Some aspects of ivermectin resistance in gastrointestinal nematodes of goats and sheep

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ABSTRACT

Experiments were conducted to determine whether sheep are at risk from grazing pasture previously grazed by goats harbouring ivermectin-resistant *Ostertagia* spp. by monitoring the level of resistance with faecal egg count reduction tests and larval development assays. Ivermectin resistance emerged rapidly in goats grazed on the paddocks previously grazed by goats or sheep. In contrast, resistance was never consistently identified in sheep grazed on paddocks previously grazed by sheep although ivermectin resistance emerged after about 5 months in sheep grazed on paddocks previously grazed by goats.

Ivermectin resistance was suspected in *Trichostrongylus colubriformis* and *Ostertagia* species on a goat farm in Northland, New Zealand. A controlled efficacy study was conducted in lambs (n=12) and young Angora goats (n=10) with this isolate. The establishment rate of *T. colubriformis* and *Ostertagia* spp. was similar ($p>0.05$) in the sheep and goats. Following ivermectin treatment (0.2 mg/kg b.wt. per oral) to half of the lambs and goats, the burdens of *T. colubriformis* were reduced by 39% and 13% in lambs and goats respectively whereas *Ostertagia* spp. burdens were reduced by 33% and 0% in lambs and goats respectively.

In series of larval development assays with ivermectin aglycone, ivermectin and avermectin B_2, this isolate of *T. colubriformis* had a resistance ratio of 37, 4-5 and 3-4 respectively indicating ivermectin aglycone is the drug of choice for detecting ivermectin resistance in *T. colubriformis*. However, both ivermectin and avermectin B_2 were still able to discriminate between resistant and susceptible *T. colubriformis* under controlled experimental conditions. The LC_{50} values of ivermectin were found to be influenced by the age of the infection of *T. colubriformis* in sheep. These LC_{50} values were relatively constant at 23 to 37 days after infection, then rose about four fold to peak between 51 to 58 days post infection, followed by a decline close to the original starting values by 72 days post infection. The resistance ratios recorded with thiabendazole were also high (14 to 15) suggesting that the ivermectin-resistant strain of *T. colubriformis* was also resistant to benzimidazoles.

The LC_{50} values of ivermectin for *T. colubriformis* in a larval development assay for the two reciprocal first generation (F_1) crosses of ivermectin-resistant and -susceptible

parents were slightly lower than the LC_{50} values for ivermectin-resistant parents but greater than the ivermectin-susceptible parents suggesting that ivermectin resistance in *T. colubriformis* is inherited as an incompletely dominant trait.

The fitness of the ivermectin-resistant strain of *T. colubriformis* was investigated and there was no significant differences ($p>0.05$) in infectivity, longevity of infection, fecundity and development of eggs to larvae under natural or laboratory conditions between the ivermectin-resistant strain and two susceptible field isolates. The survival of ivermectin-resistant strain larvae was intermediate between the two susceptible field isolates at $10^{O}C$, $20^{O}C$, $25^{O}C$ and $30^{O}C$ under laboratory conditions.

An efficacy study in sheep indicated that the moxidectin oral formulation (0.2 mg/kg b.wt.), moxidectin injectable formulation (0.2 mg/kg b.wt.) and ivermectin oral formulation were 98%, 4% and 62% effective against this ivermectin-resistant strain of *T. colubriformis* respectively. These findings indicate that formulation of an anthelmintic plays an important role in the efficacy against resistant nematodes.

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CHAPTER ONE

INTRODUCTION AND LITERATURE REVIEW

1.1. INTRODUCTION

One of the major limitations to increasing productivity following intensification of the grazing livestock industries has been the greater range and severity of animal health problems, of which diseases caused by nematode parasites have been prominent (Waller and Prichard, 1986). Up to 1938, before the discovery of phenothiazine, few useful drugs existed for the treatment of helminth infections. Since then, and particularly over the past 30 years, considerable advances have been made in the development of safer and more effective drugs. Potentially toxic chemicals, in use less than half a century ago, have been replaced with modern highly effective anthelmintics that offer greater efficacy with higher safety margins and ease of application. The introduction of thiabendazole in 1961, the first of the benzimidazoles, saw the beginning of broad-spectrum anthelmintic therapy that has led to the development of drugs with combined activity against nematodes, cestodes and trematodes. The discovery of the avermectins in 1976, followed by the introduction of ivermectin with its broad-spectrum antiparasitic activity that also includes ectoparasites has taken the stage one step further to the single-molecule 'endectocide'.

Integrated helminth control programs utilising various systems of rotational grazing and natural resistance may, under certain conditions, reduce losses due to internal parasites in livestock. The success of any internal parasite control program is usually significantly improved by incorporating anthelmintics. Therefore, most of the helminth control programs rely largely on regular or pre-planned anthelmintic treatments or the integration of such treatments with grazing management. However, the selection for anthelmintic resistance occurs rapidly where repeated use of anthelmintics is commonly utilised (Smith, 1990). Currently, the value of the control strategies involving anthelmintics is under threat because of the development of anthelmintic resistance in nematodes of sheep and goats. Endoparasite resistance to anthelmintics is unfortunately now a potential problem affecting sheep and goat

industry worldwide, especially that operating under intensive grazing conditions. Resistance to benzimidazole drenches has been recorded since the 1960's (Smeal *et al.*, 1968). Since then there has been steady progression with the development of new action families and subsequent evolution of resistance to these by the endoparasites of sheep. The problem has reached alarming proportions in the sheep and goat industries in Australia, New Zealand, South Africa and South America. Many farmers have now limited options for broad-spectrum endoparasite control. Resistance has now been recorded in the field to every class of anthelmintic (Conder and Campbell, 1995). Some parasites are resistant to two or more of the three broad spectrum anthelmintic groups (Sangster *et al.*, 1979; Kettle *et al.*, 1983; Barton *et al.*, 1985; Edwards *et al.*, 1986; Van Wyk and Malan, 1988; Badger and McKenna, 1990; Watson and Hosking, 1990; Maingi, 1991; Reinecke *et al.*, 1991; Love *et al.*, 1992; Pomroy *et al.*, 1992; Uppal *et al.*, 1992; Varady *et al.*, 1993; Mwamachi *et al.*, 1995; Singh and Yadav, 1997; Van Wyk *et al.*, 1997; Waruiru *et al.*, 1998). However, there is no report of isolation of ivermectin-resistant strain of *Trichostrongylus colubriformis* in the field. Moreover, there are a number of reports of ivermectin-resistant *Ostertagia circumcincta* in goats in New Zealand (Badger and McKenna, 1990; Pomroy *et al.*, 1992) but only reported recently in sheep (Mason *et al.*, 1999) despite mixed grazing. The studies reported in this thesis concentrate on the isolation of an ivermectin-resistant strain of *T. colubriformis*, mode of inheritance of this resistance, the fitness status of this strain and the efficacy of moxidectin against this strain. Results are also reported of a mixed grazing trial of sheep and goats designed to investigate if sheep are at risk from grazing pasture contaminated with goat derived ivermectin-resistant *Ostertagia* species.

1.2. ANTHELMINTIC GROUPS

Anthelmintics can be classified into several groups depending upon their mode of action, range of activity against different parasites or their chemical structure. Based on their range of activity, they can be classified as either broad-spectrum or narrow spectrum drugs. A summary of anthelmintic classification and their use in sheep and goats is given by Prichard *et al.* (1980), Marriner and Armour (1986), Bogan and Armour (1986) and Taylor (1992). Table 1.1 provides a summary of anthelmintics currently available for sheep and goats, along with their spectrum of activity.

Table 1.1: Anthelmintic groups

Group	Range of Activity* Mode of action	Anthelmintic	Activity								
			Gutworms			Lung Worms	Tape Worms	Liverfluke			Ecto-parasite
			Ad	DL	AL			<6w	>6w	Ad	
Benzimidazoles	Broad* Tubulin Binding	Thiabendazole	44 mg/kg								
		Oxibendazole	10 mg/kg								
		Fenbendazole	5 mg/kg								
		Mebendazole	15mg/kg								
		Oxfendazole	5 mg/kg								
		Albendazole	5 mg/kg							7.5mg/kg	
		Febentel	5 mg/kg								
		Thiophanate	50 mg/kg			100 mg/kg					
		Netobimin	7.5 mg/kg (20 mg)							20 mg/kg	
		Triclabendazole						12 mg/kg			
Imidazothia-zoles/tetrahydr-oxypyrimidines	Broad* Cholinergic Agonists	Levamisole Morantel	7.5 mg/kg								
Macrocyclic Lactones	Broad* Glutamate-gated chloride channel potentiation	Ivermectin Moxidectin	200 mcg/kg								
Substituted Salicylanilides	Narrow* Uncouple Oxidative phosphory-Lation	Oxyclozanide								15 mg/kg	
		Nitroxynil							10 mg/kg		
		Rafoxanide							7.5 mg/kg		
		Closantel							10 mg/kg		
		Brotianide							6 mg/kg		

Active against a parasite group

Variable activity against a parasite group

Ad = Adult; DL = Developing Larvae; AL = Arrested Larvae; w = weeks

1.2.1. BROAD-SPECTRUM ANTHELMINTICS

1.2.1.1. Benzimidazoles

McKellar and Scott (1990) made a comprehensive review of this group of anthelmintics. The first benzimidazole introduced was thiabendazole in 1961, followed by parbendazole in 1967, oxibendazole in 1973, fenbendazole in 1974, oxfenbendazole in 1975, albendazole in 1976, triclabendazole in 1981 and ricobendazole in 1987. The other chemicals included in this group are febantel, netobimin and thiophanate, which are referred to as probenzimidazoles because they are metabolised in the body by ruminal and hepatic pathways into a variety of benzimidazole compounds. The range of activity of this group of drugs is shown in Table 1.1.

Following oral dosing in sheep and goats, the drug usually passes to the rumen, which acts as a reservoir allowing gradual release and absorption of the drug into the blood stream (Marriner and Bogan, 1981). Thereafter, drug concentrations of the circulatory metabolites can be found in the plasma, from where they are extensively recycled between the circulation and the gut wall, down the whole length of the intestinal tract (Prichard *et al.*, 1981). Nematodes attached to the gut wall may be more exposed to the recycled drug than to the drug present in the digested food. The oral administration of anthelmintic solutions may result in oesophageal groove closure in some animals, with subsequent bypass of the rumen and delivery of the drug into the abomasum (Prichard, 1980; Hennessy, 1985). The anthelmintic is absorbed very quickly from the abomasum, so its metabolism and excretion may be more rapid, resulting in reduced exposure of the parasite to the drug and lowered efficacy. The solubilization of relatively insoluble drugs may be reduced in some disease and nutritional states and thus the absorption and efficacy of the drugs may be reduced (Prichard, 1985).

Mode of action

Benzimidazoles bind to β-tubulin, a constituent protein present in microtubules, plasma and mitochondrial membranes (Prichard, 1986). This leads to inhibition of microtubule production. Microtubules are usually associated with the formation of mitotic spindles during cell division, maintenance of cell shape, cell motility, cellular

secretion, nutrient absorption and intracellular transport (Lacey, 1988). Inhibition of microtubules leads to a disruption of these functions. There is also a reduction in enzyme activity such as acetylcholinesterase secretion, and carbohydrate catabolism by the fumarate-reductase system (Prichard, 1973).

1.2.1.2. Imidazothiazoles/tetrahydropyrimidines

Levamisole is a different chemical type to the morantel, pyrantel and oxantel group (Prichard, 1978) but for practical purposes they are often linked together. The work of Coles *et al.*, (1975) indicates that these anthelmintics act on the same or a closely adjacent site. The paralysis they produce may be reversible. Rew and Fetterer (1986) indicated that both these groups of anthelmintics appear to act as cholinergic agonists at the neuromuscular junction.

1.2.1.3. Macrocyclic lactones

1.2.1.3.1. Ivermectin

Ivermectin is a member of the avermectin group of compounds, which are isolated as fermentation products from the actinomycete *Streptomyces avermitilis* (Miller *et al.*, 1979). Ivermectin is the 22, 23-dihydroxy derivative of the natural product avermectin B_1. Ivermectin was the first of the new wave of macrocyclic lactone anthelmintics, and it has been reviewed many times (e.g. Baker and Swain, 1989; Goa, *et al.*, 1991)

Ivermectin is an off-white powder, largely insoluble in water but very soluble in organic solvents. With the use of detergents, micellar aqueous solutions can be prepared, and are in commercial use. Ivermectin has a high therapeutic index, not because of extraordinary safety in mammals but because of extraordinary potency against parasites. In rodents, it is fetotoxic only at dosages that approximate the maternotoxic dosage. Fetotoxicity was not seen in extensive trials done in target species for registration purposes. There is now an extensive record of the safety of ivermectin when given to domestic animals at dosages of 200-500 μg/kg body weight (Conder and Campbell, 1995).

The rate of absorption of ivermectin depends on the route of administration and the formulation used. With the oral formulation for sheep at a dose rate of 200 μg/kg body

weight, for example, the biological half-life obtained from the terminal portion of the bioavailability curve was 2.5 days whereas the biological half-life after subcutaneous administration was 3.7 days (Steel, 1993).

Antiparasitic Spectrum

Administration of ivermectin results in efficacy against the immature and mature stages of many nematodes in sheep and these are listed in Table 1.2.

Table 1.2: Parasites of sheep for which the use of ivermectin (oral formulation) has been approved in New Zealand.

GASTRO-INTESTINAL NEMATODES	OTHER NEMATODES	NASAL BOTS	ITCH MITES
Haemonchus contortus[a] (adult and immature) *Haemonchus placei*[a] (adult) *Ostertagia circumcincta*[a] (adult and immature) *Trichostrongylus axei* (adult and immature) *Trichostrongylus colubriformis* (adult and immature) *Trichostrongylus vitrinus* (adult and immature) *Cooperia curticei* (adult and immature) *Cooperia oncophora* (adult) *Gaigeria pachyscelis* (adult and immature) *Oesophagostomum columbianum* (adult and immature) *Oesophagostomum venulosum* (adult) *Nematodirus filicollis* (adult and immature) *Nematodirus spathiger* (adult and immature) *Strongyloides papillosus* (adult and immature) *Chabertia ovina* (adult and immature) *Trichuris ovis* (adult)	*Dictyocaulus filaria* (adult and immature)	*Oestrus ovis* (all 3rd instars)	*Psorergates ovis*

[a] Including hypobiotic larvae

1.2.1.3.2. Moxidectin

Moxidectin is a derivative of nemadectin, a naturally occurring product of the filamentous bacterium *Streptomyces cyaneogriseus noncyanogenus*. These compounds are structurally similar to the milbemycins, and they differ from avermectins in the absence of a side-chain (disaccharide substituent) at the C-13 position. Moxidectin and nemadectin differ from the milbemycins in having an unsaturated C-25 chain (Carter, 1989).

Nemadectin is a white fluffy solid when obtained by freeze-drying from *t*-butanol. It is soluble in organic solvents and nearly insoluble in water (Carter, 1989). Moxidectin, the derivative in commercial use, has a broad spectrum of antiparasitic activity and a margin of a safety is up to 5 times the recommended dose (Conder and Campbell, 1995).

Moxidectin is commercially available in two formulations for sheep, which are oral and injectable. Following a single oral or subcutaneous dose of 0.2 mg/kg body weight in sheep, the drug is absorbed at a significantly faster and higher rate after oral administration as compared to subcutaneous administration. However, there is more mean residence time in plasma after subcutaneous administration (16.80 days) as compared to oral drench (12.55 days) (Alvinerie *et al.*, 1998). Similarly, a study has demonstrated the higher persistent activity of moxidectin injectable formulation as compared to moxidectin oral formulation against natural infestations of *H. contortus*, *O. circumcincta* and *T. colubriformis* (Bairden *et al.*, 1995). Both formulations have been shown to have almost 100% efficacy against natural infestations of *T. colubriformis*, *Ostertagia* species and *H. contortus* (Dorchies *et al.*, 1996). The persistent activity of both formulations against the later two species is up to 35 days (Dorchies *et al.*, 1996). However, the persistent activity of moxidectin is short against *T. colubriformis* than against *O. circumcincta* and *H. contortus* (Bairden *et al.*, 1995; Kerboeuf *et al.*, 1995). Moxidectin oral formulation is more persistent than ivermectin given by the same route (Lanusse *et al.*, 1997). It has been suggested that such a difference could be related to higher liposolubility of the moxidectin compared with ivermectin (Alvinerie *et al.*, 1996).

Antiparasitic Spectrum

Treatment of sheep with oral drench, a formulation containing 0.1% moxidectin, at a dose rate of 200 μg/kg body weight, provides efficacy against a broad spectrum of parasites listed in Table 1.3. Administration of the injectable product at a dose rate of 200 μg/kg body weight also gives high efficacy against many nematodes and arthropod parasites, and these are listed in Table 1.3.

Table 1.3: Parasites of sheep for which the use of moxidectin (oral and injectable formulations) has been approved in New Zealand.

GASTROINTESTINAL NEMATODES	PULMONARY WORMS	NASAL BOT	EXTERNAL PARASITES
Haemonchus contortus[a] *Ostertagia circumcincta*[a] *Ostertagia trifurcata*[a] *Trichostrongylus axei*[a] *Trichostrongylus colubriformis*[a] *Trichostrongylus vitrinus*[a] *Cooperia curticei* (adults only) *Cooperia oncophora* (adults only) *Cooperia punctata* (adults only) *Oesophagostomum venulosum* (adults only) *Nematodirus spathiger* *Nematodirus filicollis* *Strongyloides papillosus* (adults only) *Chabertia ovina* *Trichuris ovis* (adults only)	*Dictyocaulus filaria* (adults and immature)	*Oestrus ovis**	*Psorergates ovis*

[a] including inhibited larvae; * injectable only

Mechanism of action of macrocyclic lactones

The mechanism of action of the macrocyclic lactones is not fully understood. Previously, it was thought that the activity of macrocyclic lactones against nematodes was due to inhibition of chitin metabolism (Calcott and Fatig, 1984), based on studies done with a fungus, *Mucor miehei*, and the brine shrimp, *Artemia salina*. However, this possibility was later proven to be invalid when it was demonstrated avermectin B_{1a} of commercial grade did not inhibit chitin metabolism of fungi (Onishi and Miller,

1985) or insects (Gordnier *et al.*, 1987). Similar grade Milbemycin D also had no effect on chitin metabolism (Gordnier *et al.*, 1987). Later, it was suggested that these compounds exert their effect by irreversibly opening chloride channels in muscle membranes (Martin and Pennington, 1989) and these chloride channels were associated with γ-aminobutyric acid (GABA) receptors (Turner and Schaeffer, 1989). Contrary to this report, Geary *et al.* (1992a) indicated that there is no GABA association. It has been proposed that the anthelmintic activity of the macrocyclic lactones is mediated by an interaction with a glutamate-gated chloride channel; this conclusion is based on electrophysiological examination of membrane currents recorded from *Xenopus laevis* oocytes injected with *Caenorhabditis elegans* RNA (Arena *et al.*, 1992). It is unclear where the receptors relevant for anthelmintic activity for the macrocyclic lactones are located. A study predicted that altered pharynx function may be the actual site of anthelmintic action within the host as opposed to somatic musculature function, since ivermectin inhibits pharyngeal pumping more potently than motility in *H. contortus* (Geary *et al.*, 1993). A similar pattern of ivermectin sensitivity in pharyngeal versus somatic muscle has been demonstrated for *T. colubriformis* (Bottjer and Bone, 1985) and *C. elegans* (Laughton *et al.*, 1997). Furthermore, laser ablation of all neurons in the circumesophageal ganglion of *C. elegans* does not block pharyngeal pumping action, which suggests the macrocyclic lactone receptors are located in muscle membrane, as opposed to neurons that innervate it.

However, in insects, ivermectin interacts with both GABA receptors (Duce and Scott, 1985; Scott and Duce, 1985; Deng and Casida, 1992) and glutamate-gated chloride channels (Scott and Duce, 1985; Cully *et al.*, 1996) suggesting that the multiple targets for avermectins may be present in insects.

