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Phenotypic Characterization of Two *Ancylostoma caninum* Isolates with Different Susceptibilities to the Anthelmintic Pyrantel[▽]

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The anthelmintic pyrantel plays an important role in the control of gastrointestinal helminths of humans and domestic animals. Despite the demonstration of pyrantel resistance in several helminth species over the last 20 years, the resistance mechanism remains unclear. It has been hypothesized that resistance may arise as a consequence of changes to the relative proportions of subpopulations of nicotinic acetylcholine receptors (nAchRs). To test this hypothesis, we examined the responses of two isolates of the canine hookworm *Ancylostoma caninum* with low-level resistance (isolate NT) and high-level resistance (isolate PR) to pyrantel to nicotinic agonist drugs reported to be selective for three nAchR subtypes. We used larval motility and conformation assays and force transduction experiments with adult worms. Pyrantel and levamisole were less potent against larvae of isolate PR than larvae of isolate NT (up to an 18-fold increase in the 50% inhibitory concentration); on the other hand, bephenium was more potent against larvae of isolate PR than larvae of isolate NT (twofold) and nicotine had the same potency against larvae of both isolates. In adults, pyrantel, levamisole, and nicotine were less potent against isolate PR than isolate NT (two- to threefold), but the potency of bephenium against the two isolates was equivalent. Our data indicate a complex pattern of nAchRs in this species and suggest that the two isolates differ in their relative sensitivities to agonists targeting different nAchRs.

The cholinergic anthelmintic pyrantel is used to control important nematodes in small-animal, equine, porcine, and human medicine. Despite the competition from newer anthelmintics, particularly those in the ivermectin/milbemycin and benzimidazole classes, pyrantel's market penetration in these fields remains significant (13). It is comparatively inexpensive and has an excellent safety profile, and it is therefore likely to hold a place in the pharmacopeia well into the 21st century for use in people in regions where intestinal nematode infections are endemic, as well as animals. Continued use is, however, dependent upon the target nematodes remaining susceptible to the drug. Resistance to pyrantel has now been described in nematodes of horses (3, 4), pigs (25), and canines (6, 12); and a single report of pyrantel failure against a human hookworm, *Ancylostoma duodenale*, in Australia exists (20).

Some progress has been made toward the development of a laboratory-based bioassay for the detection of pyrantel resistance. A larval migration assay has been shown to discriminate between pyrantel-susceptible and -resistant isolates of the porcine nematode *Oesophagostomum dentatum* (19), and a larval development assay has been reported to be able to detect pyrantel resistance in some livestock species (15). In addition, a larval motility assay (LMA) has been observed to discriminate between isolates of the canine hookworm (*Ancylostoma caninum*) with different susceptibilities to pyrantel, but the biphasic nature of the dose-response curves obtained by this assay complicates mathematical interpretation (11). Recently, a novel bioassay, the larval arrested morphology assay (LAMA), has been developed for the detection of resistance to pyrantel in *A. caninum* (11). While more work is required to properly assess the performance of this assay with a comprehensive range of isolates, it shows promise as a tool for the detection of resistance to pyrantel in hookworm species. Although they were primarily developed to detect resistance, these bioassays also offer tools that can be used to investigate mechanisms of resistance.

Pyrantel is one of the cholinergic nicotinic agonist group of anthelmintics, which also includes levamisole, oxantel, and morantel. These anthelmintics target the nematode nicotinic acetylcholine receptor (nAchR), a ligand-gated ion channel that is a significant component of nematode neurotransmission (16). Nicotinic agonist drugs are selective for particular subpopulations of nAchRs (18). For example, the porcine roundworm (*Ascaris suum*) has at least three distinct pharmacological subpopulations of nAchR on muscle according to the L-/B-/N-subtype model: an L subtype against which pyrantel and levamisole are most selective, a B subtype against which bephenium is most selective, and an N subtype against which nicotine and oxantel are most selective (23). Whether these subtype designations apply to all nematode parasites of veterinary and medical importance is uncertain (28).

Under a model of distinct pharmacological nAchR subpopulations, a number of important issues arise. Should resistance to a particular anthelmintic nAchR agonist result from the loss of specific target receptors, it is likely that susceptibility to anthelmintics acting on other nAchR subtypes would be re-

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tained. A further possibility is that the expression of alternate subtypes may be increased in the face of a reduction in the target subtype as a means of compensation for the biological cost associated with a reduction in a single subset of nAchRs at the neuromuscular junction. In resistant worms, this could have the effect of increasing susceptibility to agonists selective for alternate subtypes.

The canine hookworm (*Ancylostoma caninum*) is likely to be of use to those investigating the mechanisms of pyrantel resistance in hookworms infecting people. Given the difficulties encountered in studying human hookworms, namely, the detection of anthelmintic resistance and access to and characterization of biological material, *A. caninum* is well placed to serve as an excellent model for the human hookworms. These species, *Ancylostoma duodenale* and *Necator americanus*, represent major components of soil-transmitted geohelminthiasis throughout much of the world, infecting close to 1 billion people (2). The impacts of these nematodes on human health are far-reaching: iron deficiency anemia, mental retardation, and death in infants and elderly people are commonly observed sequelae of infection (1). Since anthelmintic administration programs are the cornerstone of preventative health programs in regions where hookworms are endemic, it is important to understand the potential for the development of drug resistances in human hookworms, with the canine hookworm likely to be useful in this regard.

