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Prepared by: Malcolm Knox

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In vitro larval assays for anthelmintic resistance in cattle nematodes

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Abstract

Determining anthelmintic resistance (AR) by means of a Faecal Egg Count Reduction Test (FECRT) is laborious and time-consuming. A number of *in vitro* techniques exist for measuring AR, of which the Larval Development Assay (LDA) is most widely used. The LDA is known not to be suitable for evaluating the susceptibility of *Teladorsagia circumcincta*, the Small Brown Stomach-worm of sheep, to macrocyclic lactones (MLs). Through a process of isolation, infection and challenge with oral ivermectin, ML resistant isolates for three of the primary nematode parasites of cattle in sub-tropical and temperate Australia, *Cooperia oncophora*, *Cooperia punctata/pectinata* and *Haemonchus placei* were obtained. A similar process did not produce a reliably ML resistant isolate of *Ostertagia ostertagi*. All four species were subjected to comparative tests with susceptible isolates of the same species to determine if the LDA and larval migration assay (LMA) could be used for routine diagnostic purposes. For *C. punctata/pectinata* and *H. placei,* results for the LDA with ivermectin aglycone and the LMA with eprinomectin suggest these tests could potentially be used in a routine diagnostic to determine ML resistance status of field populations of these nematode species. For *C. oncophora*, results suggested that the *in vitro* methods used could not be reliably used for diagnosis of ML resistance for this species. For *O. ostertagi*, *in vitro* analyses using the LDA and LMA did not discriminate between the available isolates, which were all susceptible. The project was terminated due to the inability to find an ML-resistant isolate.

Executive summary

Internal parasite infections constitute an important endemic disease of cattle in Australia. The mainstay of worm control in cattle production systems is chemical anthelmintics (drenches or pour ons), but increasingly, resistance of all the major cattle parasites to all the currently available chemical groups is being reported from around the world. Although there are recent reports of reduced cattle anthelmintic efficacy in Australia, the information is incomplete and highlights the need for a simple and inexpensive test with which to monitor drench efficacy.

The Faecal Egg Count Reduction Test (FECRT) can estimate anthelmintic efficacy by comparing worm egg counts before and after treatment. It is crude, laborious and time-consuming and hence, not widely used. A number of *in vitro* techniques have been developed in an attempt to address these shortcomings, such as an egg-hatch assay, larval motility/migration assays, biochemical detection of resistance genes and the Larval Development Assay (LDA). The LDA is convenient in that it requires the collection of only one faecal sample and is effective in diagnosing resistance in the three major worm parasites of sheep (Barber's Pole Worm – *Haemonchus contortus*, Black Scour Worm – *Trichostrongylus colubriformis* and Small Brown Stomach Worm – *Teladorsagia circumcincta*) to the white and clear drenches. It yields inconclusive results when trying to measure resistance of *T. circumcincta* to the macrocyclic lactone (mectin, ML) drenches.

The LDA has been used for nematode parasites of horses and pigs and, to a limited extent, some worms of cattle. The test needed to be further validated for the different chemical groups against parasites expressing the resistant phenotype. Through a process of isolation, infection and challenge with oral ivermectin, ML resistant isolates for three of the primary nematode parasites of cattle in sub-tropical and temperate Australia, *Cooperia oncophora*, *Cooperia punctata/pectinata* and *Haemonchus placei* were obtained. A similar process did not produce a reliably ML resistant isolate of *Ostertagia ostertagi*.

All four species were subjected to comparative tests with susceptible isolates of the same species to determine if the LDA and larval migration assay (LMA) could be used for routine diagnostic purposes. For *C. punctata/pectinata* and *H. placei,* results for the LDA with ivermectin aglycone and the LMA with eprinomectin suggest these tests could potentially be used in a routine diagnostic to determine ML resistance status of field populations of these nematode species. For *C. oncophora*, only the LMA with eprinomectin showed a low level discrimination between susceptible and resistant isolates suggesting that the *in vitro* methods used could not be reliably used for diagnosis of ML resistance for this species. For *O. ostertagi*, *in vitro* analyses using the LDA and LMA did not discriminate between the available isolates, which were all susceptible, and no diagnostic method can be recommended at this stage.It was decided to terminate the project because no *O.ostertagi* isolate resistant to ML could be found.

