**Dose-Response Assay Templates for *In vitro* Assessment of Resistance to Benzimidazole and Nicotinic Acetylcholine Receptor Agonist Drugs in Human Hookworms**

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**Abstract.** With the implementation of mass drug administration programs for the control of human hookworms, there is a need to monitor for the emergence of drug resistance. We have therefore examined *in vitro* assays for monitoring sensitivity to benzimidazoles (egg hatch assay) and nicotinic acetylcholine receptor agonist drugs (motility and morphology assays), with a view to developing tools for monitoring drug sensitivity in the field. We have performed assays with *Necator americanus* and *Ancylostoma ceylanicum*, and combined this with published data on *N. americanus* and *Ancylostoma caninum*, to indicate the breadth of the responses of various hookworm species and isolates in these *in vitro* assays. This has allowed us to generate assay templates covering the known range of responses, with scope to cover any shift in response that may be indicative of resistance. These assays will have immediate applicability in monitoring for the emergence of drug resistance in human hookworm populations.

**INTRODUCTION**

Soil-transmitted helminths (STHs) have a major impact on human health in many tropical and subtropical countries. Control of these parasites relies on the periodic mass administration of anthelmintics. Drugs recommended by the World Health Organization (WHO) for the control of STH infections are the benzimidazoles, mebendazole, and albendazole, and the nicotinic acetylcholine receptor (nAChR) agonist drugs, levamisole and pyrantel. The use of these drug groups for control of gastrointestinal nematode parasites in livestock has lead to the emergence of significant levels of resistance. Hence, there is a need to manage the use of the drugs used against human STHs to guard against the emergence of resistance, because the number of drugs available for rotation is small, and there cannot be reliance upon continuous development of novel compounds. An important part of managing drug use for the control of human STHs will be an ability to detect resistance should it emerge. The identification of resistance in a population will indicate the necessity of using an alternative drug or drug combinations against that worm population to maintain effective levels of control, and to prevent further selection pressure on the initial drug group to which resistance is appearing. Monitoring sensitivity to both the alternative and initial drugs would then be important for the management of further drug use in that human population.

The tests available to monitor drug resistance in nematode parasites fall into three broad groups: 1) the Fecal Egg Count Reduction Test (FECRT) in which the fecal egg counts in pre- and post-drug treatment human samples are compared to indicate the percentage reduction in egg count as a result of the drug treatment; 2) phenotypic assays in which the effects of drugs on free-living life cycle stages are examined with *in vitro* bioassays; and 3) molecular tests in which the genotypic changes associated with drug resistance are monitored using polymerase chain reaction (PCR)-based methods. The FECRT is the most immediately available of these tests for diagnosis of resistance in human STHs, and is currently being assessed in this role by the WHO. However, it suffers from a number of weaknesses, which may confound the ability to detect resistance; for example, effects of pre-treatment infection levels on observed fecal egg count reductions, and density-dependent fecundity effects. Phenotypic tests are used in the livestock industries with some drug groups, most notably egg hatch assays with benzimidazoles. Molecular tests are also available for detecting resistance to benzimidazole drugs in some livestock nematode species. For the nAChR agonist drug group, phenotypic tests have been described for livestock and companion animal gastrointestinal nematode species. The only evidence for the molecular basis of resistance to this drug class in a parasitic nematode species indicates that gene transcription levels may be involved rather than gene polymorphisms. Whereas phenotypic tests would appear to be directly transferable from the livestock to the human field because they examine direct drug effects on whole worms *in vitro*, the use of molecular tests for the human STHs requires more information on the likely polymorphisms or other molecular changes that may be associated with resistance in these species. Hence, the application of phenotypic tests to human STHs may offer a means to monitor drug sensitivity in these parasites in the short term, while more sensitive molecular tests are being developed and validated.

Therefore, we have sought to develop templates for phenotypic assays for use in the field to measure drug sensitivity in human hookworms. We have examined egg hatch assays for the benzimidazole drug group, as well as motility and morphology assays for the nAChR agonist drug group. Egg hatch assays have been widely used with nematodes of livestock to detect resistance, and several studies have tested human hookworm populations using this type of assay. These previous human field studies involved a population that was predominantly *Necator americanus*, with some *Ancylostoma duodenale* also present, or *N. americanus* alone. We have measured the response of laboratory-maintained drug-susceptible isolates of the human hookworm *N. americanus*, as well as *Ancylostoma ceylanicum*, in egg hatch assays with thiabendazole, and combined these data with those from the published literature on human and canine hookworm egg hatch assays to devise a drug concentration range that may be useful for field monitoring of human hookworm responses to the benzimidazole drug group. Similarly, we have examined the laboratory isolates of *N. americanus* and *A. ceylanicum* with pyrantel motility and...
morphology assays, alongside data from the literature describing the use of these tests with canine hookworms showing some resistance to this drug to devise a suitable concentration range for this drug with human hookworms. In addition, for the egg hatch assay, we have used the livestock parasite *Haemonchus contortus* to compare an agar-based method with the water-based format, which is traditionally used for this assay with livestock nematodes. This component of the present study aimed to compare the resistance-diagnosing abilities of the two assay systems as an important determinant of which system may be most useful for field studies with human hookworms.