We have characterized the responses of two *A. caninum* isolates, one with high-level pyrantel resistance and one with low-level pyrantel resistance, to nicotinic agonist drugs selective for different subtypes under the L-/B-/N-subtype model. The responses at the larval stage were examined by using both LMA and LAMA, and the responses in adult worms were examined by using whole-worm cannulation and force transduction. The aims of these studies were to determine whether both life stages are responsive to agonists selective for each receptor subtype and to assess whether the response of the highly pyrantel-resistant isolate to other putative non-L-type-selective agonists differs from that observed in the isolate with low-level resistance to pyrantel.

MATERIALS AND METHODS

***Ancylostoma caninum* isolates.** Two isolates of *A. caninum* were used for these experiments: an isolate with high-level resistance to pyrantel, designated isolate PR, and an isolate with low-level resistance to pyrantel, designated isolate NT. These isolates have previously been described in detail (11). Briefly, isolate PR was sourced from a pool of isolates recovered from dogs in metropolitan Brisbane, Australia, while isolate NT was sourced from a puppy in an aboriginal community in the Northern Territory, Australia. A direct in vivo comparison of these isolates by the use of an abbreviated critical trial (7) revealed that the clinical efficacy of pyrantel was 25% against isolate PR and 71% against isolate NT on the basis of the proportion of adult worms expelled during the trial (11).

Larvae and adult worms were recovered as described previously (11). Briefly, larvae at the L3 stage (L3 larvae) were cultured from feces and stored at room temperature in BU buffer (50 mM Na_2HPO_4, 22 mM KH_2PO_4, 70 mM NaCl, pH 6.8) containing 10 μl/ml penicillin (5,000 U/ml)-streptomycin (5 mg/ml) and 100 μl/ml amphotericin B (250 μg/ml) for no more than 2 weeks before use in the assays. Donor dogs were euthanized with intravenous pentobarbital, and adult worms were immediately removed from the intestine and placed in handling buffer warmed to 37°C. Six days prior to euthanasia, the donor dog harboring PR worms was administered a standard dose (14.4 mg/kg of body weight) of pyrantel embonate to eliminate susceptible individuals. The handling buffer used was based on that described by Sangster and Mettrick (27) and contained 130 mM NaCl, 2.8 mM KCl, 2.8 mM CaCl, 4.5 mM MgCl, 100 mM glucose, and 10 mM Tris. Adult worms were used for the force transduction studies within 6 h of collection.

Drugs. The following nicotinic agonist drug salts were used in these experiments: pyrantel embonate, levamisole hydrochloride, nicotine, and bephenium hydroxynaphthoate. All drugs were purchased from Sigma-Aldrich, Sydney, Australia. These drugs were chosen as representative putative L-subtype (pyrantel and levamisole), N-subtype (nicotine), and B-subtype (bephenium) agonists (18). Stock solutions of each drug were prepared in dimethyl sulfoxide (for LAMA and LMA, pyrantel was used at 10 mg/ml, levamisole was used at 10 mg/ml, bephenium was used at 50 mg/ml, and nicotine was used at 100 mg/ml; for force transduction, pyrantel was used at 500 μg/ml, levamisole was used at 324 μg/ml, bephenium was used at 1,600 μg/ml, and nicotine was used at 1,215 μg/ml).

LAMA and LMA. LAMA was performed as described previously (11) by using the agar matrix technique in 96-well plates. Drug gradients were prepared by serially diluting stock concentrations twofold in dimethyl sulfoxide. A 2-μl aliquot from every second or third dilution was placed in each well such that each row of the plate comprised a gradient of 10 dilution points. The first two wells of each row were left blank as control wells. All wells were then filled with 200 μl of 2% agar (powdered agar of grade J; Davis Gelatin Co.), which was allowed to set before the plates were stored in the dark at 4°C. All plates were used within 3 weeks of production. To undertake the assay, the plates were equilibrated to room temperature for 2 h before 30 μl of larval suspension (1,500 larvae/ml in H_2O with 10 μl/ml penicillin-streptomycin and 100 μl/ml amphotericin B) was dispensed onto the surface of the agar in each well; this amount represented approximately 45 larvae per well. The plates were incubated for 48 h at 25°C in plastic zip-lock bags. The morphology of quiescent larvae following incubation (LAMA) was assessed as described previously (11). The effects of the drugs on larval motility were then assessed on the same assay plates (LMA) (11, 14). To stimulate motility, 40 μl of water heated to 50°C was added to each well. Larvae moving with a smooth sinusoidal motion were counted as normally motile, while twitching or immotile larvae were not counted. Assays were performed in nine replicates for each drug and each isolate.

Force transduction. A force transducer was constructed from an analogue moving-coil ammeter, as described by Sangster et al. (26). As shown in Fig. 1, the needle of the meter acts as a sensitive transducer arm to which a worm can be anchored. A correcting current modulated by a feedback circuit maintains the position of the needle when a force is applied and is proportional to the magnitude of that force. The transducer was interfaced to a laptop computer via a USB connection, which allowed the correcting current to be monitored and logged by using a data-recording program created by the numerical programming package MATLAB (The MathWorks). Prior to each use, the transducer was calibrated by using a set of standard weights (20 mg, 50 mg, 150 mg, 300 mg).