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1. Background

1.1. Drench resistance in cattle worms

Resistance to the so-called white (benzimidazole – BZ), clear (levamisole – LEV) and mectin (macrocyclic lactone – ML) anthelmintics is increasing for nematode parasites of cattle in the USA (Kaplan, 2004: Gasbarre and Smith, 2009 a,b), Europe (Coles, 2002; Demeler et al, 2009), South America (Borges et al, 2005; Soutello et al, 2007; Suarez and Cristel 2007; Condi et al, 2009) and New Zealand (Pomeroy, 2006; Waghorn et al, 2006). The species of parasites involved vary between locations but include *Cooperia* spp. (BZ, LEV and ML), *Ostertagia ostertagi* (BZ, LEV and ML), *Oesophagostomum radiatum* (ML) and *Haemonchus placei* (BZ, ML).

In Australia, there is a paucity of information on the status of cattle anthelmintic resistance (Besier, 2008). Until recently, the only confirmed cases of anthelmintic resistance were two cases of BZ resistance in *Trichostrongylus axei* (Eagleson and Bowie, 1986; Eagleson et al., 1992). In May 2008 at a MLA sponsored workshop and symposium at the Australian Veterinary Association conference in Perth, evidence of reduced efficacy of treatment of nematodes with ML anthelmintics was presented from locations in Victoria (Rendel, 2008) and South East Queensland (Ehrlich et al, 2008; Lyndal-Murphy et al, 2010). Reports of reduced efficacy of treatment of nematodes with ML anthelmintics have also emerged from locations on the Central Tablelands and North Coast of NSW (S. Love, pers. comm.). In some regions, ML products are not only used for the control of nematode parasites but are sometimes used to target ectoparasitic buffalo fly and bush, scrub and cattle ticks (MLA, 2005), potentially increasing selection for resistance in helminths occurring in those areas. These indications that resistance may be emerging in Australia highlight the need for a simple test to allow graziers to detect the presence of resistance in their herd. The availability of a low cost means of diagnosing anthelmintic resistance will enable individual producers to monitor resistance levels and identify the most effective anthelmintic for use within their enterprise.

1.2. Measuring anthelmintic efficacy

The so-called "gold standard" for determining the efficacy of an anthelmintic is to compare the post mortem worm burdens of treated animals and untreated controls. There are various experimental designs for this procedure and worm infestations can either be naturally acquired, or induced. This is expensive and not applicable to the monitoring of drench performance in the field. The next best *in* vivo alternative is the FECRT, which amounts to a comparison of faecal worm egg counts before and after treatment. A faecal larval culture after treatment may be required for more accurate identification of the surviving (presumed resistant) worms. This is a laborious and time-consuming process.

Several laboratory (*in vitro*) techniques have been developed. They require faecal samples to be collected only once and reflect the modes of action of the various chemical groups, such as the egg hatch assay (best suited to the BZs), the larval migration/motility assay (LMA) and the LDA. Molecular biological techniques, seeking to demonstrate the presence of resistance genes, continue to receive attention, but require more refinement.

The LDA is very effective in demonstrating resistance in the three major pathogenic sheep nematodes (Barber's Pole Worm – *Haemonchus contortus*, Black Scour Worm – *Trichostrongylus colubriformis* and Small Brown Stomach Worm – *Teladorsagia*

circumcincta) to the BZ and LEV drenches. It is also effective in determining resistance in the former two species to the ML drenches, but yields inconclusive results when trying to measure resistance in *T. circumcincta*.

Although demonstrating some preliminary success in overseas testing, use of the LDA in cattle, especially for measuring resistance of *O. ostertagi* (Small Brown Stomach-worm of cattle) to MLs, needed further testing and validation.

2. Project objectives

1. Determine whether the LDA is a practical option for estimating the presence of anthelmintic resistance to currently available chemical groups in the major nematode parasite species of cattle.
2. If the LDA proves unsuitable for resistance detection with a particular worm species, examine other assay alternatives, for example, the larval migration assay (performed on L3 stage worms).
3. If the above studies do not demonstrate substantial differences between resistant and susceptible isolates, further selection with drug will be required until higher level resistance is demonstrated in the *in vitro* tests.
4. Validate the performance of the most suitable larval assay (from 1. and 2.) for each species and define resistance factors (RF_{LDA}) for each drug and detection limits for resistance.
5. Compare results of *in vitro* larval assays on field samples with FECRT and determine if results correlate.
6. Establish methodology within diagnostic laboratories and promote use by cattle producers in areas where resistance is suspected.