It is likely that all macrocyclic lactone anthelmintics share the same mechanism of action, although most of the work has been carried out using ivermectin. Ivermectin, Milbemycin D and moxidectin induced similar ionotropic effects on membrane conductance in a shore crab muscle fiber preparation (Bowman *et al.*, 1991) which was reversed by subsequent addition of picrotoxin, a potent blocker of chloride channels. These data and similar data for several other macrocyclic lactones (Bowman

et al., 1991) support the idea of a common mechanism of action for the members of macrocyclic group of anthelmintics.

1.2.2. NARROW-SPECTRUM ANTHELMINTICS

1.2.2.1. Salicylanilides and substituted phenols

The members of salicylanilides and substituted phenols group of anthelmintics binds extensively to plasma proteins (Mohammed-Ali and Bogan, 1987). The mode of action appears to be due to uncoupling of oxidative phosph orylation (Van den Bossche *et al.*, 1979).

1.2.2.2. Organophosphates

Several organophosphate products were available in the market for use in domestic animals. The mode of action of organophosphates is due to phosphorylation of the enzyme acetylcholinestrase, which regulates the concentration of the neurotransmitter, acetylcholine, at neuromuscular junctions (Lee and Hodsden, 1963). This results in paralysis of the worm by interrupting neuromuscular transmission.

1.2.2.3. Piperazine

Diethylcarbamazine is available in some countries for control of lungworm in cattle and sheep. Piperazine products are mainly used for small animals but are also available for pigs and poultry. The mode of action of piperazine is thought to be as a GABA agonist (Martin, 1982). Diethylcarbamazine acts by increasing the flow of chloride ions into the cell, resulting in hyperpolarization of neuronal post-synaptic membranes (Martin, 1982).

1.2.2.4. Phenothiazine

The mode of action of phenothiazine is thought to be similar to the mode of action of the benzimidazole group of anthelmintics (Rew and Fetterer, 1986). The benzimidazole-resistant worms may be cross-resistant to phenothiazine (Kelly *et al.*, 1981b).

1.3. PHARMACOKINETICS OF ANTHELMINTICS (SHEEP VS GOATS)

Although nematode genera infecting goats are similar to those of sheep, many anthelmintics appear to be less effective in the former host. Hall *et al.* (1981) originally made the suggestion that the dose rates of anthelmintics for sheep were inappropriate for goats. It has also been suggested that reduced efficacy of these drugs in goats could promote development of drug resistance (Charles *et al.*, 1989) which might then be transferred to sheep (Kettle *et al.*, 1983). It is well established that there are distinct differences between the behaviour of anthelmintics in these two host species (as there are between them and cattle). In general, anthelmintics are cleared more rapidly from the systemic circulation in goats compared with sheep (Swan and Gross, 1985) which implies that use of sheep dose rates in goats is actually underdosing them by comparison with sheep.

Following oral or intraruminal dosing the maximum serum concentrations of oxfendazole and its respective metabolites are lower and the drug is cleared more rapidly in goats than sheep (Sangster *et al.*, 1991; Bogan *et al.*, 1987). Bogan *et al.* (1987) have shown that following oral administration of 10 mg/kg oxfendazole, the area under the curve of serum concentrations of oxfendazole and its metabolites in goats was about 42% of that in sheep. Similarly, the systemic availability of albendazole following its oral administration was lower in goats than sheep. It was suggested that albendazole might have sequestered to a greater extent in the liver of goats than of sheep which would have resulted in a lower concentrations of its metabolite in plasma and abomasal fluid (Hennessy *et al.*, 1993a). Dwarf/pygmy goats appear to constitute a distinct group that metabolises some drugs such as benzimidazoles quickly than some other breeds (Baggot and McKellar, 1994).

The peak plasma levels and biological half-life of levamisole following oral administration in goats is only about 60% of the level in sheep (Galtier *et al.*, 1981). Although the maximum plasma levels for closantel were similar in sheep and goats, the drug is cleared at a rate 2-3 times greater in goats, which would reduce the protection from reinfection with *H. contortus* for 28 days in sheep to only 10 days in goats (Hennessy *et al.*, 1993b). The clearance of ivermectin from plasma after oral administration was faster in goats than in sheep, with detectable levels being present

up to 5 days in goats compared with 11 days in sheep (Scott *et al.*, 1990a). The biological half-life of ivermectin in plasma is similar in cattle and sheep but because of a larger volume of distribution, plasma clearance is more rapid in sheep (Steel, 1993).

There is evidence that differences in pharmacokinetics of anthelmintics are reflected in differences in efficacy, particularly with partly resistant strains of nematodes and this may lead to a misdiagnosis of resistance when the drug is still effective against the same isolate in sheep. Hall *et al.* (1981) could not effectively remove *H. contortus* and *Ostertagia* spp. from goats with treatment with benzimidazoles and levamisole but experimentally these drugs were found to be effective in sheep against these strains. A reduced efficacy of levamisole has also been reported against induced immature and mature infections of nematodes in goats (Coles *et al.*, 1989a). Similarly, Gillham and Obendorf (1985) showed that levamisole was not effective in removing an isolate of *T. colubriformis* in experimentally infected goats but this drug was effective in sheep infected with the same strain. Oxfendazole at 20 mg/kg was less effective in reducing benzimidazole-resistant *T. colubriformis* in goats than sheep but there was no significant difference when lower dose rates were used. Mwamachi *et al.* (1995) suggested that a dose rate of at least 1.5 times that recommended for sheep should be implemented for goats. However, the range of dose rates used showed similar efficacy against a benzimidazole-resistant *T. colubriformis* in both host species (Sangster *et al.*, 1991). Similarly, McKenna and Watson (1987) observed no significant differences in oxfendazole efficacy between sheep and goats infected with a benzimidazole-resistant isolate of *H. contortus*.

In addition, other factors such as disease or diet can also play a significant role in the pharmacokinetics of drugs. Taylor *et al.* (1992) demonstrated that fenbendazole and ivermectin when administered to pastured lambs and calves had significantly lower plasma concentration and area under the curve than did housed animals fed hay and concentrate ration.

1.4. DEFINITIONS OF RESISTANCE

Shoop (1993) proposed the following definitions of anthelmintic resistance.

Resistance - a change in the gene frequency of a population that is produced by drug selection whereby more drug is required to exact some effect than was required prior to selection.

Side-resistance - a state in which a drug-selected population has a gene(s) coding for a mechanism that defeats the toxicity of drugs within a mode of action family.

Cross-resistance – a state in which a drug-selected population has a gene(s) coding for a mechanism that defeats the toxicity of drugs from different mode of action families.

Multiple resistance – a state in which a population has been selected independently by drugs from different mode of action families to produce different but concurrent mechanisms of evasion, as used herein, or it is sometimes used as a synonym of cross-resistance.

Reversion – a state in which there is a return to or towards drug susceptibility.

Tolerance – innate lack of susceptibility that does not result from drug selection.

Multiple resistance can be either multidrug resistance or multigeneric resistance. Multidrug resistance occurs when the same nematodes are resistant to two or more anthelmintic groups, either as a result of selection by each group independently or as a result of cross resistance. Multigeneric resistance occurs when a mixed population of nematodes is resistant to one or more anthelmintic drugs. Multigeneric resistance can be multidrug resistance and vice versa.

1.5. INCIDENCE OF ANTHELMINTIC RESISTANCE IN SHEEP AND GOATS

Anthelmintic resistance is usually only noticed when worm control policies fail dramatically. Throughout the world, resistance has been detected most commonly among the gastrointestinal nematodes of sheep and goats. Most reports of anthelmintic resistance in sheep and goats originate from the Southern Hemisphere, in particular the large pastoral sheep-rearing areas of Australia, New Zealand, South Africa and South America. Resistance was first reported against phenothiazine (Drudge *et al.*, 1957a) although since then it has been widely reported against all the groups of anthelmintics.

Countries where anthelmintic resistance has been reported are shown in Tables 1.4, 1.5, 1.6, and 1.7.

Table 1.4: Incidence of benzimidazole resistance recorded from different countries.

LOCALITY	PARASITE	REFERENCE
Argentina	*Haemonchus* spp.	Eddi *et al.* (1996)*
	Ostertagia spp.	Eddi *et al.* (1996)*
	Trichostronglyus spp.	Eddi *et al.* (1996)*
Australia	*Haemonchus contortus*	Le Jambre *et al.* (1979a); Green *et al.* (1981)
	Ostertagia circumcincta	Barton *et al.* (1985); Beveridge *et al.* (1990)
	Ostertagia trifurcata	Martin *et al.* (1985); Beveridge *et al.* (1990)
	Ostertagia davtiani	Beveridge *et al.* (1990)
	Trichostronglyus colubriformis	Barton *et al.* (1985); Love *et al.*(1992)
	Trichostrongvlus axei	Beveridge *et al.* (1990)
	Trichostrongvlus vitrinus	Beveridge *et al.* (1990)
	Nematodirus spathiger	Beveridge *et al.* (1990); Obendorf *et al.* (1991)
	Nematodirus filicolis	Martin *et al.* (1985); Beveridge *et al.* (1990)
	Nematodirus abnormalis	Beveridge *et al.* (1990); Obendorf *et al.* (1991)
	Oesophagostomum spp.	Edwards *et al.* (1986)*
Belgium	*Haemonchus contortus*	Geerts *et al.* (1990)
Brazil	*Haemonchus contortus*	Charles *et al.* (1989); Echevarria *et al.* (1991)
	Ostertagia spp.	Echevarria *et al.* (1996)*
	Trichostronglyus spp.	Echevarria *et al.* (1996)*
	Nematodirus spathiger	da Costa *et al.* (1985)
Czechoslov-akia	*Ostertagia* spp	Varady *et al.* (1993)*
	Trichostronglyus spp.	Varady *et al.* (1993)*
Denmark	*Ostertagia* spp.	Bjorn *et al.* (1991)*; Maingi *et al.* (1996)*
	Trichostronglyus spp.	Bjorn *et al.* (1991)*; Maingi *et al.* (1996)*
France	*Haemonchus contortus*	Kerboeuf *et al.* (1988); Hubert *et al.* (1991)
	Ostertagia circumcincta	Kerboeuf *et al.* (1988); Hubert *et al.* (1991)
	Ostertagia trifurcata	Hubert *et al.* (1991)
	Trichostronglyus colubriformis	Kerboeuf *et al.* (1988); Hubert *et al.* (1991)
	Trichostrongvlus axei	Kerboeuf *et al.* (1988)
	Nematodirus spp.	Kerboeuf *et al.* (1988)*
	Cooperia curticei	Hubert *et al.* (1991)
	Oesophagostomum venulosum	Kerboeuf *et al.* (1988)

French West Indies	*Haemonchus contortus*	Barre *et al.* (1997)
	Trichostrongylus spp.	Barre *et al.* (1997)*
	Oesohagostomum spp.	Barre *et al.* (1997)*
Germany	*Haemonchus contortus*	Bauer *et al.* (1987); Duwel, (1991)
	Ostertagia circumcincta	Duwel (1991)
	Trichostronglyus colubriformis	Duwel (1991)
India	*Haemonchus contortus*	Yadav (1990); Uppal *et al.* (1992)
Kenya	*Haemonchus contortus*	Njanja *et al.* (1987); Mwamachi, *et al.* (1995)
	Trichostronglyus colubriformis	Maingi (1991)
Malaysia	*Haemonchus contortus*	Dorny *et al.* (1993); Chandrawathani *et al.* (1999)
Martinique	*Haemonchus contortus*	Gruner *et al.* (1986)
Netherlands	*Haemonchus contortus*	Boersema *et al.* (1982); Boersema *et al.* (1987)
	Ostertagia circumcincta	Boersema *et al.* (1987)
	Trichostronglyus colubriformis	Boersema *et al.* (1987)
	Cooperia curticei	Borgsteede (1986); Borgsteede *et al.* (1991)
New Zealand	*Haemonchus contortus*	Kettle *et al.* (1982); McKenna (1989)
	Ostertagia circumcincta	McKenna (1989); Pomroy *et al.* (1992)
	Ostertagia trifurcata	McKenna (1989); Pomroy *et al.* (1992)
	Trichostronglyus colubriformis	Kettle *et al.* (1982); McKenna (1989)
	Trichostrongylus vitrinus	McKenna (1989)
	Nematodirus spathiger	Middelberg and McKenna (1983)
	Nematodirus filicolis	McKenna (1989)
	Cooperia spp.	Hughes (1988)*
	Oesophagostomum venulosum	McKenna (1989)
	Chabertia spp.	Hughes (1988)*
	Strongyloides spp.	McKenna (1989)*
Paraguay	*Haemonchus* spp.	Maciel *et al.* (1996)*
	Ostertagia spp.	Maciel *et al.* (1996)*
	Trichostronglyus spp.	Maciel *et al.* (1996)*
South Africa	*Haemonchus contortus*	Berger (1975); Van Wyk and Malan (1988)
	Ostertagia circumcincta	Van Schalkwyk and Shroder (1989)
Spain	*Ostertagia circumcincta*	Requejo-Fernandez *et al.* (1997)
Sri Lanka	*Haemonchus contortus*	Van Aken *et al.* (1989)
Switzerland	*Haemonchus contortus*	Jordi (1980)
Tanzania	*Haemonchus contortus*	Ngomuo *et al.* (1990)

UK	*Haemonchus contortus*	Taylor and Hunt (1988); Scott *et al.*(1989)
	Ostertagia circumcincta	Coles *et al.* (1991); Mitchell *et al.* (1991)
	Trichostronglyus colubriformis	Scott *et al.* (1990b)
	Cooperia curticei	Hunt *et al.* (1992)
	Oesophagostomum columbianum	Scott *et al.* (1990b)
	Chabertia spp.	Scott *et al.* (1990b)*
USA	*Haemonchus contortus*	Conway (1964); Lyons *et al.* (1993)
	Ostertagia circumcincta	Herd *et al.* (1984)
	Trichostronglyus colubriformis	Herd *et al.* (1984)
Uruguay	*Haemonchus* spp.	Nari *et al.* (1996)*
	Ostertagia spp.	Nari *et al.* (1996)*
	Trichostronglyus spp.	Nari *et al.* (1996)*
Zimbabwe	*Haemonchus contortus*	Boersema and Pandey (1997)
	Cooperia spp.	Mukaratirwa *et al.* (1997)*

* Species not identified

Table 1.5: Incidence of Levamisole/Morantel/Pyrantel resistance.

LOCALITY	PARASITE	REFERENCE
Argentina	*Haemonchus* spp.	Eddi *et al.* (1996)*
	Ostertagia spp.	Eddi *et al.* (1996)*
	Trichostronglyus spp.	Eddi *et al.* (1996)*
Australia	*Haemonchus contortus*	Green *et al.* (1981); Edwards *et al.* (1986)
	Ostertagia circumcincta	Sangster *et al.* (1979); Le Jambre (1979)
	Ostertagia trifurcata	Le Jambre (1979)
	Trichostronglyus colubriformis	Sangster *et al.* (1979); Love *et al.* (1992)
	Trichostrongylus vitrinus	Beveridge *et al.* (1990)
Brazil	*Haemonchus contortus*	Santiago *et al.* (1979); Charles *et al.* (1989)
	Ostertagia circumcincta	Santiago *et al.* (1979)
	Trichostronglyus colubriformis	Santiago and da Costa, (1979)
	Oesophagostomum columbianum	Charles *et al.* (1989)
	Strongyloides papillosus	Charles *et al.* (1989)
Czechoslova-kia	*Ostertagia* spp.	Varady *et al.* (1993)*
	Trichostronglyus colubriformis	Varady *et al.* (1993)*
Denmark	*Ostertagia* spp.	Bjorn *et al.* (1991)*; Maingi *et al.* (1996)*
	Trichostronglyus spp.	Maingi *et al.* (1996)*
India	*Haemonchus contortus*	Uppal *et al.* (1992); Yadav *et al.* (1993)

Kenya	*Haemonchus* spp.	Maingi (1991)*
	Trichostrongylus colubriformis	Waruiru *et al.* (1998)
	Oesophagostomum spp.	Waruiru *et al.* (1998)*
Malaysia	*Haemonchus contortus*	Chandrawatani *et al.* (1999)
New Zealand	*Haemonchus* spp.	Kettle *et al.* (1983)*
	Ostertagia circumcincta	Pomroy *et al.* (1992)
	Ostertagia trifurcata	Pomroy *et al.* (1992)
	Trichostronglyus colubriformis	McKenna and Seifert (1985)
	Nematodirus spathiger	Middelberg and McKenna (1983)
Paraguay	*Haemonchus* spp.	Maciel *et al.* (1996)*
	Ostertagia spp.	Maciel *et al.* (1996)*
	Trichostronglyus spp.	Maciel *et al.* (1996)*
South Africa	*Haemonchus contortus*	Van Wyk *et al.* (1989b); Van Wyk *et al.* (1997)
	Ostertagia circumcincta	Van Schalkwyk and Shroder (1989)
	Trichostronglyus colubriformis	Van Wyk *et al.* (1990)
UK	*Ostertagia* spp.	Britt (1986)*
	Trichostronglyus axei	Britt (1986)
USA	*Haemonchus contortus*	Miller *et al.* (1987)
Uruguay	*Haemonchus* spp.	Nari *et al.* (1996)*
	Ostertagia spp.	Nari *et al.* (1996)*
	Trichostronglyus spp.	Nari *et al.* (1996)*
Zimbabwe	*Haemonchus contortus*	Boersema and Pandey (1997)

* Species not identified

Table 1.6: Incidence of Ivermectin resistance.

LOCALITY	PARASITE	REFERENCE
Argentina	*Haemonchus* spp.	Eddi *et al.* (1996)*
	Ostertagia spp.	Eddi *et al.* (1996)*
	Trichostronglyus spp.**	Eddi *et al.* (1996)*
Australia	*Haemonchus contortus*	Le Jambre (1993a)
	Ostertagia spp.	Besier and Wroth (1993)*
Brazil	*Haemonchus contortus*	Echevarria *et al.* (1991); Vieira *et al.* (1992)
	Trichostronglyus spp.**	Echevarria *et al.* (1996)
Czechoslova-kia	*Ostertagia* spp.	Varady *et al.* (1993)*
	Trichostronglyus spp.**	Varady *et al.* (1993)*
Denmark	*Ostertagia* spp.	Maingi *et al.* (1996)*
	Trichostronglyus spp.**	Maingi *et al.* (1996)*
Kenya	*Haemonchus contortus*	Mwamachi *et al.* (1995); Waruiru *et al.* (1998)

Malaysia	*Haemonchus contortus*	Chandrawathani *et al.* (1999)
New Zealand	*Ostertagia circumcincta*	Pomroy *et al.* (1992); Watson *et al.* (1996)***
	Ostertagia trifurcata	Pomroy *et al.* (1992)
Paraguay	*Haemonchus* spp.	Maciel *et al.* (1996)*
	Ostertagia spp.	Maciel *et al.* (1996)*
	Trichostronglyus spp.**	Maciel *et al.* (1996)*
South Africa	*Haemonchus contortus*	Van Wyk *et al.* (1989a)
	Ostertagia circumcincta	Reinecke *et al.* (1991)
UK	*Ostertagia circumcincta*	Jackson *et al.* (1992)
USA	*Haemonchus contortus*	Craig and Miller (1990)
Uruguay	*Haemonchus contortus*	Nari *et al.* (1996)*

* Spp. not identified; ** small percentage of larvae in faecal cultures; *** Side resistance to moxidectin

Table 1.7: Incidence of Phenothiazine and Salicylanilides resistance.

