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A novel approach for combining the use of in vitro and in vivo data to measure and detect emerging moxidectin resistance in gastrointestinal nematodes of goats

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Abstract

Ivermectin and moxidectin are closely related avermectin/milbemycin anthelmintics and available data suggest that side resistance occurs with these two drugs. However, moxidectin remains effective against many species of ivermectin-resistant worms due to its higher potency. The larval development assay (LDA) is routinely used to diagnose ivermectin resistance in *Haemonchus contortus* but laboratory diagnosis of moxidectin resistance is hampered by the lack of any validated in vitro tests. The objective of this study was to measure the relative susceptibility/resistance of *H. contortus* to moxidectin on goat farms in Georgia, and to validate the DrenchRite® LDA for detecting resistance to moxidectin. Fecal egg count reduction tests (FECRT) were performed at five different moxidectin dose levels and DrenchRite® LDAs were performed in duplicate on nine meat goat farms in Georgia, USA. To improve our ability to make inferences on the relative levels of resistance between farms, FECRT data were first analysed using a linear mixed model, and then Tukey's sequential trend test was used to evaluate the trend in response across dose levels. LDA data were analysed using log-dose logit-response and probit models. Using these statistical results, we were able to rank the nine farms from the least to the most resistant, and to develop a set of criteria for interpreting DrenchRite® LDA results so that this assay can be used to diagnose both clinically apparent moxidectin resistance, as well as sub-clinical emerging resistance. These results suggest that our novel approach for examining these types of data provides a method for obtaining an increased amount of information, thus permitting a more sensitive detection of resistance. Based on results of the LDA, moxidectin-resistant farms had resistance ratios, compared with an ivermectin-sensitive farm, ranging from 32 to 128, and had resistance ratios of 6–24 compared with an ivermectin-resistant/moxidectin naive farm. Moxidectin resistance was diagnosed both in *Haemonchus* and *Trichostrongylus* on almost half of the farms tested, despite this drug only being used on these farms for 2–3 years.

Keywords: *Haemonchus contortus*; *Trichostrongylus colubriformis*; DrenchRite; Larval development assay; Linear mixed model; Tukey's trend test; Anthelmintic resistance; Moxidectin

1. Introduction

In the southern United States (US) and throughout much of the warm temperate, subtropical and tropical regions of the world, *Haemonchus contortus* is the parasite species of primary concern in sheep and goats. A 7-year review (1993–2000) of clinical cases at Auburn University Veterinary Medical Teaching Hospital (Auburn, Alabama, USA) demonstrated that parasitic disease was the primary reason that 91% of goats were examined and treated by hospital clinicians (Pugh and Navarre, 2001). Over the past

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40 years, the primary means of controlling *H. contortus* has been the frequent administration of anthelmintics. Unfortunately, the intensive use of, and virtual total reliance on, drugs for the control of gastrointestinal nematodes in small ruminants has led to the worldwide development of anthelmintic-resistant nematode populations, which are reaching alarming proportions throughout much of the world (Kaplan, 2004). The inability to control multiple-drug-resistant worms currently threatens the future viability of continued small ruminant production in many countries (Waller, 1999). In the southern US, greater than 90% of all goat farms tested had resistance to two of three drug classes (ivermectin and albendazole) and about 30% of farms had worms resistant to all three drug classes (ivermectin, albendazole and levamisole) (Mortensen et al., 2003). Moxidectin was the only drug that was effective on all farms tested (mean reduction in fecal egg counts (FECs) = 99%), though on some farms there was evidence that early resistance may be developing. In other areas of the world, similar patterns exist; severe multiple-drug resistance, with moxidectin remaining as the most efficacious drug. However, in recent years moxidectin resistance is being reported with increased frequency (Love et al., 2003; Hughes et al., 2004; Thomaz-Soccol et al., 2004; West et al., 2004).

Ivermectin and moxidectin are closely related drugs belonging to the avermectin/milbemycin class of anthelmintics (commonly referred to as macrocyclic lactones), though moxidectin is more potent against many species of parasitic nematodes. It is generally recognised that resistance to one drug in an anthelmintic class confers resistance to all of them, a phenomena referred to as side resistance (Shoop et al., 1995; Sangster, 1999). Though precise mechanisms are not well understood, and some minor differences almost certainly exist (Molento et al., 2004), most published data suggest that these two drugs have very similar mechanisms of action and resistance (Conder et al., 1993; Forrester et al., 2004; Njue et al., 2004). Side resistance was confirmed in several studies demonstrating that development of resistance to one avermectin/milbemycin, simultaneously results in resistance to another avermectin/milbemycin, and that similar resistance ratios (dose required to kill resistant worms:dose required to kill susceptible worms) exist for both ivermectin and moxidectin (Shoop et al., 1993; Molento et al., 1999; Ranjan et al., 2002). This suggests strongly that ivermectin-resistant worms are technically also moxidectin-resistant. However, at recommended dosages moxidectin remains effective against many ivermectin-resistant nematode species, and a difference in inheritance patterns between ivermectin- and moxidectin-resistant *H. contortus* have been described (Le Jambre et al., 2005). It is therefore quite likely that moxidectin selection of ivermectin-resistant nematodes results in the acquisition of additional 'resistance alleles' of important genes. It is not known how many additional alleles are required to make the jump from ivermectin to moxidectin resistance, or how rapidly this process can occur, but there are grounds for concern considering the extremely common background of ivermectin-resistant *H. contortus* on the farms where moxidectin is being applied in many areas of the world. Of additional concern is the extremely long and persistent activity of moxidectin, (Abbott et al., 1995; Lanusse et al., 1997) a characteristic that may be important in the development of resistance via 'tail' selection of incoming L_3s during the residual phase (Le Jambre et al., 1999).