Prior to the attachment of a selected adult female, the worm was prepared under a dissecting microscope in accordance with the previous description by Sangster et al. (26). A small transverse incision was made in its cuticle slightly posterior to the midbody using a razor blade. A smaller incision was also made anterior to the midbody. The internal contents of the worm were removed through the larger posterior incision by using fine thumb forceps. A fine cannula (internal diameter, 0.4 mm), which was prepared by heating and drawing a 0.5-μl pipette tip to a curved taper, was gently introduced into the pseudocoelomic cavity through the posterior incision. Nylon suture material (no. 0; Ethilon; Johnson and Johnson, Australia) was secured to the arm of the transducer and was then attached with cyanoacrylate (Loctite Prism 406 instant adhesive; Henkel Australia Pty. Ltd., Sydney, Australia) near the anterior region of the worm. The posterior region of the worm was anchored to a plastic weigh boat in the same fashion. The weigh boat was secured (Blu-Tak adhesive; Bostik) to a heating mat (37°C), and the transducer was positioned to impart a baseline tension of 20 mg on the worm.

The drug gradients used in the force transduction experiments were prepared by serially diluting stock solutions in handling buffer. Concentration ranges were based upon the mean minimum effective concentrations previously reported for levamisole-susceptible and -resistant *Haemonchus contortus* worms (26). The drugs were injected into the worm through a pipette in 20-μl volumes. Each worm received a series of injections from the lowest to the highest drug concentrations, with 20 μl of handling buffer injected between drug injections. The injection of handling buffer was performed only once a stable contractile response had been obtained. At the conclusion of the drug injections, 20 μl of a 50 mM KCl solution was injected to invoke a maximal contraction (26). The maximal contraction of each worm was used to standardize the responses between worms by expressing the contraction force at each drug concentration as a percentage of the force measured following the injection of KCl. The percent contraction at each drug concentration was used to calculate the 50% inhibitory

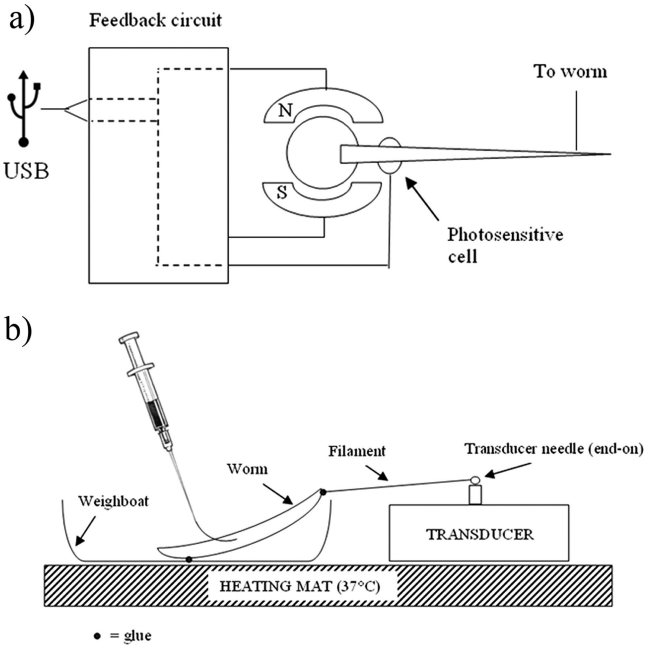

FIG. 1. Force transducer circuitry and experimental apparatus. (a) The photosensitive cell is capable of sensing minute deflections of the needle upon contraction of the worm. A feedback circuit assesses the signal from the photosensitive cell and applies a modulating current to maintain the position of the needle against the contraction force. The correcting current applied to the needle can be continuously monitored by use of a USB interface and is proportional to the force applied to the needle (N, north; S, south). (b) Cannulated adult female worms were glued to the base of a plastic weigh boat and then attached to the transducer needle through the use of a nylon filament attached to the anterior region of the worm.

concentrations (IC_{50}s). Experiments were performed with five worms of each isolate for each drug.

Statistical analysis. The LAMA, LMA, and force transduction data were analyzed by using GraphPad Prism software. All data, with the exception of the pyrantel LMA data, were fitted with variable-slope sigmoidal dose-response curves, which enabled the calculation of the IC_{50}s. The pyrantel LMA data were fitted with hand-drawn curves, and the IC_{50}s could not be calculated. For the LAMA and LMA data, the IC_{50} represents the concentration of drug required to cause abnormal quiescence or motility in 50% of the larvae, respectively. For the force transduction data, the 50% effective concentration (EC_{50}) represents the concentration of drug required to cause a force of contraction half that of the maximal contraction induced by KCl. The maximal contraction value used was the mean for each isolate. Significant differences in IC_{50}s and EC_{50}s were determined by using an unpaired *t* test.

Animal ethics. This experiment was approved by the University of Queensland's Animal Ethics Committee (approval number SVS/654/06/).

RESULTS

LAMA and LMA. The dose-responses to all four agonist drugs were observed by using both LAMA and LMA (Fig. 2 and 3, respectively). For LAMA, the dose-responses for all agonists tested could be described with sigmoidal curves. For LMA, a biphasic response for pyrantel was observed. The response comprised an initial decline in motility and a subsequent increase in motility, followed by a complete decline in motility over a small concentration range. Data for the other three agonists obtained by this assay were sigmoidal in nature,