LOCALITY	DRUG	PARASITE	REFERENCE
India	Rafoxanide	*Haemonchus contortus*	Singh *et al.* (1996)
South Africa	Rafoxanide	*Haemonchus contortus*	Van Wyk and Gerber (1980)
	Closantel	*Haemonchus contortus*	Van Wyk *et al.* (1982)
	Nitroxinil	*Haemonchus contortus*	Van Wyk *et al.* (1997)
	Dinitrophenol	*Haemonchus contortus*	Van Wyk *et al.* (1997)
United States	Phenothiazine	*Haemonchus contortus*	Drudge *et al.* (1957a)
Zimbabwe	Rafoxanide	*Haemonchus contortus*	Boersema and Pandey (1997)

1.6. MULTIPLE RESISTANCE

Anthelmintic resistance is now not limited to a single parasite or single anthelmintic class, and there are now numerous reports of multigeneric resistance (Barton *et al.*, 1985; Martin *et al.*, 1985; Edwards *et al.*, 1986; McKenna, 1989; West *et al.*, 1989; Watson and Hosking, 1990; Hong *et al.*, 1992; Varady *et al.*, 1993; Mwamachi *et al.*, 1995) and/or multidrug resistance (Sangster *et al.*, 1979; Kettle *et al.*, 1983; Barton *et al.*, 1985; Edwards *et al.*, 1986; Van Wyk and Malan, 1988; Badger and McKenna, 1990; Watson and Hosking, 1990; Maingi, 1991; Reinecke *et al.*, 1991; Love *et al.*, 1992; Pomroy *et al.*, 1992; Uppal *et al.*, 1992; Varady *et al.*, 1993; Mwamachi *et al.*, 1995; Singh and Yadav, 1997; Van Wyk *et al.*, 1997; Waruiru *et al.*, 1998).

1.7. MECHANISM OF ANTHELMINTIC RESISTANCE

1.7.1. BENZIMIDAZOLES

The mechanism of resistance to benzimidazoles has been under intensive investigation in the past few years, and some progress in its understanding has been achieved. It is now clear that the mechanism of resistance in nematodes is based on the alteration in the benzimidazole-tubulin interaction. It has been observed that when given to nematode-infected animals at therapeutic dose rates, benzimidazole anthelmintic causes microtubules to disappear from intestinal cells of benzimidazole-susceptible, but not from benzimidazole-resistant nematodes (Sangster *et al.*, 1985). Tubulin from resistant isolates of *H. contortus* and *T. colubriformis* has been shown to bind less benzimidazole than susceptible strains of the same parasite (Lacey and Prichard, 1986; Lacey and Snowdon, 1988). These findings were further supported by Lubega and Prichard (1990) who showed that binding of various benzimidazole compounds to nematode tubulin could be resolved to high-affinity and low-affinity components and they associated resistance in *H. contortus* with a loss of high affinity benzimidazole receptors on tubulin. Roos *et al.* (1990) extended this concept by using restriction mapping with cloned α- and β-tubulin genes from *H. contortus* as probes and they found no differences in α-tubulin from benzimidazole-susceptible and -resistant populations but they did find differences in β-tubulin. They identified between two and six β-tubulin fragments, designated as isotype I, in susceptible worm populations whereas only one to two of isotype I β-tubulin fragments were present in resistant worms. Further, they found that a 9 kb fragment was always present in resistant populations and in some susceptible populations. However, further selection of this resistant strain again produced a single 9 kb fragment (Kwa *et al.*, 1993). Therefore, a higher level of resistance could not be correlated with additional change in β-tubulin, suggesting other mechanisms may play a role.

A second β-tubulin isotype, designated as isotype 2, was isolated by Geary *et al.* (1992b). But they could not differentiate in bands between resistant and susceptible populations using an isotype 2 probe in restriction mapping analysis. However, Kwa *et al.* (1993) using an isotype 2 probe found that in the most resistant populations, isotype 2 genes were deleted. Similar results with *T. colubriformis* demonstrated that

highly resistant worms deleted the gene coding for isotype 2 (Le Jambre, 1993b; Roos *et al.*, 1995).

Molecular studies, using an allele-specific polymerase chain reaction (PCR), have demonstrated that all benzimidazole-resistant isolates of *H. contortus*, *O. circumcincta* and *T. colubriformis* examined have a phenylalanine (Phe) to tyrosine (Tyr) mutation at amino acid 200 in isotype 1 β-tubulin (Kwa *et al.*, 1994; Elard *et al.*, 1996). A similar change of Phe to Tyr at position amino acid 200 sequence of the corresponding gene has been shown to confer resistance to benzimidazole anthelmintics in the free living nematode *C. elegans* and in the fungus *Aspergillus nidulans* (Kwa *et al.*, 1993; 1994).

It is concluded that benzimidiazole pressure selects for change in β-tubulin in two ways i.e. (1) selection of a pre-existing 9 kb isotype 1 β-tubulin gene (2) elimination of isotype 2 genes in worms with higher level of resistance.

1.7.2. LEVAMISOLE

The mechanism of levamisole resistance in nematodes in not fully understood. A reduced number of acetylcholine binding sites were found in mutant strains of *C. elegans* resistant to levamisole and morantel, compared with susceptible strains (Lewis *et al.* 1980; Lewis *et al.*, 1987). A similar reduction in acetylcholine binding sites was found in levamisole-resistant *H. contortus* (Sangster *et al.*, 1988). In a binding experiment with *H. contortus*, Sangster *et al.* (1998) further extended this concept and they found two binding sites; high affinity binding site and low affinity binding site. In contrast to *C. elegans*, the high affinity binding site did not appear to be altered in resistant isolates of *H. contortus*. On the other hand, affinity to a second, lower affinity binding site was altered. Resistant worms had a lower affinity for drug at this site but also had more binding sites than susceptible worms. It was concluded that this resistant isolate of *H. contortus* could overcome the effects of levamisole by having more receptors (low affinity) available for normal transmitter function.

In a molecular approach, sequences of ligand binding receptor subunits of acetylcholine of levamisole-susceptible *T. colubriformis* (Wiley *et al.* 1996) and *H. contortus* (Hoekstra *et al.* 1997) have been compared with sequences from levamisole-

resistant worms of the same species. Resistant worms had no mutations, which would be responsible for resistance. However, Fleming *et al.* (1997) found that a point mutation was present in a non-ligand binding subunit of the acetylcholine receptor from the levamisole-resistant strain of *C. elegans*.

It is concluded that the alteration in low affinity site and abundance in homogenates of levamisole resistant *H. contortus* compared with levamisole susceptible worms may reflect true differences between the resistant and susceptible isolates, which are relevant to the mechanism of resistance. Confirmation of the relevance of these changes requires further physiological and molecular studies.

1.7.3 IVERMECTIN

The mechanism of ivermectin resistance in nematodes is not fully understood and limited literature is available on a possible mechanism of ivermectin resistance. In a binding experiment, Rohrer *et al.* (1994) found no difference in the affinity or density of ivermectin binding sites of ivermectin-resistant and ivermectin-susceptible *H. contortus*. Ivermectin/milbemycins appear to have two sites of action in parasites, one is the pharynx and another is somatic muscle (Gill and Lacey, 1998). Different phenotypic responses were demonstrated, using larval motility and larval development assay, in ivermectin-resistant strains of *H. contortus, O. circumcincta* and *T. colubriformis*, both between and within species (Gill and Lacey, 1998). It has also been suggested that selection with sub optimal doses produces a different mechanism of avermectin resistance as compared to selection with the recommended dose (Gill *et al.*, 1998). Their study demonstrated that in the two laboratory-selected ivermectin-resistant isolates of *H. contortus*, the LC_{50} or LP_{50} values of different analogues of avermectins were almost similar to ivermectin-susceptible strain in a larval development and larval motility assay. However, the third ivermectin-resistant field isolate showed a marked increase in LC_{50} or LP_{50} values as compared to a susceptible strain with several different analogues of avermectins in a larval development and larval motility assay. Surprisingly, in one laboratory-selected ivermectin-resistant strain of *T. colubriformis*, resistance was not associated with the somatic muscle site in larval motility assay, but worm expulsion following ivermectin treatment appeared to involve effects on somatic muscle (Gill and Lacey, 1998). The above information

suggests that there are two non-equivalent ivermectin receptor sites but avermectin resistance could manifest itself in different ways.

In a study with insects, Scott (1989) has suggested that two mechanisms are effective in producing abamectin-resistance in one strain of *Musca domestica*. The two mechanisms are decreased cuticular penetration of the drug and increased drug metabolism. However, Scott *et al.* (1991) were unable to demonstrate a metabolism component associated with resistance in a second resistant strain of *M. domestica* and they suggested the high level of resistance was not typical of a decreased cuticular penetration mechanism. Therefore, another altered target site was suggested for resistance.

Recently, molecular studies have shown that an alteration in P-glycoprotein gene structure and/or its transcription may be involved in resistance to ivermectin in *H. contortus* (Xu *et al.*, 1998; Le Jambre *et al.*, 1999; Sangster *et al.*, 1999). P-glycoproteins are conserved transmembrane proteins of the adenosine triphosphate binding cassette superfamily which have a role in transporting compounds, including drugs across membranes.

The mechanism of ivermectin resistance is quite complex, with mechanism varying both within and between species. Therefore, no firm conclusions can be drawn from the available literature. Further work is needed to understand the multiple mechanism of ivermectin resistance.

1.8. DIAGNOSIS OF ANTHELMINTIC RESISTANCE

Anthelmintic resistance has been detected in most parts of the world, including New Zealand, and the need for diagnostic techniques which can estimate both the extent and the development of the resistance has been emphasied by Presidente (1985) and Taylor and Hunt (1989).

1.8.1. *IN VIVO* TESTS

Two *in vivo* tests have been used to diagnose anthelmintic resistance; faecal egg count reduction test and controlled efficacy test.

1.8.1.1. Faecal egg count reduction test

The faecal egg count reduction test (Boersema, 1983; Presidente, 1985) estimates anthelmintic efficacy by comparing the faecal egg counts of animals before and after treatment. An untreated control group may also be included to monitor the changes in egg count during the treatment period. This test is widely used as a measure of treatment success and this continues to be a mainstay due to their relative ease and versatility. The World Association for the Advancement of Veterinary Parasitology has recommended that faecal egg count reduction and egg hatch assays should be used in monitoring for resistance in nematodes (Coles *et al.*, 1992).

Nevertheless, because of poor sensitivity, faecal egg count reduction tests are not reliable tests for detecting the level of anthelmintic resistance. Sangster *et al.* (1979) found a good correlation between faecal egg counts and worm counts of *H. contortus* but not for *T. colubriformis*. The correlation was not as good for *O. circumcincta* (Martin *et al.*, 1985). Resistance for minor species in the mixed population may be missed using this technique when major species vastly outnumber them (Presidente, 1985; Martin *et al.* 1985). Egg counts for *Nematodirus* spp. are generally low and may not be a reliable indicator of infection (Kingsbury, 1965). If the interval between the treatments is less than 10 days (longer interval may be required for ivermectin; Jackson, 1993), egg production may be suppressed, leading to overestimation of anthelmintic efficacy (Martin *et al.*, 1985). Hence, the precision of faecal egg count reduction tests has been questioned (Obendorf *et al.*, 1991). Larval cultures can be used in conjunction with faecal egg count reduction tests to determine the species involved with resistance (McKenna, 1997), but culture conditions may favour the development of one species over another (Presidente, 1985; Dobson *et al.*, 1992b) and highly fecund nematode species may mask species with low fecundity (West *et al.*, 1989).

Different methods for calculating faecal egg count reduction have been described (Presidente, 1985; Vizard and Wallace, 1987; Anderson *et al.*, 1988; Dash *et al.*, 1988; Butler, 1989; Coles *et al.*, 1992; Bjorn *et al.*, 1991). These methods differ in four main principles: (1) the use of arithmetic versus geometrical mean faecal egg counts, (2) the use of pre-treatment and post-treatment egg counts compared to the use of only post-

treatment egg counts, (3) inclusion of a control group egg counts and (4) level of faecal egg count reduction at which resistance is declared.

Dash *et al.* (1988) and McKenna (1990) have considered the use of arithmetic mean versus geometrical mean in the calculation of faecal egg count reductions. Dash *et al.* (1988) concluded that the arithmetic mean was the most appropriate in the calculation of faecal egg count reductions for detection of anthelmintic resistance. This was supported by McKenna (1990) who suggested that not only is the arithmetic mean simpler to calculate and better suited for comparative purposes, it is also likely to provide a more conservative and truer measure of anthelmintic efficacy.

The level of faecal egg count reduction, at which resistance is declared, is determined from a cut-off point. If geometrical mean egg counts are used in the calculation, a reduction of less than 90% is generally accepted as indicating resistance (Edwards *et al.*, 1986; Vizard, 1986), and reductions of 90-96% suggest emerging resistance and the need for further testing (Vizard, 1986). The same cut-off point of 90% is suggested when arithmetic mean egg counts are used (Riffkin *et al.*, 1984; Webb and Ottaway, 1986; Coles *et al.*, 1992).

Another important parameter is the interval between the collection of pre-treatment and post-treatment faecal samples. Benzimidazoles may temporarily reduce nematode egg output, so sampling before 7 days could give a falsely favorable impression of anthelmintic efficacy (Dash *et al.*, 1988). The suppression of faecal egg counts may be even longer (at least 14 days) in some ivermectin-resistant strains of *O. circumcincta* (Jackson and Coop, 1995) and *H. contortus* (Le Jambre, 1993a) following treatment. Therefore, faecal samples taken before 14 days after treatment may not contain eggs from some nematodes, which are resistant to ivermectin.

1.8.1.2. Controlled efficacy test

The controlled efficacy test involves infecting groups of animals with third stage infective larvae and treating them with an anthelmintic when the parasites have reached the adult stage (usually after 21 days for sheep and goat nematodes). The animals are then slaughtered 10 to 14 days after treatment and their worm burdens counted. One group of animals is left untreated. By treating at different drug

concentrations, an ED_{50} (the concentration of drug required to kill 50% of parasites) can be calculated. The guidelines of the World Association for the Advancement of Veterinary Parasitology for evaluating anthelmintic efficacy using the controlled test have been published (Wood *et al.*, 1995) but do not stipulate an arbitrary level at which anthelmintic resistance is declared.

In addition to sheep and goats, other *in vivo* models like rodents have been used to assess anthelmintic resistance of parasites. The only models of this type used to date to assess resistance of suspect field strains compared to strains known to be susceptible to the drugs are jirds infected with *T. colubriformis* (Ostlind *et al.*, 1990) or *H. contortus* (Conder *et al.*, 1991) and guinea pigs infected with *T. colubriformis* (Kelly *et al.*, 1981a) or *H. contortus* (Rolfe, 1990). The resistance factors for resistant strains relative to susceptible strains for these *in vivo* models are more closely parallel to the situation in the field than the *in vitro* tests. Although, these models are available for all classes of drugs, they are expensive and labour intensive relative to *in vitro* tests. Moreover they require animal use (Conder and Campbell, 1995) and are not available for all parasites.

The controlled efficacy test is a reliable method of assessing anthelmintic efficacy against mixed nematode infections but is also the most expensive and time consuming (Boersema, 1983). In addition, efficacy tests, as they are generally run, are incapable of detecting resistance until it is well established. Therefore, it cannot be recommended for the routine diagnosis of anthelmintic resistance.

1.8.2. *IN VITRO* TESTS

Many *in vitro* tests have been developed to diagnose the degree of anthelmintic resistance. These vary in their utility and applicability to either field diagnosis of resistance or specific research studies. The following *in vitro* tests have been used to detect anthelmintic resistance.

1.8.2.1. Egg hatch test (Benzimidazole)

The principle of the egg hatch test is based on the ovicidal properties of the benzimidazoles and the ability of eggs from resistant strains to embryonate and hatch in higher drug concentrations than the susceptible strains. The percentage of eggs that

hatch (or die) at each concentration is determined and a dose response curve of percentage egg hatch (or die), corrected for the natural mortality of untreated eggs, is plotted against drug concentration. Transformation of data either by log probit (Healy, 1988), or arcsine (Cawthorne and Whitehead, 1983) is required to obtain a linear regression from which ED_{50} (Concentration of the drug required to kill 50% of nematode eggs) can be calculated. When log probits are used, the drug concentration is usually logarithmically transformed and the ED_{50} is referred to as LC_{50} (the log concentration that prevent 50% eggs from hatching). By maintaining reference strains of known susceptible and resistant parasites, the responses of test isolates can be compared and the resistance ratio calculated. The culture of recovered first stage larvae to third stage allows the species of nematode involved to be determined.

A number of egg hatch assays/embryonation assays have been developed for detection of resistance to benzimidazole anthelmintics (Le Jambre, 1976; Coles and Simpkin, 1977; Hall *et al.*, 1978 and Whitlock *et al.*, 1980). Boersema (1983) has reviewed the variations between the methodology. The comparison between these benzimidazole egg hatch assays is shown in the Table 1.8 (Johansen, 1989).

Table 1.8: Comparison between the methodologies of egg hatch assay for benzimidazole.

VARIABLES	LE J	LE J	C& S	HALL	WHIT	WHIT	DON
Single (S) or mixed species (M)	S	S	S	S	S	M	S
Fresh (F) or embryonated eggs (E)	E	F	F	F	F	F	F
Pure (P) or commercial (C) drug	P	P	P	C	P	P	P
Drug dilution (D) or fixed drug conc. (F)	D	D	D	D	F	D	D
Incubation temp. (°C)	26	26	27	26	26	26	27
Incubation time (hrs)	24	72	24	24	24	24	48
Larvae(L) or embryonated eggs and Larvae (T)	L	L	T	L	L	L	L

Le J: Le Jambre (1976), C&S: Coles and Simpkin (1977), Hall: Hall *et al.* (1978): Whit: Whitlock *et al.* (1980), Don: Donald *et al.* (1980)

The main disadvantage of egg hatch assay in routine diagnosis, has been the requirement for undeveloped eggs. This is because the benzimidazoles act during the

early part of development. As embryonation proceeds beyond the ventral indentation stage, a false positive result may be obtained because the eggs become less sensitive to this group of anthelmintics. Thus the egg hatch test should be carried out within 4 hours of faecal collection (Coles and Simpkin, 1977). As benzimidazoles affect anaerobic metabolism, the reduced susceptibility of embryonated eggs to benzimidazole anthelmintics (thiabendazole) could be due to the fact that embryonated eggs have a greater capacity for, and do utilize aerobic metabolism, to a greater extent than do unembryonated eggs (Weston *et al.*, 1984). Whitlock *et al.*, (1980) recommended recovery of nematode eggs by sugar floatation and their incubation with a range of concentrations of anthelmintic in McCartney flasks before transportation back to the laboratory. A different approach to prevent the development of nematode eggs in transit to the laboratory is by storing faecal sample at 4°C for up to 3 days (Smith-Buijs and Borgsteede, 1986). Comparable results have been obtained by storing the faecal samples in polythene bags with air excluded (Presidente, 1985). An anaerobic storage of nematode eggs for up to seven days has been described by Hunt and Taylor (1989).

Another important variation that should be considered during interpretation of egg hatch assays is whether both embryonated eggs and larvae, or simply only larvae are to be counted to evaluate the effects of the drug. All the workers apart from Coles and Simpkin (1977) counted only the number of hatched larvae, which is a faster procedure but can give misleading results. Boersema (1983) found that even in a susceptible strain, only a fraction of embryonated eggs actually hatched and this proportion decreased with increasing drug concentration, whereas in a resistant strain nearly all the eggs which embryonated succeeded in hatching. Therefore, if only a percentage of hatched eggs is counted, the resistance ratio (EC_{50} resistant/EC_{50} susceptible) is likely to be much lower than if a percentage of embryonated eggs and larvae are counted.

The use of thiabendazole as the test drug in egg hatch assays to detect anthelmintic resistance has been justified because side resistance occurs within this group of anthelmintics (Martin *et al.*, 1985). It has been found that eggs from susceptible nematodes rarely hatch at concentrations of more than 0.1 μg/ml of thiabendazole (Whitlock *et al.*, 1980). This concentration is often used as the discriminating dose for

determining whether a parasite is susceptible or resistant to the benzimidazole group of anthelmintics. Fenbendazole is not suitable for use in this test because of low solubility (Lacey and Prichard, 1986). The test was also found to be unsuitable for oxfendazole, to which fenbendazole is metabolised in the host. An ovicidal effect on a benzimidazole-resistant strain of *H. contortus* could not be detected up to a concentration of 320 μg/ml oxfendazole, and at concentrations greater than 160 μg/ml a precipitate formed (Boersema *et al.*, 1982).