It is generally accepted that successful implementation of nematode control programs designed to limit the development of anthelmintic resistance depends to a large degree on the availability of effective and sensitive methods for its detection and monitoring (Taylor et al., 2002). The emergence of widespread moxidectin resistance could seriously threaten the bourgeoning goat industry in the US and established small ruminant industries throughout the world; therefore, it is very important that assays be developed and validated to monitor the efficacy of this drug. The larval development assay (LDA) is a commonly used in vitro test for the diagnosis of resistance in nematodes of sheep and goats (Johansen and Waller, 1989). The LDA is available as a commercial test called DrenchRite®, (Microbial Screening Technologies, New South Wales, Australia) which is designed to detect resistance to all three major drug classes (benzimidazoles, imidozothiazoles/tetrahydropyrimidines, avermectin/milbemycins) commonly used to treat nematode infections of livestock. Unfortunately, this assay has not been optimised or validated to detect resistance to moxidectin and no other assays have been validated for this purpose. Thus, there is no means to detect emerging resistance to moxidectin prior to ultimate treatment failure. However, with sufficient in vivo efficacy data collected in parallel with the LDA data, it should be possible to use the LDA data for ivermectin to measure resistance to moxidectin. The objectives of this study were to establish relevant diagnostic values for moxidectin resistance using the DrenchRite® LDA, and to determine if resistance to moxidectin is emerging as an important problem on goat farms in Georgia following only 2–3 years of use. To achieve these objectives we performed DrenchRite® LDA in concert with FEC reduction tests (FECRT) using multiple dose levels of moxidectin. To improve our ability to make inferences on the relative levels of resistance between farms, FECRT data were first analysed using a linear mixed model, and then linear combination of the least square mean values derived from the mixed model analysis were used to evaluate the trend in response across dose levels using a Tukey's trend test (Tukey et al., 1985). This novel approach for measuring resistance in the field may have important applications for studies designed to investigate factors involved in the evolution of anthelmintic resistance.

2. Materials and methods

In this study, we examined the efficacy of moxidectin in 294 meat-type goats of various breeds on nine privately owned farms in Georgia, USA. Seven of these farms served

as 'test' farms, each had documented ivermectin resistance and a history of regular moxidectin treatment (of varying frequency) over the previous 2–3 years (Farms C_n, C_s, F_v, J_s, M_c, M_y and W_t). Six of these seven farms (C_n, C_s, F_v, M_c, M_y and W_t) also participated in our 2001 resistance prevalence study, (Mortensen et al., 2003) so we had historical data on ivermectin resistance and moxidectin susceptibility. Two additional farms served as controls. One of these farms was a closed herd with known sensitivity to ivermectin (based on data from the 2001 study) and no history of either moxidectin or ivermectin use in recent years (C_1, ivermectin-sensitive/moxidectin-sensitive control). The other farm was a closed herd with known ivermectin resistance but no history of moxidectin use (C_2, ivermectin-resistant/moxidectin-sensitive/naive control).

2.1. FECRT

On each of the nine farms we performed FECRTs using moxidectin at varying dose levels. Pre-treatment FECs were performed on each goat using a modified McMaster method with a sensitivity of 50 eggs per gram (EPG). Goats with a FEC < 200 were excluded from the study. All goats with a FEC of at least 200 EPG were ranked by FEC, blocked into groups of six, and within a block were assigned randomly to a treatment group. Six goats were assigned to each treatment group but in some groups on some farms only four or five goats were available for sample collection on the post-treatment collection date (for a variety of reasons). The following treatments were administered: no treatment (control), and moxidectin (Cydectin Pour-On for cattle, Fort Dodge Animal Health, Princeton, New Jersey, USA) at four different dose levels; M_1 (10 or 25 μg/kg), M_2 (25 or 50 μg/kg), M3 (100 μg/kg), and M4 (400 μg/kg) (At the time that this study was performed, the only available formulation of moxidectin for ruminants in the US was the Cydectin Pour-On for cattle. Though not approved for use in sheep and goats, it was routine veterinary practice to recommend oral administration of this formulation in these species). In addition, an ivermectin-treated (Ivomec Sheep Drench, Merial Ltd., Duluth, Georgia, USA) group (400 μg/kg) was included on the two control farms to confirm the ivermectin susceptibility/resistance status. Moxidectin dosages were selected based on data from a previous study that tested the efficacy of four different dosages of moxidectin on a goat farm in Oklahoma, USA (Pomroy, W.E., Hart, S., Min, B.R., 2002. Titration of efficacy of ivermectin and moxidectin against an ivermectin-resistant *Haemonchus contortus* derived from goats in the field. In: Novel Approaches – A Workshop Meeting on Helminth Control in Livestock in the New Millenium, Edinburgh, UK). On the first four farms tested (C_1, C_2, C_n, M_e), we used 10 and 25 μg/kg for the M_1 and M_2 groups, respectively; but then based on the observed results, we increased the doses for the M_1 and M_2 groups to 25 and 50 μg/kg, respectively, for farms J_s, M_y and W_t. On farm C_s only the M_2 group (50 μg/kg) was included and on farm F_v only the M_1 group (25 μg/kg) was included in the study.

