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Further characterization of a cattle nematode population with demonstrated resistance to current anthelmintics

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ABSTRACT

We previously documented the appearance of cattle nematode parasites resistant to avermectins, milbemycin, and a benzimidazole at the end of a grazing season in a backgrounding operation in the upper Midwestern US. To further characterize the pattern of drug resistance, we extended the study to (1) monitor the animals over the course of the grazing season; (2) increase the number of animals slaughtered at the end of the season to minimize the effect of potential outlying observations; (3) increase the time interval between treatment and slaughter to ensure sufficient time for drug action; (4) utilize repeated fecal sampling in the fecal egg reduction test to minimize procedural variation; (5) increase the number of drugs tested. The results of the present study were in agreement with those of the previous study and demonstrated that during the course of the grazing season the pastures harbored significant numbers of parasites that were refractory to avermectins, milbemycin, and a benzimidazole at the label recommended doses. As seen previously, *Haemonchus contortus* resistant to all these anthelmintics were present over the course of the study period. In contrast, *Cooperia* sp., mainly *punctata*, and *Haemonchus placei* were resistant only to the macrocyclic lactones. There was no apparent resistance against the older anthelmintic levamisole, which had not been used for >20 years in the operation. However, animals treated with this drug continued to harbor small but measurable numbers of *Ostertagia ostertagi*.

1. Introduction

Previous studies on a beef cattle backgrounding operation in the upper Midwestern US had demonstrated the existence of cattle nematode parasites resistant to avermectins, milbemycin, and a benzimidazole (Gasbarre and Smith, 2009). In the course of that study, *Cooperia punctata* and *Haemonchus placei* resistant to avermectins and milbimycin, and *Haemonchus contortus* resistant to all three classes of drug, were demonstrated on pastures at the end of the grazing season. To confirm that observation and to further characterize the pattern of drug resistance, a second study was performed 1 year later on the same pastures.

In this second study, a number of changes were made in the experimental protocol to address questions arising from the results of the initial study. These changes included: (1) a more thorough examination of fecal egg patterns in the cattle prior to their placement on pasture; (2) testing of the efficacy of a broader variety of strategic treatment options, including the older anthelmintic levamisole and the use of combinations of drug classes; (3) a lengthening of the period between drug treatment and slaughter to insure sufficient time for the drug to act upon the worms; (4) replicate fecal sampling to reduce variance; (5) a doubling of the size of the slaughter groups to lessen the impact of outliers of the mean worm recovery values for each test group; (6) separation of treatment

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groups to prevent transfer of pour-on products between groups. In this report we outline the results of that study.

2. Materials and methods

2.1. History of animals

Female or castrated male beef or beef cross calves used for this study were purchased in the fall/winter of 2003–2004 by order-buyers at auction barns in the southeastern United States at a weight of 175–225 pounds. When the animals were assembled, they were dewormed with Dectomax® injectable, and then placed either directly with various contract grazers or sent to preconditioning lots for 30–60 days in Alabama and Mississippi. When the animals left the preconditioning lots to the various contract grazers they were dewormed with Cydectin®. While on pasture, contract grazers dewormed the animals once or twice with either IvomecPlus®, Dectomax®, or Cydectin®. During the last week of April 2004, all animals were assembled from the grazers, sorted according to weight, and dewormed with levamisole injectable, after which they were shipped to Wisconsin. There the 172 lightest animals were placed on the study pasture for the summer 2004.

The pasture forage used for grazing was primarily bluegrass with red and white clover. An oat by-product (oat hulls), corn screenings and a salt-mineral containing Rumensin® were available free choice.

2.2. History of pasture

Only cattle had been grazed on this pasture for the previous 40 years. Since ~1980, all animals grazed on this pasture had been dewormed at turnout with either fenbendazole or one of the avermectins, then strategically twice more during the grazing season. In this study, Dectomax® injectable was used to deworm all animals on 15 June and 21 July 2004. Random fecal samples were taken on the following days (Table 1): 10 samples on 21 May (2 weeks after turnout); 24 on 15 June (day of treatment); 16 on 29 June (14 days after treatment); 24 on 7 July (day of treatment); 25 on 4 August (14 days after treatment).

