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High-level pyrantel resistance in the hookworm *Ancylostoma caninum*

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Abstract

While anthelmintic resistance is now a widely recognized issue in the livestock industries, its existence within companion animal medicine has been rarely established conclusively. We undertook a placebo-controlled *in vivo* trial to measure the efficacy of pyrantel embonate against pooled isolates of the hookworm *Ancylostoma caninum* from Brisbane, Australia. A statistically significant fall in adult worm burden was observed among dogs in the pyrantel treatment group compared to the control dogs (178.0 ± 24.5 versus 239.7 ± 14.0; $p = 0.02$), equating to an efficacy of just 25.7% (95% CI, 15.0–35.1%), as based upon reduction in mean worm burden. Analysis of faecal egg count trends through the course of the study revealed that egg counts rose in both control and pyrantel-treated dogs, with a greater rise observed in the latter group (11.6 ± 8.3% versus 17.3 ± 7.6%; $p = 0.04$), despite the decrease in adult worm numbers in this group. Our results indicate that high-level anthelmintic resistance does occur in companion animal medicine, and highlight the need for greater vigilance and more judicious use of anthelmintics in small animal practice. They further indicate that the faecal egg count reduction test needs to be used with caution with this parasite.

Keywords: *Ancylostoma caninum*; Dog; Pyrantel; Resistance; Australia

1. Introduction

The canine hookworm, *Ancylostoma caninum*, is one of the most pathogenic gastrointestinal parasites of dogs (Bowman et al., 2003), and in addition poses a significant public health risk to humans (Prociv and Croese, 1996). In Australia, regular anthelmintic treatment of dogs is advocated as a preventative health measure for canine and human populations. For many years, most of the canine anthelmintic preparations used in this country relied upon the activity of the tetrahydropyrimidine anthelmintic pyrantel for treatment of hookworm, and pyrantel-based products continue to be widely used.

At the time of its introduction in the 1970s, pyrantel was highly effective against *A. caninum*, with trials revealing therapeutic efficacies exceeding 95% (Klein et al., 1978; Todd et al., 1975). In 1987, an apparent treatment failure of a pyrantel/oxantel combination product against *A. caninum* in a greyhound imported into New Zealand from Australia was reported. Subsequent investigations provided evidence supporting the existence of pyrantel resistance (Jackson et al.,

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1987). Hopkins and Gyr (1991) later reported a clinical efficacy of 75.1% for pyrantel embonate against *A. caninum* using a critical trial approach.

A potential impediment to the study of anthelmintic resistance in *A. caninum* is the ability of hookworms to modulate their individual egg output proportionate to intestinal population density (Krupp, 1961). Thus, the utility of the faecal egg count reduction test (FECRT) as a measure of changes in adult worm burden following drug treatment is uncertain. The objective of this study was to investigate the efficacy of pyrantel against Brisbane isolates of *A. caninum* using a placebo-controlled *in vivo* trial with measurement of reduction in adult worm burden as the endpoint. By monitoring egg count trends throughout the study we also sought to determine the suitability of the FECRT as a measure of drug efficacy against *A. caninum*.

2. Materials and methods

2.1. A. caninum isolates

Faeces were obtained from 10 Brisbane dogs collected from local dog refuges and screened by density floatation for the presence of hookworm eggs (Kassai, 1999). The faeces of six dogs positive for hookworm eggs were subject to coproculture for harvest of infective larvae, as described previously (Kotze et al., 2004). Larvae were counted and combined, with equal numbers from each isolate contributing to the final pool. The pooled Brisbane sample was stored in darkness in BU buffer (Tissenbaum et al., 2000). To confirm that the isolates were *A. caninum* and not contaminated with other hookworm species that could potentially be present in Brisbane dogs (*A. braziliense* or *A. ceylanicum*), larvae were identified using a polymerase chain reaction (PCR)-based assay (Traub et al., 2004).

2.2. Recruitment of trial dogs

Twelve sex-matched Brisbane pound dogs were recruited for the pyrantel efficacy trial. These animals were of varying breeds, and aged between 3 and 6 months based upon dental examination (Harvey, 1985). All dogs were treated with fenbendazole (Panacur Vetguard Wormer for Dogs®, Intervet Australia Pty. Ltd.) at 50 mg/kg/day for 3 days to clear any intestinal nematode infection prior to the commencement of the trial. The faeces of all dogs were examined by faecal floatation daily for 15 days following fenbendazole administration to ensure that dogs were free of intestinal nematode infection.

