying pad followed by the 8, 1.2, 0.45, and 0.22 μm final filter. The OGC was recovered sterile and used as a supplement. The experimental design as well as specific details can be found in Tables 1 and 2.

The 2nd series of experiments compared the growth and development of each strain of *H. contortus* in API-1 culture medium supplemented with 1.38 mM ascorbic acid and 3.2 mM cysteine. The gas phase for the 100 ml culture vessels was 85% N_2: 5% O_2: 10% CO_2 at pH 6.4 for the 6 days of incubation (DIC) (39°C). It was important that the culture vessels be sealed tightly. This 2nd series of experiments compared the growth

Table 1. ***Haemonchus contortus*—development of susceptible (BPL) and drug-resistant (CR) strains from artificially exsheathed larvae to advanced stages in API-1 culture medium supplemented with Fildes' reagent and ovine gastric contents (OGC).**

Strain of *H. contortus*	Time (days) for development to stage							
	Parasitic 3rd	3rd molt	Early 4th	4th molt	Young adult		Mature adult	
					Male	Female	Male	Female
In vitro*								
BPL	0	3–5	3–5	19	21	25	28	35
CR	0	2–5	3–5	14	21	25	28	35

* Two trials, each trial consisting of 2 culture vessels.

and development of the 2 strains in: (1) the presence of a graded concentration of a drug (TBZ) to which they had developed resistance (Tables 3, 4); and, (2) the presence of a graded concentration of levamisole (LV) to which they had not developed resistance. Control cultures did not have the drugs. The cultures were diagnosed at 6 DIC because the growth characteristics of these 2 strains are similar up to about 6 DIC when the nematodes are in the mid-4th larval stage.

The methods of Douvres et al. (1966) were used to test sterility of all stocks of media and to examine and evaluate the development of the nematodes. Cultures were judged free of contamination if there was no visible sign of fungi and bacteria. The larval stages, adults, and eggs were identified according to Veglia (1915) and from original observations.

Drug preparation and delivery

Both thiabendazole-hydrochloride (Merck and Co., Rahway, NJ) and levamisole (American Cyanamid, Princeton, NJ) were dissolved in deionized water at concentrations ranging from 10 to 0.01 μg/ml of the drug when 0.1 ml of the drug was added to 9.9 ml of the culture medium at the beginning of each experiment. One-tenth ml of deionized water was added to each control culture. The amount of drug available to the worms in the culture medium was then about 1% less than that added because of the dilution factor when 0.1 ml of the larvae were added to the culture vessel. Only data for concentrations of the drug ranging from 2.5 to 0.1 μg/ml are presented in Tables 3 and 4. Where pilot studies were run at 10, 5, and 0.01 μg/ml to work out the most useful concentrations of the drug, those data are reported in the text where they provide useful information.

Results and Discussion

Growth and development of BPL and CR strains of *H. contortus* in vitro

In general, growth and development of BPL and CR strains in vitro and in vivo were similar to those results reported previously by Stringfellow (1986). Both strains of *H. contortus* developed from infective larvae to mature egg-laying females in 35 days (Table 1) in API-1 supplemented with Fildes' reagent and ovine gastric contents (OGC). There was no apparent difference in the rate of development of males and females of both strains up to about the early to mid-4th stage; however, beyond that point the CR strain grew better than the BPL strain at least to the 4th molt and young adult stage. In general, males of both strains underwent the 4th molt a few days before the females. The largest mature adult male and female BPL strain worms grown in vitro were 9 mm and 12–14 mm long, and the adults of the CR strain measured 10 mm and 15 mm, respectively.

Data on survival and yields of advanced stages of both strains of *H. contortus* are given in Table 2. The cultures were terminated at 42 DIC. There was an average of 29 males (0.07%) and 29 egg-laying and egg-producing females (0.07%). All worms of both strains were alive at 14 DIC and were at the 4th stage. At 21 DIC, 14% of the larvae of the CR strain and 0% of the larvae of the BPL strain had reached the young adult stage. By 28 DIC, 5% of the males and females of the BPL strain had reached the young adult stage and only 1% of the CR strain had reached the adult stage. In general, the results obtained here for both strains of *H. contortus* were similar to those previously reported for the BPL strain (Stringfellow, 1986).

