AN IN VITRO LARVAL MOTILITY ASSAY TO DETERMINE ANTHELMINTIC SENSITIVITY FOR HUMAN HOOKWORM AND STRONGYLOIDES SPECIES


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Abstract. With the implementation of programs to control lymphatic filariasis and soil-transmitted helminths using broad spectrum anthelmintics, including albendazole and ivermectin, there is a need to develop an in vitro assay for detection of drug resistance. This report describes an in vitro assay for measuring the effects of ivermectin and benzimidazoles on the motility of larvae of the hookworm species Ancylostoma ceylanicum, A. caninum, and Necator americanus, and Strongyloides species including Strongyloides stercoralis, and S. ratti. A dose-response relationship was demonstrated with each of the parasite species, with distinct differences observed between the various species. In pilot field testing of the assay with N. americanus larvae recovered from human fecal samples, a dose-response relationship was observed with ivermectin. While the assay has demonstrated the ability to determine drug responsiveness, its usefulness in resistance detection will require correlation with the clinical outcome among individuals infected with parasite strains showing different drug sensitivities.

INTRODUCTION

The recognition of the significant morbidity attributable to infection with the lymphatic filarial parasites Wuchereria bancrofti and Brugia malayi, along with studies demonstrating the efficacy of community-based distribution of antifilarial chemotherapy given annually or semiannually as a single dose, has led to the implementation of a World Health Organization (WHO)—sponsored program to eliminate lymphatic filariasis.1 The strategy relies on the use of diethylcarbamazine (DEC), either as a single agent, or in combination with albendazole. In areas where loiasis or onchocerciasis are endemic, ivermectin is used in combination with albendazole.1

A further benefit of such programs is that these regimens result in a significant reduction in prevalence and intensity of intestinal nematode infections, including hookworm (Necator americanus and Ancylostoma duodenale), Ascaris lumbricoides, Trichuris trichuria, and Strongyloides stercoralis. In addition to the well recognized clinical benefits attributable to treatment of these intestinal geohelminth infections, such as the reduction in iron deficiency anemia, and gastrointestinal disturbance, some studies have indicated that such programs result in significant overall improvements in well-being, including school performance and growth among children, outcomes that cannot be accounted for by direct nutritional effects.2,3

With the growing use of broad spectrum anthelmintics, which has occurred with ivermectin in the onchocerciasis control program (OCP), and is now occurring with albendazole in the filariasis elimination programs, an important question emerges as to whether selection pressure will lead to the development of drug resistance. Although resistance to ivermectin has not been reliably documented in the OCP, mathematical modeling indicates that ivermectin distribution will be required for at least an additional 10 years in endemic regions of Africa for effective control.4 While the long life cycle of filarial parasites, and the involvement of an intermediate insect host mitigate against the development of resistance, such a prolonged period of drug use is likely to increase the chances of the development of resistance.4

The potential for the development of resistance among intestinal nematode parasites may be greater than that for filarial parasites: the parasite density is greater in the bowel where the (adult) sexual stages of the parasites reside, and the parasite life cycle is significantly shorter. This is highlighted by experience in veterinary practice, where clinically significant resistance to benzimidazoles drugs and ivermectin has emerged.5,6 Three studies have recently been published indicating pyrantel and benzimidazole resistance in human hookworm infection in Africa and Australia.7–9 Although these studies have been criticized for methodologic weaknesses,10 they further highlight the issue of anthelmintic resistance in human gut nematodes.

To lessen the impact of drug resistance, there is a need to develop tools to monitor for its emergence. In this way, selection for resistance may be reduced by, for example, the use of appropriate drug rotation strategies when resistance to one drug group is detected. The fecal egg count reduction test (FECRT), which measures changes in fecal parasite egg counts following chemotherapy, is currently the standard method for determining the therapeutic efficacy of anthelmintic chemotherapy in humans.11 However, studies using this methodology are costly and cumbersome to perform, and are subject to a number of significant confounding factors, including the over-dispersion of parasite burden, leading to sample bias. In veterinary practice, while the FECRT is also the standard method for measurement of resistance, considerable efforts have been made to develop simple in vitro tests as more cost-effective alternatives.11,12 These tests have, in some cases been widely applied in surveys for anthelmintic resistance (e.g., see Palmer and others13). A commercial larval development assay (Drenchrite®; Horizon Technology, Roseville, New South Wales, Australia) is currently marketed in Australia for testing resistance in gastrointestinal nematodes of sheep and goats to benzimidazoles, levamisole, and macrocyclic lactones (MLs), including ivermectin. While such tests for human intestinal nematode infection are conceptually very similar, and a need for their development has been articulated,10 to date little work has been undertaken in this area.