2.3. Controlled trial

Dogs were placed in individual cages and each animal was fed 300 infective larvae from the pooled Brisbane isolates combined with a small meal of commercial dog food. Dogs were examined to ensure the entire meal was consumed. The health of dogs was assessed each day following infection by observing appetite, demeanour, faecal consistency, including presence or absence of blood (*i.e.* normal/diarrhoea/dysentery) and mucous membrane colour and refill. Following the establishment of stable patent hookworm infection in all dogs for 5 days (as judged by stable faecal egg counts over a 3-day period), they were allocated into control and treatment groups, with the six dogs in each group matched for age, sex, weight and total egg output (Table 1). Treatment group dogs were each administered the standard therapeutic dose of pyrantel embonate (14.4 mg/kg) as oral syrup (Exelpet Palatable Puppy Worming Suspension®, Effem Foods Pty. Ltd.) with a small meal in accordance with the manufacturer's instructions. Control group dogs received an equivalent amount of distilled water with a small meal. All faeces from each dog were collected and weighed daily for the duration of the trial and faecal egg counts recorded. Each faecal egg count was performed in triplicate using the McMaster counting chamber technique (Hall, 1987). On the 6th day following drug administration, all dogs were euthanased using intravenous pentobarbital. The small intestine, from the pylorus to the ileocaecal valve was removed, incised longitudinally and carefully examined for the presence of adult hookworms. Those present were removed from the mucosa and placed in 70% alcohol for later counting and sexing. A representative sample of 20 worms from each dog was subject to microscopic examination to confirm mature genital morphology to ensure that they comprised mature adult worms. Worms recovered from each of the treatment and control groups were then pooled, and 100 females were randomly

Table 1
Baseline characteristics of control and treatment groups following establishment of stable patency

Characteristic	Treatment group	Control group
Sex (M/F)	3/3	3/3
Age[a] (range)	6 months (5–7)	6 months (5–8)
Weight[a] (range)	10 kg (6.5–12)	11 kg (5.5–20)
FEC[a,b]	7650 epg (7650–7650)[c]	6850 epg (6700–6950)[c]
Egg output/day[a,b]	5,061,500	4,925,500

[a] Median.
[b] Calculated from three consecutive daily counts (days 22–24).
[c] Range of medians over 3-day period.

selected from each pool and individually measured (length and width) and weighed, to assess the effect of intestinal crowding upon worm size.

2.4. Statistical analysis

Daily individual egg counts were determined by calculating the arithmetic mean of counts from three replicates. Total faecal egg output was calculated by weighing the faeces produced daily by each dog and multiplying this by the daily individual egg count. Efficacy (expressed as a percentage) was calculated as previously reported (Jacobs et al., 1994) based on arithmetic means. The 95% confidence interval for the efficacy estimate based upon arithmetic means was calculated using the method described by Coles et al. (1992), adjusted to reflect degrees of freedom appropriate to our sample size. These calculations were carried out using the statistical software package Stata® and Microsoft Excel®. Investigation for significant differences between treatment and placebo groups was undertaken using the non-parametric Mann–Whitney *U*-test using Stata®.

2.5. Animal ethics

This experiment was approved by the University of Queensland's Animal Ethics Committee, approval number SVS/293/05/.

3. Results

The baseline characteristics of the study population are shown in Table 1. Control and treatment groups were matched for age, sex, weight and total egg output per day. As determined by PCR assay, *A. braziliense* and *A. ceylanicum* were not present in any of the infections (data not shown).

Trends in egg counts for the entire trial, along with clinical signs, are shown in Fig. 1. Eggs were first detected in faeces on day 15 post-infection, and counts increased rapidly over the following 2 days when counts began to plateau. All dogs experienced at least 1 day of diarrhoea in the prepatent period; however there was no single day when all 12 dogs were diarrhoeic. Diarrhoeic samples containing blood (dysentery) were observed in seven different dogs during the prepatent period, peaking 12–14 days following infection. A single instance of dysentery occurred following patency, on day 19 of the trial. All other clinical indicators remained normal in all dogs throughout the course of the trial.