All drugs were administered orally and all animals were weighed on a portable scale to determine appropriate dosage. Feces were collected for FECs 1–3 days prior to treatment for use in making treatment assignments, on the day of treatment, and again 14–18 days after treatment. Pre- and post-treatment fecal cultures were performed on pooled fecal samples from each treatment group to determine species-specific FEC reduction levels.

2.2. DrenchRite® LDA

DrenchRite® LDAs were performed in duplicate on nematode eggs isolated from pooled feces collected on the day of treatment, following directions of the manufacturer (DrenchRite® Users Guide, 1996, Horizon Technology, Australia) with minor modification. DrenchRite® LDA plates contain eight wells with no drugs that serve as controls and 11 wells with doubling concentrations of a drug across the plate, such that well 2 contains the lowest, and well 12 contains the highest concentration of a drug. After 7 days the assays were terminated by adding Lugols iodine to each well, and the contents of all wells were transferred to clean 96-well flat bottomed plates. All eggs and larvae (L_1/L_2, L_3) in each well were counted using an inverted compound microscope at 100× or 200×, and all L_3s in the ivermectin wells were identified to genera (M.A.F.F., 1977). DrenchRite plates are manufactured with two different ivermectin analogs, but experience with this test in our laboratory has shown that ivermectin-2 (ivermectin-aglycone) yields higher resistance ratios than ivermectin-1 (ivermectin monosaccharide) making it a better choice for detecting resistance for *H. contortus* and *Trichostrongylus colubriformis*, which are the primary parasitic pathogens we see. Therefore, we used only the ivermectin-2 data in our analyses.

2.3. Data analysis

FECR data were analysed using arithmetic means and the formula FECR (%) = 100 $(1 - T_2/T_1 \times C_1/C_2)$, where T, C, 1, and 2 refer to treated, control, pre-treatment, and post-treatment mean FECs, respectively (Dash et al., 1988). Essentially, what this formula produces is a calculation for the relative change for the quantity X, where X is the ratio of the FECR for the treated group to that of the control group. Because the results are in the form of a ratio, the magnitude of the counts, and hence the amount of variation in the data, is not directly addressed. Therefore, a linear mixed model was used to fit the quantity X for each farm and the animals were treated as random effects. The linear mixed model fitted was: Response = overall mean + dose effect + animal effect + error. In this model, animal effects were included as random effects to account for variations in animals and analysis was carried out for each farm. A goodness of fit for the proposed model was also carried out. Data for FECR were also analysed using the RESO

FECRTv4 program (Cameron, A. RESO fecal egg count reduction analysis spreadsheet. AusVet Animal Health, available for download at http://www.vetsci.usyd.edu.au/sheepwormcontrol/index.html under Site Map) to allow direct comparison with a previous study. We also used a Tukey's trend test (Tukey et al., 1985) to evaluate the trend in response across dose levels. This test uses a linear combination of the least square means (*Ls* means) to assess an overall trend in the response with increasing doses of a compound. All effects were evaluated at a 5% significance level. All statistical analyses were performed using SAS version 9.1.2 (Cary, North Carolina). Farms were ranked from the least to the most resistant by comparing both the *Ls* means values for FECR and the significance of the trend test values.

Statistical Analysis of the LDA data was performed using two methods. Firstly, we used a probit model for fitting a dose–response curve for each farm separately using PROC GENMOD in SAS version 9.1.2 (SAS-Publication, 2004). Ninety-five percent confidence intervals (CIs) were then constructed for the 95th percentile and the median of the dose response curve. Second, we used a log-dose logit-response model (Waller et al., 1985; Dobson et al., 1987; SAS-Publication, 2004) to produce dose–response curves and values for LC_{50} and LC_{95} for ivermectin-2. Data were also examined empirically to estimate the critical well (approximating the LC_{50}) and the well containing the 5% delineating dose (approximating the LC_{95}). The critical well is defined as the well where development to the L_3 stage is inhibited by 50% compared with controls (DrenchRite Users Guide, 1996, Horizon Technology, Australia). The 5% delineating dose is defined as the well containing the highest drug concentration where greater than or equal to 5% of larvae developed to the L_3 stage (Tandon and Kaplan, 2004).

3. Results

3.1. FECRT

Moxidectin was highly effective on both control farms but ivermectin was only effective on control farm C_1 (99% reduction) and not control farm C_2 (70% reduction). On farm C_1, moxidectin was 100% effective in reducing FEC at both the 100 and 400 μg/kg doses, whereas on control farm C_2, moxidectin was 97% effective in reducing FEC at 100 μg/kg and 100% at 400 μg/kg (Table 1). At the 100 μg/kg dose, the seven test farms demonstrated a mean FECR for *H. contortus* of 38% with a range between 0% and 91% (Table 1), and a mean FECR of 49.3% with a range between 0% and 99.8% for *T. colubriformis* (Table 2). At the 400 μg/kg dose, the seven test farms had mean FECRs of 76% and 65% for *H. contortus* and *T. colubriformis*, respectively, with a range for FECRs of between 0% and 100% for both species. Using a cutoff value for resistance of less than 95% reduction in FEC at the 100 μg/kg dose, all seven of the farms demonstrated resistance in *H. contortus* and six of seven demonstrated resistance in *T. colubriformis*. For both nematode species, three of seven farms demonstrated resistance at the 400 μg/kg dose, and on one farm (M_y) resistance to moxidectin at the 400 μg/kg dose was seen in both *H. contortus* and *T. colubriformis* (Tables 1 and 2). Post-treatment fecal cultures revealed large changes in the relative percentage of *H. contortus* and *T. colubriformis* larvae recovered as the moxidectin dose increased (Fig. 1).