2.3. Fecal sampling and animal identification

A new plastic sleeve was used on each of the 172 animals on this pasture to obtain rectal fecal samples on October 2004 and 2006 for a pretreatment mean eggs per gram (EPG) measurement and again on October 25 and 26 for the fecal egg reduction test 14 days after treatment. A uniquely numbered ear tag was also placed in one ear of each animal at the first sampling.

Table 1
Fecal egg counts from subset of calves on G pasture at selected times during grazing season. All calves in group were treated with Dectomax® on 6/15 and 7/21.

Date	No. of animals	Mean EPG ± SD	Range
5/21	10	1 ± 2	0–4
6/15	24	15 ± 26	0–112
6/29	16	4 ± 5	0–18
7/21	24	25 ± 50	0–242
8/4	25	24 ± 27	0–92

2.4. Fecal examination

A modified Wisconsin technique was used; 5.0 g feces were mixed with a small amount of tap water. The mixture was screened through a coarse sieve, poured into a 15 ml centrifuge tube, and spun at 1×10^3 rpm for 10 min. The supernatant was discarded; 12 ml of concentrated sugar solution (1200 g sugar/l 400 ml distilled water/2 g phenol) was added, and then mixed to break up the pellet. More sugar solution was added to form a convex meniscus. A cover slip was placed on the meniscus and the tube centrifuged again at 1000 rpm for 10 min, or allowed to stand for 30 min. The cover slip to which the eggs adhered was removed from the tube and placed on a microscope slide. The ova counts were divided by 5 and reported as EPG.

2.5. Allocation to treatment

Each animal's EPG counts were averaged and the animals were ranked from highest to lowest EPG. Based on decreasing EPG counts, replicates of nine animals were formed. Within each replicate, drawing numbers from a container randomly assigned animals to treatment. The study was designed to have a minimum of 19 animals per treatment group.

Each of the following dewormers constituted a treatment group:

Ivermectin (Ivomec Plus Injectable®)	Lot MBCD0890	Exp. 10/2005
Eprinomectin (Eprinex Pour-on®)	Lot NBJ 1100	Exp. 04/2006
Dormectin (Dectomax injectable®)	Lot K4D00612	Exp. 04/2007
Moxidectin (Cydectin Pour-on®)	Lot 268887	Exp. 07/2007
Fenbendazole (Panacur® Oral liquid)	Lot UFE1	Exp. 05/2008
Levamisole (Levasole® injectable)	Lot 3111303	Exp. 11/2005
Eprinomectin + Levamisole	Lot and date as indicated previously	
Eprinomectin + Fenbendazole	Lot and date as indicated previously	
No medication to control group		

Animals were individually weighed before treatment on a Tru-Test® scale on 11 October 2004.

Certified weights were used to check the scale for accuracy before the first and after the last animal was weighed. The weight range at necropsy was 388–827 pounds.

One individual calculated dose according to the specifications of the manufacturer; another individual did the same and confirmed the calculation before dosing. Injectable products (ivermectin, doramectin, levamisole) were administered subcutaneously in the neck area with a 16 gauge–1 inch Monoject® needle attached to a 12 cm^3 syringe accurate to 0.2 ml. The calculated dose was

rounded up to the next 0.2 ml. A new syringe and needle were used on each animal. Pour-on products (eprinomectin and moxidectin) were poured from the withers to the tailhead using a 35 ml Monoject® syringe accurate to 1 ml. The calculated dose was rounded up to the next ml. Fenbendazole was administered orally using a 35 ml Monoject® syringe accurate to 1 ml. The calculated dose was rounded up to the next ml.

Control animals were processed the same as the treated animals, but without drug treatment.