The in vitro techniques for detection and monitoring of anthelmintic resistance in veterinary practice include 1) egg...
hatch assays, 2) larval paralysis, migration, and motility tests, 3) larval development tests, 4) adult development tests, and 5) metabolic tests. While each of these tests has specific strengths and weaknesses, a number of important factors apply in the selection of an assay methodology suitable for human parasites. These include the ability to apply the assay in a field situation with relatively little specialized equipment, a requirement for relatively low numbers of parasites, and, ideally, a method to readily purify parasites from infected stool samples. The aim of this project was to develop an in vitro test for defining drug sensitivity of human hookworm and Strongyloides spp. isolates. The assay was based on assessment of drug effects on the motility of infective-stage larvae. Factors that led us to select an assay based on motility effects included the need for an assay that would be suitable for use in field settings, where the ease of parasite collection from fecal cultures is a major consideration, as well as previous reports of its application to detect resistance to both the benzimidazoles and macrocyclic lactone drug groups.

**MATERIALS AND METHODS**

**Parasites. Hookworm.** Since fecal material containing human hookworm species was not available in quantity, assay development and optimization were undertaken using the dog hookworm species *Ancylostoma caninum*. Fresh feces from dogs with known *A. caninum* infection were collected. Diagnosis of other parasite infections and intensity of infection was undertaken by standard microscopic techniques. To isolate infective-stage larvae, feces were mixed with water and homogenized by shaking in a two liter container. An equal volume of vermiculite was added and mixed with the fecal suspension. Additional water was added until the mixture was uniformly moist. Two layers of surgical gauze were placed on the mixture, and the container was then placed in a humidified incubator at 26°C for five days. The top layer of gauze was then removed, placed into a 50-mL conical tube containing 30 mL of water, and vigorously shaken by hand. The gauze was removed, and the tubes were subjected to centrifugation at 1,000 × g for five minutes. To reduce bacterial and fungal contamination that may hamper reading of motility assays, the larvae were resuspended in water containing amphotericin B (0.25 μg/mL, Fungizone®; Bristol-Myers Squibb, New York, NY) and ceftriaxone (20 μg/mL, Rocephin®; Roche, Basel, Switzerland), and incubated for one hour at ambient temperature. The water from the petri plates was poured off and larvae recovered from the fluid were then centrifuged, washed one additional time in water, and counted.

The origin and maintenance of *N. americanus* and *A. ceylanicum* in adult laboratory hamsters has been previously described. Infective larvae of both species were recovered from fecal cultures as described for *A. caninum*. For the pilot field study using human hookworm, stool samples were collected from subjects resident in a village in Madang Province, Papua New Guinea, known to be endemic for *N. americanus*. Fecal samples (approximately five grams taken from fecal collections shown by microscopic examination to be positive for hookworm eggs) were subject to coproculture using the Harada-Mori technique. Briefly, approximately 10 grams of feces was placed on a strip of filter paper and placed in a 50-mL conical tube. Five milliliters of water was added to the bottom of the tube. After 72 hours, the water was poured off and larvae recovered from the fluid by low speed centrifugation (2,000 rpm for 10 minutes). Microscopic examination was undertaken to verify that the species of larvae was hookworm and not the co-endemic nematode Strongyloides falleborni.

**Strongyloides ratti.** Since fecal samples from humans with *S. stercoralis* infection were not available (except for a single case described later in this report), assay development was undertaken using samples from the related rodent species *S. ratti*. The lifecycle of *S. ratti* was established in Wistar rats from live infective-stage parasites kindly provided by Dr. Mark Viney (University of Bristol, University, Bristol, United Kingdom). Infective-stage larvae were harvested as previously described. Briefly, fecal pellets from infected rats were collected into 9-cm diameter watch glasses and moistened. They were placed into 9-cm diameter square plastic petri plates containing 25 mL of water and maintained at ambient temperature. The water from the petri plates was collected after 5–7 days, and the larvae were harvested and purified as described earlier in this report.