3.2. DrenchRite® LDA

Analysis of DrenchRite® LDA data for ivermectin-2 using a log-dose logit-response model demonstrated a wide variability between farms in the dose–response (Fig. 2). A best-fit curve using a one-population model could not be fitted for farm F_v, but LC_{95} was estimated using a two-population model (Dobson et al., 1987). In a separate analysis using a probit model, 95% CIs were constructed for the 95th percentile and the median of the dose–response curve for each farm, and the LDA well containing this drug concentration value (to the nearest 0.5 well) was determined. These values were then used to calculate resistance ratios (RR) compared with farm C_1 (Table 3). The RR for the ivermectin-resistant/moxidectin-naive control farm C_2 compared with C_1 were 5.3 and 16.0 for the median and

Table 1
Least square (*Ls*) mean values (and SEM) for fecal egg count reduction following treatment with moxidectin at variable dosages for *Haemonchus contortus*, and relative ranks of moxidectin sensitivity from the least (1) to most (9) resistant

Farm	*Ls* means 400 μg/kg	Rank 1st iteration	*Ls* means 100 μg/kg	Rank 2nd iteration	*Ls* means 50 μg/kg	*Ls* means 25 μg/kg	*Ls* means 10 μg/kg
C_1	100.0 (15.6)	1	100.0 (14.0)	1		N/A	N/A
C_2	100.0 (21.3)	1	96.9 (26.1)	2		N/A	N/A
C_n	96.7 (245.0)	6	31.0 (245.0)	5		−254.9 (245.0)	−391.2 (245.0)
C_s	99.9 (25.7)	4	83.0 (25.7)	4	−50.26 (25.7)		
F_v	98.2 (36.4)	5	3.9 (31.5)	6		59.66 (33.7)	
J_s	−72.2 (346.5)	9	−197.7 (346.5)	9	−515.9 (346.5)	−761.4 (346.5)	
M_e	100.0 (21.2)	1	91.1 (21.2)	3		−15.3 (19.4)	47.9 (21.2)
M_y	46.4 (75.6)	8	19.5 (70.0)	8	−38.8 (70.0)	−76.7 (75.6)	
W_t	93.1 (45.3)	7	33.8 (45.3)	7	−21.7 (45.3)	34.7 (45.3)	

N/A, fecal cultures for the two low-dose treatment groups on farms C_1 and C_2 failed to yield usable results so that species-specific reductions could not be calculated.

Table 2
Least square (*Ls*) mean values (and SEM) for fecal egg count reduction (FECR) following treatment with moxidectin at variable dosages for *Trichostrongylus colubriformis* and for total egg counts without regard to species

Farm	*Ls* means for *Trichostrongylus*					*Ls* means for total FECR				
	400 μg/kg	100 μg/kg	50 μg/kg	25 μg/kg	10 μg/kg	400 μg/kg	100 μg/kg	50 μg/kg	25 μg/kg	10 μg/kg
C_1	100.0 (15.6)	100.0 (14.0)		N/A	N/A	100.0 (15.6)	100.0 (14.0)		72.6 (12.7)	82.6 (12.7)
C_2	100.0 (21.3)	96.9 (26.1)		N/A	N/A	100.0 (21.3)	96.9 (26.1)		51.7 (21.3)	27.4 (21.3)
C_n	11.7 (172.9)	−48.6 (172.9)		−40.4 (172.9)	−246.7 (172.9)	79.1 (229.0)	15.1 (229.0)		−212.0 (229.0)	−362.3 (229.0)
C_s	−259.7 (903.7)	−328.0 (903.7)	−127.7 (903.7)			93.3 (31.1)	24.0 (31.1)	−38.6 (31.1)		
F_v	99.9 (26.7)	83.7 (23.1)		−4.3 (24.7)		98.9 (27.8)	38.1 (24.1)		35.0 (25.7)	
J_s	100.0 (213.6)	73.9 (213.6)	77.1 (213.6)	−45.7 (213.6)		48.3 (251.3)	−7.6 (251.3)	−100.8 (251.3)	−546.0 (251.3)	
M_e	100.0 (9.3)	99.8 (9.3)		88.5 (8.5)	70.5 (9.3)	100.0 (13.3)	96.5 (13.3)		49.1 (12.2)	61.9 (13.3)
M_y	46.4 (254.4)	−196.9 (235.6)	−110.4 (235.6)	−551.6 (255.0)		46.4 (79.0)	15.6 (73.2)	−40.2 (73.2)	−86.2 (79.0)	
W_t	99.6 (8.1)	87.8 (8.1)	80.5 (8.1)	71.6 (8.1)		92.9 (49.0)	28.0 (49.0)	−30.9 (49.0)	21.4 (49.0)	

N/A, fecal cultures for the two low-dose treatment groups on farms C_1 and C_2 failed to yield useable results so that species-specific reductions could not be calculated.