2.6. Selection of animals for necropsy

Six animals per treatment group were selected for necropsy, removed from the pasture and not treated until they arrived at the holding area. This insured no inter-animal transfer of the pour-on products during transportation. At the holding area, animals were placed by treatment group in concrete floored pens with solid partitions between pens to prevent contact between treatment groups to prevent transfer of drug between treatment groups. The highest EPG animals were selected for necropsy to insure maximal numbers of worms for species identification.

2.7. Segregation of animals left on pasture

The pasture was divided into four sections after treatment. The Eprinex® pour on and the Eprinex® + Levamisole treatment groups were placed in one section, the Cydectin® treated animals in a different section, the Levamisole, Ivomec Plus®, Dectomax® and fenbendazole treated groups in the third section, and the untreated controls in another section. This partitioning was done to prevent transfer of the pour on products between groups (Sallovitz et al., 2005).

2.8. Nematode identification

Nematodes were cleared in phenol–alcohol (80 parts melted phenol crystals and 20 parts absolute ethanol) for study in temporary wet mounts on glass microscope slides. Interference-contrast light microscopy was used to study the synlophe (pattern of surface longitudinal cuticular ridges) and other characters at a magnification of 200–400×. Specimens of *C. punctata*, *C. spatulata* and *C. oncophora* were identified on the basis of spicule morphology and characteristics of the synlophe described by Lichtenfels (1977). Specimens of *H. contortus* and *H. placei* were identified on the basis of spicule length (Lichtenfels et al., 1988a,b) and morphology, and characteristics of the synlophe (Lichtenfels et al., 1994). Specimens of *Nematodirus helvetianus* were identified on the basis of characteristics of the spicules and synlophe (Lichtenfels and Pilitt, 1983). The specimens of *Ostertagia ostertagi* were identified on the basis of synlophe characteristics (Lichtenfels et al., 1988a,b; Lichtenfels and Hoberg, 1993). The characters of the synlophe made it possible to identify males and females of all species. Fourth-stage larvae were identified to genus. The specimens have been deposited in the U.S. National Parasite Collection (Nos. 96824–96974).

2.9. Statistical analyses

Data were analyzed using Sigma Stat® (Point Richmond, CA). Data were analyzed for normality using the Kolmogorov–Smirnov test. Data found to be normally distributed were analyzed by one-way ANOVA and differences in mean values among the treatment groups compared using the Holm–Sidak method. For data not adhering to the assumption of normality, analysis was performed using the Kruskal–Wallis one-way analysis of variance on ranks. For groups of equal size, multiple range testing was performed using the Tukey test. For groups of unequal size, pairwise comparison used Dunn's method.

3. Results

3.1. Fecal egg counts over the grazing season

Treatment of calves at turnout on pasture reduced parasite burdens, as samples taken shortly after turnout had very few trichostrongylid nematode eggs (1 ± 2; Table 1). As the grazing season progressed, the efficacy of treatment with the avermectin, Dectomax® was progressively less efficacious. Mean fecal egg counts from 25 animals on August 4, 2 weeks after drug treatment, had the same value as samples from 24 animals immediately prior to treatment (25 ± 50 vs. 24 ± 27).

3.2. Fecal egg reduction test

The mean group EPG values for all treatment groups were identical at the time of treatment (Table 2). Two weeks following treatment, the group mean fecal egg reduction was ≥95% only in those groups given levamisole either alone or in conjunction with eprinomectin, and these were the only groups whose % reduction were significantly different from the untreated animals. In contrast, groups given milbemycin, fenbendazole, or a combination of eprinomectin and fenbendazole resulted in an intermediate EPG reduction, but this was not significantly different from either the untreated group or the groups that showed significant egg reduction. Finally, EPG

Table 2
Results of Fecal egg reduction test for all animals on study. Fecal EPG values were determined approximately 1 week prior to treatment and again 14 days after treatment by average of counts from 2 consecutive days.