During the course of the study, a single batch of larvae isolates of *S. stercoralis* from an anonymous infected patient was provided by a local diagnostic laboratory. These were collected by agar plate culture.

**Drug sensitivity assay.** The assay methodology was adapted from a 96-well microtiter plate assay described by Gill and others for measuring the effects of avermectins on the motility of third-stage larvae of the ruminant gut parasite *Haemonchus contortus*. Stock solutions of ivermectin, thiabendazole, and albendazole (all obtained from Sigma Aldrich, St. Louis, MO) were prepared at 10, 40, or 20 mg/mL, respectively, in dimethylsulfoxide and were serially diluted either two-fold (ivermectin and albendazole) or four-fold (thiabendazole) to produce a series of drug dilutions. Aliquots were added at a dilution of 1% to molten agar in a total volume of 200 μL in individual wells of a 96-well microtiter plate. The final drug concentrations in the assay plates consisted of two-fold serial dilutions starting at 1.6 μg/mL, 0.4 mg/mL, and 0.2 mg/mL for ivermectin, thiabendazole, and albendazole, respectively. Approximately 30 worms (in 30 μL of water) were placed into each well, and the plate was incubated in the dark at 25°C for 48 hours.

The number of separate assay wells used for each series of drug tests varied according to the availability of larvae at different times during the course of the study (see Figure legends and Table footnotes). All assays consisted of at least two wells at each drug concentration (this was increased up to six wells in some cases). Drug sensitivity was generally determined over a series of approximately 10 drug dilutions. However, in some cases, only 4–5 separate drug concentrations were assessed. The number of control wells (no drug added) in each assay varied from 6 up to 21.

The effect of the drugs on worm viability was assessed by counting the numbers of motile larvae after the 48-hour incubation period. Prior to counting, the worms were stimulated to move using hot water as described by Satou and others for the assessment of motility in *S. ratti* and *S. venezuelensis* following in vitro exposure to drugs. Very little movement was apparent prior to stimulation. However, after the addition of 40 μL of water at 50°C to each well, control worms and those unaffected by drug were observed to move in a rapid sinusoidal motion. In contrast, drug-affected worms showed a twitching motion, or remained motionless. Criteria 609
for distinguishing between motile and non-motile larvae were developed in the first stage of this study, and are described in the Results. Only motile worms were counted in each well. Preliminary experiments showed that the total number of worms in separate wells was almost identical (a repetitive dispensing pipette was used to load larvae into assay plates). Thus, it was considered only necessary to count motile worm numbers to calculate the percent motility relative to a number of control (no drug) wells. The worms were counted under 250× magnification.

Pilot experiments were undertaken to determine the effect on assay results of holding worms for up to five days in water at temperatures of 20, 25, and 29°C prior to undertaking the assay (48-hour assay duration at 25°C), and the effect of varying the temperature that the plates were held at during the 48-hour incubation period (20, 25, and 29°C).

**Statistical analyses.** Dose-response data was analyzed using non-linear regression (sigmoidal dose-response, GraphPad Prism®; GraphPad Software, Inc., San Diego, CA). Drug sensitivity data were expressed as LC₅₀ values (with 95% confidence intervals). These were defined as the lethal concentrations of drug required to decrease the numbers of motile worm infections intervals). These were defined as the lethal concentrations of drug required to decrease the numbers of motile worms to 50% of that observed in control wells of the microtiter plate.

**Ethical approval.** Approval for maintenance of the life cycle of *S. ratti* in laboratory rats was obtained from the Animal Ethics Committee of the Queensland Institute of Medical Research. *Necator americanus* and *A. ceylanicum* were maintained in hamsters at the University of Nottingham with the approval of the University of Nottingham Ethics Committee and under a license from the British Home Office. Ethical approval to obtain the infected human feaces was obtained from the Human Ethics Committee of the Queensland Institute of Medical Research, and from the Medical Ethics Advisory Committee of the Government of Papua New Guinea.