Growth and development with and without drugs

There was no noticeable effect on the larvae of either the BPL or the CR strains when 0.1 ml of deionized water was added to the control cultures. Larval growth in API-1 medium plus reducing agents was similar to growth obtained in the "optimal" system (Table 1); however, both BPL and CR strains developed slightly faster in the 100-ml vessels (Table 3). Larvae of the BPL strain gradually stopped moving when concentrations of thiabendazole (TBZ) were added to the cultures. Exsheathed BPL larvae did not develop beyond the parasitic 3rd larval stage when

Table 2. *Haemonchus contortus*—survival and yields of advanced stages that developed from artificially exsheathed infective larvae* in roller culture bottles consisting of API-1 culture medium supplemented with Fildes' reagent and ovine gastric contents.

Days in culture	Strain of *H. contortus*	Total inoculum alive (%)	Live (%) worms in stage					
			Parasitic 3rd	3rd molt	4th stage	4th molt	Young adult	
							Male	Female
7	BPL	100	8	1	91	0	0	0
	CR	100	6	3	91	0	0	0
14	BPL	100	0	0	100	0	0	0
	CR	100	0	0	100	0	0	0
21	BPL	74	0	0	91	7	0	0
	CR	90	15	0	47	24	6	8
28	BPL	82	0	0	65	31	3	2
	CR	61	0	0	64	33	1	0

* Forty thousand larvae per culture.

TBZ was added at 2.5 μg/ml (Fig. 1). Some BPL larvae did reach the 3rd molt and early 4th stage when concentrations of TBZ were 1.0 and 0.1 μg/ml; larvae reaching early 4th stage were in poor condition. BPL larvae grew like the controls at 0.01 μg/ml concentrations of TBZ. The CR strain developed to the mid-4th larval stage in concentrations of TBZ from 0.01 to 2.5 μg/ml. They remained at the parasitic 3rd larval stage at 5 μg/ml. Survival and yields of both strains of larvae are presented in Table 4.

In general, although the BPL larvae did not develop and remained at the parasitic 3rd larval stage, they were viable; thus the high rate of survival at 2.5 μg/ml. The survival and yields of the CR strain were similar to the controls when the concentration of TBZ ranged from 0.01 to 2.5 μg/ml.

Table 3. *Haemonchus contortus*—development of susceptible (BPL) and drug-resistant (CR) strains from artificially exsheathed larvae to mid-4th stage in API-1 culture medium with and without thiabendazole (TBZ) or levamisole (LV).

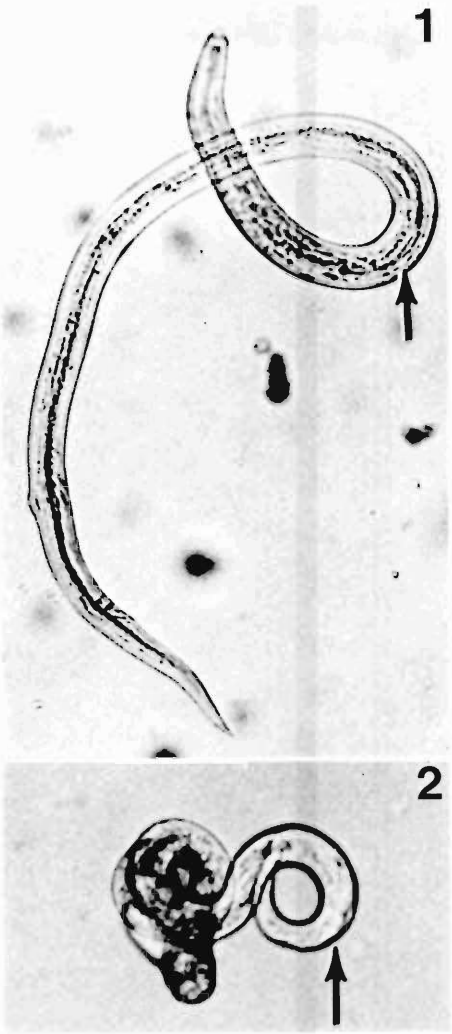
Strain of *H. contortus*	Drug concentration (μg/ml)	Time (days) for development to mid-4th stage*			
		Parasitic 3rd	3rd molt	Early 4th	Mid-4th
BPL					
	0	0	3	3	6
TBZ	2.5	0	0	0	0
	1.0	0	2	3	6
	0.1	0	2	3	6
LV	2.5	0	0	0	0
	1.0	0	2	3	6
	0.1	0	2	3	6
CR					
	0	0	2	3	6
TBZ	2.5	0	2	3	6
	1.0	0	2	3	6
	0.1	0	2	3	6
LV	2.5	0	0	0	0
	1.0	0	2	3	6
	0.1	0	2	3	6