Treatment	Mean ± SD		%Reduction (no. ≥ 95%/ no. of treated)
	Before Trt*	Post-Trt	
None	36 ± 61^{a}	69 ± 79^{c}	−95 (0/20)[b]
Eprinex® + Levasole®	37 ± 72^{a}	0.2 ± 0.4^{a}	99 (16/19)[a]
Levasole®	40 ± 92^{a}	0.2 ± 0.4^{a}	99 (16/19)[a]
Eprinex® + Panacur®	35 ± 64^{a}	8 ± 9^{bc}	77 (2/19)[ab]
Cydectin®	38 ± 64^{a}	9 ± 15^{bc}	77 (4/19)[ab]
Panacur®	42 ± 91^{a}	13 ± 52^{ab}	68 (7/19)[ab]
Eprinex®	40 ± 75^{a}	43 ± 81^{bc}	−7 (1/19)[b]
Dectomax®	39 ± 77^{a}	53 ± 99^{bc}	−35 (1/19)[b]
IvomecPlus®	37 ± 65^{a}	66 ± 87^{c}	−78 (0/20)[b]

* Values with same superscript do not differ at $p \geq 0.05$ by Kruskal–Wallis one way.

Table 3
Results of Fecal egg reduction test for 6 animals from each group selected for slaughter. Fecal EPG values were determined approximately 1 week prior to treatment and again 14 days after treatment by average of counts from 2 consecutive days.

Treatment	Mean ± SD		% Reduction (no. ≥ 95%)
	Before Trt*	Post-Trt	
None (6)	95 ± 85^{a}	136 ± 85^{b}	-44 (0)^{b}
Eprinex® + Levasole®	110 ± 100^{a}	0.1 ± 0.1^{a}	>99 (6)^{a}
Levasole®	109 ± 149^{a}	0.4 ± 0.5^{a}	>99 (6)^{a}
Eprinex® + Panacur®	93 ± 92^{a}	16 ± 12^{ab}	83 (1)^{ab}
Cydectin®	102 ± 85^{a}	12 ± 18^{ab}	89 (3)^{ab}
Panacur®	115 ± 143^{a}	40 ± 92^{ab}	66 (4)^{ab}
Eprinex®	109 ± 109^{a}	109 ± 124^{b}	-1 (1)^{b}
Dectomax®	107 ± 114^{a}	114 ± 151^{b}	-7 (0)^{b}
IvomecPlus®	100 ± 90^{a}	153 ± 105^{b}	-54 (0)^{b}

* Values with same superscript do not differ at $p \geq 0.05$ by Kruskal–Wallis one-way analysis of variance on ranks with pairwise multiple comparisons using Tukey test.

reduction following treatment with any of the avermectins was not significantly different from the non-treated group.

When applied to only those animals selected for slaughter (Table 3), the fecal egg reduction assay gave results identical to those from the entire grazing group. Again, the initial group mean EPG values were identical, and because of the selection criteria for the slaughter animals, were approximately 3 times higher than those of the entire treatment group. Only those groups given levamisole, either singly or in combination, showed fecal egg reductions that were significantly different from the untreated animals. As in the entire group, the use of an avermectin appeared to have no effect on fecal egg counts 2 weeks after drug treatment.

3.3. Numbers of parasites recovered

The results of worm recoveries were consistent with the results of the fecal egg reduction test (Table 4), although some minor differences were noted. In the abomasum only treatment with levamisole, either alone or in combination with eprinomectin, resulted in a statistically signification reduction in parasite numbers compared to the control group. The combination of levamisole and eprinomectin was marginally better than levamisole alone, but this treatment was not significantly different than most of the other treatments, with the exception of doramectin (Table 4).

A slightly different pattern was seen in the small intestine (Table 4). Treatment with a drug combination, either levamisole and eprinomectin or fenbendazole and eprinomectin, resulted in significantly fewer worms than did treatment with ivermectin plus clorsulon. Because of the variation seen among animals within a group, no statistically significant differences could be demonstrated between any treatment group and the untreated control group.

When abomasal and small intestinal counts were combined, the differences in worm numbers closely mirrored those seen in the fecal egg reduction test. Treatment with the combination of levamisole and eprinomectin resulted in a significant reduction in worm recoveries when compared to the untreated and the ivermectin plus clorsulon treated groups (Table 4). The other treatment groups were found to have an intermediate level of reduction in worm numbers, but again owing to within group variation the differences were not statistically significant.