Borgsteede and Couwenberg (1987) suggested that the LC_{50} is not suitable for indicating the level of resistance of adult worms. They recorded changes in LC_{50} in an *in vitro* egg development assay during the patent period of susceptible and resistant strains of *H. contortus* in sheep. The LC_{50} followed a parabolic pattern in both susceptible and resistant strains. Another experiment showed similar results with a *H. contortus* strain with no previous exposure to benzimidazole compounds (Kerboeuf and Hubert, 1987).

A number of questions concerning the interpretation of egg hatch assay were raised by Kerboeuf *et al.* (1989) in relation to the age of the parasite and levels of infectivity. They studied the susceptibility of eggs from parasites of varying infectivity. Storing larvae at 4°C for different periods resulted in different infectivity levels. The highest LC_{50} values were recorded after one month of storage before infection and changes in the LC_{50} values were observed during the course of infection as described previously (Borgsteede and Couwenberg, 1987; Kerboeuf and Hubert, 1987).

The LC_{50} values of thiabendazole against susceptible *H. contortus* strains used by different workers were compared by Boersema (1983) and he found variations in LC_{50} values from 0.023 to 1.1 ppm. Le Jambre (1976) found some between-day differences in the LC_{50} and attributed it to between-day differences in the ambient temperature during the hour spent collecting the faeces from sheep. Repeated trials within laboratories using the same resistant nematode strain showed variations in the standard error of resistant ratios from 8 to 37% (Boersema *et al.*, 1982). This was due to variations in the methods used and the use of different susceptible strains as controls. It has been proposed that a standard reference strain should be used, which could act as a valid means for inter-laboratory comparisons (Waller and Prichard, 1986).

An egg development assay for evaluation of benzimidazole resistance in *Nematodirus spathiger* was developed (Obendorf *et al.*, 1986) using basic protocols of the egg hatch test of Whitlock *et al.* (1980). Further work can be undertaken using field isolates of *Nematodirus* species to determine its usefulness as a means of diagnosing benzimidazole resistance in *Nematodirus* species.

The egg hatch assay (benzimidazole) is simple, fast and the results are obtained in 1-3 days. The qualitative method (Whitlock *et al.*, 1980) has been evaluated as a routine diagnostic technique in mixed nematode populations and was found to be more rapid and economical (Presidente, 1985). Under field conditions, the egg hatch assay provides a good correlation with faecal egg count reduction test (Maingi *et al.*, 1996). The egg hatch assay has been claimed to be the only practical definitive test for confirming benzimidazole resistance in equines (Whitlock *et al.*, 1980). On the other hand it is better suited for research than routine diagnosis because it is technically demanding, presents difficulties with interpretation when mixed infections are involved and requires freshly collected or well stored eggs (Donald, 1985).

1.8.2.2. Egg hatch assay (Levamisole)

A levamisole egg hatch assay was developed by Dobson *et al.* (1986). The technique was tested using known levamisole-resistant and -susceptible strains of sheep trichostrongylid nematodes. The percentage of eggs failing to hatch at each drug concentration was calculated and results were subjected to probit analysis. Corrections were made both for presence of unhatched eggs in control wells and for eggs, which had hatched before the addition of levamisole. A variable incubation period depending on the species tested is required for the eggs to develop to fully embryonated pre-hatch stage before drug dilutions are added. They suggested that the optimum time for the addition of drug is approximately 1 hour prior to commencement of hatching, which is when the first stage become transparent and larvae are actively moving within the egg envelope. However, if hatching has occurred then it should be estimated and corrected for, in the analysis of data. This pre-hatch stage can be achieved in 15 hr for *Ostertagia* spp. and 16 hr for *Trichostrongylus* spp. and *H. contortus* at an incubation temperature of 26°C. Following the addition of anthelmintic, another 6 hr incubation is required before the assay is terminated by snap freezing for 5 minutes prior to adding a

drop of chilled formaldehyde to each well and the assays are stored at 4°C. Chilling is required to prevent the larvae from curling which make it difficult to distinguish them from unhatched larvae, and storage at 4°C is required because it takes some time for formaldehyde to exert its toxic effect. Waller *et al.* (1986) substituted morantel for levamisole in this assay to determine the level of resistance to morantel but the toxic effects on eggs unrelated to resistance occurred at concentrations exceeding 64 μg/ml of morantel.

The levamisole egg hatch assay is rapid, takes two days to obtain the results and is accurate and sensitive but timing of addition of the drug and termination is crucial. There are difficulties in interpretation of results with mixed infections and inter-laboratory comparisons of results are not valid. Thus, in its present form, it cannot be recommended for field screening for resistance (Johansen, 1989).

1.8.2.3. Larval paralysis and motility assay

A larval paralysis assay to detect levamisole and morantel resistance was first developed by Martin and Le Jambre (1979). The principle of the assay is to determine the percentage of paralysed third stage larvae following exposure to serial dilution of anthelmintic for 24 hours. A dose response curve is plotted and compared with resistant strains. There are a number of disadvantages of this technique. Firstly, high concentrations of levamisole are less effective than lower concentration in paralysing third stage larvae (Barton, 1983) because of reversibility of paralysis at high concentrations of levamisole (Boersema, 1983). Secondly, there is some subjectivity in judging whether or not a larva is paralysed and the assay cannot be preserved for counting at a later time. Thirdly, the test is time dependent. The optimum incubation time for this assay is 24 hr (Martin and Le Jambre, 1979), but deviations from this, particularly if a longer time is chosen, will result in a rapid change in the percentage of motile larvae between susceptible and resistant strains (Johansen, 1989). However, Geerts *et al.* (1989) reported good repeatability of the test with any differences being attributed to the age of larvae. Their results indicated that incubation time (24, 48 or 72 hr), incubation temperature (20 or 25°C) and the observation period of larvae (5 or 15 second) had no statistically significant influence on the test results.

In an another study, Gill *et al.* (1991) described a larval paralysis/motility assay for detection of ivermectin resistance in *H. contortus*. They detected inhibition of larval motility of L_3 larvae incubated in the dark on agar matrix containing ivermectin, by failure of affected larvae to move when stimulated by exposure to light. Ivermectin potency was quantified after three cycles, each involving storage in the dark for 24 hr followed by brief exposure to light. A 50% inhibition of motility (LP_{50}) was achieved with ivermectin concentrations between 0.30 and 0.49 μm when an ivermectin-susceptible strain was used, while LP_{50} of ivermectin-resistant strain ranged from 0.8 to 2.6 μm depending on the *in vivo* resistance status of the isolate.

A modification of the larval paralysis assay, suitable for detecting thiabendazole resistance was described by Sutherland and Lee (1990). Infective larvae of trichostrongyle nematodes were incubated in eserine, an acetylcholinesterase inhibitor. The thiabendazole-resistant larvae were paralysed more slowly than susceptible strain. These differences were related to the presence of higher levels of acetylcholinesterase in the thiabendazole-resistant strains than susceptible strains.

The micromotility meter, an instrument for measuring the motility of larvae and adult nematodes after incubation with anthelmintics was developed by Bennet and Pax (1986). The instrument uses microprocessor technology to measure light reflection at the miniscal interface. Movement of larvae in the solution is claimed to alter the angle of light refraction entering the photodiode. The deviation is measured on a computer to give a motility index. Micromotility meter was used to ascertain drug effects of four commercial anthelmintics on *H. contortus* larvae (Folz *et al.*, 1987). Albendazole, levamisole and ivermectin were active at four concentrations (50, 100, 150 and 200 μg/ml). The fourth anthelmintic, coumaphos, was active at only the two highest concentrations. All four anthelmintics significantly affected the motility.

Another study (Coles *et al.*, 1989b) made electronic measurements of motility of third stage larvae of a susceptible and a levamisole/benzimidazole resistant strain of *H. contortus* after incubation in solutions of cambendazole, fenbendazole and levamisole for 24 hrs. Results confirmed resistance to benzimidazole but failed to show differences in response to levamisole.

The main disadvantage of the micromotility meter in testing anthelmintic activity is that in some cases the host metabolism may be involved in activating a compound. Conversely host metabolism can also deactivate some compounds. However, all major anthelmintics, when exposed to helminths maintained in culture, will depress their motility (Bennett and Pax, 1986). Moreover, although the instrument measures helminth activity quantitatively, it is not capable of differentiating between helminth paralysis and mortality and both effects would give a significant response in the assay. Theoretically a drug could alter the parasite function and not motility; consequently, initial minor effects may not be observed, although chronic major effects would usually result in a significant motility (motor activity) change, the later being the situation with all effective anthelmintics (Folz *et al.*, 1987). The micromotility meter does not appear to have been developed further.

Another movement assay, migration inhibition assay, based on the ability of treated larvae to move from one chamber through a mesh sieve into second chamber was described by Wagland *et al.* (1992) and Rothwell and Sangster (1993). In this assay culture-derived late third stage or fourth stage larvae of *H. contortus* were used. This assay was useful for detecting resistance to each of the three classes of modern broad spectrum anthelmintics, as well as salicylanides for which none of the other *in vitro* assay has proven useful (Rothwell and Sangster, 1993). Unfortunately the assay's complexity may limit its use.

1.8.2.4. Larval development assay

The general principle of the larval development assay is to incubate the eggs in serial dilutions of a range of anthelmintics. The number of eggs, first, second and third stage larvae are counted and their percentage is determined. Variations of the larval development assay have been described by Coles *et al.*, (1988), Giordano *et al.*, (1988), Stringfellow (1988), Lacey *et al.*, (1990), Taylor (1990), Hubert and Kerboeuf (1992) and Gill *et al.*, (1995). Most of these have included successive refinement to the assay protocol.

The technique was originally described by Ibarra and Jenkins (1984). In this technique the media used for cultivation of larvae consisted of one part distilled water and one part faecal suspension of rat faeces rich in bacteria. They found that the development

of larvae was effective and assay was suitable for assessing activity of broad-spectrum anthelmintics including benzimidazoles but unsuitable for narrow-spectrum anthelmintics. Another study described the use of a nutritive medium consisting of Earle's balanced salt solution and yeast extracts (Hubert and Kerboeuf, 1984). They found the development of larvae was better than using rat faeces. Another variation in the larval development assay was described by Coles *et al.* (1988) in which first stage larvae were cultured to third stage larvae in the presence of heat treated lyophilized *Escherichia coli*, as a food source and the anthelmintic under test. The test was able to differentiate between benzimidazole-resistant and -susceptible strain of *H. contortus*. This was further successfully used for detection of ivermectin resistance (Giordano *et al.*, 1988).

A microlarval development assay was developed for the detection of anthelmintic resistance in *H. contortus*, *O. circumcincta* and *T. colubriformis* (Hubert and Kerboeuf, 1992). Eggs were cultured to first stage larvae in the presence of Earle's balanced salt solution, yeast extract and bacteria in a total volume of 100 µl. Then 50 µl of serially diluted anthelmintic was added in each tube and the development was continued up to the third stage. They could generate good dose response curves with thiabendazole, levamisole, pyrantel tartrate and ivermectin allowing the determination of 50% lethal concentration and a resistance factor when resistant strains were available.

A modification of this test using yeast extract as a food source was described by Taylor (1990). The utilization of agar matrix containing the drug by Lacey *et al.* (1990) and Gill *et al.* (1995) apparently increases the value of the assay for compounds such as ivermectin where solubility in the liquid medium as used in other assays of this type may be a problem. The nematode eggs were placed on an agar matrix containing serial dilutions of an avermectin in the wells of a microtitre plate (Gill *et al.*, 1995). Development was allowed to proceed for 6 days at 25°C by which time larvae in the control wells had reached the infective stage (L_3) stage. The number of eggs, L_1, L_2 and L_3 larvae at each drug concentration were counted after staining with Lugol's iodine. This was corrected against the number in the control wells and a log-dose response curve was fitted to calculate the LC_{50} values. Ivermectin-susceptible *H. contortus* isolates were equally sensitive to inhibition by avermectins.

In contrast several ivermectin-resistant isolates of *H. contortus* showed reduced sensitivity to avermectins inhibition of development. The resistance ratio (LC_{50} of resistant strain/ LC_{50} value of susceptible strain) for ivermectin-resistant isolates was dependent on avermectin structure, with avermectin B_2 the most sensitive probe of the analogues tested.

The main limitation in interpreting the results of larval development assay is the variation in the LC_{50} values during the course of infection. Such variations were demonstrated with ivermectin against *T. colubriformis* (Hoza, 1998) and *O. circumcincta* (Amarante *et al.*, 1997). This may have some implications for the use of this assay in the field. However, this limitation can be overcome with the use of avermectin analogues, which discriminate better between ivermectin-resistant and ivermectin-susceptible strains. It has been shown that avermectin B_2, ivermectin aglycone and ivermectin monosaccharide discriminate better between ivermectin-resistant and susceptible strains of *H. contortus*, *O. circumcincta* and *T. colubriformis*, respectively as compared to other avermectin analogues (Gill *et al.*, 1995; G.C. Coles personal communication; Gill and Lacey, 1998).

The larval development assay besides having the ability to detect resistance to all three classes of modern anthelmintics, has an added advantage of not requiring undeveloped eggs and of more easily allowing identification of the resistant species. (Coles *et al.*, 1988; Gill *et al.*, 1995). As eggs are easily obtainable in large numbers from faeces, it is possible for field workers to determine resistance for themselves. Since the test relies on the effect of anthelmintic on growth of first stage larvae, the age of the faecal sample is not critical as in the egg hatch assay. The faecal samples may be preserved up to 7 days and yet their eggs remain viable for the test. The identification and specification of resistant individuals is more easily performed on third stage larvae in this test than on eggs or first stage larvae (Coles *et al.*, 1988; Taylor, 1990). The larval development assay may be applicable to screen benzimidazole resistance in the field even if a mixed population is involved (except for *Nematodirus* spp.) (Johansen, 1989; Taylor, 1990). However, problems of bacterial overgrowth of the agar in the wells and a poor dose response with levamisole may cause difficulties with interpretation of results.

1.8.2.5. Adult development assay

Nematode culture techniques now permit the development of infective third stage larvae to adult parasite *in vitro* for several species of nematodes (Taylor and Hunt, 1989). Stringfellow (1984, 1986) cultured *H. contortus* to adult egg laying stages. An *in vitro* adult development assay for detecting benzimidazole resistance in *H. contortus* is based on these *in vitro* techniques and has been described by Stringfellow (1988). Using susceptible and resistant strains of *O. circumcincta* and *H. contortus*, the incorporation of an anthelmintic into growth medium at the early fourth stage of development, suppressed further development of the susceptible strains of the parasites whereas the resistant parasites were able to complete their development to late fourth and adult stages (Taylor and Hunt, 1989).

1.8.2.6. Biochemical tests

A tubulin binding assay was developed by Lacey and Snowdon (1988). In this assay, a crude extract of tubulin was made from infective larvae and then incubated with tritiated labeled benzimidazole until the equilibrium was reached. The free unbound drug was then removed with charcoal leaving tritium-benzimidazole-tubulin complex to be estimated using liquid scintillation spectrometry. Bound radioactivity was calculated and standarized to pmole mg^{-1} for each of the isolate, by dividing by the protein concentration assayed. The extent of resistance was expressed as susceptibility factor (SF) i.e. ratio of resistant isolate binding to susceptible binding using McMaster susceptible strains as the standard (SF=1.00). Different susceptible and resistant isolates of *H. contortus*, *T. colubriformis* and *O. circumcincta* were examined and the assay showed lesser SF for resistant isolates and higher SF for susceptible isolates.

The tubulin binding assay is very rapid, sensitive and accurate. The test can be adopted in field conditions as it is standardized and inter-laboratory comparisons can be made with greater confidence than other *in vitro* techniques. However, there are disadvantages of this test. The test requires the use of expensive laboratory instruments and work with radioactive isotopes and is very time consuming.

Another study (Sutherland and Lee, 1989) described a simple colorimetric assay in which samples were compared either by visual examination or through the use of a

densitometer, involving a comparison of nonspecific esterases and acetylcholinesterases of benzimidazole-resistant and -susceptible trichostrongylid strains. The assay showed significantly more nonspecific esterase of acetylcholinesterase activity in the infective stage larvae of benzimidazole-resistant strains compared with susceptible strains.

1.8.2.7. Genetic assays

Genetic assays are limited and only available for detecting benzimidazole resistance where resistant populations have been identified using cloned β-tubulin probes with restriction mapping (Le Jambre, 1990; Roos *et al.*, 1990) or by isoenzyme analysis using isoelectric focussing (Sutherland *et al.*, 1988). An isoelectric analysis could be useful in identifying ivermectin-resistant strains of nematodes (Echevarria *et al.*, 1992). However, this was not possible if benzimidazole resistance was also present as elevated enzyme levels due to the benzimidazole resistance masked the difference between ivermectin-susceptible and -resistant strains.

A new molecular technique for the diagnosis of benzimidazole-resistant natural populations of *O. circumcincta* was developed by Elard *et al.* (1999). The technique was based on the use of the polymerase chain reaction and allowed the genotyping of individual resistant (rr) or susceptible (rS and SS) adult worms or larvae.

None of the available biochemical or genetic tests are conducive to field use, due to the expense and technical requirements. Being very specific in nature, most of these assays are also not useful in assessing resistance to the spectrum of drugs available (Conder and Campbell, 1995).

Waller (1986) compared the then available techniques for detection of anthelmintic resistance. The evaluation was based on a literature review and was presented in tabular form whereby scores corresponding to advantages were ascribed to each technique. Johansen (1989) also made an evaluation but these two did not differ significantly. Waller (1986) tended to give a high rating to the levamisole egg hatch assay and tubulin binding assay and a lower rating to egg hatch assay (benzimidazole) but Johansen (1989) gave a higher rating to egg hatch assay (benzimidazole) compared

with levamisole egg hatch assay and tubulin binding assay. They both gave larval development assay a lower rating (Table 1.9).

More recently, Varady and Corba (1999) compared the six *in vitro* assays in determining benzimidazole and levamisole resistance in *H. contortus* and *O. circumcincta*. The degree of resistance to thiabendazole and levamisole was compared by (1) an egg hatch assay, (2) an egg hatch paralysis assay, (3) a larval development assay, (4) a larval paralysis assay, (5) a larval paralysis assay with physostigmine and (6) larval micromotility assay. They concluded that the egg hatch assay and larval development assay were the most suitable *in vitro* methods for field screening of anthelmintic resistance in sheep.

Table 1.9: Evaluation of different techniques used to detect anthelmintic resistance (Waller, 1986 and Johansen, 1989).

The higher the numbers better the procedure in relation to particular attribute. The numbers in left parenthesis are the ratings given by Johansen (1989) and number in the right parenthesis are the ratings given by Waller (1986).