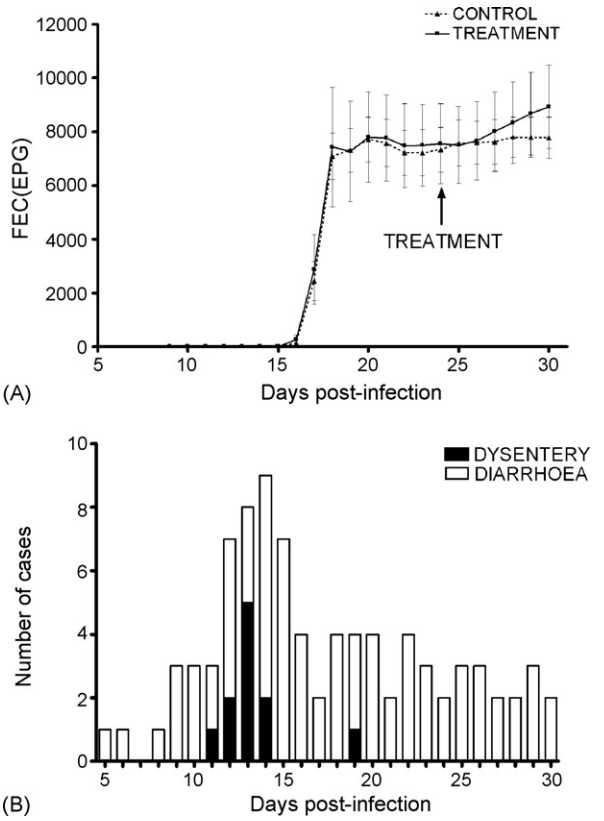

Fig. 1. (A) Mean egg count trends for the control and treatment group from infection (day 1) until euthanasia (day 30), (B) clinical signs noted through the course of the trial.

Table 2
Changes to FEC in treatment and control groups following the administration of pyrantel and placebo

Group	Dog code	% Change in FEC[a]	Group mean ± S.E.M. (%)
Control	HOOK 01	−2.1	+11.6 ± 8.3
	HOOK 03	+21.0	
	HOOK 04	+15.7	
	HOOK 07	+4.2	
	HOOK 09	+10.0	
	HOOK 11	+6.1	
Treatment	HOOK 02	+26.5	+17.3 ± 7.6[b]
	HOOK 05	+21.9	
	HOOK 06	+16.7	
	HOOK 08	+21.4	
	HOOK 10	+24.6	
	HOOK 12	+5.3	

[a] Calculated by comparing final egg counts on day 30 (7 days after drug treatment) to baseline egg counts as established in Table 1.
[b] $p = 0.04$.

Table 3
Number of adult *Ancylostoma caninum* found within the intestines of dogs at necropsy

Group	Dog code	Adult worms present at necropsy
Control	HOOK 01	253
	HOOK 03	235
	HOOK 04	246
	HOOK 07	255
	HOOK 09	220
	HOOK 11	229
	Mean ± S.E.M.	239.7 ± 14.0
Treatment	HOOK 02	140
	HOOK 05	174
	HOOK 06	187
	HOOK 08	170
	HOOK 10	215
	HOOK 12	182
	Mean ± S.E.M.	178.0 ± 24.5

Changes to egg counts among individual dogs are summarized in Table 2. While a rise in the egg output of both control and pyrantel-treated dogs was observed, a significantly greater rise in the egg output was observed among the pyrantel treated dogs (17.3 ± 7.6% versus 11.6 ± 8.3%; $p = 0.04$). The adult worm burden among study dogs is presented in Table 3. The adult worm burden among dogs in the pyrantel treatment group was significantly lower than that observed among control dogs (178.0 ± 24.5 versus 239.7 ± 14.0; $p = 0.02$). However, this reduction in worm burden was small, calculated at 25.7% (95% confidence interval 15.0–35.1%). Neither difference in the ratio of female to male worms was observed between groups (1.2:1 for both groups), nor was there any significant difference in mean length, width or weight of female worms recovered from control or treatment dogs (data not shown).

4. Discussion

In this placebo-controlled trial we have identified high-level pyrantel resistance in Brisbane isolates of *A. caninum*, with an efficacy of just 25.7%. This is a substantial reduction compared to an earlier report of 75.1% (Hopkins and Gyr, 1991). Such a dramatic escalation in the resistance of *A. caninum* to pyrantel over the intervening 15 years should be of great concern to companion animal parasitologists and veterinary clinicians. Pyrantel was demonstrated to be highly effective against *A. caninum* on its introduction in the 1970s, as measured by critical and controlled studies (Klein et al., 1978; Todd et al., 1975). Widespread pyrantel use over the ensuing 30 years has clearly produced significant levels of resistance in *A. caninum* in Brisbane. This experience parallels that reported in human hookworm infection with *A. duodenale*, where, following a period of heavy pyrantel use, therapeutic efficacy was observed to fall (Reynoldson et al., 1997).

The finding that the most severe signs of disease occurred prior to patency has important implications for clinical practice, where the diagnosis of hookworm disease generally relies upon the observation of eggs in a faecal sample. Miller (1966a,b) previously reported the development of clinical signs in canine hookworm infection from as early as 7 days post-infection, well before eggs are shed in the faeces. While this aspect of our findings is therefore not novel, it reinforces the fact that a negative faecal egg count alone should not be used to eliminate hookworm from differential diagnoses, particularly in situations where acute infections are possible.