**MATERIALS AND METHODS**

**Parasite isolates.** Sheep infected with *H. contortus* were housed at the McMaster Laboratory, CSIRO Livestock Industries, Armidale, New South Wales (NSW), Australia. Three isolates were used.

Kirby 1982 – isolated from the field at the University of New England Kirby Research Farm in 1986.17

Wallangra 2003 – a multidrug resistant strain isolated from the New England region in 2001.18

Gold Coast 2004 – an isolate from the Gold Coast hinterland of coastal southern Queensland.19

*Necator americanus* and *A. ceylanicum* were maintained in hamsters at the University of Nottingham. The isolates have not been exposed to anthelmintics since isolation from humans and dogs, respectively, in India over 30 years ago.

Approval for the use of sheep was obtained from the CSIRO Livestock Industries Armidale Animal Ethics Committee, approval number 08/20. Procedures for the care of the sheep adhered to the CSIRO guidelines for animal husbandry. The maintenance of hookworms in hamsters was conducted under a British Home Office Project License (Ref: 40/2621), after initial approval by the Ethical Review Committee (Animals) of the University of Nottingham and confirmed by the Inspectorate of the Home Office under the Animals (Scientific Procedures) Act 1986. Animals were maintained and treated in strict accordance with the standard operating procedures in force in the Biomedical Services Unit of the University.

**Preparation of eggs.** Feces from *H. contortus*–infected sheep were sent by courier from Armidale to the Queensland Biosciences Precinct laboratory at St. Lucia, Brisbane, in airtight bags. Nematode eggs were recovered from the feces by passage through a series of fine sieves (250 and 75 μm) followed by centrifugation in a stepwise sucrose gradient (10%, 25%, and 40% w/w sucrose). The eggs were recovered from the interface between the 10% and 25% sucrose layers, and washed over a 25-μm sieve with water to remove residual sucrose. The eggs were diluted in distilled water at a concentration of 70–90 eggs per 30 μL after the addition of amphotericin B (final concentration 25 μg/mL), and used immediately for egg hatch assays.

Feces from either *N. americanus*– or *A. ceylanicum*–infected hamsters were divided between 6 × 20 mL plastic tubes, and 10 mL saturated saline solution was added, and the tubes were rotated to mix for 1 hour. The solution was then passed through a sieve into a small beaker, and the sieve washed with 10 mL of saturated saline. The solution was then poured into plastic test tubes and centrifuged at 300 × g for 5 minutes. The top 1 cm of solution from each tube was transferred to 20 mL plastic tubes and topped up with 15 mL of distilled water and centrifuged for 10 minutes at 2,000 rpm. The supernatant was poured away and the pellets were resuspended in a little water and pooled together. The concentration of eggs was determined by counting those present in at least three 10 μL aliquots, and the eggs were diluted in distilled water at a concentration of 30–35 eggs per 30 μL and used immediately for egg hatch assays.

**Preparation of L3 stage worms.** *Ancylostoma ceylanicum.* Feces were collected overnight on damp paper, transferred to a beaker and mixed together with equal parts of fine granules of activated charcoal, and then mixed with a small amount of distilled water to form stiff slurry. A small portion was very thinly smeared on a 70-mm circle of Whatman filter paper (Whatman, Kent, UK) in a plastic Petri dish. The dishes were placed in a plastic box lined with damp paper and incubated at room temperature for 7 days. The cultures were checked frequently to ensure they remained moist. Infective larvae were harvested by lifting the filter paper and rinsing the back of the paper into the Petri dish and collecting the solution. The solution was centrifuged at 300 × g for 2 minutes, and the supernatant was removed, leaving a volume of 5 mL in the bottom of the tube. The larvae were re-suspended by agitation and the numbers of larvae present was ascertained by counting an aliquot (100 μL).

*Necator americanus.* The culture of larvae for this species was similar to that described previously for *A. ceylanicum,* but using an adaption of the method of Harada and Mori.20 Briefly, strips of filter paper smeared with feces were placed into glass boiling tubes. Moisture was maintained by adding water (5 mL) to the tubes and sealing them with petroleum jelly and cling film. Larvae were collected after 7 days at 26°C by removing the filter paper and rinsing the sides of the tube with distilled water. Centrifugation and measurement of the concentration of larvae were performed as described previously for *A. ceylanicum.*

**Egg hatch assay.** Egg hatch assays were performed with *H. contortus* in a series of experiments in which agar-based (modified from Kotze and others,15 and Lacey and others16) and water-based (modified from Coles and others6) methods were compared, as well as in a set of experiments with *N. americanus* and *A. ceylanicum* in which only the agar-based method was used.