PROCEDURE	ACCURACY	SENSITIVITY	SIMPLICITY	RAPIDITY	LABOUR	EQUIPMENT	APPLICABILITY
FECR	(2) (2)	(2) (2)	(3) (3)	(2) (1)	(2) (1)	(4) (3)	(4) (4)
EHT	(3) (3)	(3) (3)	(3) (2)	(4) (3)	(3) (2)	(3) (2)	(2) (2) BZ only
EHL	(2) (3)	(2) (3)	(3) (2)	(3) (3)	(2) (2)	(3) (2)	(2) (2) Lev only
TBA	(3) (3)	(3) (3)	(2) (2)	(2) (3)	(2) (3)	(1) (1)	(2) (2) BZ only
LM	(1) (1)	(1) (1)	(4) (4)	(2) (4)	(2) (2)	(2) (2)	(3) (3)
FC/LD	(3) (3)	(3) (3)	(3) (2)	(2) (1)	(3) (2)	(3) (2)	(4) (2)
CAET	(4) (4)	(4) (3)	(2) (3)	(1) (1)	(1) (1)	(1) (1)	(4) (4)

FECR = Faecal egg count reduction test; EHT = Egg hatch assay-thiabendazole; EHL= Egg hatch assay-levamisole

TBA = Tubulin binding assay; LM = Larval motility assay; FC/LD = Faecal culture/larval development assay

CAET = Controlled anthelmintic efficiency test

1.9. GENETICS OF ANTHELMINTIC RESISTANCE

Anthelmintic resistance develops in a population of nematodes because of selection acting on the variation between individuals within the population. If a dose rate of chemical is used which discriminates between individuals within the normal phenotypic distribution for resistance, then one can predict a genetic response that is polygenic. This response occurs because there are many genes responsible for differences in resistance between individuals of phenotypic distribution. An example of this type of selection is the frequent use of non-persistent anthelmintics in the field. Anthelmintic treatment at the recommended dose rate generally removes 99% of a previously unselected population. A small number of worms may survive treatment, probably as the result of the ability to reduce energy demands, using other reserves of energy, switching to alternate energy pathways or by reducing uptake of anthelmintics (Le Jambre, 1985). The survivors are the individuals, which have the greatest proportion of resistance alleles in the population. If the treatment with the same anthelmintic at the same dose rate is repeated a number of times then it will lead to build-up of resistance alleles. The changes in resistance status over subsequent generations will reflect changes in the frequency at several loci leading to the development of polygenic resistance. This has been shown in populations of worms, which are benzimidazole resistant (Le Jambre *et al.*, 1979b; Martin *et al.*, 1988b). However, genetic studies with levamisole resistance in *T. colubriformis* (Martin and McKenzie, 1990) and ivermectin resistance in *H. contortus* (Dobson *et al.*, 1996; Le Jambre *et al.*, 2000) suggest that resistance to these anthelmintics in these species is controlled by single gene. Therefore, selection from within the normal distribution does not preclude the chance of a single gene inheritance of resistance.

Laboratory selection usually results in a polygenic response where the selecting dose rate used is lethal to about 95% of the population (McKenzie, 1985). In addition, laboratory populations are of limited size and thus chance of a rare allele for resistance being present is much less than in large field populations.

Doses of the drug selecting outside the normal phenotypic distribution are expected to select for single gene response. This results because selection acts on rare variants which lie outside the normal distribution of phenotypes for resistance (McKenzie,

1985). An example of this type of selection is the action of persistent insecticides used in the field. At the time of treatment, the concentrations used are lethal to 100% of a susceptible population. Over time the concentrations of the insecticide decreases which allows individuals carrying a rare mutation for resistance to survive (McKenzie, 1985). Single gene responses are usually seen in field selection.

The resistant gene may be dominant or recessive with respect to fitness in the presence of the anthelmintic, and this may be determined by the dose rate used. If a dose rate is used that kills susceptibles but allows resistant heterozygote and homozygote individuals to survive then resistance is effectively dominant with respect to fitness. If a higher dose rate is used, which leaves only homozygous-resistant individuals surviving, then resistance is recessive with respect to fitness. The latter is preferred in terms of worm control programs, as this will limit the rate at which resistance evolves from a susceptible population (Georghiou and Taylor, 1986). An example of these type of responses is the action of ivermectin and moxidectin against nematodes. It has been suggested on the basis of data from efficacy studies of ivermectin and moxidectin against ivermectin-resistant strains *of H. contortus* and *O. circumcincta* that resistance with respect to fitness in the presence of anthelmintic is dominant with ivermectin and recessive with moxidectin in these species (Barger, 2000). It appears that the gene that controls ivermectin resistance also controls moxidectin resistance (Shoop *et al.*, 1993). However, it does not confer an equal degree of resistance to the two anthelmintics. On the basis of published reports, Barger (2000) calculated an average efficacy of 44% for ivermectin against ivermectin-resistant strains of *H. contortus* and *O. circumcincta* but moxidectin was 95-100% effective. Therefore, ivermectin has a markedly reduced efficacy against heterozygotes and leaves behind homozygotes, allowing the heterozygotes to pass their resistance to the next generation. However, moxidectin removes most of heterozygotes and leaves behind homozygotes. Hence with ivermectin, the resistance exhibited is a dominant trait but with moxidectin it is a recessive trait (Barger, 2000).

Studies on genetics of resistance in nematodes are few compared to studies that have been performed in arthropods. The study of the genetics of anthelmintic resistance in nematodes has been made easier with the development of *in vitro* assays, such as the larval development assay which monitors inhibition of development in the free living

larval stages (Gill *et al.*, 1995). These assays are cheaper and easier to perform than *in vivo* efficacy studies.

1.9.1. BENZIMIDAZOLES

Resistance to benzimidazoles was the first to be studied genetically and an earlier report has shown it to be inherited as an incompletely dominant trait in *H. contortus* (Le Jambre *et al.*, 1979b). Another report suggested cambendazole resistance in *H. contortus* was probably inherited recessively (Herlich *et al.*, 1981). Different conclusion reached between these studies probably reflects the influence the genetic and environmental factors contribute to the resistance. Thiabendazole resistance in *T. colubriformis* is inherited as an incompletely recessive character with an apparent maternal effect (Martin *et al.*, 1988b). All these studies indicated that more than one gene was involved in the fullest expression of resistance to the benzimidazoles. More recent work has shown that at least two loci are involved in benzimidazole resistance in *H. contortus* and *T. colubriformis* and resistance is a recessive trait (Roos *et al.*, 1993; Grant, 1994).

1.9.2. LEVAMISOLE

Further anthelmintic selection on a levamisole-resistant strain of *T. colubriformis* supported the view that resistance to levamisole was under the control of a single dominant gene (Waller *et al.*, 1985). Using a statistical model, Dobson *et al.* (1987) also concluded that a single dominant gene determined levamisole resistance. However, it was previously recognised that the data may also fit a polygenic model (Dobson and Griffiths, 1985). Another study concluded that the high level of levamisole resistance in *T. colubriformis* is inherited as a sex-linked recessive character and is probably controlled by a single gene or tightly linked group of genes, whereas under low selection pressure levamisole resistance showed a polygenic control mechanism (Martin and McKenzie, 1990).

The different conclusions reached between these studies indicate some limitations in the modelling approach or perhaps there was a similar response to selection unrelated to the degree of dominance/recessiveness of the resistance alleles and their association with the X chromosome. Moreover, Waller *et al.* (1985) and Dobson *et al.* (1987)

measured the response to selection using an *in vitro* egg hatch assay on sexually undifferentiated eggs and larvae, so different responses between males and females could not be detected.

The resistance to levamisole is inherited differently in *H. contortus* as compared to *T. colubriformis*. Dobson *et al.* (1996) showed that levamisole resistance in *H. contortus* is inherited as an autosomal recessive trait and is not sex linked. Although levamisole resistance is inherited as recessive trait in both *T. colubriformis* and *H. contortus* but It is observed that levamisole resistance is common in *T. colubriformis* but is rare in *H. contortus* in the field (Waller *et al.*, 1995). Male nematodes are heterogametic having one X-chromosome and no homologue (XO) and females are homogametic having two X-chromosomes (XX). This means the sex linked recessive character is recessive in females but effectively dominant in males as they have only one copy of the X-chromosome. The completely recessive character as found with levamisole-resistant *H. contortus* responds to selection much less effectively than does the sex-linked recessive resistances (Dobson *et al.*, 1996). This difference in inheritance of levamisole resistance between *T. colubriformis* and *H. contortus* may be responsible for the difference in prevalence of levamisole resistance found for *T. colubriformis* and *H. contortus*.

1.9.3. IVERMECTIN

Because ivermectin resistance has arisen relatively recently, few genetic studies have been performed with resistant nematodes. Studies with *H. contortus* indicate that, in contrast to benzimidazoles and levamisole resistance, in this species at least, ivermectin resistance is inherited as a completely dominant trait and is controlled by a single gene (Dobson *et al.*, 1996; Le Jambre *et al.*, 2000). The level of resistance in the first generation (F_1) of the reciprocal crosses of resistant and susceptible nematodes in a larval development assay were indistinguishable from that of the ivermectin-resistant parent which indicated that the trait is completely dominant. Another study on the basis of limited data has indicated that resistance is also inherited as a completely dominant character in *O. circumcincta* (Coles, 1997). In a laboratory selected ivermectin-resistant strain of *T. colubriformis*, resistance is suggested to be inherited as a partially dominant trait that does not appear to be under the control of a

single gene (Gill *et al.*, unpublished in Gill and Lacey, 1998). However, given the selection process which led to the selection of this isolate, the possibility that more than one resistant mechanism is represented among the individuals in this isolate cannot be discounted (Gill and Lacey, 1998).

1.10. LIFE CYCLE OF NEMATODE PARASITES

It is important to review the life cycle of *T. colubriformis* and *O. circumcincta* in order to understand their population dynamics in the host and on pasture.

The life cycle of nematode parasites is well documented (Soulsby, 1982). Nematodes have both free-living and a parasitic stage in their life cycle (Fig. 1.1). Sexually mature males and females mate in the gut of the host. Females produce eggs, which are passed in the faeces of host. The free-living (pre-parasitic) stages then commence and development from the eggs to the unhatched larval stages occurs quickly if the environment is moist (about 80% relative humidity) and warm (optimum temperature 25 -27^{O}C). The eggs then hatch into first stage larvae (L_1). If the conditions are unfavorable for hatching, *Trichostrongylus* spp. and *Ostertagia* spp. can survive at the unhatched egg stages for several weeks (Anderson, 1983; Young, 1983). The first larval stages feed on bacteria, grow and moult into second larval stages (L_2). The second stage larvae undergo a further process of feeding, growing and an incomplete moult in which the cuticle separates but it is not shed resulting in the ensheathed third stage larvae (L3) which cannot feed. These larvae survive on stored energy. The time for development from eggs into infective larvae (L_3) varies greatly, from a number of days to numbers of weeks, and depends on the species of nematode and environmental conditions.

Migration of third stage larvae from the faeces onto the herbage occurs by way of water films and under the influence of light (Rogers and Sommerville, 1963). From here they can be ingested by the grazing host (e.g. Sheep) and the parasitic stage begins. The gastrointestinal tract of the host provides a chemical stimulus, so that the protective sheath of the larvae is shed and larvae become associated with the mucosal lining of gut (Dunn, 1978). Inhibition of larvae may occur, a process whereby the larvae remain dormant within or on the surface of the mucosa for up to several months with no further development. A proportion of these larvae will eventually mature to

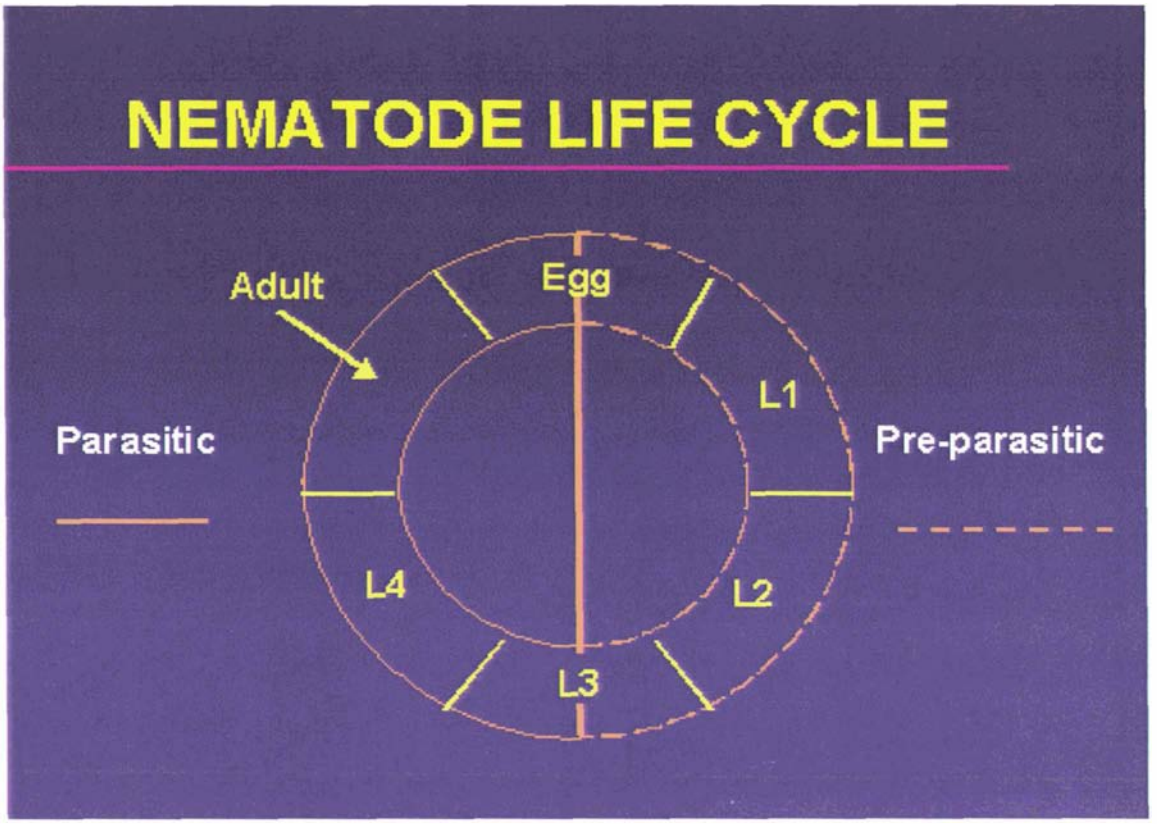


Figure 1.1: Life cycle of nematode parasites

adults. Inhibition allows the population to survive during environmental periods that are unfavorable to the free-living stages. *Ostertagia* inhibition commonly occurs during the winter/spring and *T. colubriformis* inhibits in winter (Waller *et al.*, 1981).

The larvae then feed, grow and moult into [illegible] that feed, grow, develop and moult into mature adults. Male and female worms mate and female worms lay eggs to complete the life cycle. The pre patent period is 18-21 days (Soulsby, 1982).

1.11. OSTERTAGIA VS TELADORSAGIA

The Generic level systematics of the sub-family Ostertagiinae remains complicated and, to a large extent, unresolved. A considerable divergence in opinion exists over the number of genera in the Ostertagiinae. Some studies recognised 17 genera (Gibson and Khalil, 1982), whereas others included only five to eight genera (Durette-Desset, 1983, 1989; Durette-Desset *et al.*, 1999) or seven genera (Jansen, 1989 cited in Lichtenfels and Hoberg, 1993). Current confusion relates to opinions over genera to be

included or excluded from Ostertagiinae and proposed synonymies for a number of genera.

The species of *Ostertagia circumcincta* was first placed by Drozdz (1965) (cited in Lancaster *et al.*, 1983) into the genus *Teladorsagia* according to the priority rule after proposing that the genus *Stadelmannia* (into which *O. circumcincta* had been placed by Sarwar (1956) was a synonym of *Teladorsagia*. However, by means of cross breeding experiments, Lancaster *et al.* (1983) demonstrated that *Ostertagia ostertagi*, *O. circumcincta* and *Ostertagia leptospicularis* are polymorphic species. The alternative morphs to these species are *Ostertagia lyrata* and *Ostertagia trifurcata*. A similar study (Daskalov, 1974) suggested that *O. circumcincta*, *Teladorsagia davtiani* and *O. trifurcata* are polymorphic species. In view of these findings, Lancaster *et al.* (1983) suggested that genus *Teladorsagia* should be abandoned and genus *Ostertagia* should be retained.

However, other studies (Gibson and Khalil, 1982; Durette-Dessett, 1983, 1989; Durette-Dessett *et al.*, 1999) placed *O. circumcincta* in genus *Teladorsagia* rather than in genus *Ostertagia*. These studies suggested that the *Ostertagia* spp. had a bursal configuration of 2-1-2 with middle ray longer than the one behind it (Fig. 1.2) and a prominent proconus e.g. *O. ostertagi/O. lyrata*, *O. leptospicularis/Ostertagia kolchida*, *Ostertagia gruehneri/Ostertagia arctica*, *Ostertagia mossi/Ostertagia dikmansi*, and *Ostertagia bisonis*. In contrast, *Teladorsagia* spp. had a copulatory bursal ray pattern of 2-2-1 (Fig. 1.2) and absence of a proconus e.g. *O. circumcincta*, *O. trifurcata*, *T. davtiani*. But the use of bursal ray configuration has been questioned by Lichtenfels and Hoberg (1993) who indicated that other genera with 2-2-1 configuration would be *Hyostrongylus*, *Spiculopteragia* and *Mazamastrongylus*, distinguished by the configuration of the spicules, accessory bursal membrane and cuticular ridge patterns (Jansen, 1986; Lichtenfels *et al.*, 1993). The use of the cuticular ridge patterns (Synlophes) has been suggested to differentiate the species of the genus *Ostertagia* and *Teladorsagia* (Lichtenfels *et al.*, 1988b). The synlophe of species (*Teldorsagia circumcincta*, *Teladorsagia trifurcata* and *T. davtiani*) in genus *Teldorsagia* was identical to each other but was different from other species (*O. ostertagi* and *O. leptospicularis*) in genus *Ostertagia*. The synlophe of *O. ostertagi* and *O. leptospicularis* was also different from each other suggesting that more characters and

more species should be studied before any attempt is made at sorting out the generic groups of Ostertagiinae. It was recommended to use *Ostertagia*, *Marshallagia* and *Teladorsagia* genera in subfamily Ostertagiinae and the validity of other genera e.g. *Hyostrongylus*, *Spiculopteragia* and *Mazamastrongylus* needs to be firmly established by studying synlophe, genital cone, bursa and esophagus (Lichtenfels *et al.*, 1993).

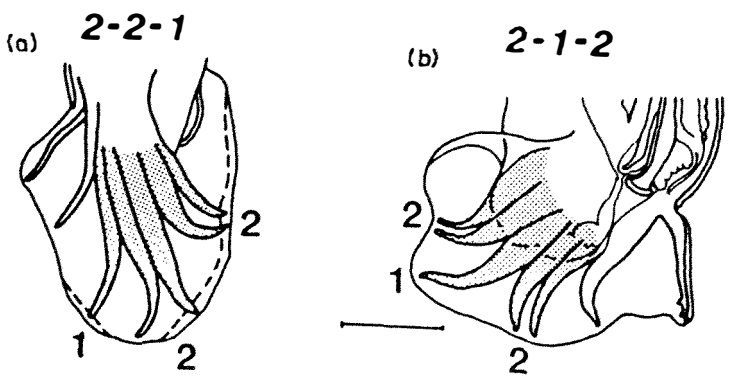


Figure 1.2: Bursal ray patterns in the Ostertagiinae. (a) Right lateral view showing 2-2-1 pattern of lateral rays as in *Teladorsagia* spp. (b) Left lateral view showing 2-1-2 pattern of lateral rays as in *Ostertagia* spp. (Lichtenfels *et al.*, 1988a).

Similarly, breeding experiments (Suarez and Cabaret, 1992) demonstrated interbreeding between *O. ostertagi* & *O. lyrata* and *O. ostertagi* & *O. leptospicularis* suggesting that they belong to same genus. However, interbreeding of *T. circumcincta* was not successful with *O. ostertagi* and *O. leptospicularis* but was successful *with T. trifurcata* suggesting that *T. circumcincta* and *T. trifurcata* belong to same species.

The validity of individual species within the genus *Teladorsagia* has been investigated using allozyme electrophoresis (Andrews and Beveridge, 1990). The absence of allelic differences among the species of genus *Teladorsagia* (*T. circumcincta*, *T. davtiani* and *T. trifurcata*) suggests that they belong to a single species. However in this study no comparison was made with the species of genus *Ostertagia*. Another allozyme study on the basis of allozyme frequencies suggested that species in genus *Ostertagia* (*O. ostertagi*, *O. lyrata*, *O. leptospicularis*, *O. kolchida*) were similar to each other but significantly different from *T. circumcincta* (Gasnier *et al.*, 1993). The

species of genus *Teladorsagia* (*T. circumcincta* and *T. trifurcata*) were similar to each other. This concept was further extended by using second internal transcribed spacer (ITS-2) sequence of members of genus *Teladorsagia* (*T. circumcincta*, *T. davtiani* and *T. trifurcata*) and of genus *Ostertagia* (*O. ostertagi* and *O. leptospicularis*)(Stevenson *et al*., 1996). They found no difference in ITS-2 consensus sequences for the three taxa within the genus *Teladorsagia* but there was a 13-15% difference between members of genus *Teladorsagia* and *Ostertagia* suggesting that *T. davtiani* and *T. trifurcata* are morphs of *T. circumcincta* and *Ostertagia* and *Teladorsagia* are different genera. However, they also recorded a 9% difference in ITS-2 consensus sequences for the two taxa within the genus *Ostertagia*. Therefore, the validity of these genera and other genera, and species composition of each genera still remains unresolved. Much work remains to be done before the definite conclusions are reached. It is hoped the molecular studies will provide some information in future.