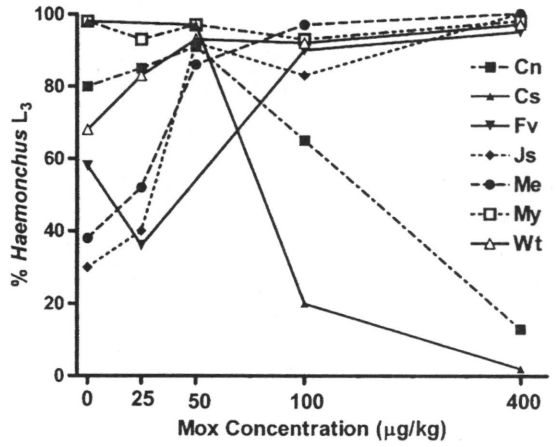

Fig. 1. Change in the percentage of *Haemonchus* L_3s recovered from fecal cultures. Four or five groups of goats were administered increasing doses of moxidectin (MOX) on seven goat farms. On all farms *Trichostrongylus* was the only other genera of nematode larvae identified in significant numbers. Individual farms are designated by the abbreviations C_n, C_s, F_v, J_s, M_e, M_y, and W_t.

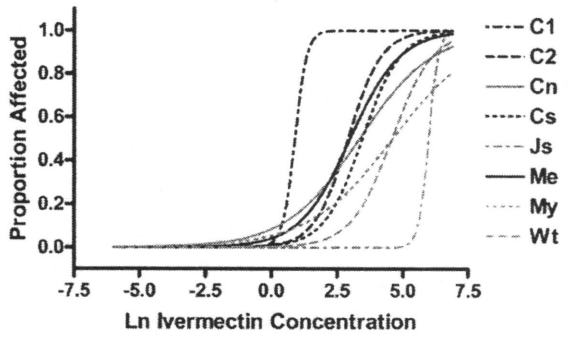

Fig. 2. Log-dose logit-response model curves for ivermectin-2 (ivermectin aglycone) for eight goat farms. C_1 and C_2 are the control farms and C_n, C_s, J_s, M_e, M_y, and W_t are the test farms. Farm C_1 had known sensitivity to ivermectin and no history of either moxidectin or ivermectin use in recent years. Farm C_2 had known ivermectin resistance but no history of moxidectin use. Farms C_n, C_s, J_s, M_e, M_y, and W_t each had documented ivermectin resistance and a history of regular moxidectin treatment (of varying frequency) over the previous 2–3 years. A best-fit dose–response curve could not be fitted for farm F_v (not shown).

the 95th percentiles, respectively. RR for the next most sensitive farm were twofold higher than for C_2, and RR for the most resistant farm were 128 at both measures. All farms classified as moxidectin-resistant had RRs of $\geqslant$ 32 and 96 for the median and the 95%th percentiles, respectively, compared with C_1, and RRs calculated and compared with the ivermectin resistant, moxidectin naive control farm (C_2) were $\geqslant$6.0 for both measures for all farms classified as moxidectin-resistant.

3.3. Relative resistance rankings and diagnostic criteria for resistance

Farms were ranked from least to most resistant for *H. contortus* based on the *Ls* means of the FECRT for

Table 3
Wells of the DrenchRite® larval development assay plate containing the 95% confidence intervals for the median and 95th percentile of the ivermectin concentration (approximating the LC_{50} and LC_{95}), and corresponding resistance ratios (RR) for each farm compared with control farm C_1, based on drug concentrations that correspond to the given wells

Farm	Well containing median	RR median	Well containing 95th percentile	RR 95th percentile
C_1	3.5	N/A	5	N/A
C_2	6	5.3	9	16.0
C_n	7.5	16.0	10.5	48.0
C_s	7	10.7	11	64.0
F_v	9	42.7	11	64.0
J_s	10.5	128.0	12	128.0
M_e	7	10.7	10	32.0
M_y	9	42.7	>12	>128.0
W_t	8.5	32.0	11.5	96.0

the 400 and 100 μg/kg doses as well as the significance of the Tukey trend test values (Tables 1 and 4). Rankings were then performed using values for the 95% CIs for the 95th percentile of the LDA, and a third ranking was performed using the median of the dose–response curve of the LDA. The mean of the three different rankings was then calculated to reveal a consensus ranking (Table 5). These data demonstrate there are clear and distinct levels of sensitivity to the avermectin/milbemycin drugs on different farms, and that these differences can be detected and measured using the DrenchRite® LDA. Taken together, the analyses for the in vivo and in vitro data for *H. contortus* were used to establish criteria for the DrenchRite® LDA for estimating the relative sensitivity of worms on a farm to moxidectin, and for making a definitive diagnosis of moxidectin resistance (Table 6).