For agar-based assays, a stock solution of thiabendazole (5 mg/mL) was prepared in dimethyl sulfoxide (DMSO) and serially diluted 2-fold in the same solvent. Aliquots (2 μL) from a series of dilutions (including the stock) were added to 96-well microtitre plates, such that each row of the plate comprised a gradient of 10 dilutions. The first two wells of each row were used as control wells (received 2 μL of DMSO only). Each drug concentration was present in quadruplicate (*H. contortus*) or triplicate (*N. americanus, A. ceylanicum*) wells on each plate. The 200 μL of 2% agar (Davis Gelatine Co., Botany, New South Wales, Australia; powdered agar Grade J) was dispensed into each well of the plate and allowed to set. Plates were placed into plastic press-seal bags stored at 4°C for no more than 3 months. Prior to use, plates were equilibrated to room temperature for 2 h before 30 μL of egg suspension in H₂O was dispensed onto the surface of the agar in each well, distributing 70–90 *H. contortus,* or 30–35 *N. americanus* and *A. ceylanicum* eggs into each well. Plates were then returned to a bag and incubated for 48 h at 26°C. Lugol’s iodine (10 μL) was then added to each well. For the *H. contortus* assays, the numbers of larvae present in each drug well were counted using an inverted microscope.
and numbers of larvae and unhatched eggs were also counted in 12 control wells for each experiment. For *N. americanus* and *A. ceylanicum*, the amount of fecal material in each well prevented direct counting within the well. The contents of each well were therefore pipetted onto a slide for counting of larvae in drug and control wells. *Haemonchus contortus* experiments were repeated three times, whereas *N. americanus* and *A. ceylanicum* experiments were performed twice.

In a separate experiment, we prepared a batch of assay plates, reserved a single plate for use immediately, and placed the others into press-seal bags (12 × 21 cm) along with a sheet of absorbent cloth (Chux Superwipes, Clorox Australia Pty. Ltd., Padstow, New South Wales, Australia; cut into 11 × 7 cm pieces), which had been soaked in a solution of 2.5 μg/mL of amphotericin B. The plates were placed into incubators at either 4 or 26°C. Freshly prepared *H. contortus* Kirby1982 eggs were assayed in the single plate on the day of preparation of the plates, and then again at various times afterwards using plates stored for up to 78 days at the two temperatures.

The water-based assay used a 96-well format rather than the previously described 24-well format. Into each drug well were deposited 0.43 μL of the series of thiabendazole solutions described previously, and 20 μL of distilled water, and the plates were agitated for 30 minutes on a plate shaker. Control wells received DMSO only. Egg solution (30 μL) (*H. contortus* only) was then added, and the plates were sealed in plastic bags and incubated at 26°C for 48 hours. Numbers of larvae and/or eggs were then counted as described previously for the agar assay.

**Larval morphology/motility assays.** These assays were performed with *N. americanus* and *A. ceylanicum* larvae in 96-well microtitre plates using the agar-matrix technique described by Kopp and others. A stock solution of pyrantel citrate (40 mg/mL) was prepared in DMSO, and serially diluted 4-fold in the same solvent. Aliquots (2 μL) from a series of 20 dilutions were added to 96-well microtitre plates, such that each row of the plate comprised a gradient of 10 dilutions. The first two wells of each row were used as control wells (received 2 μL of DMSO only). Each drug concentration was present in triplicate assay wells on each plate. The 200 μL of 2% agar (Davis Gelatine Co., powdered agar Grade J) were dispensed into each well of the plate and allowed to set. Plates were stored at 4°C in lightproof containers for no more than 3 months. Prior to use, plates were equilibrated to room temperature for 2 h before 30 μL of larval suspension in H2O (with 50 U/mL penicillin, 50 μg/mL streptomycin, and 25 μg/mL amphotericin B) were dispensed onto the surface of the agar in each well, distributing ~25–35 larvae into each well. Plates were then placed in plastic press-seal bags and incubated for 48 h at 25°C.

The first assessment of drug effects was based on the observation of Kopp and others that the presence of pyrantel causes an alteration in the posture shown by *A. caninum* larvae in the quiescent state, which they adopt when left in aqueous solutions at room temperature (the “larval arrested morphology assay” [LAMA]). Larvae that were quiescent without any kinking or with only very mild deviations about the head or tail were counted as “normally quiescent.” Coiled larvae, or larvae with extensive kinking throughout the body were not counted. The effect of pyrantel at each concentration was thus quantified by comparing the percentage of “normally quiescent” larvae in each drug well to the average shown by control wells.