* Six DIC, 4 trials of 1 culture each.

Table 4. *Haemonchus contortus*—survival and yields of larval stages of a susceptible (BPL) and drug-resistant (CR) strain with and without thiabendazole (TBZ) or levamisole (LV).

Strain of *H. contortus*	Drug concentration (μg/ml)	Total inoculum alive (%)	Live (%) worms in stage			
			Parasitic 3rd	3rd molt	Early 4th	Mid-4th
BPL						
	0	100	75	11	7	8
TBZ	2.5	100	100	0	0	0
	1.0	86	48	6	42	3
	0.1	88	44	2	47	6
LV	2.5	84	100	0	0	0
	1.0	100	91	0	5	5
	0.1	100	83	0	8	8
CR						
	0	100	6	0	12	81
TBZ	2.5	100	27	1	40	32
	1.0	100	22	17	43	18
	0.1	100	24	10	38	28
LV	2.5	70	96	4	0	0
	1.0	100	49	0	22	30
	0.1	100	50	0	23	26

* Six DIC, 4 trials of 1 culture each.



Figures 1, 2. 1. BPL strain of *Haemonchus contortus* parasitic 3rd-stage larvae (arrow) exposed to 2.5 μg/ml thiabendazole. Larvae are viable. ×125. 2. CR strain of *Haemonchus contortus* parasitic 3rd-stage larvae (arrow) exposed to 2.5 μg/ml levamisole. Larvae abort the 3rd molt. ×125.

When levamisole (LV) was added to the culture medium, the larvae of both BPL and CR strains tended to round up and stop moving although they appeared to be alive. The exsheathed BPL larvae did not develop beyond the parasitic 3rd stage when LV was added at 2.5 μg/ml (Tables 3, 4). The larvae developed beyond the 3rd molt to the mid-4th stage when the concentrations of LV were 1.0 and 0.1 μg/ml, similar to the controls (Table 4). Similar results were obtained with the CR larvae indicating that the LV was active against both the BPL and the CR strains. The CR larvae did not develop beyond the 3rd molt (frequently aborted the 3rd molt) remaining as parasitic 3rd-stage larvae at LV concentrations of 2.5 μg/ml (70% survival) (Fig. 2). At LV concentrations of 1.0 and 0.1 μg/ml a slightly greater percentage of CR larvae developed to mid-4th stage than in the control group, reflecting the fact that CR larvae develop better in 100-ml vessels. Survival and yields of both strains of larvae are presented in Table 4.

Conclusions

Thiabendazole, the imidazole used in the present study to test for drug resistance, interferes with energy generation (Rew, 1978). It was used in the present study in place of cambendazole because it is more water soluble, and there were no synergistic effects of the solvent and the drug on the nematode. Levamisole, which interferes with neuromuscular transmission (Rew, 1978), was used in the present study as a positive control. The older methods for detecting drug resistance using animals are very effective (Kates et al., 1973; Colglazier et al., 1974); however, they are also very expensive. In those tests the nematode was grown to the adult stage in the host; the hosts were treated with the drug and the efficacy of the drug was determined by comparing the number of worms recovered from treated and control groups. In recent years several methods have been developed to detect resistance of nematode parasites to drugs. Some of these methods are economically appealing. Le Jambre (1976) and Dobson et al. (1986) found that thiabendazole- and levamisole-resistant strains of *H. contortus* hatched at higher concentrations of the drugs than susceptible strains. Gerald C. Coles (pers. comm., July, 1985) and coworkers, in a test of the growth and development of larvae of cambendazole-resistant and susceptible strains of *H. contortus,* found that their larval test was more sensitive than the egg-hatch test. More recently, Sangster et al. (1985) reported that benzimidazoles affected microtubule-dependent acetylcholine esterase secretion, the formation of microtubules in intestinal cells, and colchicine binding in susceptible versus resistant strains of *Trichostrongylus colubriformis.* Also, Jenkins et al. (1986) have reported that the aggregation response of *T. colubriformis* was useful for screening anthelmintics.