## RESULTS

**Assay development.** In initial experiments, a set of criteria for motility assessment was developed and validated using *S. ratti* and *A. caninum* larvae (Table 1). A worm was considered to be motile if it moved in a sinusoidal motion when stimulated by water at 50°C. The pattern of the motion differed between the hookworm and *Strongyloides* species: *S. ratti* larvae showed rapid motion that could be described as a thrashing motion, whereas *A. caninum* larvae moved with a smoother, considerably slower motion than *S. ratti* larvae.

The appearance of drug-affected non-motile worms differed between the two species and the two drug classes drugs tested. For *S. ratti* larvae with ivermectin, as the drug concentration increased, a marked and rapid change from motile to non-motile was observed. This enabled a clear definition of the LC₅₀. Such a clear definition of the LC₅₀ was less apparent for *S. ratti* with thiabendazole because the larvae continued to show movement at drug concentrations significantly higher than the LC₅₀. However, the pattern of movement of thiabendazole-affected worms differed significantly from that observed in controls, thus allowing these worms to be scored as non-motile. The transition in pattern of motion from motile to non-motile involved a change from rapid and sustained sinusoidal motion to an only transient sinusoidal movement that was sustained for no more than 3 seconds. The transition in pattern of motion differed significantly after addition of hot water that it was difficult to distinguish between this drug-induced twitching motion and normal sinusoidal movement of control worms. The amount of rapid twitching motion was so considerable immediately after addition of hot water that it was difficult to distinguish between this drug-induced twitching motion and normal sinusoidal movement of untreated worms. However, after 30 seconds, the extent and velocity of twitching decreased significantly.

**Criteria used for assessing the effects of thiabendazole and ivermectin on the motility of *Strongyloides ratti* and *Ancylostoma caninum*"**

<table>
<thead>
<tr>
<th>Species</th>
<th>Motility rating</th>
<th>Criteria for motility rating</th>
<th>Other observations</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>S. ratti</em></td>
<td>Motile</td>
<td>Worms moving in a rapid sinusoidal thrashing motion</td>
<td>Most motile worms at or near surface of liquid</td>
</tr>
<tr>
<td>Non-motile</td>
<td></td>
<td>Worms twitching Parts of body appear stiff If sinusoidal motion is apparent, it occurs only briefly following addition of hot water (3–4 strokes) before twitching resumes Sinusoidal motion may occur briefly in half of body, with other half appearing to be stiff</td>
<td>Most worms near bottom of liquid</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>Thiaebendazole:</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>Worms affected by thiabendazole show a lot of twitching (non-sinusoidal) motion. This twitching continues at concentrations significantly higher than the LC₅₀</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>Ivermectin:</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>No movement at concentrations slightly higher than the LC₅₀</td>
</tr>
<tr>
<td><em>A. caninum</em></td>
<td>Motile</td>
<td>Worms moving in a smooth sinusoidal motion (slower than <em>S. ratti</em>)</td>
<td>Thiaebendazole:</td>
</tr>
<tr>
<td>Non-motile</td>
<td></td>
<td>Around the LC₅₀² Worms moving with a sinusoidal motion with an arc less than half that shown by control (no drug) worms</td>
<td>Worms affected by thiabendazole show very little motion</td>
</tr>
<tr>
<td></td>
<td></td>
<td>At a higher concentration: No movement</td>
<td>Ivermectin:</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>No movement at concentrations slightly higher than the LC₅₀</td>
</tr>
</tbody>
</table>

* LC₅₀ = 50% lethal concentration.
† For *S. ratti*, the motility rating is made 30 seconds after addition of hot water to assay well. For *A. caninum*, the motility rating is made immediately after addition of hot water.
assume the characteristic restricted movement described above (for non-motile worms), thus enabling the operator to score the worms as healthy (motile) or drug-affected (non-motile). Healthy motile larvae showed smooth sinusoidal motion immediately upon addition of the hot water, and maintained this motion for a period of at least two minutes. This procedure, whereby 30 seconds were allowed to elapse between addition of hot water and assessment of worm motility, was therefore adopted routinely for *S. ratti*. After some practice, the operator could add hot water to one well, use the next 30 seconds to score the previous well (that had received hot water 30 seconds earlier), and continue in this fashion to read the assay, pausing at the end of each row of the plate if necessary.