3. Methodology

3.1 Cattle nematode isolate collection

At the commencement of the project it was essential to extend CSIRO's cattle nematode isolate collection from 2 *Cooperia punctata* and 2 *H placei* isolates to include isolates of *Cooperia oncophora* and *O. ostertagi*. Contact was made with researchers and field veterinarians who had reported an interest in this field of work. Collection of isolates proceeded from FECRTs from Western Victoria, Northern NSW and WA. Approaches made to the Southern Tablelands of NSW yielded no samples. In addition, VHR and Novartis also provided isolates that are routinely used in their research.

After receipt of faecal material from collaborators (primarily from FECRTs), considerable effort and resources were directed towards the separation of species of interest into monospecific infections through single passage through sheep or young calves, targeted collection of faeces to coincide with prepatent periods, and through focusing on cultures with high incidence of particular species. Multiplication of cultures then proceeded to a level where larvae were available for infections and, if possible, cryogenic preservation. Table 1 describes the isolates obtained.

The following isolates were added to the cattle nematode collection described above after passage through calves and/or sheep and purification to monospecific infections:

Cooperia punctata Dayboro	Sourced from FECRT by VHR
Haemonchus placei Dayboro	Sourced from FECRT by VHR
Cooperia oncophora DAFWA	Sourced from FECRT by DAFWA
Ostertagia ostertagi VHR Susceptible	Isolated by VHR
Trichostrongylus axei DRF	Sourced from FECRT by Rendell

3.2 Selection of isolates for ML resistance

Cooperia oncophora

After purification and multiplication in donor calves or sheep, the 3 resistant isolates (Novartis, DRC, WA BOV 005&009) were combined in equal proportions and given to calves to form our RESMIX strain. After establishment, RESMIX was then challenged with ivermectin at 200 µg/kg liveweight resulting in a 75% reduction in FEC. Resultant larvae were then established in additional calves and challenged with 200 µg/kg ivermectin, with no detectable impact on FEC. Comparison of RESMIX in LDA and LMA plates was with a susceptible isolate derived from material provided by VHR (VHR SUS).

Cooperia punctata/pectinata

The *C. punctata/pectinata* Dayboro isolate was obtained from VHR in late 2011 and was produced from FECRTs using ivermectin, eprinomectin and moxidectin challenges of a field infection. This culture was multiplied in 2 calves and faeces collected between 14 and 18 days of infection was cultured in order to produce pure *Cooperia* spp L3. Further collections from 25 -30 days yielded mixed cultures of *Cooperia* spp. and *Haemonchus* spp. Challenge of this infection with 200 µg/ kg ivermectin saw no change in FEC at 5 days and an increase in FEC by 9 days post dosing.

Generations of the *C. punctata/pectinata* Dayboro isolate were then established in a further 2 groups of calves with drug challenges with ivermectin at 200 µg/kg for each generation and no observable reduction in FEC, before *in vitro* testing in LDA and LMA plates proceeded against the susceptible Taree isolate.

Ostertagia ostertagi

Attempts to isolate ML resistant *O. ostertagi* from larvae provided by DAFWA (WA BOV 003, 005, 008 and 009) from FECRTs, proved unsuccessful although the DAFWA *C. oncophora* was isolated from these same cultures. This was probably due to the low numbers of *O. ostertagi* larvae collected and age of cultures when delivered to Armidale for infection into calves. Regular contact with VHR and Novartis Animal Health did not yield any isolates of *O. ostertagi* with suspected resistance to ML anthelmintics. Approaches made to NSWDPI staff on the Central Tablelands did not yield any samples for culture as the owner of the farm where ML resistance was suspected had treated all cattle with alternative anthelmintics to remove the infection. No further suspect cases were advised.

Further selection of the Victorian isolate (DR-C OFZ) was attempted. Initially established infections were challenged with 200 µg/kg ivermectin as this concentration was used in the FECRT on the same farm and indicated a low level of ML resistance. This challenge dose of drug eliminated the established infection. Further calves were infected and challenged with 100 µg/kg ivermectin which reduced the FEC by 93%. Large quantities of faeces were then cultured and yielded

enough L3 to infect one calf but this infection did not establish. Three calves were then infected with the parent *O. ostertagi* isolate and, once established, the infection was challenged with 50 μg/kg ivermectin. This dose removed the infection from 2 calves and reduced the FEC in the other by 85%. Larvae produced by culturing large quantities of this calf's faeces were then given to two immunosuppressed calves but infections failed to establish.

As no increase in the *in vivo* resistance of this isolate appeared to be possible, work commenced on *in vitro* selection (per Kaminsky et al, 2008) of the parent isolate but after two attempts at 3 different drug concentrations (1-4 pg/ml in 2 mL agar) negligible numbers of L3 were produced and this attempt was abandoned.