Table 4
Mean number of worms recovered from necropsy 7 days after treatment.

Treatment	Mean abomasum*	Mean small intestine	Total
None	1575^{c}	8563^{ab}	$10{,}138^{c}$
Eprinex® + Levasole®	35^{a}	0^{a}	35^{a}
Levasole®	171^{ab}	38^{ab}	209^{ab}
Eprinex® + Panacur®	548^{abc}	0^{a}	548^{abc}
Cydectin®	448^{abc}	763^{ab}	$1{,}211^{abc}$
Panacur®	333^{abc}	17^{ab}	350^{abc}
Eprinex®	1733^{bc}	988^{ab}	$2{,}721^{bc}$
Dectomax®	909^{abc}	$2{,}725^{ab}$	$3{,}634^{bc}$
Ivomec Plus®	993^{abc}	$14{,}792^{b}$	$15{,}785^{c}$

* Values with same superscript do not differ at $p \geq 0.05$ by Kruskal–Wallis one-way analysis of variance on ranks with pairwise multiple comparisons using Tukey test.

Table 5
Percentage by species of worms recovered from the abomasum.

Treatment	Worm number	*H. contortus*	*H. placei*	*O. ostertagi*
None	1575	25	45	30
Eprinex® + Levasole®	35	83	0	17
Levasole®	171	5	5	90
Eprinex® + Panacur®	548	98	2	0
Cydectin®	448	95	5	0
Panacur®	333	86	0	14
Eprinex®	1733	57	43	0
Dectomax®	909	67	33	0
Ivomec Plus®	993	42	56	2

3.4. Species of parasites recovered

Analyses of the species of worms recovered from the treatment group revealed a number of interesting points. For example, *H. contortus* was the most commonly recovered worm from the abomasum in all groups except the untreated controls, the levamisole treated group and the ivermectin plus clorsulon treated group (Table 5). In contrast, *H. placei*

Table 6
Percentage by species of worms recovered from the small intestine.

Treatment	Worm number	*C. punctata*	*Cooperia* sp.	*N. helvetianus*
None	8,563	86	12	2
Eprinex® + Levasole®	0	NA	NA	NA
Levasole®	38	100	0	0
Eprinex® + Panacur®	0	NA	NA	NA
Cydectin®	763	100	0	0
Panacur®	17	100	0	0
Eprinex®	988	99	1	0
Dectomax®	2,725	74	22	4
Ivomec Plus®	14,792	80	20	<1

was the most commonly recovered worm in the abomasa from the untreated and ivermectin plus clorsulon groups. Abomasal worms recovered from the levamisole group were almost entirely *O. ostertagi*.

In the small intestine (Table 6), the dominant species recovered was *C. punctata*. In both combination drug groups, no worms were recovered from the small intestine. In all other groups, *C. punctata* accounted for at least 74% of all worms recovered.

4. Discussion

This study extends and confirms the earlier observation of the appearance of anthelmintic resistant parasites in a commercial cattle operation in the US. While anthelmintic resistance is increasingly common throughout the world in small ruminants (see Waller, 1997), there have been documented reports in cattle in New Zealand (Vermunt et al., 1995; Hosking et al., 1996; Waghorn et al., 2006), England (Stafford and Coles, 1999) and South America (Anziani et al., 2004), and recently in the US (Gasbarre and Smith, 2009). Because anthelmintic resistance has not been previously reported in US cattle despite the widespread use of the drugs, and also because of the difficulty in accurately determining the presence of such parasites, the previous study was repeated with several important changes in the protocol. Although the first study adhered to the general guidelines used for determining the presence of drug resistant nematodes (Coles et al., 1992), some unique aspects of the cattle–nematode–drug interaction warranted replication and expansion of the study.