With all three hookworm species examined (*A. caninum*, *A. ceylanicum*, and *N. americanus*), the distinction between drug-affected non-motile larvae and healthy larvae could be readily scored in the same manner as described earlier for *S. ratti* larvae exposed to ivermectin. Thiabendazole- and ivermectin-affected hookworm larvae showed very little movement at concentrations slightly higher than the LC50, thus readily enabling the LC50 region to be determined. The degree of movement in thiabendazole- or ivermectin-affected worms was clearly distinguishable from healthy motile worms immediately after addition of hot water to the assay wells. Therefore, it was not necessary to allow a 30-second period to elapse before scoring the motility as was required for *S. ratti* and thiabendazole.

The assay was unsuitable for examination of sensitivity to albendazole due to the poor solubility of this compound. Precipitated drug was visible in wells containing the drug at concentrations greater than 50 μM. As the drug concentration increased further, the percentage inhibition of motility increased slowly without reaching 100%; at 380 μM, 40% of the *A. caninum* and 15% of the *S. ratti* remained motile.

The results of assays performed after a period of 24, 48, or 72 hours incubation are shown in Figure 1. As each assessment required the addition of 40 μL of water, the concentration of drug and larvae was altered, thus precluding repeated assessment of the same assay well at the different timepoints. Therefore, measurements at each timepoint were independently recorded from separate wells. In all cases, as expected, the LC50 decreased as the assay duration increased. The most marked effect of assay duration on LC50 was with the *S. ratti*

![Figure 1](https://example.com/figure1.png)

**Figure 1.** Determination of 50% lethal concentration (LC50) values for *Strongyloides ratti* (A) and *Ancylostoma caninum* (B) larvae after exposure to thiabendazole (A1 and B1) or ivermectin (A2 and B2) for 24, 48, or 72 hours (hr). Larvae were placed onto assay plates containing thiabendazole or ivermectin at a range of concentrations, and held at 25°C for the stated times before motility was assessed; n = number of separate assays; error bars indicate standard errors.
thiabendazole combination (Figure 1 A1), where the LC₅₀ after 48 hours of incubation was approximately 8% of the value obtained after 24 hours of incubation. Significantly, for the *S. ratti* thiabendazole assay, interassay variation in the LC₅₀ was also lower at the 48-hour timepoint compared with the 24-hour timepoint (SE = 46% of the mean at 24 hours versus 33% of the mean at 48 hours). Responses of both *S. ratti* and *A. caninum* larvae to ivermectin were more similar at the 48-hour and 72-hour timepoints than for the comparison of 24 hours with 48 hours. Given the finding of reduced interassay variability for both species at an incubation duration of 48 hours, this interval was selected as the standard duration of incubation for future assays.

The effect of storage of worms prior to testing in the assay is shown in Figure 2. *Ancylostoma caninum* larvae were significantly more tolerant of storage than *S. ratti* larvae. Motility of *S. ratti* larvae decreased significantly when worms were held for three days at either 25°C or 29°C prior to the 48-hour assay (at 25°C) (Figure 2 A1), whereas storage of *A. caninum* larvae for 5 days at 28°C (prior to a 48-hour assay) had no

**FIGURE 2.** Effects of storage at various temperatures on motility of *Strongyloides ratti* (A) and *Ancylostoma caninum* (B) larvae and determination of 50% lethal concentration (LC₅₀) values with thiabendazole and ivermectin. Larvae were held for various times at 20°C (■), 25°C (□), or 29°C (▲) and then placed onto drug assay plates and held for 48 hours at 25°C before assessment of motility in control (A1 and B1) or drug-effected wells of the plate (A2, A3, B2, and B3). For A1 and B1, data shown are the mean ± standard error, n = 10–21 separate wells. For A2, A3, B2, and B3, data shown are the LC₅₀ calculated using motility data from duplicate or triplicate assay wells at each of 4–5 drug concentrations.
adverse effects on motility (Figure 2 B1). Despite the markedly decreased motility observed in control S. ratti larvae following storage, the LC50 derived from these assays was similar to that obtained with fresh larvae (up to 1.65-fold less than the LC50 at zero storage time). That is, the LC50 varied little when the number of motile larvae were expressed as a percentage of motile control worms even though the controls were also adversely affected by storage. For A. caninum, storage of worms prior to testing had a similar effect on LC50 (approximately a two-fold variation in ivermectin LC50 after five days storage). Thus, aging of larvae prior to testing did not disproportionately affect the response to anthelmintic.