If the information gained from the different studies of these species is added to characters used in their generic classification (Fig. 1.3) then species in the *Teladorsagia* genus appear to be more closely related to *Ostertagia* than some species of *Ostertagia* are to each other. Therefore, many researchers are still using genus *Ostertagia* for species *O. circumcincta* while others are using *Teladorsagia*. In the present studies the genus *Ostertagia* has been used for the species *O. circumcincta* and *O. trifurcata*.

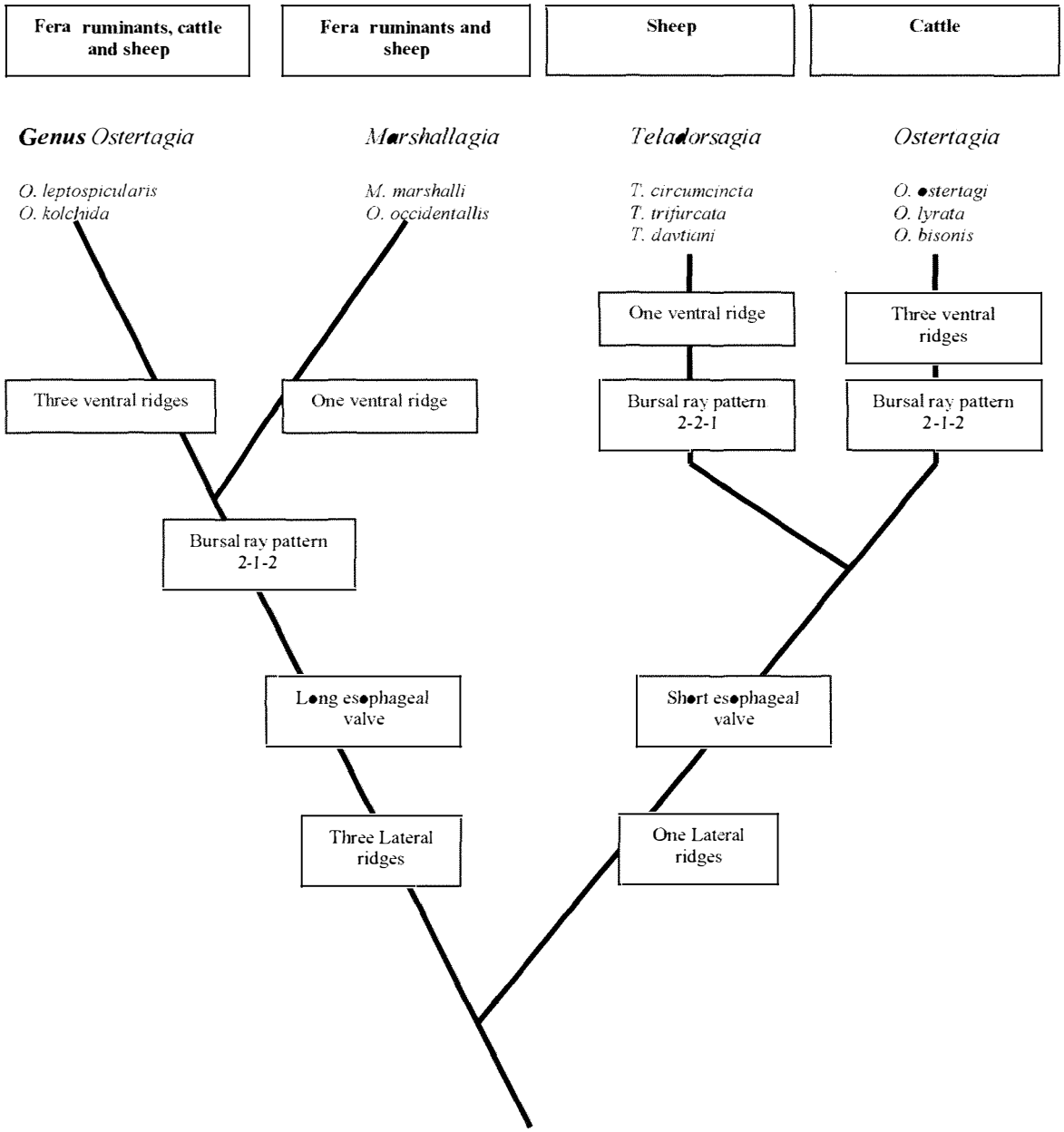


Figure 1.3: Dendrogram showing one possible classification of 10 species of Ostertagiinae parasitic in cattle, sheep and goats in North America, and characters employed (Lichtenfels *et al.*, 1988a).

1.12. POPULATION DYNAMICS OF *TRICHOSTRONGYLUS COLUBRIFORMIS* IN THE SHEEP

As the immune response develops, the nature of dynamics of *T. colubriformis* in sheep changes.

1.12.1. ESTABLISHMENT RATE

A detailed series of models to describe the population dynamics of *T. colubriformis* in sheep has been published (Dobson *et al.*, 1990d) based on several experiments (Dobson et al., 1990a, 1990b, 1990c). An establishment rate of approximately 65% of L_3 given in the first week in 21 weeks old previously uninfected sheep has been recorded (Dobson *et al.*, 1990a). This fell to low levels (<5%) after 7, 10, 14 weeks of continuous L_3 intake for high (2000 larvae/day for 5 days a week), medium (632 larvae/day for 5 days a week) and low (200 larvae/day for 5 days a week) infection rates respectively. At the low infection rate, establishment remained at maximum levels for the first 4 weeks but then fell at a rate similar to that observed for high infection rates. These results indicated that a threshold of worm exposure is required before resistance to establishment is developed (Dobson *et al.*, 1990a). The threshold worm burden (established adults) from the model of Dobson *et al.* (1990a) was estimated to be 3532. In lambs sensitised with irradiated larvae, Windon *et al.* (1984) estimated the threshold vaccine dose to be 4400 irradiated *T. colubriformis* given twice. However, only female worms establish after irradiation and so their threshold represented an established worm burden of approximately 2000-4000 female worms. Similarly, Dineen (1963) and Donald *et al.* (1964) also suggest that resistance to establishment occurs once the threshold of worm exposure has been reached. A similar threshold phenomenon was observed with *Trichostrongylus vitrinus* in lambs (Waller and Thomas, 1981). Donald and Waller (1982) also concluded from the data of Gibson *et al* (1970) that a single anthelmintic treatment of grazing sheep on contaminated pasture may remove a large *Trichostrongylus* spp. adult population which is not replaced and subsequent production losses may be reduced despite continuing exposure.

This concept of a threshold of antigenic stimulus for induction of immunity was further explored by Emery *et al.* (1992). They exposed sheep to multiple normal

infections of 30,000 *T. colubriformis* larvae over an 8 weeks infection period. The sheep showed suppressed establishment of larvae to reinfection. However, a series of larval infections with 10^5 *T. colubriformis* larvae truncated at 4 days period did not protect sheep against reinfection. It was suggested that there is a need for exposure to a critical amount of antigen, which is produced in larger quantities by adult worms before immunity to establishment is produced.

The age of the host also plays an important role in determining the establishment rate of *T. colubriformis* in sheep (Dobson *et al.*, 1990b). In their study, sheep of 12-36 weeks of age reared worm free in pens were infected with 2000 L_3 of *T. colubriformis* each weekday. The decrease in establishment rate was found to be faster in the older host compared to the younger host. Moreover, arrested development of the exsheathed L_3 stage was found to be less marked in older lambs. Sheep, which had experienced natural infection on pasture up to 20 weeks of age before exposure, in pens, to the same experimental infection as their worm-free counterparts reared in pens, showed a similar response. However, at 36 weeks of age pasture reared sheep had acquired a high level of resistance to infection with *T. colubriformis*.

The physiological status of the host has also been acknowledged to influence the establishment rate of *T. colubriformis* in sheep. The increased establishment rate to 65% around lambing is observed (Barnes and Dobson, 1990) and in normal conditions a return into full immunity (i.e.<1% establishment rate) takes most, if not all, of the lactation period (Barger, 1997). Similarly, another Australian study (O'Sullivan and Donald, 1973) showed that in pregnant and lactating Merino ewes, the establishment of *T. colubriformis* was consistently high (80-90%) over a 9-week period commencing 3-6 weeks prior to lambing. This is probably due to a prolonged loss of immunity to *T. colubriformis* over lactation in Merino ewes (Gibbs and Barger, 1986). In contrast to these observations, the establishment rate of *T. colubriformis* never exceeded 2% in Romney ewes around lambing (Leathwick *et al.*, 1999) after a single challenge. A study has demonstrated that loss of immunity around parturition is greater in the Merino than in other breeds of sheep (Southcott *et al.*, 1972). New Zealand breeds such as Romney lambs appear to be more resistant to parasitic infections than Merino lambs (Barger, 1997).

1.12.2. NEMATODE FECUNDITY

The quoted estimates of *T. colubriformis* egg production are 500-1200 eggs (Soulsby, 1982), 262 eggs (Coyne *et al.*, 1991) and 600 eggs (Dobson *et al.*, 1990a) per female worm/day. As the immune response develops fecundity declines. Parasite egg production, expressed as eggs per gram (EPG) of faeces was proportional to infection rate but estimates of fecundity in eggs per female/day showed the opposite relationship with infection rate (Dobson *et al.*, 1990a). In their study, fecundity stayed high for 5 weeks at a low infection rate (200 larvae/day for 5 days a week) but only maintained this level for 3 weeks and 1 week at medium (632 larvae/day for 5 days a week) and high infection rates (2000 larvae/day for 5 days a week), respectively (Dobson *et al.*, 1990a). These results suggest that fecundity, like establishment rate, is affected at threshold levels of immunological recognition. In contrast to some other nematodes, no density dependent effects on fecundity were observed (Dobson *et al.*, 1990d).

In single infections, under experimental conditions, Hoza (1998) observed low levels of egg production at the start of infection, which rose to a peak by 7-12 weeks after infection and then declined to low levels again. These findings reflect that immature and older nematodes produce less eggs than the mature nematodes.

1.12.3. EXPULSION OF ADULT WORMS

The rejection of adult *T. colubriformis* in lambs given 2000, 1124, 632 or 200 L_3 per day, 5 days per week for up to 20 weeks was examined (Dobson *et al.*, 1990c) and rejection of adult worms was observed over a period of about 9 weeks commencing when the establishment of incoming larvae declined to approximately 1%. The rejection of worms started 7-9 weeks after infection in the first three infection groups. There was a delay of about 5 weeks before the worm expulsion commenced in the lowest infection group and reached the same levels to those observed in high infection rates. The worm expulsion and decreased fecundity started at the same time (Dobson *et al.*, 1990d). In contrast, Chiejina and Sewell (1974) indicated that decreased fecundity preceded worm expulsion. The death rate of worms was largely controlled by host-derived factors and not worms age (Dobson *et al.*, 1990c). Therefore, once the threshold level is reached, the worm death rate will be independent of infection rate

and worm burden. In contrast, Dineen, (1963) indicated that *T. colubriformis* infection would be continually tolerated.

Similarly, another study with *T. colubriformis* suggested that worm loss occurred between 8-15 weeks (Chiejina and Sewell, 1974). In a study with a related parasite, six month old sheep were infected with 1000 *T. vitrinus* L_3 per day (Seaton *et al.*, 1989a) and total worm burdens were found to be 16910, 16054 and 1050 after 4, 8 and 12 weeks of infection, respectively. These findings suggested loss of adult *T. vitrinus* worms occurred between 8 weeks to 12 of infection.

1.13. POPULATION DYNAMICS OF *OSTERTAGIA CIRCUMCINCTA* IN THE SHEEP

As the immune response develops, the nature of dynamics of *O. circumcincta* in sheep changes.

1.13.1. ESTABLISHMENT RATE

Resistance to establishment of *O. circumcincta* occurs between four to eight weeks and the lambs become almost completely immune to incoming larvae by 12 weeks after infection. The first indication of immunity is the inhibition of developing *O. circumcincta* worms (Seaton *et al.*, 1989b). In this respect initial host response to *O. circumcincta* differs from that seen with *T. colubriformis* where resistance to establishment is the first sign of immunity. Inhibition of worms at the early L_4 stage is a well-recognised consequence of continuous infection with *O. circumcincta* (Gibson and Whitehead, 1981; Callinan and Arundel, 1982; Hong *et al.*, 1987).

The population dynamics of *O. circumcincta* in lambs following single infections of 3000, 10000 or 30000 third stage larvae was studied by Hong *et al.* (1986). The authors used the measurements of female worm lengths and observations on the incidence of reduced vulval flaps to show that the development of some of the worms was initially arrested and that subsequently adult worms which were lost were replaced by arrested forms resuming their development. In another study in lambs following daily infection with either 250, 500 or 1000 L3 of *O. circumcincta*, Hong *et al.* (1987) indicated that the numbers of worms were related to rate of intake of larvae. The observations of female worm length and incidence of reduced vulval flaps

indicated that the population of worms turned over rapidly. They observed a rapid decline in worm numbers and suggested that resistance to establishment of worms develops very rapidly. This differs from the development of a protective immunity in calves to *O. ostertagi* where a lengthy exposure is required before they become refractory (Michel *et al.*, 1973).

It has been shown that the concurrent burdens of *T. colubriformis* dramatically increase the worm burdens of *O. circumcincta*, possibly indicating a reduction in host resistance to the latter species due to the larger populations of *T. colubriformis* (Sykes *et al.*, 1988). However, Dobson *et al.* (1992a) demonstrated that population dynamics (establishment, worm burdens, egg counts) of neither *T. colubriformis* nor *O. circumcincta* was affected by concurrent infection with the other species. These authors showed that infection with *T. colubriformis* alone did not protect against establishment of *O. circumcincta*, but infection with *O. circumcincta* alone provided slight protection against establishment of *T. colubriformis*. They also indicated that there was no difference in the population dynamics of either species between single or concurrent infections at the infection rate and time used in their trial. In contrast to these findings, Douch (1989) immunized lambs with *T. colubriformis* L_3 and obtained 87% protection against *T. colubriformis* and 42% protection against *Ostertagia* spp. acquired during grazing.

The relaxation of immunity to *O. circumcincta* during lactation does not occur in Coopworth ewes (McAnulty *et al.*, 1991). However, the establishment rate of *O. circumcincta* was higher (6.1%) in recently lambed Romney ewes, than was establishment rate of *T. colubriformis* (1.6%) (Leathwick *et al.*, 1999) indicating that the relaxation of immunity is more profound with the abomasal than the small intestinal parasite species. Nevertheless, immunity in the lambing Romney ewes to *O. circumcincta* declines, it does not dissipate completely.

Recently, Niezen *et al.* (1998) have demonstrated that the establishment of *O. circumcincta* is also affected by the diet of the animal. In this study, lambs fed lotus (*Lotus pedunculatus*) had lower egg counts, lower *O. circumcincta* worm burdens and fewer female *O. circumcincta* than lambs fed ryegrass (*Lolium perenne*). The reasons for reduced establishment of *O. circumcincta* with the lotus diet are unknown.

1.13.2. NEMATODE FECUNDITY

In the lambs infected with 1000, 3000 and 5000 L_3 of *O. circumcincta* per day for 8 weeks, the mean eggs per female per day after 8 weeks was 274, 171 and 87 respectively (Coop *et al.*, 1977). Similarly, Michel (1969) has found that eggs per female of *Ostertagia ostertagi* reduced at higher infection rates and egg counts declined as infection progressed. In a study with *O. circumcincta*, the egg count was independent of infection rate (Symons *et al.*, 1981) and declined after 8 weeks. Similar decline in egg counts in lambs infected daily with 50, 250 or 2500 L_3 of *O. circumcincta* was observed (Gibson and Everett, 1978). During a study following daily infection with either 250, 500 or 1000 L_3 of *O. circumcincta* in lambs, peaks in egg counts followed by a rapid decline in egg counts were observed (Hong *et al.*, 1987). A similar pattern in mean egg count after an initial infecting dose of 1000 L_3 per day was observed with *O. circumcincta* by Seaton *et al.*, (1989b). It was suggested that both reduced fecundity and loss of adult worms were occurring simultaneously (Hong *et al.*, 1987). In contrast to these findings, Callinan and Arundel (1982) did not observe any trend in egg count and eggs per female.

1.13.3. EXPULSION OF ADULT WORMS

In a study of naturally acquired burdens of *O. circumcincta* in sheep, Waller and Thomas (1978a and b) observed that worm burdens of both tracer and setstocked lambs corresponded closely to the rise and fall in pasture larval availability and that over the season, tracer lambs continued to harbour worm burdens with females exhibiting prominent vulval flaps while the continuously grazed setstocked lambs were increasingly populated with worms with reduced flaps as the grazing season progressed. They concluded that worms were constantly being replaced and the turnover period was approximately at monthly intervals. Similarly, another experiment reported fewer large size worms at eight weeks than at four weeks after a daily infection of 1000 L_3 of *O. circumcincta* (Seaton *et al.*, 1989b) and suggested that worm loss and recruitment occurred simultaneously during this period. However, since most lambs were almost totally immune to incoming larvae by 12 weeks (Seaton *et al.*, 1989b), the duration of this turnover must had been short in the majority of individuals.

The results of an experiment in which lambs were given a single inoculum of *O. circumcincta* larvae (Hong *et al.*, 1986) suggested that a constant proportion of the worms are lost per unit of time. A study with *O. ostertagi* has proposed that populations in calves are regulated by a turnover of adult nematodes rather than by an increase in resistance to the establishment of newly ingested infective larvae (Michel, 1969). This may well apply to *Ostertagia* spp. in sheep.

1.14. POPULATION DYNAMICS OF *T. COLUBRIFORMIS* AND *O. CIRCUMCINCTA* ON PASTURE

1.14.1. DEVELOPMENT OF LARVAE

Many factors influence the development and survival of the free-living stages of nematodes but oxygen, temperature and moisture are amongst the most important limiting factors. Moisture is much less of a limiting factor than temperature (Gibson and Everett, 1967). There are many other factors, which influence the development of eggs and larvae such as whether a particular egg or larvae within a faecal pellet is located near the center or near the surface. Even outside the faeces a difference of a centimeter in distance above the soil surface, a difference in whether a larvae happen to be on grass, clover, dandelion or some other plant, a difference in exposure to sunlight, would influence development and survival of free living stages. All these factors have to do with what may be called nanoclimate rather than microclimate. These nanoclimate factors undoubtedly help to determine why some eggs fail to hatch and remain embryonated during warm periods (Levine and Andersen, 1973).

The macroclimatic meteorological parameters, which are generally recorded to measure the larval development, do not correspond to the microclimate conditions in the faeces or in the sward. Recently, a study in New Zealand has suggested that the temperature in the faecal mass during summer months is higher than the air temperature, which may influence the development and survival of larvae (Familton and McAnulty, 1995).