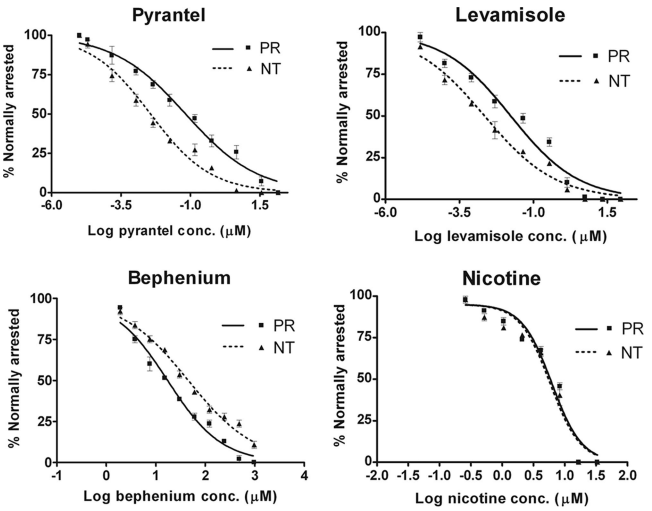

FIG. 2. Dose-responses determined by LAMA. The graphs depict the reduction in the percentage of normally arrested larvae with increasing drug concentration. Error bars represent standard errors.

permitting the calculation of IC_{50}s and the subsequent IC_{50} ratios (Table 1).

By LAMA, the pyrantel IC_{50} for isolate PR was 0.066 μM, whereas the IC_{50} was 0.0037 μM for isolate NT, representing a ratio of 18.3 (Table 1). While the calculation of IC_{50}s of this drug by LMA was not possible due to the biphasic nature of the response, the following points of discrimination were observed: the initial minimum of the response curve was approximately 70% for isolate PR and 50% for isolate NT, the subsequent recovery peaked at approximately 90% for isolate PR and 70% for isolate NT, and a significant divergence between the response curves was evident over a pyrantel concentration range of 0.001 to 0.58 μM (log −3.0 to log 0).

While the responses to levamisole were similar by both assays, a greater degree of discrimination was observed between

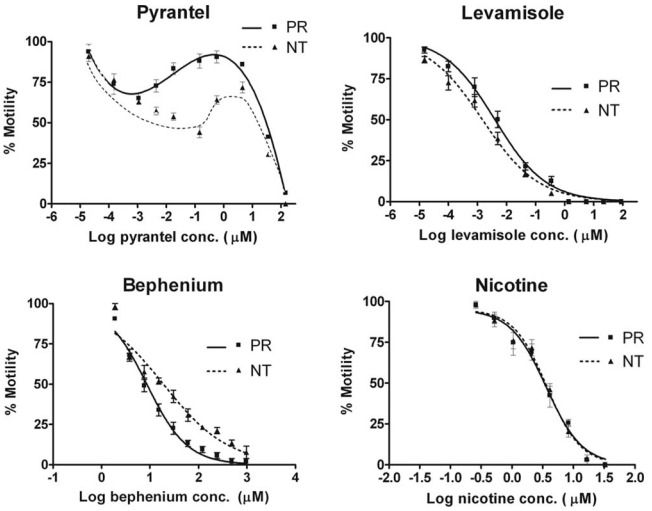

FIG. 3. Dose-responses determined by LMA. Pyrantel responses describe fourth-order polynomial curves that were drawn by hand. The responses to the other drugs were fitted with sigmoidal curves. Error bars represent standard errors.

TABLE 1. IC_{50}s and IC_{50} ratios determined by LAMA and LMA

Assay	Drug	IC_{50} (μM [95% CI])		IC_{50} ratio
		NT	PR	
LAMA	Pyrantel	0.0037 (0.0026–0.0052)	0.066 (0.045–0.099)	18.3[a]
	Levamisole	0.0022 (0.0015–0.0032)	0.017 (0.011–0.028)	7.2[a]
	Nicotine	5.8 (5.1–7.6)	6.2 (4.8–7.1)	1.07
	Bephenium	45 (39–52)	17 (14–21)	0.46[a]
LMA	Pyrantel	—[b]	—	—
	Levamisole	0.0015 (0.0011–0.0020)	0.0040 (0.0029–0.0054)	2.7[a]
	Nicotine	3.7 (3.2–4.3)	3.6 (3.0–4.3)	0.95
	Bephenium	19 (14–25)	8.6 (7.5–9.8)	0.45[a]

[a] The values for isolates NT and PR are significantly different ($P < 0.05$).
[b] —, not determined (see the text).

isolates PR and NT by LAMA (IC_{50} ratio, 7.2) compared to the LMA (IC_{50} ratio, 2.7). The difference in the IC_{50} was, however, significant when either assay was used. The dose-responses for nicotine did not differ between isolates by either LMA or LAMA. The responses to bephenium were the inverse of those observed with pyrantel and levamisole, with higher IC_{50}s observed for isolate NT relative to that for isolate PR, giving ratios of the IC_{50} for PR to the IC_{50} for NT of 0.46 and 0.45, respectively (Table 1).

Force transduction. Representative force transduction traces for the responses of isolates PR and NT to pyrantel are shown in Fig. 4. The contraction force was seen to increase as the drug concentration increased, with the magnitude of the response to each drug concentration being greater in isolate NT than in isolate PR. The maximal KCl-induced contraction was similar for both isolates. Force transduction dose-response plots, which depict the force of contraction against the drug concentration, are shown in Fig. 5, and the EC_{50}s and the ratios of the EC_{50} for PR to the EC_{50} for NT shown in Table 2. The EC_{50} of pyrantel for adult PR worms was observed to be 3.1-fold greater than that for adult NT worms. The relative responses of the two isolates to levamisole and nicotine were similar, with the ratios of the EC_{50} for PR to the EC_{50} for NT being 2- and 2.1-fold, respectively. The responses to bephenium were similar for both isolates (EC_{50} ratio, 0.97).