Given that the LAMA required observation only, the same plates and larvae could subsequently be used for the larval motility assay (LMA) as previously described. This was undertaken by adding 40 μL of water heated to 50°C to assay wells to stimulate the worms to move. Larvae moving with smooth sinusoidal motion were counted, whereas immotile or twitching larvae were not counted. Numbers of larvae moving normally in each well were expressed as a percentage of the mean for control wells.

Each experiment was conducted with a single batch of larvae recovered after 7 days of incubation and used immediately. The numbers of replicate drug wells used for each assay varied according to the availability of larvae on particular days. Each experiment consisted of single or duplicate assay wells at a range of drug concentrations for *N. americanus*, and duplicate or triplicate assay wells at each drug concentration for *A. ceylanicum*, with ~25 or 30–35 larvae added to each well for *N. americanus* and *A. ceylanicum*, respectively. The numbers of control (no drug) wells on each plate varied from 4 to 6 for *N. americanus*, and was 8 for *A. ceylanicum*. Two separate experiments were conducted for each species.

**Statistical analyses.** The percentage egg hatch for each worm isolate in control wells of water- and agar-based egg hatch assays were compared using t tests (*P* = 0.05). Dose responses for egg hatch, larval morphology, and larval motility experiments were examined using non-linear regression (GraphPad Prism software, San Diego, CA). The IC50 values were defined as the concentration of drug required to inhibit egg hatch to 50% of that observed in controls, or cause abnormal quiescence or motility in 50% of larvae relative to controls. Log-transformed IC50 data were analyzed using one-way analysis of variance (ANOVA), and significant differences in IC50 values were determined using Tukey-Kramer Multiple Comparison Tests, except for motility data for which an unpaired t test was used (as only two nematode isolates were examined for this assay). Differences were considered significant at *P* < 0.05. For the thiabendazole stability experiment, overlap of 95% confidence intervals (CI) was assessed for the single IC50 values obtained at each time point for each temperature.

**RESULTS**

Agar- and water-based egg hatch assays with thiabendazole were compared using three *H. contortus* isolates. The mean egg hatch in control (no drug) wells was over 90% in all cases (*N* = 3 independent experiments with each isolate, each experiment with 12 control assay wells), and there were no significant differences between hatch in agar- and water-based assays in control wells for each isolate (*P* = 0.05, pooled data from 3 experiments). Dose response data for thiabendazole against the Kirby1982 and GoldCoast2004 isolates are shown in Figure 1, with IC50 values for these isolates and Wallangra2003 given in Table 1. Dose responses were sigmooidal in each case. The dose response was shifted to the right for each agar assay relative to the respective water-based assay. This shift amounted to an increase in IC50 of between 1.6- and 2.8-fold. In terms of resistance diagnosis, the two assays performed very similarly, with the two drug resistant isolates showing resistance ratios (IC50s relative to Kirby) of 29 or 21 for GoldCoast, and 21 or 26 for Wallangra.

Agar-based egg hatch assays with thiabendazole were conducted with *N. americanus* and *A. ceylanicum*. The results
alongside data taken from Albonico and others\textsuperscript{14} (\textit{N. americanus}) (using a water-based method) and Kotze and others\textsuperscript{15} (\textit{N. americanus} and \textit{A. caninum}) (using an agar-based method) are shown in Figure 2, with IC\(_{50}\) values in Table 2. The various field populations and laboratory isolates showed sigmoidal dose responses, with IC\(_{50}\) values varying over a 4.6-fold range. Of note however were the differences between data sets for \textit{N. americanus}. The populations from the two field studies\textsuperscript{14,15} showed significantly higher IC\(_{50}\) values than the laboratory-resistant \textit{N. americanus} isolate. The IC\(_{50}\) for \textit{A. caninum} was similar to the laboratory \textit{N. americanus}, whereas that for \textit{A. ceylanicum} was similar to the two field \textit{N. americanus} populations. Consideration of the various dose-responses enabled the selection of a range of 10 2-fold serially diluted drug concentrations, which represented a potentially useful range for a standardized assay template, centered on the data sets for the two field populations of \textit{N. americanus}. This range is shown by the arrows below the x-axis in Figure 2. Its lower point was 0.01 \(\mu\)g/mL, which was 0.6-fold lower than the IC\(_{50}\) for the laboratory isolate of \textit{N. americanus}, and up to 7.8-fold lower than the IC\(_{50}\) for two field \textit{N. americanus} populations, ranging up to 5.0 \(\mu\)g/mL, representing a 64-fold increase above the highest observed IC\(_{50}\). This upper limit of 5.0 \(\mu\)g/mL was 15-fold higher than the highest concentration at which hatching occurred with the laboratory \textit{N. americanus} isolate (0.34 \(\mu\)g/mL), and 11-fold higher than the IC\(_{99}\) value derived from Albonico and others\textsuperscript{14} (data not shown).