Varying the temperature at which the assay plates were held during the 48-hour incubation led to some changes in LC50 values (Table 2). With S. ratti, 2.4- and 1.8-fold decreases in LC50 were observed with thiabendazole and ivermectin, respectively, as the temperature increased from 20°C to 29°C (95% confidence intervals at 20°C and 29°C did not overlap for either drug). The LC50 for ivermectin with A. caninum also showed a similar 1.6-fold decrease; however, the thiabendazole LC50 remained unchanged.

**Drug sensitivity of three hookworm species, S. ratti, and S. stercoralis.** Mean LC50 results obtained for thiabendazole and ivermectin with S. ratti, A. caninum, A. ceylanicum, N. americanus, and S. stercoralis using the 48-hour assay are shown in Table 3 (insufficient S. stercoralis were available for assays with ivermectin). Typical dose-response curves are shown in Figure 3. There were marked differences in the relative sensitivity of various species to the drugs. Larvae of S. ratti were the most susceptible to thiabendazole and the most tolerant of ivermectin. Necator americanus larvae showed an LC50 to ivermectin approximately two-fold higher than the other hookworm species, and were markedly more tolerant of thiabendazole than all the other species; approximately 50% of N. americanus larvae remained motile at the highest drug concentration that could be examined due to the solubility limitation of the drug (2 mM).

**Field testing of the drug sensitivity assay.** Testing of N. americanus larvae (isolated from human fecal samples in Madang Province, Papua New Guinea) with ivermectin demonstrated a clear dose-response (Figure 4) with an LC50 value of 0.072, similar to that obtained for the University of Nottingham isolate of this species (Table 3). Some tolerance was noted when the larvae were assayed with thiabendazole, with up to 28% motility noted in several wells at the highest drug concentration. However, insufficient larvae were recovered from fecal cultures to allow the completion of full dose-response experiments with both drugs.

### Table 2

Effects of assay temperature on sensitivity of *Strongyloides ratti* and *Ancylostoma caninum* larval motility to inhibition by thiabendazole and ivermectin

<table>
<thead>
<tr>
<th>Species</th>
<th>Drug</th>
<th>20°C (LC50 ± SE)</th>
<th>25°C (LC50 ± SE)</th>
<th>29°C (LC50 ± SE)</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>S. ratti</em></td>
<td>Thiabendazole</td>
<td>16 (11–24)</td>
<td>8.6 (5.9–13)</td>
<td>6.6 (4.2–10)</td>
</tr>
<tr>
<td></td>
<td>Ivermectin</td>
<td>1.2 (1.1–1.4)</td>
<td>0.96 (0.80–1.16)</td>
<td>0.68 (0.58–0.80)</td>
</tr>
<tr>
<td><em>A. caninum</em></td>
<td>Thiabendazole</td>
<td>182 (160–208)</td>
<td>184 (148–228)</td>
<td>196 (175–220)</td>
</tr>
<tr>
<td></td>
<td>Ivermectin</td>
<td>0.084 (0.077–0.091)</td>
<td>0.055 (0.050–0.061)</td>
<td>0.054 (0.014–0.203)</td>
</tr>
</tbody>
</table>

*50% lethal concentration (LC50) values were determined after 48 hours of exposure to drug using worms recovered fresh from fecal cultures. Dose-response data consisted of four replicate wells at each two-fold serially diluted drug concentration.

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**DISCUSSION**

This study describes the development of an *in vitro* assay that enables assessment of the response of larval stages of hookworm and *Strongyloides* parasites to anthelmintic drugs. A clear dose-response to thiabendazole, a representative member of the benzimidazole class of anthelmintics, and to ivermectin, the only ML anthelmintic registered for human use, was demonstrated.

For the assay to demonstrate utility for diagnosis of drug resistance, it will be necessary to establish a correlation between results of the *in vitro* assay and the sensitivity of a parasite species to a drug *in vivo*. That is, it will be important to compare *in vitro* data with therapeutic efficacy as measured by fecal egg count reduction assay, to determine whether differences in the LC50 or LC95 correspond to different rates of parasite clearance. Such a relationship has been established in the veterinary field, where a reduced therapeutic response to ivermectin among certain isolates of *H. contortus* correlates with a reduced sensitivity to ivermectin among larval stages of the parasite as measured by larval motility.15 This association between *in vivo* efficacy and the results of a conceptually similar larval development assay has enabled the development and marketing of a commercial test (Drenchrite®; Horizon Technology) to define the resistance status of nematode parasites of sheep and goats towards the benzimidazole, levamisole, and ML drug groups.