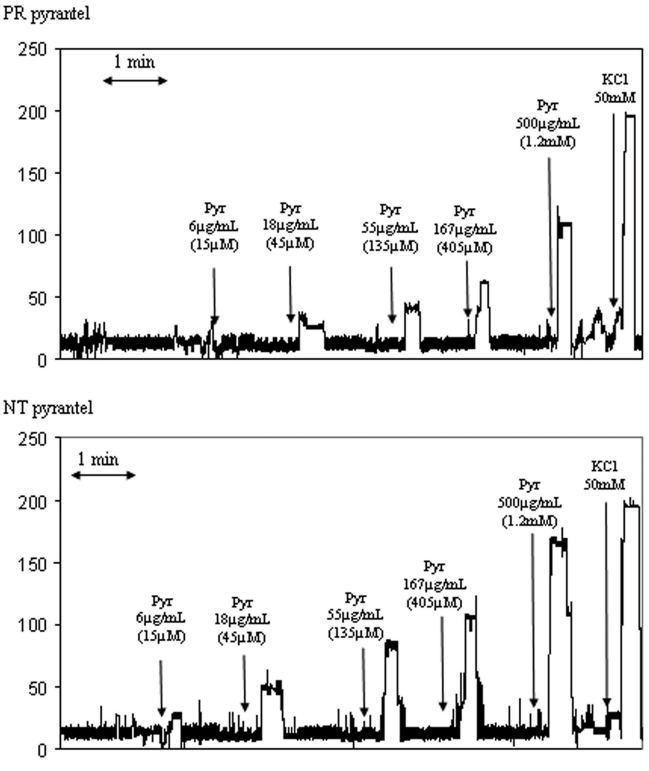

FIG. 4. Graphical output from force transducer experiments. Force transducer traces for pyrantel against a PR and an NT adult worm. Pyr, pyrantel.

DISCUSSION

The present study utilized larval bioassays and force transduction measurements to characterize two *A. caninum* isolates with significantly different susceptibilities to pyrantel. The differences in the responses between L3 larvae of isolates PR and NT observed provide some support to the L-, N-, and B-subtype nAchR model proposed for *A. suum* by Martin and Robertson (18). Despite the substantial difference in the IC_{50}s for the putative L-subtype agonists pyrantel and levamisole for

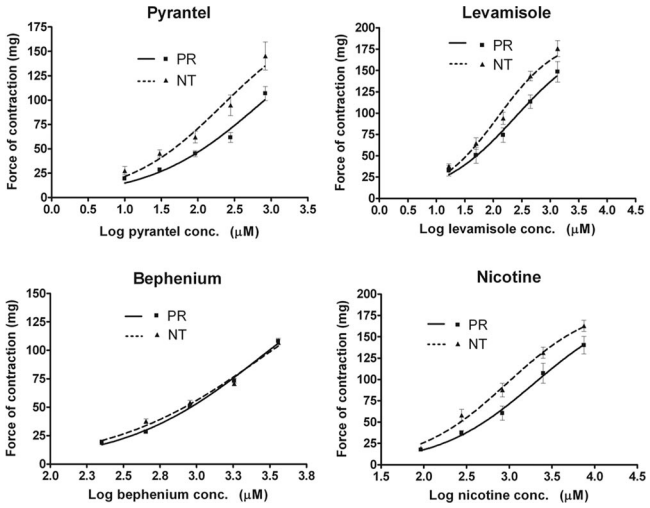

FIG. 5. Force transduction dose-response plots for the four nicotinic agonists tested. Error bars represent standard errors.

TABLE 2. EC_{50}s and EC_{50} ratios for force transduction experiments

Drug	EC_{50}[a] (μM [95% CI])		EC_{50} ratio
	NT	PR	
Pyrantel	217.2 (154.8–304.8)	681.3 (516.2–899.4)	3.1[b]
Levamisole	120.5 (105.9–137.2)	244.3 (209.4–284.9)	2.0[b]
Nicotine	941.6 (849.2–1044)	1,963 (1,739–2,214)	2.1[b]
Bephenium	2,879 (2622–3161)	2,791 (2,603–2,991)	0.97

[a] The EC_{50} represents the concentration of drug required to produce a contraction force that is 50% of that induced by KCl.
[b] The values for isolates NT and PR are significantly different ($P < 0.05$).

these two isolates, no difference in response to the N-subtype agonist nicotine was observed, suggesting that resistance to pyrantel and levamisole is accompanied by retained susceptibility to nicotine in this life stage. In addition, the relative responses to pyrantel/levamisole and bephenium were quite different between the two isolates, again suggesting the involvement of different receptor subtypes. The biphasic response to pyrantel as determined by LMA has previously been reported in this species (11).

The marked difference in drug concentrations required to elicit responses in larvae and adults is likely to be due to the difference in the time of drug exposure used in our experiments. While larvae were bathed in the drug solutions for 48 h prior to assessment, the responses in cannulated adults were observed soon (within minutes) after exposure. In experiments whose findings have not been published, the authors have observed that adult *A. caninum* worms respond to pyrantel and levamisole at concentrations comparable to those reported here for the larval bioassays when the worms were incubated in the presence of these agonists for 48 h. A proportion of the difference in sensitivity might also be attributed to the comparative sizes of L3 larvae (length, <1 mm) and adult female worms (length, approximately 10 mm).