### Figure 1
Thiabendazole egg hatch assay dose responses of \textit{Haemonchus contortus} isolates. Data from water-based assays shown with solid lines and from agar-based assays with dashed lines. For clarity, the Wallangra2003 isolate is not shown. Data are pooled from three separate experiments, each with quadruplicate assay wells at a range of drug concentrations.

### Figure 2
Dose responses of various hookworm species/isolates in egg hatch assays with thiabendazole. Some data taken from Albonico and others\textsuperscript{14} and Kotze and others\textsuperscript{15}. Arrows below x axis indicate concentration range subsequently chosen as suitable for standard thiabendazole assay format (see text for details).

### Table 1

<table>
<thead>
<tr>
<th>Isolate</th>
<th>IC(_{50}) ± SE ((\mu)g/mL)</th>
<th>RR†</th>
<th>IC(_{50}) ± SE ((\mu)g/mL)</th>
<th>RR†</th>
</tr>
</thead>
<tbody>
<tr>
<td>Kirby1982 water</td>
<td>0.035 ± 0.004 a</td>
<td></td>
<td>0.078 ± 0.005 b</td>
<td></td>
</tr>
<tr>
<td>GoldCoast2004 water</td>
<td>1.01 ± 0.27 cd</td>
<td>29</td>
<td>1.60 ± 0.04 de</td>
<td>21</td>
</tr>
<tr>
<td>Wallangra2003</td>
<td>0.74 ± 0.11 c</td>
<td>21</td>
<td>2.06 ± 0.16 e</td>
<td>26</td>
</tr>
</tbody>
</table>

*IC\(_{50}\) = concentration of drug required to reduce egg hatch to 50% of that observed in control wells; mean ± SE, N = 3 separate experiments, each with quadruplicate assay wells at a series of drug concentrations; IC\(_{50}\) values followed by the same letter are not significantly different at P = 0.05.

† RR (resistance ratio) = IC\(_{50}\) of drug-resistant isolate/IC\(_{50}\) of Kirby1982.

### Table 2

<table>
<thead>
<tr>
<th>Data source/hookworm species</th>
<th>IC(_{50}) ± SE ((\mu)g/mL)</th>
</tr>
</thead>
<tbody>
<tr>
<td>This study, \textit{N. americanus}</td>
<td>0.017 ± 0.001 (2) a</td>
</tr>
<tr>
<td>Albonico and others\textsuperscript{14}, predominantly \textit{N. americanus}</td>
<td>0.078 ± 0.002 (4) b</td>
</tr>
<tr>
<td>Kotze and others\textsuperscript{15}, \textit{N. americanus}</td>
<td>0.076 ± 0.014 (8) b</td>
</tr>
<tr>
<td>This study, \textit{Ancylostoma ceylanicum}</td>
<td>0.056 ± 0.006 (2) b</td>
</tr>
<tr>
<td>Kotze and others\textsuperscript{15}, \textit{Ancylostoma caninum}</td>
<td>0.018 ± 0.001 (5) a</td>
</tr>
</tbody>
</table>

*IC\(_{50}\) = concentration of drug required to reduce egg hatch to 50% of that observed in control wells; mean ± SE, n = number of separate experiments, each with 2-3 assay wells at a series of drug concentrations; IC\(_{50}\) values followed by the same letter are not significantly different at P = 0.05.
IN VITRO HOOKWORM DRUG SENSITIVITY ASSAYS

In a separate experiment to examine the stability of thiabendazole egg hatch assay plates, we assessed the effect of storage of plates at 4 or 26°C, using eggs of the *H. contortus* Kirby1982 isolate. The assay performed similarly over a 78-day period, with IC₅₀ values at Days 1 and 78 showing overlapping 95% CIs (data not shown) for both temperatures, and the IC₅₀ values at the seven time points examined in the experiment varying over only a 2.2-fold range. No drying or cracking was observed in the agar, and there were no signs of bacterial or fungal growth.

Morphology and motility assays were performed with pyrantel against the L3 stages of the laboratory isolates of *N. americanus* and *A. ceylanicum*. These data, alongside the morphology assay data from Kopp and others for two isolates of *A. caninum* showing different sensitivities to the drug (pyrantel efficacies 28% and 71% for pyrantel resistant (PR), and northern territory (NT), respectively), are shown in Figure 3 and Table 3. *Necator americanus* showed sigmoidal dose responses in both the morphology and motility assays. The IC₅₀ for *N. americanus* in the morphology assay was similar to the *A. caninum* PR isolate. *Ancylostoma ceylanicum* was significantly less susceptible to the effects of this drug than the other species/isolates in the morphology assay. The percentage of “normally” arrested *A. ceylanicum* larvae decreased to almost zero at the second highest drug concentration assayed, before increasing to ~30% at the highest concentration. As described previously for thiabendazole egg hatch assays, the pyrantel concentrations, which represent a useful range for a standardized assay template, focusing primarily on the *N. americanus* response in both L3 stage assays, are shown by arrows below the x axis in Figure 3.