Haemonchus placei

Larvae from the Dayboro cultures described above, were given to 2 sheep (to purify the isolate – since *C. punctata/pectinata* does not establish well in this host species) and once infection was established 200 μg/ kg ivermectin was given with no effect on FEC. Faecal collection and culture proceeded prior to further drug challenge with 200 μg/kg moxidectin which reduced FEC by 98%. Further faecal collections were then made and sufficient *H. placei* L3 harvested for additional infections. This isolate was then subjected to 2 further generational challenges with drug in calves before final infections were established in sheep for use in LDA and LMA *in vitro* assessments of resistance status. For comparison the *H. placei* Bremner isolate was also established in sheep.

Trichostrongylus axei

Larvae from Victoria (DR-Falk Control) containing 49% *T. axei* were passaged twice through sheep and the resultant culture was identified as 100% *T. axei*. As the property of origin had observed low level resistance to oxfendazole in FECRT, this isolate was challenged with 4.53 mg/kg oxfendazole which reduced the FEC by 100%. Further BZ selection was not attempted. *In vitro* comparison of this isolate with susceptible *T. colubriformis* McMaster by LDA indicated possible BZ resistance but this would need confirmation by comparison with a known susceptible *T. axei* isolate. As no other isolates of *T. axei* were available, no further work was conducted as assessment of the other nematode species was deemed to be higher priority.

3.3 Assessment of resistance using the CSIRO LDA method

The agar plate LDA method developed by Gill et al (1990) was successfully applied in the Drenchrite assay adopted by Australian and international diagnostic laboratories. This technology is no longer used for sheep in Australia due to the inconsistent results obtained for *T. circumcincta*. This method of assessing anthelmintic activity of commercial drugs and other compounds remains a useful tool for research into other species of sheep, horse, pig, dog and human nematodes. We investigated whether this system was adaptable to assessing anthelmintic resistance in cattle nematode parasites.

Initial studies using the current CSIRO methodology with *Cooperia* spp. showed that the method was not directly applicable due to low rates of egg hatch and failure of larval development through to L3. Experimentation then commenced to determine the components that were inhibiting egg hatch and larval development through a step by step process for each assay component including assessment of impacts of concentration changes or component elimination. This process indicated:

a) Harvesting eggs using the sucrose gradient method was possible provided at least 200 epg were present in the faeces. Below this concentration contaminants tended to produce overgrowth in culture wells and render the plates unreadable. Efforts to extend the method through use of alternative sucrose gradients, salt flotation, centrifugation and passage through additional sieves during processing and combinations of these, improved the cleanliness of the sample but did not substantially reduce the overgrowth problems.
b) The bleaching step found necessary to reduce overgrowth on sheep nematode LDA plates (Kotze, pers. comm.) was found to inhibit egg hatch of *Cooperia* spp. and *O. ostertagi*.
c) Tylosin appeared to inhibit larval development of *Cooperia spp*. and *O. ostertagi* beyond L1 or L2 stages.
d) Adding *E. coli* tended to result in complete overgrowth of wells in the culture plates and inhibit larval development of *Cooperia spp*. and *O. ostertagi* beyond L1 or L2 stages.
e) Changes to the concentration of growth medium (GM) resulted in negative impacts on larval development when *Escherichia coli* was added but when *E. coli* was not added 5-10μl of GM was necessary.
f) An LDA system without bleaching, Tylosin or supplementary *E. coli* could be used for *Cooperia spp*. and *O. ostertagi*, provided 10μl of GM was added 24 hours after set up; this gave >90% progression to L3 in control plates after 7 days.
g) The standard CSIRO method was suitable for use with *H. placei* isolates from cattle or sheep faeces.

The modified CSIRO LDA method was used to assess the relative resistance status of the difference species of cattle nematodes available during the period. Problems with insufficient numbers of larvae growing through to L3 in control wells of the plates in September and October 2012 led to the necessity to repeat these plates. Subsequent plates included the addition of *E. coli* with growth media after the initial 24 hours of incubation to ensure sufficient nutrients were available to the L1 and L2 nematode larvae for development to L3. The following tables (Table 1 and 2) show the results of LDA assessments of the cattle nematode isolates described above.