The force transduction observations reported in the present study are similar to those of Sangster et al. (26), who examined the responses to nicotinic agonists in levamisole-susceptible and -resistant *Haemonchus contortus* worms. Levamisole-resistant *H. contortus* worms were found to be less susceptible to pyrantel and nicotine, while the results for bephenium were equivocal. While the earlier study used minimum effective concentrations rather than EC_{50}s to compare the responses, the data are comparable to those from the present study because similar drug concentrations were required to elicit minimum responses in *A. caninum* worms. The similarities of the responses to the different nicotinic agonists in the two studies suggest that resistance to levamisole and pyrantel is likely to occur through similar mechanisms.

A key finding was the inverse relationship between sensitivities to pyrantel and bephenium in larval assays. As measured by LAMA and LMA, highly pyrantel-resistant isolate PR was approximately twofold more susceptible to bephenium than isolate NT, which had low-level pyrantel resistance. Even though bephenium is recognized as a weak anthelmintic in its own right, the possibility that susceptibility to the drug is increased in the face of pyrantel resistance suggests that drugs targeting the receptor subtype sensitive to bephenium may be useful in overcoming resistance to pyrantel. Martin and Robertson (18) have previously suggested that the clinical exploitation of the different pharmacological responses of the subpopulations might be a feasible option in the face of resistance. The inverse relationship between the two drugs in the present study indicates that resistance to pyrantel may involve a reduction in pyrantel-sensitive receptors in the resistant isolate, alongside a compensatory increase in bephenium-sensitive nAchR subtypes, in order to maintain a net nAchR population of sufficient quantity to permit normal transmission in the neuromuscular system. Changes in the relative levels of different receptor subtypes have been demonstrated in high-resolution electrophysiological studies with susceptible and resistant *Oesophagostomum dentatum* worms by Robertson et al. (22).

The relationship between the various agonists showed some differences in the results of the force transduction experiments with adults compared to those of the dose-response assays with L3 larvae. The observations made during the force transduction experiments may be of more clinical significance because they focus upon the adult life stage, against which the drugs act for worm control in vivo. While the results for pyrantel and levamisole by the force transduction experiments were in agreement with those of the bioassays with larvae, the responses for nicotine and bephenium were somewhat different. Even though there were no differences in the response to nicotine between isolates PR and NT in the larval stage, PR adults were more than twofold less sensitive to nicotine than their adult isolate NT counterparts, suggesting that pyrantel and nicotine may show some overlap in their effects on the nAchR subtypes in adult worms. In the case of bephenium, no difference in response was seen at the adult stage, despite the increased susceptibility of isolate PR observed at the L3 stage. Hence, the potential value of using drugs that target the bephenium-sensitive receptor to overcome pyrantel resistance, as suggested on the basis of the data for L3 larvae, is not supported by the results of the force transduction experiments with adult worms. The direct examination of the mortality of adult worms in clinical studies with pyrantel and bephenium is required to resolve this issue. The difference in the responses between life stages observed in these experiments highlights the complexity and plasticity of cholinergic pharmacology.

Data on the in vivo level of resistance (ED_{50}) for isolates NT and PR are limited. Pyrantel showed an efficacy of 71% against isolate NT when the isolate was first recovered from the field (11). It has undergone no further drug selection since its collection. Hence, it represents a mixture of drug-susceptible and -resistant individuals, with a predominance of susceptible individuals. Pyrantel showed only 25% efficacy against isolate PR when the isolate was first collected. The adult PR worms used in the present study survived a dose of pyrantel given to their host animal 6 days prior to euthanasia, indicating that each worm used in the study was fully resistant to the drug. The possession of more complete in vivo data for isolates PR and NT, at least for pyrantel and levamisole, would be useful in interpreting the in vitro data generated in the present study. However, when investigators are dealing with a companion animal parasite, financial constraints and animal welfare considerations make this extremely difficult to achieve. Hence, while *A. caninum* is undoubtedly a useful model for human hookworms, this modeling is likely to be enhanced in certain areas by input from the livestock industries. The greater ac-

cessibility to livestock animals, as well as the greater number of isolates among some livestock nematode species with defined levels of resistance to anthelmintics, will most likely be useful in overcoming some of the limitations faced by those working with companion-animal models, such as models of *A. caninum* infection.

Aside from the comparative responses between the isolates used in the current experiments, the general response of *A. caninum* L3 larvae and adult worms to nicotine relative to the responses determined from previous data for other veterinary nematodes is worthy of attention. The nicotine concentration ranges required to elicit dose-response curves in *A. caninum* larvae were in excess of 1,000-fold higher than those required to inhibit migration in *Oesophagostomum dentatum* worms (17). While some of this disparity might be attributable to differences in the assays used, this is unlikely to fully explain its magnitude. Similarly, the mean minimum excitatory concentration of nicotine for cannulated adult *Haemonchus contortus* worms reported by Sangster et al. (26) is almost fourfold lower than that required to elicit a similar response in adult *A. caninum* worms. These observations may be indicative of a lower representation of the N-subtype acetylcholine receptor in *A. caninum*, since another agonist reported to be selective for this subtype, oxantel, has poor activity against this species in vivo (5, 24). *A. caninum* L3 larvae show no response to oxantel by LMA or LAMA (S. R. Kopp et al., unpublished data), suggesting that oxantel is not active against this life stage. We did not possess sufficient adult worms to assess the responses to oxantel using the force transducer.