The relative tolerance observed in the University of Not-

### Table 3

Dose-response data for *Strongyloides ratti*, *Ancylostoma caninum*, *A. ceylanicum*, *N. americanus*, and *S. stercoralis* with thiabendazole and ivermectin

<table>
<thead>
<tr>
<th>Species</th>
<th>Drug</th>
<th>LC50 ± SE (μM)*</th>
<th>(n)†</th>
<th>Slope</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>S. ratti</em></td>
<td>Thiabendazole</td>
<td>3.5 ± 1.2</td>
<td>(13)</td>
<td>−2.7 ± 1.1</td>
</tr>
<tr>
<td></td>
<td>Ivermectin</td>
<td>0.49 ± 0.09</td>
<td>(8)</td>
<td>−9.4 ± 2.9</td>
</tr>
<tr>
<td><em>A. caninum</em></td>
<td>Thiabendazole</td>
<td>148 ± 25</td>
<td>(4)</td>
<td>−3.6 ± 0.6</td>
</tr>
<tr>
<td></td>
<td>Ivermectin</td>
<td>0.069 ± 0.018</td>
<td>(4)</td>
<td>−3.4 ± 0.3</td>
</tr>
<tr>
<td><em>A. ceylanicum</em></td>
<td>Thiabendazole</td>
<td>28.5 ± 4.9</td>
<td>(3)</td>
<td>−0.6 ± 0.1</td>
</tr>
<tr>
<td></td>
<td>Ivermectin</td>
<td>0.051 ± 0.01</td>
<td>(3)</td>
<td>−1.2 ± 0.1</td>
</tr>
<tr>
<td><em>N. americanus</em></td>
<td>Thiabendazole</td>
<td>2,180 ± 290</td>
<td>(3)</td>
<td>−0.5 ± 0.02</td>
</tr>
<tr>
<td></td>
<td>Ivermectin</td>
<td>0.12 ± 0.02</td>
<td>(3)</td>
<td>−1.5 ± 0.2</td>
</tr>
<tr>
<td><em>S. stercoralis</em></td>
<td>Thiabendazole</td>
<td>246 (134–451)</td>
<td>(1)</td>
<td>−1.7</td>
</tr>
</tbody>
</table>

*50% lethal concentration (LC50) values were determined after 48 hours of exposure to drug using worms stored previously at 20°C for <2 days after recovery from fecal cultures.
† n = number of separate determinations, each with 2–6 assay wells over a range of drug concentrations; except for *S. stercoralis* where data is derived from a single assay with duplicate assay wells at each of four drug concentrations (95% confidence intervals for the single LC50 value are shown).
N. americanus isolate towards thiabendazole may limit the usefulness of the assay for the detection of resistance to the benzimidazole class of anthelmintics in this species. Only 50% of the larvae were affected by the highest concentration of thiabendazole that could be used in the assay format. Despite this, an apparent dose-response relationship was evident up to a drug concentration roughly equivalent to the LC50 (Figure 3). Thus, the assay may still be an effective indicator of resistance if the resistance is associated with a significant change in the LC50. While it may be possible to detect benzimidazole resistance in this species by observing a reduction in sensitivity at the highest thiabendazole concentrations, this would represent only the response of the most drug-sensitive individuals among the population. The more tolerant individuals in both susceptible and resistant populations will clearly be unaffected by the drug in the assay. In contrast, for all other drug/parasite combinations examined, a full dose-response relationship over a range up to the complete inhibition of motility was readily demonstrated, and therefore, would enable the detection of changes in LC95. It will be important to determine the dose response towards thiabendazole of N. americanus isolated from humans to assess the potential usefulness of the assay for field measurements of drug susceptibility with this drug/parasite combination.

A variety of in vitro assays have been described for monitoring drug sensitivity of parasites. The motility assay described in the present study needs to be examined alongside egg hatch assays and larval development assays to determine the most suitable assay for monitoring the development of resistance in human hookworm and Strongyloides species. Parallel assessments of each assay with susceptible and resistant parasite strains will be required.