Inclusion of the post-FECRT Dayboro isolates of *C. punctata/pectinata* and *H. placei* into the cycle of at least three generations of drug challenge has improved the resistance ratio's (RR) for these species when compared to those indicated in the previous milestone report. In particular, the ivermectin aglycone results suggest a reasonable *in vitro* resistance test may be possible. The same cannot be suggested for the *C. oncophora* isolates tested even with the combined RESMIX isolate which LDA results indicate is no different to the known susceptible VHR isolate despite surviving repeated drug challenge. For the DR-C OFZ *O. ostertagi* isolate used, *in vivo* resistance to ivermectin could not be firmly established and this was supported in the lack of difference between this isolates and the VHR SUS isolate used in the LDA tests.

3.4 Assessment of liquid culture LDA method

A liquid culture method for *C. oncophora* and *O. ostertagi* was recently published by Demeler et al. (2010). After correspondence with this group, we attempted to utilise this method for assessment of larval development in parallel with the assessment of the CSIRO method. Initial studies were not successful as development did not proceed for any species beyond L1 or L2 larval stages and the majority of larvae died

within 3-4 days of plate setup. Electronic discussion with Dr Demeler did not resolve the problem and a visit to our laboratory was proposed for late January 2011. Soon after this visit ambiguities in the published methodology were resolved and we were able to successfully apply the method to *C. oncophora* and *H. placei*.

3.5 Assessment of a larval migration assay for cattle nematodes

A number of larval migration assays (LMA) have been applied to assessing the anthelmintic resistance status of sheep nematodes. In 2006 Kotze *et al.* developed an improved method for differentiating between resistant and susceptible *H. contortus* isolates. Demeler *et al.* (2010) developed an alternative methodology for use with *C. oncophora* and *O. ostertagia* but concluded that differentiation between resistant and susceptible isolates was not sufficient for routine use. Kotze's method involved the use of 96 well 20μm filter plates with agar plugs whereas Demeler's method used 24 well plates with 28μm filtration.

We commenced work to determine whether the Kotze method could be adapted for use with cattle nematodes. The first step in this assessment was to determine whether cattle nematode larvae (L3) could migrate through the 20μm mesh of commercially available plates (Millipore Ltd). After overnight incubation in control plates at 26°C it was found that all L3 of *C. oncophora*, *C. punctata/pectinata*, *O. ostertagi* and *H. placei* had successfully migrated through the 20μm mesh.

Comparison of migration rates of L3 for available isolates then proceeded by adding two-fold dilutions of ivermectin or eprinomectin to plates.

4. Results

4.1 Assessment of resistance using the CSIRO LDA method

Results of LDA plate assessments for *C. punctata/pectinata*, *H. placei* and *O. ostertagi* are presented in Table 2, 3 and 4 and Figure 1. These results show no clear distinction between putative susceptible and resistant isolates as resistance ratios (RR) are not substantially different from 1. The data for *O. ostertagia* are included since the very flat curve may indicate both susceptible and resistant individuals in this isolate.

4.2 Assessment of liquid culture LDA method

The results of these studies appear below in Table 5 and Figure 2 and indicate a low level of difference in resistance ratios (RR) between putative susceptible and resistant isolates.

4.3 Assessment of a larval migration assay for cattle nematodes

Results of these tests are shown in Table 6, 7 and 8 and Figure 3 and indicate the low level of difference in resistance ratios (RR) between putative susceptible and resistant isolates.The results appear to be more positive for eprinomectin plates than for ivermectin with higher RR indicated for both *Cooperia* species and *H. placei*. However, the RR for both *Cooperia* species are lower than required for a routine *in vitro* test while the RR for *H. placei* is considerably lower than that observed for isolates of *H. contortus* from sheep compared by similar methods. No difference was observed between the *Ostertagia* isolates with either drug in the LMA.

5. Discussion

Initially, little difference was observed between isolates, with resistance ratios (RR) not exceeding 2.04. However, this result is clouded to some degree by some uncertainty as to the *in vivo* resistance status of some of the worm isolates. This was largely due to their collection in the field as the progeny of worms that had survived FECRTs, with no knowledge in some cases as to the level of infection (egg count) prior to the FECRT, and therefore no accurate measure of the % efficacy of the drug treatment apart from direct comparison with untreated control animals. In other cases the FECRT data were derived from tests in which pre-treatment egg counts were very low (<100 e.p.g.), thus placing the accuracy of the FECRT in doubt.