Table 4
P-values for Tukey's sequential trend test of least square mean values for *Haemonchus contortus*, *Trichostrongylus colubriformis* and for the total without regard to species

Farm	Trend	*P*-values		
		Haemonchus	*Trichostrongylus*	Total
C_1	High-dose trend	0.2262	0.2262	0.2262
	Medium-dose trend	0.1913	0.1913	0.1913
	Low-dose trend			1
C_2	High-dose trend	0.0138	0.0138	0.0138
	Medium-dose trend	0.1263	0.1263	0.1263
	Low-dose trend			0.9267
C_n	High-dose trend	0.126	0.333	0.1451
	Medium-dose trend	0.3224	0.8334	0.3786
	Low-dose trend	0.8515	0.8076	0.8439
C_s	High-dose trend	0.0009	0.9191	0.009
	Medium-dose trend	0.6504	0.0347	0.1361
F_v	High-dose trend	0.0479	0.6349	0.0936
	Medium-dose trend	0.9713	0.9969	0.9873
J_s	High-dose trend	0.1393	0.098	0.1105
	Medium-dose trend	0.376	0.9402	0.6791
	Low-dose trend	0.8005	0.9319	0.8765
M_e	High-dose trend	0.0128	0.028	0.0144
	Medium-dose trend	0.0009	0.3748	0.0117
	Low-dose trend	0.7704	0.999	0.8556
M_y	High-dose trend	0.2161	0.145	0.2098
	Medium-dose trend	0.4171	9.6557	0.4296
	Low-dose trend	0.7963	0.4902	0.7744
W_t	High-dose trend	0.2682	0.0203	0.226
	Medium-dose trend	0.088	0.1116	0.0889
	Low-dose trend	0.365	0.3146	0.3596

P values <0.05 are considered significant.

3.4. Additional results

Empirically derived values for the critical well and the well containing the 5% delineating dose were determined and compared with the LC_{50} and LC_{95} values calculated using the log-dose logit-response model (Table 7). Results of this comparison indicate that there is little practical difference in these methods.

Results from a similar study conducted in 2001 on the same farms (Mortensen et al., 2003) were compared with results of the current study using the RESO method for calculating FECR (Table 8). These data demonstrate that on many farms there has been a dramatic reduction in the effectiveness of moxidectin after only 2 years.

4. Discussion

In this study, we have taken a novel approach for investigating the presence of anthelmintic resistance by combining in vitro drug efficacy data with in vivo field data to make inferences on the relative sensitivity/resistance to moxidectin on individual farms. On each farm DrenchRite® LDAs were performed in concert with FECRTs using multiple dose levels of moxidectin to gain data on the relative susceptibility of the worms, making it possible to compare the FECRT results with the in vitro LDA results. Because numerous factors contribute to high variability in FEC, which can impact results of FECRT, we performed a mixed model analysis that included pretreatment FEC, dose effects, and farm differences as random effects to account for variation. Linear combination of the least square mean values derived from the mixed model analysis were then used to evaluate the trend in response across dose levels by performing a Tukey's sequential trend test. This test examines whether there is an increasing trend in FECR with increasing drug dose. Results of the trend test helped us to further refine our interpretation of the least square mean values for ECR, enabling us to rank farms in terms of their susceptibility/resistance to moxidectin.

We chose to use the Tukey's sequential trend test because this is a useful method for detecting a linear trend in experiments involving increasing doses of a drug. Though we do not know of any instances where this or a similar method has been used in parasitological research,

Table 5
Farm rankings from least (1) to most (9) moxidectin-resistant and declared resistance status based on least square (*Ls*) means of fecal egg count reduction (FECR), and the 95% confidence intervals (CI) for the median and 95th percentile in the larval development assay (LDA) for *Haemonchus contortus*

Farm	% FECR *Ls* means	LDA 95% CI median	LDA 95% CI 95th percentile	Consensus ranking	Declared resistance status[a]
C_1	1	1	1	1	S
C_2	2	2	2	2	S
C_n	5	5	4	5	LR
C_s	4	4	5	4	DR
F_v	6	7	6	6	LR
J_s	9	9	8	9	R
M_e	3	3	3	3	DR
M_y	8	8	9	8	R
W_t	7	6	7	7	R

[a] S, susceptible; DR, developing resistance; LR, low resistance; R, resistant.

Table 6
Criteria for establishing a diagnosis of moxidectin resistance in *Haemonchus contortus* using the DrenchRite® larval development assay based on data from nine goat farms in Georgia, USA

Resistance status[a]	Well for LC_{50}	Well for LC_{95}
Susceptible	<6.5	<9.5
Developing resistance	6.5–7	9.5–10.5
Low resistance	7.5–9	10.5–11
Resistant	⩾8.5	⩾11.5

[a] Criteria for both LC_{50} and LC_{95} should be met to make the suggested diagnosis to resistance status. If only one of two criteria are met then the farm may fall somewhere between the proposed classifications for resistance.

Table 8
Percent reduction in fecal egg counts following treatment with moxidectin (MOX) at a dose of 400 μg/kg

Farm	Overall MOX 2001	MOX 2003		
		Overall	*Haemonchus*	*Trichostrongylus*
C_n	94	84	97	32
C_s	96	95	100	65
F_v	100	96	94	100
J_s[a]	–	7	0	100
M_e	99	100	100	100
M_y	100	59	59	59
W_t	100	86	81	99
Mean	98.2	75.3 (86.7)	75.9 (88.5)	79.3 (75.8)

Data provide a comparison of results of the present study (2003) with results from 2001 (Mortensen et al., 2003). To make data comparisons more consistent with the 2001 values, percent reductions in fecal egg counts were calculated using the same procedures (RESO FECRT v4 program) and therefore differ from values reported in Tables 1 and 2. Values for means in parentheses represent the 2003 mean reduction of the same six farms tested in 2001.