An important observation in this study was the poor correlation between faecal egg count reduction and changes in adult worm burden. While a significant reduction in adult worm numbers (25.73%, $p < 0.05$) was demonstrated in the pyrantel-treated group, faecal egg count rose in all pyrantel-treated dogs following treatment. *A. caninum* is known to modulate its egg production in response to the degree of intestinal crowding (Krupp, 1961). For example, data presented in Krupp's paper shows that female *A. caninum* present in a burden of 200 worms can produce twice as many eggs per worm than those present in a burden of 300 worms. This indicates that female *A. caninum* have a very large reproductive reserve that is only fully exploited in light infections. Our data suggests that the elimination of a proportion of the adult population following anthelmintic treatment is associated with an increase in total egg output due to increased egg production in surviving worms. This increase in egg output occurred to a greater extent in drug-treated dogs (alongside a reduction in adult numbers) compared to the control group which presumably maintained stable adult worm burdens following their placebo treatment. The slight increase in egg counts noted for the control group is likely to represent a normally maturing infection (Onwuliri et al., 1981).

The mechanism behind this modulation in egg output is not known. One might expect that females in less crowded conditions have better access to resources, grow larger and therefore produce more eggs. However, Sarles (1929) and McCoy (1931) could not demonstrate a correlation between worm burden and worm size. Likewise, we found no significant difference in size between control and treatment group worms, despite treatment group worms being less crowded.

A comparison of our faecal egg count and adult worm burden data for the pyrantel-treated animals therefore indicates that while the FECRT may assist in the detection of highly resistant or susceptible isolates, it has limited value as a quantitative measure of anthelmintic efficacy against *A. caninum* isolates of intermediate resistance status. Thus, the FECRT may still serve a purpose as a screening tool to identify potentially resistant isolates that require further investigation. There is also the possibility that the FECRT could be combined with the screening of faeces for the presence of expelled worms to improve interpretation of results.

Wolstenholme et al. (2004) identified three key factors influencing selection pressure for resistance in veterinary helminths: parasite genetics, parasite biology including the availability of refugia, and the relationship between the parasite and its host. The genetics and mechanisms of pyrantel resistance in *A. caninum* require investigation. *A. caninum* is a highly pathogenic parasite and a significant zoonosis (Bowman et al., 2003), and thus most veterinarians advocate regular prophylactic use of anthelmintics to minimize risks to both dogs and people. The use of 3-monthly worming regimes on pet dogs would be expected to exert drug selection pressure, especially in the absence of drug rotation practices, however the impact of this commonly recommended treatment frequency is off-set by poor treatment compliance amongst dog owners, which in the United States has been estimated to be below 50% (American Animal Hospital Association, 2003).

On the other hand, a large proportion of the *A. caninum* population would be expected to be in refugia at any one time and this would be expected to slow resistance development. Refugia is provided by free-living and somatic reservoir stages of the *A. caninum* life cycle as well as by parasites infecting free-ranging (stray or feral) dogs and untreated pets. Free-ranging dogs in this district are known to frequent public parks and even suburban backyards as they scavenge for food (Queensland Department of Natural Resources and Mines, 2004), and they represent an untreated reservoir host that may transmit infections to pet dogs by defecating in public areas. Given the more variable nature of anthelmintic use in urban dog population compared to the flock/herd-wide application of drugs to livestock on rural properties, it is not surprising that the development of anthelmintic resistance has been less of an issue in canine parasites than those of livestock. However, it is clear that high-level pyrantel resistance has emerged in Brisbane dogs and it can no longer be assumed that resistance is not an issue in companion animal parasitology.

At the time of writing, there are 133 anthelmintic products registered in Australia for the treatment of canine hookworm infections (APVMA, 2005). Of these, 51 products rely solely on pyrantel embonate for their activity against hookworm (including pyrantel–oxantel–praziquantel and pyrantel–low dose ivermectin combinations) and a further 15 products rely on a pyrantel–febantel combination. A synergistic interaction between febantel and pyrantel has been identified (Hopkins and Gyr, 1991), and since the mechanism of this synergism is yet to be fully ascertained our data should not be considered applicable to pyrantel when used in combination with febantel. Of the remaining registered products, 11 rely on another cholinergic anthelmintic, levamisole, for activity against hookworm. In sheep nematodes, levamisole-resistance is known to confer side resistance to pyrantel, although the reverse does not apply (Waller et al., 1986).

A surveillance system for monitoring the efficacy of these companion animal anthelmintics is urgently required in order to fully ascertain the impact that current worming protocols are having on the development of anthelmintic resistance. This will be important in identifying situations where pyrantel may be still effective, in defining the extent of the pyrantel-resistance problem, and in detecting the emergence of resistance to drugs from other groups. Animal welfare considerations preclude the widespread use of controlled or critical trials, and the FECRT is clearly unreliable with this parasite. For these reasons, *in vitro* assays such as larval motility assays and/or larval development assays (Gill et al., 1991; Kotze et al., 2004) are likely to play a key role in future investigations of anthelmintic resistance in this nematode.

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