Through a process of isolation, infection and challenge with oral ivermectin, ML resistant isolates for three of the primary nematode parasites of cattle in sub-tropical and temperate Australia, *C. oncophora*, *C. punctata/pectinata* and *H. placei* were obtained. A similar process did not produce a reliably ML resistant isolate of *O. ostertagi*. All four species were subjected to comparative tests with susceptible isolates of the same species to determine if the LDA and LMA could be used for routine diagnostic purposes.

For *C. punctata/pectinata* and *H. placei,* results for the LDA with ivermectin aglycone and the LMA with eprinomectin suggest these tests could potentially be used in a routine diagnostic to determine ML resistance status of field populations of these nematode species.

An isolate of resistant *C. oncophora* (RESMIX) was derived from mixing resistant isolates originating from field assessments by Novartis Animal Health, David Rendell (Victoria) and DAFWA (Western Australia). This isolate was then challenged with ivermectin over two generations prior to *in vitro* assessment of resistance in comparison to the susceptible VHR isolate. Neither of the LDA ivermectin analogues provided any indication of difference between the susceptible and resistant isolates while the eprinomectin LMA tended to show a low level discrimination between the isolates. At present the *in vitro* methods used could not be reliably used for diagnosis of ML resistance for this species.

Our quest to identify a resistant isolate of *O. ostertagi* was not successful and *in vitro* comparison of the sole isolate (DR-C OFZ; where low level resistance had been suspected from FECRT), with the susceptible VHR isolate showed no differences for both LDA and LMA. Until a reliable ML resistant isolate of this species can be identified the objective of using *in vitro* methods to reliably diagnose ML resistance is not possible. This led to the decision to terminate the project.

6. Tables

Table 1 Nematode isolates, their origins and ivermectin (IVM) resistance status from FECRT where known.

Isolate & Origin	% Reduction (IVM)	Cryogenically preserved
Cooperia punctata/pectinata		
Taree. Calves purchased by CSIRO.	100%	Yes
Casino (VHR – site 8). FECRT by VHR	89%*	Yes
Woodford (NOV). Drench test 2009	42%**	Yes
Cooperia oncophora		
VHR. Local susceptible isolate in mixed culture	100%	
DR-Cor Control. FECRT by Rendell in Western VIC	NA^	
DR-Mix (IVM-R). FECRT by Rendell from 3 properties Western VIC	74-95%*	
Novartis Animal Health Ltd	NA^	
Ostertagia ostertagi		
VHR. Local susceptible isolate in mixed culture	100%	
DR-Cor (OFZ)[#]. FECRT by Rendell in Western Victoria (2010)	NA^	Yes
DR- Cor (OFZ) ½ IVM. Selected from above	80% (1/2 IVM)	
Haemonchus placei		
Bremner (1970's). From CSIRO isolate collection	100%	Yes
Casino. FECRT by VHR (2010)	89%*	
Woodford (NOV). Drench test 2009	71%*	

* FECRT results for mixed species before isolation to individual species.
** These isolates originated from the QDPI and were obtained from Novartis Animal Health Ltd efficacy figures relate to a drench and slaughter trial conducted in 2009.
NA^ = Not available.
The group treated with oxfendazole produced a small number of *O. ostertagi* from culture.

Table 2 Agar plate LDA of cattle nematodes in ivermectin

Isolate	LC_{50} (µg/mL)	95% CI	RR
C. punctata/pectinata			
Taree	0.59	0.43-0.82	
Casino	1.01	0.56-1.82	1.71
H. placei			
Bremner	0.86	0.79-0.94	
Woodford	0.62	0.55-0.69	0.71
O. ostertagi			
DR Cor OFZ	10.74*	6.07-19.01	

* Note these results are from 2 replicate samples only

Table 3 Agar plate LDA of cattle nematodes with ivermectin aglycone.

Isolate	LC 50 (pg/mL)	95% CI	RR
C. punctata/pectinata			
Taree	0.56	0.44-0.73	
Dayboro	1.80	1.46-2.21	3.18
C. oncophora			
VHR SUS	1.90	1.54-2.35	
RESMIX	2.07	1.71-2.51	1.09
H. placei			
Bremner	0.58	0.49-0.70	
Dayboro	3.17	2.84-3.52	5.45
O. ostertagi			
VHR SUS	0.019	0.004-0.101	
DR-C OFZ	0.012	0.003-0.040	0.61

Table 4 Agar plate LDA of cattle nematodes with ivermectin

Isolate	LC 50 (pg/mL)	95% CI	RR
C. punctata/pectinata			
Taree	0.87	0.67-1.15	
Dayboro	1.27	0.90-1.81	1.46
C. oncophora			
VHR SUS	3.28	2.40-4.48	
RESMIX	2.23	1.82-2.73	0.68
H. placei			
Bremner	0.16	0.14-0.19	
Dayboro	0.38	0.32-0.45	2.37
O. ostertagi			
VHR SUS	0.012	0.001-0.105	
DR-C OFZ	0.005	0.001-0.025	0.37

Table 5 Liquid culture LDA results for cattle nematodes in ivermectin.