1.14.1.1. *T. colubriformis*

Amongst all the factors, oxygen, temperature and humidity are the most important factors (Andersen *et al.*, 1965). The minimum temperature for development of *T. colubriformis* lies within the range of 10-15^{O}C (Wallace, 1961; Gibson and Everett, 1967). At favorable temperatures a monthly minimum of approximately 50-mm of rainfall was necessary to permit the development of infective larvae (Gordon, 1948). The local climatic conditions play an important role in development of larvae of *T. colubriformis* to infective stages. When conditions are optimum, the maximum recovery of larvae of *T. colubriformis* takes place within 1- 2 weeks of eggs deposited in the United State of America (Andersen *et al.*, 1965; Levine and Andersen, 1973), Southern United Kingdom (Gibson and Everett, 1967) and in Northern United Kingdom (Boag and Thomas, 1970). However, the cold weather in winter months in the Australia, United Kingdom and United States of America prevented the eggs of *T. colubriformis* from developing to infective stages (Boag and Thomas, 1970; Beveridge, *et al.*, 1989; Callinan, 1979; Crofton, 1965; Gibson and Everett, 1967; Levine and Andersen, 1973; Southcott *et al.*, 1976; Waller *et al.*, 1981). But a study in New Zealand has suggested that considerable development occurs during winter months with both *T. colubriformis* and *O. circumcincta* (Familton and McAnulty, 1994). The development of the larvae during the rest of the year varies depending on the local climatic conditions.

The role of moisture in development of larvae of *T. colubriformis* to infective stages is also well established. A study in Fiji recovered fewer infective larvae of *T. colubriformis* and *H. contortus* throughout the year in the dry temperate region as compared to the moist temperate region (Banks *et al.*, 1990).

Clearly, the development of larvae is dependent on the local climatic conditions. Therefore, the worm control strategies should be based on the information obtained under local conditions and not from some quite different climatic conditions.

1.14.1.2. *O. circumcincta*

The eggs of *O. circumcincta* developed throughout the year in the United Kingdom (Gibson and Everett, 1972) even at somewhat lower temperatures than

Trichostrongylus spp. (Kates, 1950; Crofton, 1965). In a study conducted in laboratory, the eggs of *O. circumcincta* hatched in distilled water at temperatures of 4, 16, 25 and 35^{O}C. Hatching increased with increasing temperature but the optimum temperature for development to L_3 was 16^{O}C rather than 25^{O}C (Pandey *et al.*, 1989). The temperature for hatching and development also depends upon the origin of the isolate (Crofton and Whitlock, 1965). The Bristol (United Kingdom) isolate hatched between 4 and 34^{O}C whereas Ithaca (New York) isolate hatched only between 10 to 38^{O}C. The hatching period was more prolonged in the Ithaca isolate than Bristol isolate.

The local climatic conditions play an important role in development of larvae of *O. circumcincta* to infective stages. The development of the larvae of *O. circumcincta* was reduced during the winter months in the Southern United Kingdom (Gibson and Everett, 1972), whereas in Western Victoria in Australia summer was most disadvantageous (Callinan, 1978). Similarly, Kates (1950), in Maryland (U.S.A.), found that for experiments begun when weekly mean air temperatures were approximately 17, 21 and 26^{O}C, L_3 of *O. circumcincta* were first recovered from herbage at >6, 4 and 1 week but the corresponding temperatures in Victoria (Australia) were associated with yields of L_3 on herbage within 2 weeks (Callinan, 1978), if there was development at all.

Larval recoveries from indoor studies are usually high, up to 100% of the eggs used, while outdoor studies usually show low recovery rate. The maximum yield of *O. circumcincta* larvae on herbage in an outdoor experiment has been recorded to be 16% (Callinan, 1978). High yields (20%) of *Trichostrongylus* spp. have also been recorded (Donald, 1968) in outdoor trials. In contrast, Boag and Thomas (1975) have estimated that less than 1% *T. colubriformis* eggs ever become available as L_3 on herbage. The outdoor environments have marked fluctuations. Some authors have investigated the role of fluctuations in temperature in development. A study with *O. ostertagi* (Smith *et al.*, 1986) suggested that larvae took twice as long to develop in the field under fluctuating temperatures as compared to laboratory conditions at the same mean temperature. Similarly, Salih and Grainger (1982) suggested that fluctuating temperature slowed the development of *O. circumcincta* larvae even though the average temperature was the same as the constant temperature. The ultimate size,

which the larvae attain, was also affected by constant or fluctuating temperature. A study with *O. circumcincta* indicated that larvae were smaller when they were cultured from outdoors as compared to indoors (Gibson and Everett, 1972).

The season of the year also influences the availability of the type of nematode larvae on the pasture. The seasonal availability of infective larvae on New Zealand pastures was studied by using tracer lambs (Brunsdon, 1963). The succession of genera in order of peak occurrence was as follows: *Nematodirus* spp. in spring, *Ostertagia* spp. in late spring and summer, *Haemonchus* spp. in late summer and autumn, *T. axei* in autumn, *T. vitrinus*, *T. colubriformis* and *Cooperia curticei* in winter. In another study, a similar pattern was observed with the exception of *Nematodirus* in the Manawatu region in New Zealand (Tetley, 1959a, 1959b). A similar study (Southcott *et al.*, 1976) conducted in the New England region of New South Wales in Australia revealed *H. contortus* and *Nematodirus* spp. in summer, *Ostertagia* spp. in autumn, *Trichostrongylus* spp. in summer and *T. axei* in winter.

In a study which monitored the seasonal fluctuation in the number of larvae on herbage over a 3-year period, the availability of infective larvae followed a broad two-peak pattern (Vlassoff, 1973). The autumn peak was not always larger but was dominated numerically by *Trichostrongylus* spp. with *N. filicollis* next in all the three years. However, the spring peak was dominated numerically by *N. filicollis* and *Ostertagia* spp. with smaller numbers of *Trichostrongylus* spp.

1.14.2. FAECES AND ITS EFFECT ON LARVAL DEVELOPMENT AND SURVIVAL

The faecal pellets act as a store of infective larvae and larvae migrate from faeces which are responsible for maintaining the larval population on the herbage at steady levels for longer periods (Gibson and Everett, 1972; Levine and Andersen, 1973). Similarly, Callinan (1978) and Niezen *et al.* (1998) indicated that more rapid faecal degradation resulted in greater larval mortality and migration of larvae persisted as long as the faecal pellets persisted. Persistence of faecal pellets is greater in early winter when cold dry weather favours pellet preservation. The role of faeces as a reservoir of larvae of cattle nematodes has been well established (Anderson *et al.*, 1983; Barger *et al.*, 1984).

The form of sheep faeces, either pelleted, soft or liquid may have an impact on the rate and degree of development success. It has been suggested that diarrhoeic faeces are sprayed thinly on herbage or soil and the development success in dry conditions is poor, due to direct exposure to extreme temperatures and ultraviolet radiation. However, in warm, moist conditions the development success in loose faeces may be high (Shorb, 1943). Pelleted sheep faeces may fall to the base of herbage and development success may be higher because eggs and larvae are protected from ultraviolet radiation and extreme temperatures. Moreover, pellets are of such a size and moisture content that development of the eggs inside them is not restricted by lack of oxygen. In sheep faeces with a higher moisture content, which are not pelleted but still formed, larval development in the outer part of the faecal mass should proceed, but larval development deep within the mass may be reduced by lack of oxygen as occurs in cattle (Anderson *et al.*, 1983).

1.14.3. EFFECT OF HERBAGE SPECIES ON DEVELOPMENT OF LARVAE

Herbage species can significantly influence the number of larvae, which develop to third stage and migrate to herbage (Niezen *et al.*, 1998). The development success of larvae of *T. colubriformis* and *O. circumcincta* on herbage was greatest for Yorkshire fog, intermediate for ryegrass, cocksfoot, browntop and lucerne and lowest for tall fescue and white clover (Niezen, 1996). A similar study recovered more larvae from grass swards than from chicory swards after equal contamination of nematode eggs (Moss and Vlassoff, 1993). Another experiment showed high recovery rate of larvae from grass species than from legume species (Furman, 1944). However, the difference occurred only in winter, while in the summer larval recovery was greater on the white clover.

It has also been recognised that the conditions within swards play an important role in the development and survival of free-living stages (Brunsdon, 1982). As pasture plants have different growth patterns and widely contrasting morphologies (Lambrechtsen, 1981), it is likely they could alter the sward micro-climate, directly affecting larval development and survival or indirectly modifying the number of coprophagous or nematophagous organisms.

It has also been suggested that the type of ingested herbage species can affect the faecal moisture content (Niezen *et al.*, 1993), which will affect the larval development in faeces (Crofton, 1963). A study has demonstrated that adding maize grain to calf diets reduced the recovery of *T. axei* and *T. colubriformis* larvae from faeces (Ciordia and Bizzell, 1963) and they found similar effects if grains were added directly to the faeces.

1.14.4. SURVIVAL OF LARVAE

At low temperatures third stage trichostrongylid larvae survive on pasture for protracted periods, presumably due to the low rate of utilization of stored energy (Seghetti, 1948; Kates, 1950; Rose, 1963; Gibson and Everett, 1967; Donald, 1968; Boag and Thomas, 1970; Andersen *et al.*, 1970; Levine and Andersen, 1973; Gibson and Everett, 1976; Southcott *et al.*, 1976; Donald *et al.*, 1978). Similarly, a prolonged period of snow during winter tends to preserve the larvae rather than killing them (Gibson and Everett, 1967). As the temperatures and resultant metabolic rates rise, energy is utilized with increased speed, leading to a progressively reduced longevity (Kates, 1950; Levine, 1963; Rose, 1963; Andersen *et al.*, 1965; Anderson, 1972 & 1973; Callinan, 1978; Banks *et al.*, 1990). The larvae of *Ostertagia* spp. survive longer than *T. colubriformis* (Kates, 1950; Gibson and Everett, 1972; Pandey, 1972; Boag and Thomas, 1977; Waller and Thomas, 1978a; Boag and Thomas, 1985; Jasmer *et al.*, 1987). A trial carried out with *T. colubriformis* revealed that the optimum survival temperature of all free-living stages was 4^{O}C with 95% of infective larvae surviving for 312 days (Andersen *et al*, 1966). The infective larvae survive better than other free-living stages and embryonated eggs survive longer than unembryonated eggs (Stewart and Douglas, 1938; Andersen *et al*, 1966)

The infective larvae of *O. circumcincta* survive better at lower relative humidity (RH of 30 and 50%) compared to higher relative humidity (RH of 75 and 95%) at 16^{O}C, 25^{O}C and 35^{O}C (Pandey *et al.*, 1993). In other trichostrongylids such as *T. colubriformis* (Andersen and Levine, 1968) and *O. ostertagi* (Pandey, 1976), it has been shown that desiccated infective larvae survive better than non-desiccated larvae. However, age of the infective larvae (storage at 4^{O}C) of *T. colubriformis* can decrease their ability to withstand desiccation (Andersen and Levine, 1968).

Genetic differences between the different strains of the same species also influence the survival of infective larvae. The Weybridge isolate of *O. circumcincta* (Pandey *et al.*, 1993) is more resistant to low temperatures than the Washington (Jasmer *et al.*, 1987) and the California isolates (Furman, 1944).

Larvae can survive in soil and have been shown to reappear on herbage at various times (Bairden *et al.*, 1979; Al Saqur *et al.*, 1982). A study with *T. vitrinus* (Rose and Small, 1985) demonstrated that infective larvae buried in the soil of grass plots, to a depth of 10 cms, migrated to the herbage throughout the year. However, only a limited amount of upward migration of *T. colubriformis* larvae was observed (Sturrock, 1965). The type of the soil can also influence the upward migration of the larvae. In a study with *O. circumcincta*, Furman (1944) showed that larvae migrated upward most readily in sandy soil, less so in sandy loam and least in clay loam. They failed to migrate through dry soil.

1.14.5. MIGRATION OF LARVAE

Infective third stage larvae use the surface tension of a moisture films on leaves to migrate vertically on herbage (Croll, 1970). A higher relative humidity and higher temperature also favour the upward migration of larvae (Silangwa and Todd, 1964). Moisture above 0.15 ml/cm^2 on the leaf surface will obstruct the migration of most common species of gastrointestinal nematodes (Rogers, 1940). The amount of moisture on the leaf surface also have differential effect on migration of different nematode species; for example, *H. contortus* require less moisture on the leaf surface to migrate than *O. circumcincta* (Rogers, 1940). A good pasture transmission for *T. colubriformis* migration occurred when total monthly precipitation was more than 25 mm and the mean monthly temperature at the soil surface beneath 7-10 cm vegetation was above 16^{O}C (Levine and Andersen, 1973). Similarly, Wallace and Doncaster (1964) demonstrated that migration of *T. colubriformis* was most rapid in water saturated soil at 20^{O}C.

The type of herbage species has also been shown to influence larval migration. The vertical migration of L_3 of *T. colubriformis* and *O. circumcincta* was found to be better on lucerne and white clover whereas few larvae migrated up the swards of Yorkshire fog (Niezen *et al.*, 1998). These differences were considered to be due to different

amounts of moisture on the leaf surfaces of different plant species (Niezen *et al.*, 1998). Similar studies also observed that larvae could migrate further on some grass species than others (Crofton, 1948; Knapp, 1963; Silangwa and Todd, 1964; Moss and Vlassoff, 1993).

1.15. FITNESS OF ANTHELMINTIC-RESISTANT NEMATODES

There are very few reported studies of fitness differences between resistant and susceptible genotypes of nematodes. Most studies are aimed at investigating the fitness status or pattern of reversion to susceptibility, generally involves benzimidazole-resistant strains. A fitness study with benzimidazole-resistant *H. contortus* showed that the benzimidazole-resistant strains were more infective for sheep than the benzimidazole-susceptible strain. Faecal egg output, development and survival of eggs and free-living stages on pasture were also higher in the benzimidazole-resistant strains (Kelly *et al.*, 1978). Similarly, a higher establishment rate was recorded in sheep infected with phenothiazine-resistant *H. contortus* as compared to phenothiazine-susceptible strain (Drudge *et al.*, 1957b). Recently, in a fitness study of benzimidazole-resistant and -susceptible worms of *O. circumcincta*, a PCR technique was used to determine the genotypes (RR, SS, RS) and fitness of each was compared within the same strain (Elard *et al.*, 1998). There was no significant difference in egg production, development rate from to eggs to infective larvae under laboratory conditions, establishment rate at 35 days post infection, survival rate of adult worms in lambs at 60 days post infection and survival of infective larvae at 8^{O}C in laboratory conditions. In contrast to these findings, Maingi *et al.* (1990) demonstrated that thiabendazole-susceptible strain of *H. contortus* had a higher establishment rate, faecal egg count and more severe pathology than a thiabendazole-resistant strain of *H. contortus*. However, further selection of thiabendazole-resistant strain for four generations produced similar establishment rate, faecal egg counts and pathology comparable to those produced by a susceptible strain.

In a study with *H. contortus*, the control of a resistant strain on pasture was tried by replacing the resistant-strain with a susceptible strain (Van Wyk and Van Schalkwyk, 1990). Reversion to susceptibility occurred in only three out of five camps indicating that fitness of the resistant genotype was not at a selective disadvantage. Similarly, in a

field study, Martin *et al.* (1988a) did not observe any significant reversion to susceptibility of a benzimidazole-resistant strain of *Ostertagia* spp. over 4-years with no anthelmintic selection indicating that the resistant phenotype was as fit as the susceptible phenotype. A similar study showed that on a farm where benzimidazole resistance was encountered for the first time in 1980, a resistant population was still present in 1988, despite the use of levamisole for 6 years (Borgsteede and Duyn, 1989). Another experiment conducted to investigate the potential for benzimidazole-resistant strains of *H. contortus and T. colubriformis* to revert towards susceptibility monitored the changes in anthelmintic resistance status throughout 12 generations (Hall *et al.*, 1982). No reversion towards susceptibility was recorded for either nematode species. The studies with insecticide resistance (McKenzie *et al.*, 1982; McKenzie and Purvis, 1984) also suggest that accompanying intense selection, other alleles are concurrently selected to produce a reorganised genome such that the resistant phenotype is as fit as the susceptible phenotype, even in the absence of the selecting agent.

However, there are some reports of nematode populations, previously resistant to an anthelmintic group, showing some reversion towards susceptibility. Based on an egg hatch assay, Simpkin and Coles (1978) demonstrated a fall in resistance to thiabendazole in *H. contortus* and *T. colubriformis*, if worms were passaged for one or two generations without thiabendazole. However, treatment of lambs harbouring passaged strains of worms with thiabendazole resulted in resistance similar to the original unpassaged-resistant strain. A similar study aimed at investigating reversion to susceptibility in a thiabendazole-resistant strain of *H. contortus* found that first treatment with thiabendazole after five years of levamisole use removed 95% of the worms. However, after the third passage thiabendazole resistance reappeared and only 50% worms could be removed (Kelly and Hall, 1979).

Obvious reversion to susceptibility in benzimidazole-resistant *Ostertagia* spp was observed after a single dose (Donald *et al.*, 1980) or repeated doses of levamisole (Waller *et al.*, 1983). Similarly, another study demonstrated a change to benzimidazole susceptibility in a mixed population of *H. contortus* and *T. colubriformis* after two years of levamisole use (Waller *et al.*, 1989). However, benzimidazole resistance increased rapidly following the re-introduction of thiabendazole. The results of these

studies may not actually indicate reversion to susceptibility because levamisole treatment might have resulted in replacement of highly resistant benzimidazole worms by one reflecting a lower frequency of resistant individuals. Therefore, it is not clear yet to what extent reversion to susceptibility occurs.

CHAPTER TWO

OPTIMISATION OF THE LARVAL DEVELOPMENT ASSAY AND METHODS FOR LARVAL RECOVERY FROM PASTURE AND SOIL

2.1. INTRODUCTION

Some preliminary work was undertaken in order to optimise the larval development assay and the recovery of larvae from pasture and soil. The larval development assay used in this study is largely as described by Gill *et al.* (1995). In the experiments described in Chapters 3 and 4, the larval development assays were performed by using 200 µl of agar whereas in other Chapters 150 µl of agar was used in the assays. Therefore, a trial was carried out to determine whether the LC_{50} values change with the amount of agar used in the assay.

The methods used for the recovery of larvae from pasture and soil were similar to those routinely used by the Parasitology Section, AgResearch, Palmerston North. These methods were used to determine the success rate of larval recovery from a known number of larvae.

2.2. MATERIALS AND METHODS

Larval development assay

Animal

A sheep infected with 25000 infective larvae of a susceptible strain of *T. colubriformis* (Trial I, see Chapter 5) was used for this experiment. Faeces were collected from this sheep at 22, 24, 27, 31, 33 and 35 days post infection.

Procedure

The larval development assay is largely as described by Gill *et al.* (1995) and is described in detail in Appendix VIII. Briefly, stock solutions of ivermectin (24 µg/ml) were prepared in dimethyl sulphoxide (DMSO) and were serially diluted 1:2 with

DMSO to give 16 concentrations. Two µl of each drug concentration or DMSO only for controls, were dispensed into wells of 96-well microtitre plates and diluted with 200 µl or 150 µl of 2% agar (Bacto-agar, DIFCO). Each concentration was run in duplicate and the controls were run in triplicate. Nematode eggs were recovered from faeces by sieving, centrifugation and the use of 20% magnesium sulphate. Approximately 50 clean nematode eggs per well in 60 µl of distilled water, were added to the surface of the agar matrix, supplemented with 40 µl of nutrient medium per well (Hubert and Kerboeuf, 1984) and incubated at 25^{O}C for 7 days. The liquid phase from each well was removed and the numbers of eggs, L_1, L_2 and L_3 at each drug concentration were counted under a light microscope after staining with Lugol's iodine. Data were corrected for the mean number of larvae not developed in three control wells.

Pasture larval recovery method

Infective larvae

The faeces were collected from the same sheep used for the larval development assay. The faeces were cultured and larvae were recovered by using the Baermann technique. The numbers of infective larvae were counted in five aliquots of 100 µl of larval suspension (larvae and 100 ml deionised water). The mean (S.E.) number of larvae in 100 µl was 189.6 (±6.6). This was used to estimate the amount of larval suspension that contained 3030, 6060, 9090, 12120 or 15150 infective larvae.