[a] Farm J_s was not included in the 2001 study of resistance prevalence.

this methodology has been used extensively in clinical trial and toxicological settings (Antonello et al., 1993; Quan and Capizzi, 1999). In this study, we used the *Ls* means and the linear trend test to rank the farms in order of increasing resistance. Least squares means provide a summary statistic and represents the model adjusted mean. The standard error of the *Ls* means takes into account various sources of variability when testing for treatment differences. The null hypothesis in the Tukey's trend test is that there is no significant linear trend in the response to increasing doses of a drug.

Tukey's sequential trend test needs to be interpreted carefully, so we present an example. Consider an experiment in which there are four increasing doses of a drug. Call the doses control, low, medium and high. The sequential trend test works as follows: a high-dose trend is evaluated using a particular linear combination of *Ls* means using a *t*-test. If this test is not significant at the chosen level of significance, then the test stops and the conclusion

Table 7
Wells of the DrenchRite® larval development assay plate containing LC_{50} and LC_{95} as calculated by the log-dose logit-response model, the critical well and the well containing the 5% delineating dose (DD) as determined by empirical examination of the data

Farm	LC_{50} (nM)	Well containing LC_{50}	Critical well	LC_{95} (nM)	Well containing LC_{95}	Well containing 5% DD
C_1	2.5	3.5	3.5	4.7	4	4.5
C_2	18.4	6	6.5	116.6	9	9
C_n	28.5	7	7	492[b]	11	10.5
C_s	31.7	7	6.5	262.9	10	11
F_v	1.5	2.5[a]	9.5	713[b]	11.5	11.5
J_s	396.6	10.5	10.5	680.4	11.5	12
M_e	19.8	6.5	7	320.4	10.5	10
M_y	100.6	8.5	8.5	10917.3	>12	>12
W_t	97.5	8.5	8.5	963.9	12	11

[a] A best-fit curve using a one-population model could not be fitted for farm F_v, so the result generated for LC_{50} with this model is not valid.

[b] LC_{95} could not be calculated using a one-population model; data shown were generated by fitting data to a two-population model (Dobson et al., 1987).

is that there is no increasing trend in the response. If the high-dose trend is significant, then the highest dose is dropped and the remaining data are tested for an increasing trend in the response. This is sometimes called the medium dose trend. If this is not significant, then the test stops and the conclusion is that there is no increasing trend beyond the highest dose. If this is significant, then the medium dose is also dropped and the comparison is made between the control and the low dose for significance. Of course, to have any meaningful answers from Tukey's trend test or any other statistical test, the variability has to be "reasonable". This can sometimes be problematic in parasitological studies; therefore larger group sizes are desirable.

To illustrate the usefulness of the Tukey's trend test in ranking for resistance, consider the data from farms C_1 and C_2 from Table 1. The *Ls* means at a high dose are 100% for both farms. However, at a medium dose the *Ls* means for farm C_1 is 100%, but for C_2 is 96.9%. What should the ranking be? A small numerical change alone should not be used to derive the ranking, because how do we know whether 97% really is statistically different from 100%? Notice that for the farm C_1 the Tukey's trend test shows that there is no statistically significant increasing trend. This means that for farm C_1 there is not a statistically significant differences between the high and medium doses. However, for farm C_2, the high-dose trend is statistically significant, but the medium-dose trend is not statistically significant, which suggests that the 400 μg/kg dose is more effective than the 100 μg/kg dose, and hence C_2 is more resistant than C_1. Similar reasoning can be used for other farms to arrive at a consistent ranking scheme.

The farms C_n, J_s and M_y cannot be ranked statistically since the variability is very high and hence Tukey's trend test does not give much insight into ranking those. Of course, no other statistical test can yield meaningful results in those cases; for these farms, the *Ls* means data must be used alone. Results from the FECRT on these three farms demonstrate that one needs to understand the causes of excessive variability, and simultaneously use methods that reduce this variability together with statistical analyses that help to take variability into account.

We next ranked the farms on the basis of the analyses for the DrenchRite® LDAs and used the different rankings to generate a consensus ranked list of the farms in terms of relative moxidectin susceptibility/resistance in *H. contortus*. On the basis of these rankings, the actual *Ls* means data, and the analyses of the DrenchRite® LDA data, we classified individual farms on the basis of their relative levels of moxidectin resistance (Table 6). We used four classifications which we define as follows: sensitive – no evidence of resistance; developing resistance – evidence of early resistance but at the recommended use level moxidectin still is expected to be highly effective; low resistance – clear evidence of early stages of moxidectin resistance, but FECR can still be expected to be greater than 95% at the recommended use level; and resistant – obvious resistance with FECR expected to be less than, and perhaps much less than, 95%. In addition, we established diagnostic criteria for LC_{50} and LC_{95} values for each of these classifications. We believe that these data strongly support the use of the DrenchRite® LDAs for monitoring the development of, and in making a diagnosis of, moxidectin resistance in *H. contortus*.