Isolate	LC50 (μg/ml)	95% CI (μg/ml)	RR
C. oncophora			
DR Cor Control	1.63	0.87-3.07	
NOV	2.81	2.18-3.61	1.72
H. placei			
Bremner	1.38	0.97-1.96	
Woodford	2.46	1.79-3.38	1.78

Table 6 LMA results for cattle nematodes in eprinomectin.

Isolate	LC_{50} (μg/ml)	95% CI (μg/ml)	RR
O. ostertagi			
VHR	3.71	3.27-4.21	
DR Cor OFZ	5.07	3.12-8.24	1.37
DR Cor OFZ ½ IVM	3.87	3.44-4.36	1.04
C. oncophora			
DR-Mix	3.66	3.03-4.42	
NOV	2.79	2.54-3.08	0.76
C. punctata/pectinata			
Taree	3.61	2.42-5.40	
Casino	7.35	5.28-10.23	2.04
Woodford	4.59	3.81-5.52	1.27

Table 7 LMA results for cattle nematodes with eprinomectin.

Isolate	LC_{50} (μg/ml)	95% CI (μg/ml)	RR
C. punctata/pectinata			
Taree (SUS)	2.36	1.97-2.83	
Dayboro	4.68	3.57-6.15	1.98
C. oncophora			
VHR SUS	0.70	0.62-0.79	
RESMIX	1.29	0.91-1.84	1.86
H. placei			
Bremner (SUS)	3.36	2.07-5.45	
Dayboro	11.83	3.06-45.81	3.52
O. ostertagi			
VHR SUS	5.64	3.84-8.24	
DR-C OFZ	3.86	3.04-4.92	0.69

Table 8 LMA results for cattle nematodes with ivermectin.

Isolate	LC_{50} (μg/ml)	95% CI (μg/ml)	RR
C. punctata/pectinata			
Taree (SUS)	0.78	0.12-5.06	
Dayboro	1.40	0.49-4.02	1.78
C. oncophora			
VHR SUS	1.26	0.77-2.05	
RESMIX	1.65	1.42-1.91	1.31
H. placei			
Bremner (SUS)	2.31	1.82-2.94	
Dayboro	2.06	1.53-2.78	0.89
O. ostertagi			
VHR SUS	1.76	1.37-2.27	
DR-C OFZ	1.06	0.64-1.75	0.60