DISCUSSION

The present study indicates that in vitro dose responses of human hookworms to a benzimidazole and a nicotinic acetylcholine receptor agonist drug are readily assessed using agar-based assay systems. Although such egg hatch assays have been used previously with thiabendazole and human and canine hookworms, the present study is the first demonstration that the recently described *A. caninum* larval morphology assay is also effective in defining a dose response to the nicotinic agonist pyrantel in the human hookworm *N. americanus*, as well as *A. ceylanicum*. In addition, the second stage of this assay (the assessment of motility after stimulation of the larvae with hot water) gave clear sigmoidal dose responses for the latter species, in contrast to the biphasic response for *A. caninum*, which complicated its ability to quantify resistance ratios through the use of relative IC₅₀ for that species.

We compared agar- and water-based egg hatch assay systems for measuring sensitivity to the benzimidazole drug group to assess which system may be most useful for field studies with human hookworms. Using a livestock nematode species for which benzimidazole-resistant isolates are available, we have shown that there is a consistent degree of difference between agar- and water-based assays, amounting to an approximate 2-fold higher IC₅₀ value for the former. The susceptible isolate had an IC₅₀ below 0.1 μg/mL in both systems, indicating its susceptibility by WAAVP criteria. In addition, both the resistant isolates showed similar resistance ratios compared with the sensitive isolate in both assay systems, indicating that they are largely equivalent as far as drug resistance diagnosis is concerned. The 2-fold increase in IC₅₀ in the agar assays may be a result of a slight delay in the infusion of drug from the agar into the aqueous environment immediately surrounding the eggs after they are deposited onto the agar surface. This delay may allow for some embryonic development, which slightly increases the egg’s tolerance of the drug.

Given the apparently equivalent resistance-diagnosing ability of the two assay systems, the choice of assay for use with human hookworms may come down to factors such as ease of use in the field, and the ability to standardize across different laboratories and field sites. The agar-based system would seem to have an advantage in both these regards. Assay plates could

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**Figure 3.** Dose responses of various hookworm species isolates in larval morphology (A) and motility (B) assays with pyrantel. Some data taken from Kopp and others. Arrows below x axis indicate concentration range subsequently chosen as suitable for standard pyrantel assay format (see text for details).
be produced in a “quality-controlled” manufacturing environment, and then distributed widely, thereby removing the need for individual laboratories to manipulate drug solutions. The use of uniform assay plates would greatly aid in efforts to make direct comparisons of drug sensitivity data collected at different field sites, or at different time points at single sites. Use of the agar plates would also avoid the need for transporting technical grade drugs or organic solvents to field laboratories. A further advantage lies in the fact that the meniscus formed by the agar within each well improves viewing and counting of larvae by concentrating them to the center of the well. Our storage experiment showed that the toxicity of the benzimidazole within the agar did not decrease significantly over a 2-month period, and in addition, desiccation of the agar was not observed over this period.

A comparison of responses of the various *N. americanus* samples to thiabendazole indicated that the populations examined in the two field studies\(^{14,15}\) showed significantly reduced sensitivity to the drug compared with the laboratory susceptible isolate. The lack of *in vitro* thiabendazole sensitivity data for field isolates makes it difficult to interpret our observed difference in sensitivity between field and laboratory populations with any certainty; however, it is unlikely to represent drug resistance. Although the data of Albonico and others\(^{14}\) showed in Figure 2 and Table 2 represented worms recovered from children with and without drug treatment history, division of their data into two drug exposure subgroups (those previously exposed and those with no previous exposure) in the original work showed no difference in sensitivity (IC\(_{50}\) values) between the two groups of children,\(^{14}\) indicating that the egg hatch data shown in Figure 2 are equivalent to those shown by drug-naïve populations alone at their field sites. There is also no evidence that drug resistance is present at the Papua New Guinea field site sampled by Kotze and others.\(^{15}\) Hence, the differences between the laboratory and field *N. americanus* studies most likely represent factors unrelated to drug selection pressure (for example, outbred field populations versus laboratory-maintained isolates, human versus hamster hosts). Interestingly, the two field studies showed IC\(_{50}\) values not significantly different from each other.