The first major difference was an increase in the number of cattle slaughtered for worm recovery. In the initial study three animals per group were killed. Because host resistance to nematode infection is strongly influenced by host genetics (Gasbarre et al., 1990), a group size of 3 could be overly sensitive to the effect of an outlier value. Thus, group size was increased to 6 to add statistical power and to allow inclusion of potentially extreme animals in all groups. A second measure to increase the precision of the measures was to replicate fecal sampling at the time of treatment and again 2 weeks after treatment for the fecal egg reduction test. Previous studies had indicated that a simple replication of sampling on consecutive days and averaging of the two counts reduces the variance associated with the measure by 20% (Gasbarre et al., 1996). A third change was to extend the time between treatment and slaughter. It is possible that the interval of 7 days used in the initial study was insufficient to allow full expression of activity for the avermectins. In the present study, the interval between treatment and slaughter was increased to 14 days. A fourth change was to include a group treated with an additional class of anthelmintic, levamisole. Levamisole is no longer commonly used in US cattle operations, and as such it can be considered as a "positive control" for drug efficacy because of a lack of selection for resistance. The final change in protocol was to more intensely follow the animals throughout the grazing season. In the initial study, animals were assessed only at the end of the study. Fecal sampling of a subset of sufficient size during the season was thought to be able to yield information on changes in drug sensitivity over the course of the summer.

The modifications of the experimental protocol did not change the conclusions reached from the initial study. These include: (1) the commercial operation studied had *H. contortus* resistant to avermectins, milbemycin, and benzimidazoles, (2) also present were *H. placei* and *Cooperia* sp., mainly *punctata*, that were resistant to the avermectins and milbemycin, and (3) the treatment regimen practiced at this location, repeated strategic application to suppress parasite-induced weight loss, appears to have favored the selection of drug refractory parasites.

Additionally, this study extended the previous results in several areas. The inclusion of levamisole as a treatment option demonstrated that this drug, seldom used in US cattle today, is efficacious against the drug resistant parasites encountered in this study. While this provides an option for producers for whom drug-resistant parasites are problematic, the fact that the abomasa of the Levasole® treated group contained *O. ostertagi* indicates that care should be taken in complete reliance on this drug. Levamisole is generally believed to be ineffective against larval *Ostertagia*. As such, use of this drug alone might not adequately control *Ostertagia* numbers on pasture. Interestingly, in the previous study in which levamisole was not used, virtually no *Ostertagia* were found in any group, while in the present study 30% of the worms in the abomasa of the untreated group were *O. ostertagi*. Given the higher pathogenicity of *Ostertagia* compared to *Haemonchus* or *Cooperia* in cattle, reliance on levamisole alone could lead to suboptimal control. In contrast, the combination of levamisole and eprinomectin was the most efficacious treatment option. Combining levamisole and an avermectin or milbemycin might offer the best option for nematode control in circumstances like those described here.

An interesting observation in both this and the previous study was that ivermectin-treated cattle had higher numbers of *Cooperia* than the untreated control group. In neither study was this difference statistically significant owing to large within-group variation. Nonetheless, the repeated observation implies that additional studies should be performed to assess if selective removal of drug-sensitive nematode species may be altering the normal host-parasite interactions. It is well documented that the different genera of cattle nematodes elicit very different immune responses in their host (Gasbarre, 1997). Removal of drug sensitive but highly immunogenic species such as *O. ostertagi* may favor colonization and retention of less immunogenic but drug resistant genera such as *Cooperia*. While this is speculative, it is important to recognize that decades of intense anthelmintic use and selection for drug resistance may have altered the epidemiology and in-host biology of parasitic nematodes.

In conclusion, this study confirms and extends an earlier study which found that repeated strategic administration of commonly used anthelmintics has either selected for drug resistance *in situ* in a commercial beef cattle operation in the US, or has favored the expansion of drug resistant parasites brought in from another geographical area, i.e. the Southeastern US. The parasites present, depending upon the

species, show resistance to commonly used avermectins, milbemycin, and a benzimidazole. Although the extent of resistance in the US remains unknown, it is essential to recognize that anthelmintic resistance does exist in the US, and that care must be taken in recommending parasite control programs that may favor continued selection for and expansion of resistance to the drugs.

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