The motility assay described here has several advantages for testing of human intestinal nematode drug resistance. These include the requirement of a minimum of fecal manipulations (migration of infective larvae from fecal cultures to}
obviates the need to prepare eggs from feces), and its easily transportable agar matrix format. A significant advantage also is its utility in a field situation where freshly collected human parasites could be studied. Few items of equipment were required (low-speed centrifuge, 250× magnification microscope). The assay temperature and larval storage experiments in this study indicate that variations in these two parameters have little effect on assay results (LC₅₀ varied <2-fold). Ideally, to minimize the effects of assay conditions on dose-response results, parasite larvae isolated in the field would be stored for no longer than one or two days before being assayed at approximately 25–30°C. In addition, the test showed robust performance over a range of temperatures, suggesting that it would work satisfactorily at ambient temperature in tropical environments. A limitation of the initial field testing of this assay was the yield of larvae obtained from the small Harada-Mori cultures. Coproculture of larger samples of feces (complete samples rather than the 5–10 grams used for the present field study) should yield sufficient larvae, as was observed when dog feces were cultured for A. caninum larvae. Once sufficient dose-response data have been collected to define the range of drug sensitivity to be expected in wild-type, susceptible field populations, it may be possible to monitor for resistance using a single discriminating drug concentration rather than depending on a full dose-response assay. This would greatly reduce the numbers of larvae required from each fecal sample.

We observed significant differences in the responses of the five parasite species to the two test drugs, some of which do not correlate with that seen when the drugs are used in vivo. For example, the relative in vitro sensitivity of hookworm and Strongyloides larvae to ivermectin are the reverse of what is seen in vivo. Possible explanations include differences in the mode of action, and physical properties of the drugs resulting in different bioavailability. Although the motility assay has not been evaluated with drug-resistant parasite strains, its ability to discriminate between parasites species and strains showing different responses to drugs suggests its likely utility. For example, N. americanus showed an approximately two-fold higher LC₅₀ towards ivermectin than A. ceylanicum, a finding that is in agreement with previous reports showing that the former species is more resistant to this drug than the latter. However, the 300-fold difference in sensitivity seen in in vivo efficacy trials in the hamster model, and the 40–50-fold differences in sensitivity observed in an in vitro motility and ingestion assays using adult worms suggests that the larval motility assay may be a less sensitive indicator of drug sensitivity differences between these two species than in vivo or in vitro adult worm assays. However, the basis for differences in drug sensitivity between species may be unrelated to the mechanism of drug resistance shown by different isolates within a species due to the pharmacologic determinants of drug sensitivity, including cuticle penetration rates, detoxification enzyme levels, and activity of drug efflux pathways. Ultimately, the ability of an assay to detect drug resistance by whatever mechanism that emerges in field isolates of a parasite will determine its utility as a diagnostic test. Thus, assessment of the usefulness of the assay described in the present study for detection of resistance will need to await the examination of resistant strains for each particular species rather than rely on interpretation of current data on species differences.

While current WHO-sponsored efforts to control soil-transmitted helminths are based on the use of albendazole in combination with DEC or ivermectin, we found that albendazole was unsuitable for use in the motility assay. In contrast, thiabendazole performed well in providing clear dose-response data. The inability to directly assess sensitivity to albendazole with this assay may not be an impediment because cross-resistance is the rule within the benzimidazole class of anthelmintics. Thus, resistance to one drug within the class indicates the existence of resistance to members of the class in general. The suitability of thiabendazole in our assay system identifies it as a suitable drug for use in validating the assay as a drug resistance detection tool for the benzimidazole group of drugs.

While the motility assay has potential as a field-based test for resistance, alternate approaches to resistance detection include polymerase chain reaction (PCR)–based assays. Molecular diagnosis of drug resistance has the potential to detect resistance at much earlier stages compared with in vitro whole-worm assays, thereby allowing appropriate management decisions (drug rotation, etc.) to be made as resistance first appears. Molecular tests for benzimidazole resistance in parasitic species of veterinary importance have been described. Therefore, while the motility assay described in the present study is a potentially useful tool for the detection of resistance, it will also be useful for preliminary validation of PCR tests to relate PCR data directly to drug sensitivity observations.

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