When performing an LDA, a great deal of effort is required to count every larva in every well, and identify every L_3 in every well, but this is necessary to calculate an accurate LC_{50} and LC_{95}. A quicker and simpler approach is to count and identify all larvae in the four to five wells around the apparent critical well and not count any larvae in the lower concentration wells where little change in development is seen across wells. In addition, the usually small numbers of L_3s in the higher concentration wells are counted and identified. Results are then determined empirically, by calculating the critical well and the 5% delineating dose. Our data demonstrate that empirical determination of the critical well and the 5% delineating dose in the DrenchRite® LDA can be used as a fairly accurate estimate of the calculated values for LC_{50} and LC_{95} (Table 7). Thus, from a diagnostic standpoint, it is not necessary to count and identify every larva in every well to estimate the level of resistance to moxidectin in *H. contortus*. This can greatly increase the efficiency of performing these assays. However, for research purposes it would still be advisable to count and identify larvae in all wells so that a more precise measurement can be made.

Haemonchus contortus is recognised as the most prevalent and important nematode pathogen of goats in the southern US, as it is in most warm humid climates. However, *T. colubriformis* is also a pathogen of considerable importance, and must be considered when designing parasite control programs and when evaluating FECRT data. Least square means for FECR are presented for *T. colubriformis* but because *T. colubriformis* numbers were small on many farms, leading to high standard errors, no attempts were made to rank farms or to establish diagnostic criteria for the LDAs. However, it is interesting to note that in this study the results for both *T. colubriformis* and *H. contortus* were quite similar for moxidectin, whereas in our 2001 study, ivermectin resistance in *H. contortus* was much more prevalent than in *T. colubriformis*. This suggests that moxidectin may select more strongly for resistance in *T. colubriformis* than does ivermectin. Data from this study also strongly demonstrate the importance of identifying L_3s in the LDAs, and in performing post-treatment fecal cultures to determine the relative proportion of the major species present. *Haemonchus contortus* was the predominant species identified in fecal cultures of untreated goats on most farms; the overall farm mean was 71.1% and five of nine farms had more than 80% *H. contortus* L_3s. However, on two farms more than 60% of L_3s were *T. colubriformis*. These differences not only have direct clinical implications, but also have implications for the evaluation and interpretation of FECRT data. As seen in Fig. 1, dramatic changes

in the relative percentage of *H. contortus* and *T. colubriformis* L_3s occurred in response to moxidectin treatment on most farms, but not always in the same direction. The practical consequence of this phenomenon is that overall FECR percentages can be very misleading if post-treatment fecal cultures are not performed. On some farms one species is much more resistant than the other and very large changes in the relative percentage of eggs for these species are seen after treatment. Without doing pre- and post-treatment fecal cultures it is impossible to know which of the species are resistant. Likewise, *H. contortus* and *T. colubriformis* respond very differently to ivermectin in the LDAs. It is not possible to interpret LDA data for ivermectin or moxidectin without identifying the L_3s and determining the dose–response for each species separately.

In a previous study performed in Georgia, USA in 2001, post-treatment cultures were not performed, so only overall results without regard to species are reported (Mortensen et al., 2003). In this earlier study, we performed FECRTs on six of the seven test farms examined in the current study. Following only 2 years of moxidectin use of variable intensiveness, overall FECRs for these six farms (based on calculations of the RESO program to keep comparisons consistent with the 2001 study) decreased from a mean of 98.2 to a mean of 86.7, and the three farms with the lowest FECRs in the current study decreased from a mean of 98% to 76.3%. This suggests that resistance to moxidectin can develop very rapidly, particularly when used on farms where resistance to ivermectin pre-exists. These and other published data indicating seriously escalating global anthelmintic resistance in gastrointestinal nematodes of small ruminants provide strong evidence that effective long-term control of gastrointestinal nematodes of small ruminants will only be possible if anthelmintics are used intelligently with prevention of resistance as a goal. Implementation of novel, non-chemical approaches in a program referred to as 'sustainable integrated parasite management (sIPM) (van Wyk et al., 2006) are therefore becoming an increasingly high priority. Since moxidectin is the last line of chemical defense on many farms, it is critical that there be a means to monitor its effectiveness and detect resistance in the early stages. Simply measuring efficacy at a single relatively high in vivo dose and waiting for this dose to fail is clearly inadequate. We have shown that the DrenchRite® LDA is a very good tool for performing such monitoring, and present guidelines for interpreting the results of this assay. We believe that the accuracy for measuring moxidectin resistance using the DrenchRite® LDA can be further improved by increasing the drug concentration scale to provide more data points on the high-concentration end of the dose spectrum.

In summary, we have presented a novel statistical approach for combining laboratory and field data to make inferences on the relative level of resistance on individual farms. We also present parameters for interpreting DrenchRite® LDA results for ivermectin so that this assay can also be used to diagnose both clinically apparent moxidectin resistance, as well as sub-clinical emerging resistance. We believe that this approach has much value, and offers an improved method for measuring the relative levels of resistance on different farms. Using this approach, it should be possible to better measure the impact of using different management schemes for delaying the development of resistance to avermectin/milbemycin anthelmintics. Important issues for which there is much speculation but little data, such as the impact of refugia and whether ivermectin or moxidectin selects more rapidly for resistance in the field, may be addressed using similar protocols. Though the number of farms was small, the high prevalence of resistance to moxidectin we observed portends a very serious situation for control of both *H. contortus* and *T. colubriformis* in the southern US. Furthermore, considering recent reports of rapidly increasing moxidectin resistance in Australia (Love, 2006), this phenomenon is likely occurring throughout the major small ruminant production areas of the world.

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