In an interesting contrast to the situation for *A. caninum*, pyrantel has poor activity against the canine whipworm (*Trichuris vulpis*) (10, 29), yet oxantel is highly effective against this species (5, 24). It is reasonable to hypothesize that the insensitivity of *T. vulpis* to pyrantel is due to the relative underrepresentation of the L subtype in this species relative to the level of representation of the N subtype. The results of our experiments suggest that the opposite might be true for *A. caninum*. However, such hypotheses are speculative at this time.

The nematode for which there is the greatest knowledge of cholinergic pharmacology, *Caenorhabditis elegans*, is thought to possess only two subtypes of nAchR with different pharmacological responses (21), and it is therefore difficult to extrapolate this knowledge to parasitic nematode species in the context of the L/N/B model. Furthermore, the *C. elegans* genome is known to contain a very large number of acetylcholine receptor subunits (8), one of which is thought to be target of a novel anthelmintic compound (9). The fact that this compound also has activity against other clade V members (*Haemonchus contortus* and *Trichostrongylus colubriformis*) (9) suggests that parasitic nematodes of this clade may possess extensive nAchR families. This is in contrast to the recent findings of Williamson and colleagues (30), who observed that *Brugia malayi* (clade III) and *Trichinella spiralis* (clade I) possess modest ligand-gated ion channel families.

The insensitivity of *A. caninum* and *T. vulpis* to different nicotinic agonist drugs highlights their potential value as models for the investigation of resistance to cholinergic anthelmintics. Elucidation of the molecular mechanism for the different sensitivity patterns in hookworms and *Trichuris* spp. may provide leads for those attempting to characterize the basis of resistance to pyrantel, levamisole, and other nicotinic agonist anthelmintics. However, direct comparison of the clade I worm *T. vulpis* with the clade V worm *A. caninum* in terms of the molecular basis of the differences in cholinergic pharmacology observed may be difficult. Whether the inverse responses to pyrantel and oxantel seen with these two species are more generally reflective of their representative clades is something that needs to be determined.

The responses of nematodes to nicotinic agonist anthelmintics are complex, and these complex responses make it difficult to fully characterize the mechanism of resistance to this drug group. When investigators are confronted with a potentially complex resistance mechanism or series of resistance mechanisms, it is logical for them to build a directed molecular approach by characterizing the resistance phenotype at increasing levels of resolution. The present study advances our understanding of the pyrantel resistance phenotype of *A. caninum*. However, the results are observations rather than correlations. Further work should focus on the characterization of a wider range of *A. caninum* isolates and should attempt to resolve the observed responses to the nAchR single-channel level. This would include determination of whether the responses to levamisole/pyrantel, nicotine, and bephenium in this species are due to the existence of at least three physically distinct subpopulations of nAchR with different pharmacological responses.

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REFERENCES

1. **Bethony, J., S. Brooker, M. Albonico, S. M. Geiger, A. Loukas, D. Diemert, and P. J. Hotez.** 2006. Soil-transmitted helminth infections: ascariasis, trichuriasis, and hookworm. Lancet **367:**1521–1532.
2. **Bethony, J., A. Loukas, M. Smout, S. Brooker, S. Mendez, J. Plieskatt, G. Goud, M. E. Bottazzi, B. Zhan, Y. Wang, A. Williamson, S. Lustigman, R. Correa-Oliveira, S. Xiao, and P. J. Hotez.** 2005. Antibodies against a secreted protein from hookworm larvae reduce the intensity of hookworm infection in humans and vaccinated laboratory animals. FASEB J. **19:**1743–1745.
3. **Coles, G. C., S. N. Brown, and C. M. Trembath.** 1999. Pyrantel-resistant large strongyles in racehorses. Vet. Rec. **145:**408.
4. **Drudge, J. H., E. T. Lyons, S. C. Tolliver, S. R. Lowry, and E. H. Fallon.** 1988. Piperazine resistance in population-B equine strongyles: a study of selection in thoroughbreds in Kentucky from 1966 through 1983. Am. J. Vet. Res. **49:**986–994.
5. **Howes, H. L., Jr.** 1972. *trans*-1,4,5,6-Tetrahydro-2-(3-hydroxystyryl)-1-methyl pyrimidine (CP-14,445), a new antiwhipworm agent. Proc. Soc. Exp. Biol. Med. **139:**394–398.
6. **Jackson, R., D. Lance, and K. Townsend.** 1987. Isolation of anthelmintic resistant *Ancylostoma caninum*. N. Z. Vet. J. **35:**215–216.
7. **Jacobs, D. E., A. Arakawa, C. H. Courtney, M. A. Gemmell, J. W. McCall, G. H. Myers, and O. Vanparijs.** 1994. World Association for the Advancement of Veterinary Parasitology (W.A.A.V.P.) guidelines for evaluating the efficacy of anthelmintics for dogs and cats. Vet. Parasitol. **52:**179–202.
8. **Jones, A. K., and D. B. Sattelle.** 2004. Functional genomics of the nicotinic acetylcholine receptor gene family of the nematode, Caenorhabditis elegans. Bioessays **26:**39–49.
9. **Kaminsky, R., P. Ducray, M. Jung, R. Clover, L. Rufener, J. Bouvier, S. S. Weber, A. Wenger, S. Wieland-Berghausen, T. Goebel, N. Gauvry, F. Pautrat, T. Skripsky, O. Froelich, C. Komoin-Oka, B. Westlund, A. Sluder, and P. Maser.** 2008. A new class of anthelmintics effective against drug-resistant nematodes. Nature **452:**176–180.
10. **Klein, J. B., R. E. Bradley, Sr., and D. P. Conway.** 1978. Anthelmintic efficacy of pyrantel pamoate against the roundworm, *Toxocara canis*, and the hookworm, *Ancylostoma caninum*, in dogs. Vet. Med. Small Anim. Clin. **73:**1011–1013.