7. Figures

Figure 1 Agar plate LDA results for cattle nematodes in ivermectin

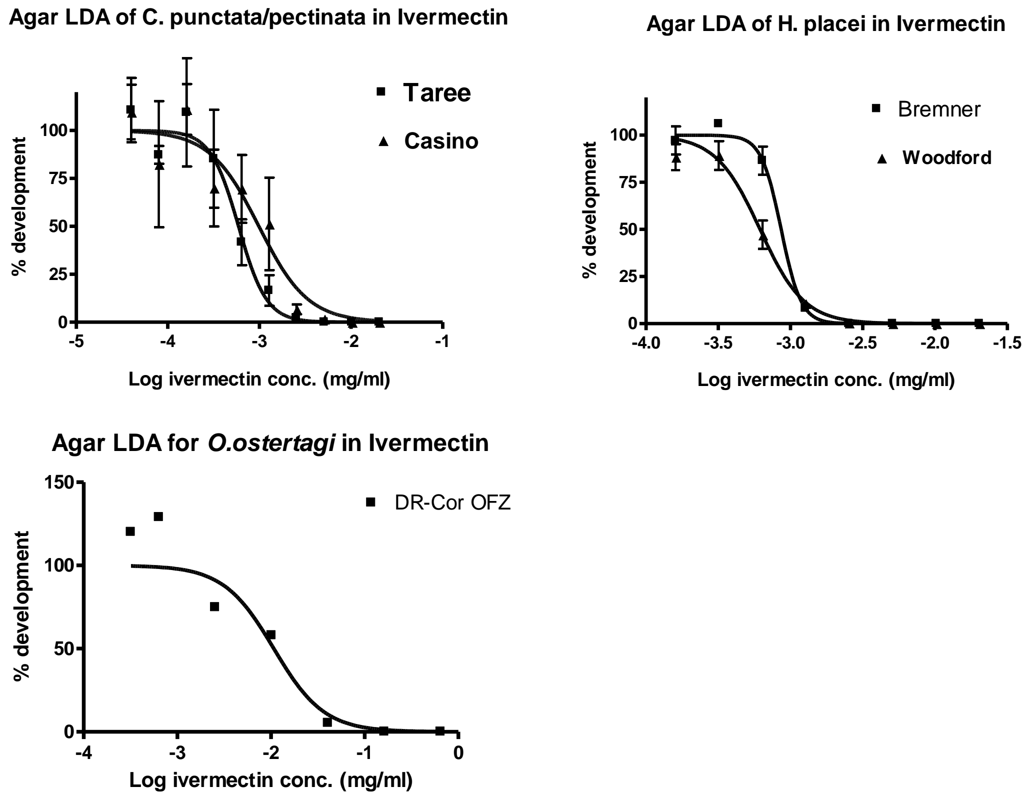


Figure 2 Results of liquid culture LDA for cattle nematodes in ivermectin.

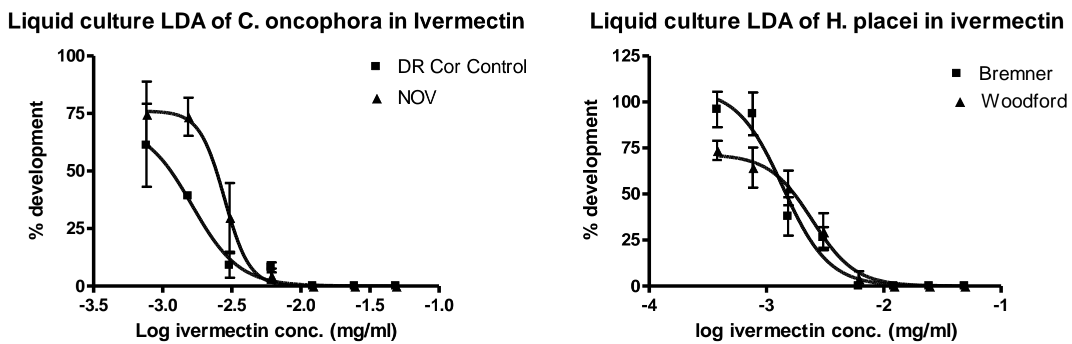
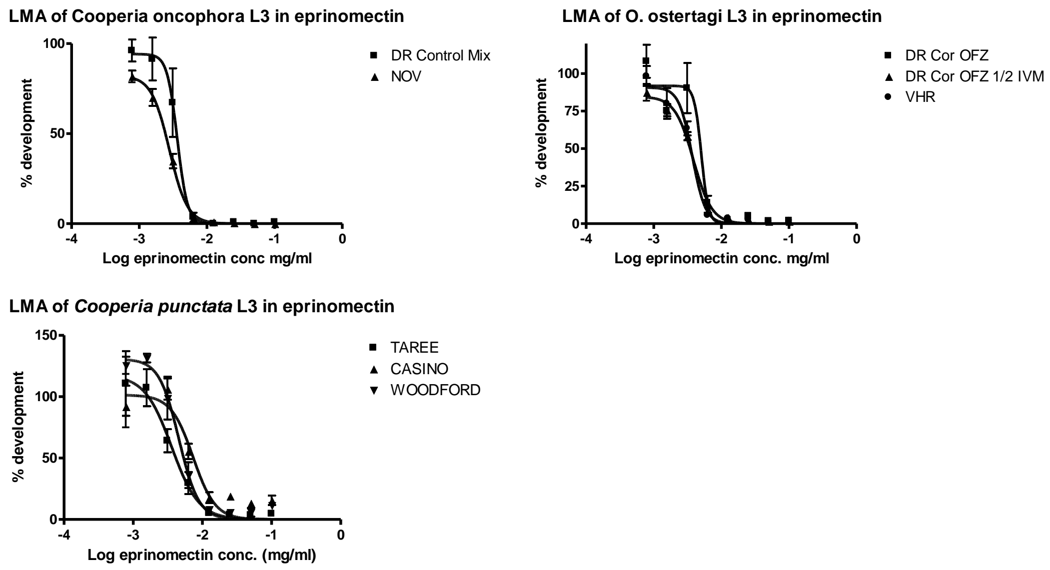


Figure 3 LMA results for cattle nematodes in eprinomectin



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