The results presented herein indicate that in

vitro procedures can be used as an assay for detecting thiabendazole resistance in *H. contortus*. The methods described are a modification of in vitro methods originally used to culture this nematode to the mature adult stages (Stringfellow, 1984, 1986). The use of in vitro culture methods provides a useful investigative tool to separate resistant from sensitive populations of this nematode. These 2 strains were easily identified with the in vitro culture techniques described in the present paper.

Literature Cited

Colglazier, M. L., K. C. Kates, and F. D. Enzie. 1974. Cambendazole-resistant *Haemonchus contortus* strain in sheep: further experimental development. Journal of Parasitology 60:289–292.

Dobson, R. J., A. D. Donald, P. J. Waller, and K. L. Snowdon. 1986. An egg-hatch assay for resistance to levamisole in trichostrongylid nematode parasites. Veterinary Parasitology 19:77–84.

Douvres, F. W. 1983. The in vitro cultivation of *Oesophagostomum radiatum*, the nodular worm of cattle. III. Effects of bovine heme on development to adults. Journal of Parasitology 69:570–576.

———, **and G. M. Malakatis.** 1977. In vitro cultivation of *Ostertagia ostertagi*, the medium stomach worm of cattle. I. Development from infective larvae to egg-laying adults. Journal of Parasitology 63:520–527.

———, **M. J. Thompson, and W. E. Robbins.** 1980. In vitro cultivation of *Ostertagia ostertagi*, the medium stomach worm of cattle. II. Effect of insect-growth-disrupting amines and amides on development. Veterinary Parasitology 7:195–205.

———, **F. G. Tromba, and D. J. Doran.** 1966. The influence of NCTC 109, serum, and swine kidney cell cultures on the morphogenesis of *Stephanurus dentatus* to fourth stage in vitro. Journal of Parasitology 52:875–889.

Jenkins, D. C., E. P. Rapson, and P. Topley. 1986. The aggregation response of *Trichostrongylus colubriformis*: a basis for the rapid interpretation of in vitro anthelmintic screens. Parasitology 93:531–537.

Kates, K. C., M. L. Colglazier, and F. D. Enzie. 1973. Experimental development of a cambendazole resistant strain of *Haemonchus contortus* in sheep. Journal of Parasitology 59:169–174.

Le Jambre, L. F. 1976. Egg hatch as an in vitro assay of thiabendazole resistance in nematodes. Veterinary Parasitology 2:385–391.

Rew, R. S. 1978. Mode of action of common anthelmintics. Journal of Veterinary Pharmacology and Therapeutics 1:183–198.

———, **C. Smith, and M. L. Colglazier.** 1982. Glucose metabolism of *Haemonchus contortus* adults: effects of thiabendazole on susceptible versus resistant strain. Journal of Parasitology 68:845–850.

Sangster, N. C, R. K. Prichard, and E. Lacey. 1985. Tubulin and benzimidazole-resistance in *Trichostrongylus colubriformis* (Nematoda). Journal of Parasitology 7:645–651.

Stringfellow, F. 1984. Effects of bovine heme on development of *Haemonchus contortus* in vitro. Journal of Parasitology 70:989–990.

———. 1986. Cultivation of *Haemonchus contortus* (Nematoda: Trichostrongylidae) from infective larvae to the adult male and the egg-laying female. Journal of Parasitology 72:339–345.

Veglia, F. 1915. The anatomy and life-history of the *Haemonchus contortus* (Rud). Third and Fourth Reports of the Director of Veterinary Research. Onderstepoort, South Africa. Pages 347–500.