It will be important to standardize the egg recovery method to be used for human hookworms. Various methods have been described for the recovery of viable eggs from feces for use in egg hatch assays: filtration and sodium chloride floatation onto a glass coverslip,\(^{6,14}\) filtration and sodium nitrate floatation, followed by further filtration of the uppermost layer,\(^{15}\) or floatation on a sucrose gradient (*H. contortus* in the present study). The method of Kotze and others\(^{15}\) had the disadvantage of producing an egg solution that was contaminated with fecal debris, thereby making it difficult to visualize larvae and eggs in the drug assay wells. On the other hand, Albonico and others\(^{14}\) did not encounter significant contamination with fecal debris with human samples using the sodium chloride floatation method, as recommended for livestock species.\(^{6}\) The sucrose gradient method still needs to be assessed with human hookworms. The preparation of “clean” suspensions of L3 larvae for pyrantel assays is readily achieved using the method described by Kotze and others.\(^{21}\) Migration of the larvae into the cloth placed above the fecal cultures ensures that they are relatively free of any contaminating fecal matter.

An examination of the dose response data for thiabendazole and pyrantel has allowed us to generate assay templates for field assessment of human hookworm sensitivities to the two drug groups. The templates consist of 10 serial dilutions of drug impregnated in agar in columns 3 to 12 of 96-well plates, with the first two columns of each plate containing agar only for control assays. The concentration ranges are as indicated in Figures 2 and 3; thiabendazole 0.01–5.0 μg/mL with 2-fold serial dilutions, pyrantel 0.001–300 μg/mL with 4-fold serial dilutions. For thiabendazole, the recommended range is centered on the two field studies,\(^{14,15}\) and extends from just below the IC\(_{50}\) of the laboratory *N. americanus* isolate examined in the present study, up to levels well above those observed to date with any of the hookworm species. For pyrantel, the range corresponds largely to the dose response shown by the laboratory *N. americanus*, with this isolate’s IC\(_{50}\) being approximately one-third of the way along the drug concentration gradient. The broader overall concentration range indicated for pyrantel (4-fold dilutions between drug concentrations) than for thiabendazole (2-fold dilutions) is a result of 1) a lack of information on the IC\(_{50}\) that may be expected for field populations of this species, given the differences noted above in IC\(_{50}\) between laboratory- and field-derived *N. americanus* in the egg hatch assays; and 2) the differences noted between the three species in the pyrantel assays (IC\(_{50}\) values over a 124-fold range in the morphology assay) compared with the more uniform egg hatch responses.

It will be important to determine whether *A. duodenale* behaves similarly to *N. americanus* in these *in vitro* drug assays. The general overlap of the responses for three hookworm species in egg hatch assays within the concentration range chosen as suitable for a standard assay format (from Figure 2) suggests that the concentration range may also suit *A. duodenale*. However, this species is known to be more susceptible than *N. americanus* to benzimidazole drugs *in vivo*,\(^{22}\) and hence it will be important to determine whether the two human hookworm species also differ in responses in the *in vitro* egg hatch assay. The responses of the three hookworm species were more spaced for the *in vitro* pyrantel assays compared with the egg hatch assay, and hence further study with *A. duodenale* will be required to determine whether our suggested range of drug concentrations for the pyrantel assays will be suitable for this species.

Of note was the increase in the percentage of normally arrested *A. ceylanicum* larvae at the highest drug concentration tested (from Figure 3A), indicating a reduced sensitivity

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**Table 3**

Dose response data for various hookworm species in agar-based larval morphology and motility assays with pyrantel

<table>
<thead>
<tr>
<th>Data source/hookworm species</th>
<th>Morphology assay IC(_{50}) ± SE (n)*</th>
<th>Motility assay IC(_{50}) ± SE (n)*</th>
</tr>
</thead>
<tbody>
<tr>
<td>This study, <em>Necator americanus</em></td>
<td>0.0445 ± 0.0144 (2) b</td>
<td>0.076 ± 0.058 (2) a</td>
</tr>
<tr>
<td>This study, <em>Ancylostoma ceylanicum</em></td>
<td>4.81 ± 2.77 (2) c</td>
<td>7.14 ± 0.37 (2) b</td>
</tr>
<tr>
<td>Kopp and others,(^{11}) <em>Ancylostoma caninum</em> NT isolate</td>
<td>0.0228 ± 0.00068 (3) a</td>
<td>†</td>
</tr>
<tr>
<td>Kopp and others,(^{11}) <em>A. caninum</em> PR isolate</td>
<td>0.0387 ± 0.0079 (3) b</td>
<td>†</td>
</tr>
</tbody>
</table>

*IC\(_{50}\) = concentration of drug required to reduce number of normally arrested larvae, or number of motile larvae, to 50% of that observed in control wells; mean ± SE; n = number of separate experiments, each with 1–3 assay wells at a series of drug concentrations; within an assay type, IC\(_{50}\) values followed by the same letter are not significantly different at P = 0.05.
† Not calculated because of the biphasic nature of the dose response.
to the drug at this concentration. Such a phenomenon has been reported for nAChR agonist drugs with a number of nematode species, and is presumed to be a type of tachyphylaxis. The phenomenon, as observed in our morphology assays with A. ceylanicum, has the potential to interfere with the assay’s ability to diagnose resistance in this species. Importantly though, we did not see the phenomenon with N. americanus, which showed no decrease in drug effects on morphology at the high drug concentrations. The percent of normally arrested larvae remained at zero at the highest drug concentration for this species, representing a 1,000-fold increase over the lowest concentration at which such a 0% was also recorded, indicating that the phenomenon would not be likely to interfere with the ability of the assay to detect significant increases in IC50 associated with drug resistance in this species.