11. **Kopp, S. R., G. T. Coleman, J. S. McCarthy, and A. C. Kotze.** 2008. Application of in vitro anthelmintic sensitivity assays to canine parasitology: detecting resistance to pyrantel in *Ancylostoma caninum*. Vet. Parasitol. **152:**284–293.
12. **Kopp, S. R., A. C. Kotze, J. S. McCarthy, and G. T. Coleman.** 2007. High-level pyrantel resistance in the hookworm *Ancylostoma caninum*. Vet. Parasitol. **143:**299–304.
13. **Kopp, S. R., A. C. Kotze, J. S. McCarthy, R. J. Traub, and G. T. Coleman.** 2007. Pyrantel in small animal medicine: 30 years on. Vet. J. [Epub ahead of print.] PMID 17720556.
14. **Kotze, A. C., S. Clifford, J. O'Grady, J. M. Behnke, and J. S. McCarthy.** 2004. An in vitro larval motility assay to determine anthelmintic sensitivity for human hookworm and *Strongyloides* species. Am. J. Trop. Med. Hyg. **71:** 608–616.
15. **Kotze, A. C., P. A. Stein, and R. J. Dobson.** 1999. Investigation of intestinal nematode responses to naphthalophos and pyrantel using a larval development assay. Int. J. Parasitol. **29:**1093–1099.
16. **Martin, R. J.** 1997. Modes of action of anthelmintic drugs. Vet. J. **154:**11–34.
17. **Martin, R. J., C. L. Clark, S. M. Trailovic, and A. P. Robertson.** 2004. Oxantel is an N-type (methyridine and nicotine) agonist not an L-type (levamisole and pyrantel) agonist: classification of cholinergic anthelmintics in *Ascaris*. Int. J. Parasitol. **34:**1083–1090.
18. **Martin, R. J., and A. P. Robertson.** 2007. Mode of action of levamisole and pyrantel, anthelmintic resistance, E153 and Q57. Parasitology **134:**1093–1104.
19. **Petersen, M. B., J. Craven, H. Bjorn, and P. Nansen.** 2000. Use of a migration assay for the separation of adult pyrantel-susceptible and -resistant *Oesophagostomum dentatum*. Vet. Parasitol. **91:**141–145.
20. **Reynoldson, J. A., J. M. Behnke, L. J. Pallant, M. G. Macnish, F. Gilbert, S. Giles, R. J. Spargo, and R. C. Thompson.** 1997. Failure of pyrantel in treatment of human hookworm infections (*Ancylostoma duodenale*) in the Kimberley region of north west Australia. Acta Trop. **68:**301–312.
21. **Richmond, J. E., and E. M. Jorgensen.** 1999. One GABA and two acetylcholine receptors function at the *C. elegans* neuromuscular junction. Nat. Neurosci. **2:**791–797.
22. **Robertson, A. P., H. E. Bjorn, and R. J. Martin.** 1999. Resistance to levamisole resolved at the single-channel level. FASEB J. **13:**749–760.
23. **Robertson, A. P., C. L. Clark, T. A. Burns, D. P. Thompson, T. G. Geary, S. M. Trailovic, and R. J. Martin.** 2002. Paraherquamide and 2-deoxy-paraherquamide distinguish cholinergic receptor subtypes in *Ascaris* muscle. J. Pharmacol. Exp. Ther. **302:**853–860.
24. **Robinson, M., F. Hooke, and K. E. Iverson.** 1976. Efficacy of oxantel pamoate and pyrantel pamoate in combination against *Trichuris Vulpis*, *Ancylostoma caninum* and *Toxocara canis* in dogs. Aust. Vet. Pract. **6:**173–176.
25. **Roepstorff, A., H. Bjorn, and P. Nansen.** 1987. Resistance of *Oesophagostomum* spp. in pigs to pyrantel citrate. Vet. Parasitol. **24:**229–239.
26. **Sangster, N. C., C. W. Davis, and G. H. Collins.** 1991. Effects of cholinergic drugs on longitudinal contraction in levamisole-susceptible and -resistant *Haemonchus contortus*. Int. J. Parasitol. **21:**689–695.
27. **Sangster, N. C., and D. F. Mettrick.** 1987. Effects of cholinergic drugs on muscle contraction in *Moniliformis moniliformis* (Acanthocephala). J. Parasitol. **73:**998–1004.
28. **Sangster, N. C., J. Song, and J. Demeler.** 2005. Resistance as a tool for discovering and understanding targets in parasite neuromusculature. Parasitology **131:**S179–S190.
29. **Todd, A. C., J. Crowley, and P. Scholl.** 1975. Critical tests with pyrantel pamoate against internal parasites in dogs from Wisconsin. Vet. Med. Small Anim. Clin. **70:**936–939.
30. **Williamson, S. M., T. K. Walsh, and A. J. Wolstenholme.** 2007. The Cys-loop ligand-gated ion channel gene family of *Brugia malayi* and *Trichinella spiralis*: a comparison with *Caenorhabditis elegans*. Invertebrate Neurosci. **7:**219–226.