The significantly greater sensitivity of N. americanus larvae compared with A. ceylanicum in pyrantel motility and morphology assays is in contrast to the previously reported similarity in the responses of the two species to this drug in vivo and in vitro adult worm motility assays. This may be a result of the existence of different nAChR subunit populations in the two life stages within each species, resulting in different relative proportions of pyrantel-sensitive receptors. Kopp and others recently showed that differences existed between life stages in terms of relative in vitro responses to different nAChR agonist drugs for two isolates of A. caninum, suggesting a degree of plasticity in receptor populations between different life stages. An increase in sensitivity to bephenium in pyrantel-resistant A. caninum larvae did not occur in adult stage worms of the pyrantel-resistant isolate. This plasticity in nAChR populations between larval and adult life stages, as judged by responsiveness in in vitro assays, may interfere with the ability of larval assays to reflect drug sensitivity of adult worms, as required for their effective use as indicators of changes in in vivo efficacy. However, both the morphology and motility assays are able to indicate relative in vitro drug sensitivities to pyrantel for A. caninum, showing their usefulness for resistance diagnosis with this species. Given the smaller size of larvae compared with adults, and the 48 hours incubation time for our larval assays compared with the 3 hours used by Richards and others for their adult assays, it would be expected that larval stages would be more susceptible to pyrantel than adults in these in vitro assays. Comparing our data with that of Richards and others shows that this is indeed the case with N. americanus (IC50 ratio adult/larvae ~37-fold), with the opposite being true for A. ceylanicum (adults more susceptible, IC50 ratio adult/larvae ~0.15). This suggests that the relative levels of pyrantel-sensitive receptors in the former species is more uniform between the life stages than the later species, hence giving encouragement that the required ability of larval responses to reflect adult responses to the drug will more likely occur with the former species than with the later. The former species, N. americanus, is of course the most relevant to our purposes of developing assays for human hookworms. It is clear that the ability of the larval assays to indicate drug resistance in the human hookworm species will need to be tested experimentally.

As noted previously, the WAAVP criteria for susceptibility to benzimidazoles in an egg hatch assay with the major livestock roundworm species is that the IC50 must be below 0.1 μg/mL, and the additional suggestion that the IC50 should also be < 0.1 μg/mL. Although the laboratory isolate of N. americanus assayed for the present study showed an IC50 value well below 0.1, the two field study IC50 values were close to the 0.1 μg/mL value despite being from populations that would be expected to be drug susceptible. Hence, a slightly higher IC50 discriminating value may need to be derived for the human hookworms. Insufficient data exist at present to assign any susceptibility cut-off for the motility or morphology assays with pyrantel. The morphology assay IC50 shown by the laboratory susceptible N. americanus was very similar to that shown by the highly drug-resistant A. caninum PR isolate, to which pyrantel shows an efficacy of just 28%. Hence, the relationships between assay parameters and pyrantel resistance, which may be derived from the morphology and motility assay responses with drug-resistant A. caninum, do not apply directly to N. americanus.

An important issue for all the assays described in the present study is the lack of known resistant isolates of the human hookworms that could be used to test the resistance diagnosing ability of the assays. Indications that the egg hatch assay may be of value for human hookworms are based solely on its use with the livestock species. For pyrantel there is at least an indication that resistance in the canine hookworm can be detected using the morphology assay, but no evidence with regard to the human hookworms. Hence, the ability of the assays to detect resistance, and the IC50 or IC99 values likely to indicate such resistance in human hookworms, remain unknown. Further studies using standardized assays at different field sites alongside efficacy data, aided by assays with resistant isolates generated through laboratory selection pressure, will be required to establish drug concentrations (IC50, IC99 values) that may be viewed as indicative of drug resistance. Similarly, an understanding of the sensitivity of the assays for detecting resistance will also require their use with known resistant isolates. However, in the current absence of any defined “resistance-indicating parameters,” the sequential use of the assays in human populations as they are exposed to benzimidazole and nAChR agonist drugs may indicate whether changes are occurring in their response over time, and comparisons between populations that are drug-naive and those repeatedly exposed to drug treatments will be important, particularly in cases where the populations are from neighboring geographic regions.

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