Procedure

The method for recovering the larvae from the pasture is described in detail in Appendix IV. Briefly, pasture samples consisting of mixture of Rye grass and Brown top were collected from a site, which had not been grazed by animals for at least 10 years. Pasture samples were weighed in aliquots of 50g each. Each aliquot was mixed in a plastic bag with either 3030, 6060, 9090, 12120 or 15150 larvae. A plastic funnel (Plate 2.1) was filled with warm water (approximately 27^{O}C) within 10 cm of the top and a drop of non-ionic detergent was added. A 50g aliquot of pasture containing larvae was put in the funnel and mixed with water thoroughly. The plastic bag was filled with warm water and emptied into the funnel. The funnels were left to stand for

24 hours and 150 ml of water containing larvae was carefully tapped off from the funnel into the beaker. These larvae were cleaned by the Baermann technique and the volume was reduced to 50 ml. The larvae were further concentrated by sedimentation to 20 ml volume for counting. The number of larvae in 0.5 ml of the suspension was calculated in duplicate after staining with Lugol's Iodine in a counting slide under a light microscope. The mean of the two counts was multiplied by 40 to get the total number of larvae recovered. The whole procedure was repeated twice.

Soil larval recovery method

Infective larvae

The faeces were collected and cultured from the same sheep used for the larval development assay. The larvae were recovered and counted in the similar way as described in the pasture larval recovery method. The mean (S.E.) number of larvae in 100 µl was 150 (±3). This was used to estimate the amount of larval solution that contained 3000, 6000, 9000, 12000 or 15000 infective larvae. The soil samples were collected from the same site as used for the pasture study.

Procedure

The method for recovering the larvae from soil is described in detail in Appendix V. Briefly, soil samples were weighed in aliquots of 800g each. Each aliquot was mixed with either 3000, 6000, 9000, 12000 or 15000 larvae in a plastic bag. One litre of water was poured into a plastic tray (40 cm×27 cm×6 cm) with an intact bottom. A further plastic tray (40 cm×27 cm×6 cm) fitted with fiberglass mesh at its bottom, was placed over the plastic tray with the intact bottom (Plate 2.2). A layer of tissue paper was placed on the mesh tray and a 800g aliquot of soil was spread out on the top. More water was added until the soil was submerged. The trays were allowed to stand for 24 hours so that the larvae could migrate into the water between the two trays and settle on the bottom of the lower tray. After 24 hours the fiberglass mesh tray containing soil was lifted with small wooden pieces from both sides and the soil was allowed to drain for 5 hours into the plastic tray underneath it. The soil sample was then discarded. The larval suspension was cleaned further by sedimentation for 24 hours in a bucket and then by the Baermann technique. The final volume was reduced to

Plate 2.1: Plastic funnels used for recovery of larvae from the pasture samples.

Plate 2.2: Plastic trays used for recovery of larvae from the soil samples.

20 ml. The larvae were counted in two sub samples of 2 ml after staining with Lugol's Iodine in a counting slide using a light microscope. The mean of two samples was taken and multiplied by 10 to get the total number of larvae. The whole procedure was repeated twice.

Statistical Analysis

The proportions of infective larvae (L_3) in larval development assay were fitted into a sigmoid curve against $\log_{10}$ transformation of ivermectin concentrations using the software programme, Slide Write Version 3.0 (Advanced Graphics Software Inc., U.S.A.). LC_{50} values were then calculated with this same software and expressed as ng/ml in the agar phase. An example of the resulting sigmoid curve is shown in Fig 2.1.

Log transformed LC_{50} values, numbers of larvae recovered from pasture and soil were compared by analysis of variance (ANOVA) and Tukey's multiple comparison test by using software programme SAS (SAS, U.S.A.).

2.3. RESULTS AND DISCUSSION

Larval development assay

There were no significant differences ($p<0.05$) in the LC_{50} values obtained with 200 µl or 150 µl of agar (Table 2.1, see Appendix 2.1 for statistical analysis). The interaction between time and amount of agar was also not significant ($p<0.05$). These results suggest that the amount of agar (200 µl or 150 µl) used in the larval development assay does not influence the LC_{50} values. Therefore, it is reasonable to compare the LC_{50} values obtained from the larval development assays performed with 200 µl or 150 µl of agar.

Table 2.1: LC_{50} values (ng/ml of the agar phase) of ivermectin for *T. colubriformis* in the larval development assay performed with 150 µl or 200 µl of agar.

DAYS POST INFECTION	LC_{50} VALUES (NG/ML OF AGAR)	
	With 200 µl agar	With 150 µl agar
22	0.501	0.496
24	0.534	0.528
27	0.518	0.510
31	0.505	0.515
33	0.543	0.492
35	0.583	0.538

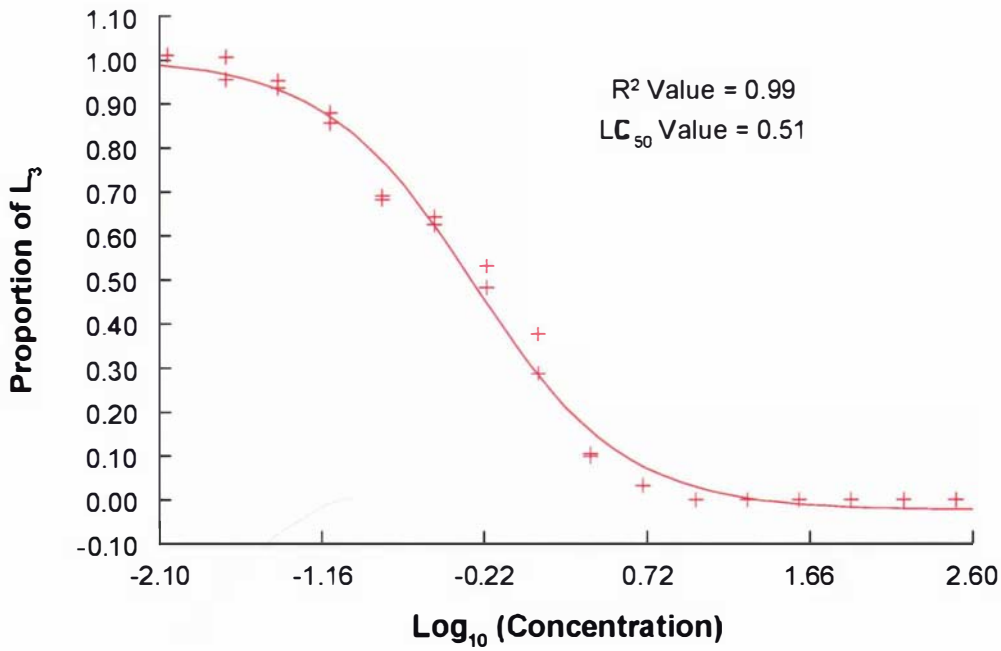


Figure 2.1: A typical dose response curve relating to the proportion of developed L_3 to logarithm concentration (ng/ml in agar phase) of ivermectin.

Pasture recovery method

The recovery rates varied between 47 and 67% with an overall mean recovery rate of 60% (Table 2.2). The number of larvae recovered increased with increasing numbers of larvae in the pasture sample. The fate of the remaining 40% of larvae is unknown. They were probably still adhered to the herbage or the sides of the funnel. Some larvae could have also been lost during the process of cleaning the larval suspension by the Baermann technique. The recovery rates were higher than those achieved by Niezen (1996). He recorded a maximum recovery rate of approximately 50% from a known number of larvae deposited on Cocksfoot and chicory. The recovery rate was only 30-40% from Yorkshire fog and ryegrass. In his study, the larvae were recovered from 50g of herbage containing 1000 *T. colubriformis* larvae by soaking the herbage in the water in a plastic container, 3-step sedimentation and then by using the Baermann technique. A number of other techniques for the extraction of infective larvae from herbage have been described in the literature (Taylor, 1939; Dinaburg 1942; Crofton, 1954; Parfitt, 1955; Durie, 1959; Thomas, 1959; Sturrock, 1961; Heath and Major, 1968; Bawden, 1969; Lancaster, 1970; Smeal and Hendy, 1972; Vlassoff, 1973; Chiejina, 1982; Couvillion, 1993). The recovery rates are variable depending on the technique used. Some of these techniques require special apparatus (Durie, 1959; Bawden, 1969; Smeal and Hendy, 1972) and are labour intensive and time consuming. The Baermann technique has also been used to extract the larvae from pasture (Shorb, 1944; Silangwa and Todd, 1964; Okon and Enyenihi, 1977; Gruner and Suryahadi, 1993) but the recovery rate from a known number of larvae in herbage is not indicated in any of these reports. The method used in the present study was easy to perform, required minimum bench space and provided comparable recovery rates to that reported in the literature.

Table 2.2: Recovery of larvae from pasture when known numbers of *T. colubriformis* larvae were added to 50g of pasture.

NUMBER OF LARVAE PUT IN THE FUNNEL ON TOP OF PASTURE	TOTAL NUMBER OF LARVAE RECOVERED			MEAN NUMBER OF LARVAE RECOVERED	PERCENT RECOVERY RATE
	Replicate I[a]	Replicate II[b]	Replicate III[ab]		
3030	1600	1266	1400	1422	46.93
6060	4066	3700	4070	3945	65.10
9090	6066	5567	5630	5754	63.30
12120	7460	5800	7340	6867	56.66
15150	11266	9533	9840	10213	67.41
Overall percent recovery of larvae from known number of larvae					**60**

[abc] row with different superscript letter is significantly different ($p<0.05$)

Soil recovery method

The recovery rates of larvae from soil varied between 9.6 and 12.4% with an overall mean recovery rate of 11.15% (Table 2.3). The number of larvae recovered increased with increasing numbers of larvae in the soil sample. The reason why more of the larvae were not recovered is not known. The larvae, which were not recovered, were probably still adhered to the soil or might have been lost during the process of cleaning the larval solution by sedimentation, syphoning or the Baermann technique. The Baermann technique has also been used to extract the larvae from soil (Shorb, 1944; Gruner and Suryahadi, 1993) but the recovery rate from a known number of larvae in soil is not indicated in any of these reports. An average recovery rate of 78% has been reported by Durie (1959) using a larval extraction apparatus. He recovered the larvae from only 50g of soil at a time, which was not practicable in the present study because the larvae were recovered from the upper 2-inch layer of soil (see Chapter 6), which weighed approximately 1600 grams. A total of 60 such plots were sampled in that study.

Table 2.3: Recovery of larvae from soil when known number of *T. colubriformis* larvae were added to 800g of soil.

NUMBER OF LARVAE PUT IN THE TRAY ON TOP OF SOIL	TOTAL NUMBER OF LARVAE RECOVERED			MEAN NUMBER OF LARVAE RECOVERED	PERCENT RECOVERY RATE
	Replicate I[a]	Replicate II[a]	Replicate III[a]		
3000	166	366	333	288	9.61
6000	500	900	833	744	12.40
9000	866	1266	1200	1111	12.34
12000	1133	1266	1300	1233	10.28
15000	1933	1433	1633	1666	11.11
Overall percent recovery of larvae from known number of larvae					**11.15**

[a] significantly not different from each other ($p>0.05$)

The consistency of the recovery rates of larvae from pasture and soil between the replicates supports the validity of the correction factors. The same recovery methods were used for the extraction of larvae from pasture and soil for the ivermectin-resistant strain and the two ivermectin-susceptible strains in the experiments described in chapter 6. Although the corrected recovery rates will not influence the comparisons of the three strains in numbers of larvae recovered from pasture and soil but the corrected recovery rates will provide a better estimate of larval migration from faeces to pasture and soil for the ivermectin-resistant and the ivermectin-susceptible strains. Therefore, it is appropriate to correct the recovery rates in the experiments described in Chapter 6.

CHAPTER THREE

ISOLATION OF FIELD ISOLATES OF *TRICHOSTRONGYLUS COLUBRIFORMIS* AND *OSTERTAGIA CIRCUMCINCTA* RESISTANT TO IVERMECTIN*

3.1. INTRODUCTION

Resistance to ivermectin has now been reported in *O. circumcincta* in Australia (Besier and Wroth, 1993), Czechoslovakia (Varady *et al.*, 1993), New Zealand (Watson and Hosking, 1990; Pomroy *et al.*, 1992), South Africa (Reinecke *et al.*, 1991) and the United Kingdom (Jackson *et al.*, 1992). Ivermectin resistance has also been reported in *H. contortus* in Argentina (Eddi *et al.*, 1996), Australia (Le Jambre, 1993a), Brazil (Echevarria and Trindade, 1989), Kenya (Mwamachi *et al.*, 1995), Malaysia (Chandrawathani *et al.*, 1999), South Africa (Van Wyk and Malan, 1988) and The United States of America (Craig and Miller, 1990). In contrast there are no published reports of field cases of ivermectin resistance in *T. colubriformis* which have been confirmed by slaughter study, although ivermectin-resistant strains have been selected in laboratories (Giordano *et al.*, 1988; Shoop *et al.*, 1990). Ivermectin-resistant *Trichostrongylus* has also been suspected to occur on the basis of faecal egg count reduction tests and larval cultures where they formed a small proportion of the larvae in post-treatment cultures (Varady *et al.*, 1993; McKenna *et al.*, 1990; Eddi *et al.* 1996; Echevarria *et al.*, 1996; Maciel *et al.*, 1996). This chapter deals with the identification of a field population of ivermectin-resistant *T. colubriformis* and *O. circumcincta* isolated from goats.

History and Background

Ivermectin resistance was suspected on an Angora goat farm in Northland, New Zealand. A faecal egg count reduction test conducted by the local veterinarian had indicated that the efficacies, at standard dose rates of ivermectin (0.2mg/kg b.wt. per

* This is a slightly edited version of a paper entitled "Resistance of field isolates of *Trichostrongylus colubriformis* and *Ostertagia circumcincta* to ivermectin", R.M. Gopal, W.E. Pomroy and D.M. West, published in the International Journal for Parasitology 29(1999):781-786.

os), albendazole (7.5mg/kg b.wt. per os), moxidectin (0.2mg/kg b.wt. per os) and a combination product (1ml/kg b.wt. per os) of levamisole (37.5 g/litre), albendazole (25.0 g/litre) and praziquantel (18.8 g/litre) were 0%, 42%, 90% and 99.9% respectively. *Trichostrongylus* was the main genus identified in a post-ivermectin treatment faecal culture. Four goats, which had not been drenched for the previous 2 months, were purchased from this Northland farm. Faecal samples were collected from these animals and cultured for larvae. These larvae were used to infect the animals in this trial.

3.2. MATERIALS AND METHODS

Trial Design

Twelve male Romney lambs and 10 male Angora goats of 10 months and 7 months age, respectively, were drenched with double dose of ivermectin and a combination product of ricobendazole and levamisole to remove existing worm burdens and housed. Faecal egg counts from all the animals were negative at 10 and 13 days after drenching indicating that the resident worms had been removed. Fourteen days after drenching, all the animals were infected with 7000 infective L_3 (89% *Trichostrongylus*, 11% *Ostertagia*) which had been cultured from the faeces of Northland goats. Twenty-eight days post infection, the lambs and goats were restrictively divided on the basis of faecal egg counts into two groups of six and two groups of five animals. One group of lambs and one group of goats were drenched orally with the recommended therapeutic dose of ivermectin (0.2mg/kg)(Ivomec for sheep and goats, MSD AgVet). The remaining six lambs and five goats were not treated and acted as controls. Seven days after treatment (35 days post infection), all animals were killed and their abomasums, small intestines and large intestines were removed for worm counting.

Parasitological techniques

Worm counts

Ten percent aliquots of abomasal contents, abomasal pepsin digests, small intestinal contents and small intestinal pepsin digests (see Appendix II for method) were sieved

through a 53 μm sieve and all the nematodes retained were counted. The whole of the large intestinal contents (unsieved) were examined for nematodes. Twenty male nematodes (if available) were retained per genus from each animal and identified to species level.

Faecal egg counts

Faeces were collected per rectum from all the animals at 28 and 35 days post infection and egg counts were estimated by a modified McMaster procedure where each egg counted represents 50 eggs per gram (see Appendix I for method).

Larval Development Assay

The susceptibility of free living stages of *Trichostrongylus* was assessed in three animals in each group at 22, 24, 26, 28, 31, 32, 34 and 35 days post infection in a larval development assay. The assay was conducted as described previously in Section 2.2 (see Appendix VIII for detailed method) except that the assay was conducted using ivermectin and avermectin B_2 and each concentration of ivermectin and avermectin B_2 was mixed with 200 μl of 2% agar in a 96 well microtitre plate. Larval development assays for a susceptible strain of *T. colubriformis* for sheep, using ivermectin and avermectin B_2 were also performed in the experiments conducted in Chapter 5 and 7. The LC_{50} values for this susceptible strain (see Appendix 3.2) were used for calculation of the resistance ratios.

Statistical Analysis

The percentage of reduction in faecal egg counts using arithmetic and geometric means was determined as described below (Presidente, 1985). Egg and worm counts were transformed to log (count) to calculate geometric means.

$$\text{Percentage reduction in faecal egg count} = 1 - \left[\frac{T2}{T1} \times \frac{C1}{C2}\right] \times 100$$

$$\text{Percentage reduction in worm count} = 1 - \left[\frac{T}{C}\right] \times 100$$

Where 1 and 2 indicate arithmetic mean egg count before and after treatment respectively. T and C indicate the treatment and the control groups respectively.

The worm counts after log transformation were analysed by t-test using software program SAS Version 6.12 (SAS, U.S.A.).

The LC_{50} values were calculated using the software programme SlideWrite Version 6 (Advanced Graphics Software Inc., U.S.A.) and expressed as ng/ml in the agar phase. The LC_{50} values after log transformation were analysed in a split-split-split plot design where the treatments were treatment (either control or treatment group), test species, individual animals, stage (either before or after treatment) and time. In the treated groups, log transformed LC_{50} values after ivermectin treatment were analysed in a two way ANOVA. The log transformed LC_{50} values of control and treatment groups before treatment were also analysed in an ANOVA. All these analysis were performed using the software programme SAS (SAS, U.S.A).

The resistance ratio was calculated as the LC_{50} value for the resistant isolate divided by the LC_{50} value for the susceptible isolate.

3.3. RESULTS

The establishment rates in control sheep and goats were similar ($p>0.05$) (see Appendix 3.1 for statistical analysis) being 22% and 21% for *Ostertagia* spp. and 20% and 28% for *T. colubriformis* in sheep and goats, respectively. The percentage of reduction in arithmetic mean faecal egg counts in the treated groups when compared with control groups 7 days after treatment was found to be 60% and 32% in lambs and goats, respectively (Tables 3.1 & 3.2). Ivermectin achieved 33% and 39% reduction in arithmetic mean worm burdens for *Ostertagia* spp. and *T. colubriformis*, respectively, in lambs whereas in goats, the arithmetic mean reduction in worm burden after ivermectin treatment was 0% and 13.5%, respectively, for *Ostertagia* spp. and *T. colubriformis* respectively (Table 3.1 and 3.2). For goats the worm counts in treated and control groups for *T. colubriformis* were also similar ($p>0.05$). However, in lambs the worm counts in treated and control groups for *T. colubriformis* were significantly different from each other ($p<0.05$).

Table 3.1: Nematode worm counts and faecal egg counts in lambs infected with ivermectin-resistant strain (89% *Trichostrongylus* spp. and 11% *Ostertagia* spp) and left untreated or dosed with oral ivermectin at dose rate of 0.2 mg/kg b.wt.

Treatment group	Nematode worm count		Faecal egg count – days after treatment	
	O. circumcincta	*T. colubriformis*	Day 0	Day 7
Control	130	1510	100	150
(Lambs)	130	1090	200	550
	250	1130	250	250
	130	1090	300	450
	150	620	200	150
	270	1340	350	350
Arithmetic Mean (S.E.)	**177** (26.7)	**1130** (122.7)	**233** (35.7)	**317** (66.7)
Geometric Mean	**167.7**	**1090.7**	**217.2**	**280.5**
Ivermectin	130	190	200	100
(Lambs)	110	1240	350	200
	110	150	350	100
	170	1860	450	400
	120	480	100	50
	70	220	200	50
Arithmetic Mean (S.E.)	**118** (13.3)	**690** (287.4)	**275** (52.8)	**150** (54.8)
Geometric Mean	**114.4**	**436.7**	**245.8**	**113.5**
% Reduction (A.M.)	33	39		60
% Reduction (G.M.)	32	60		64

A.M.- Arithmetic mean

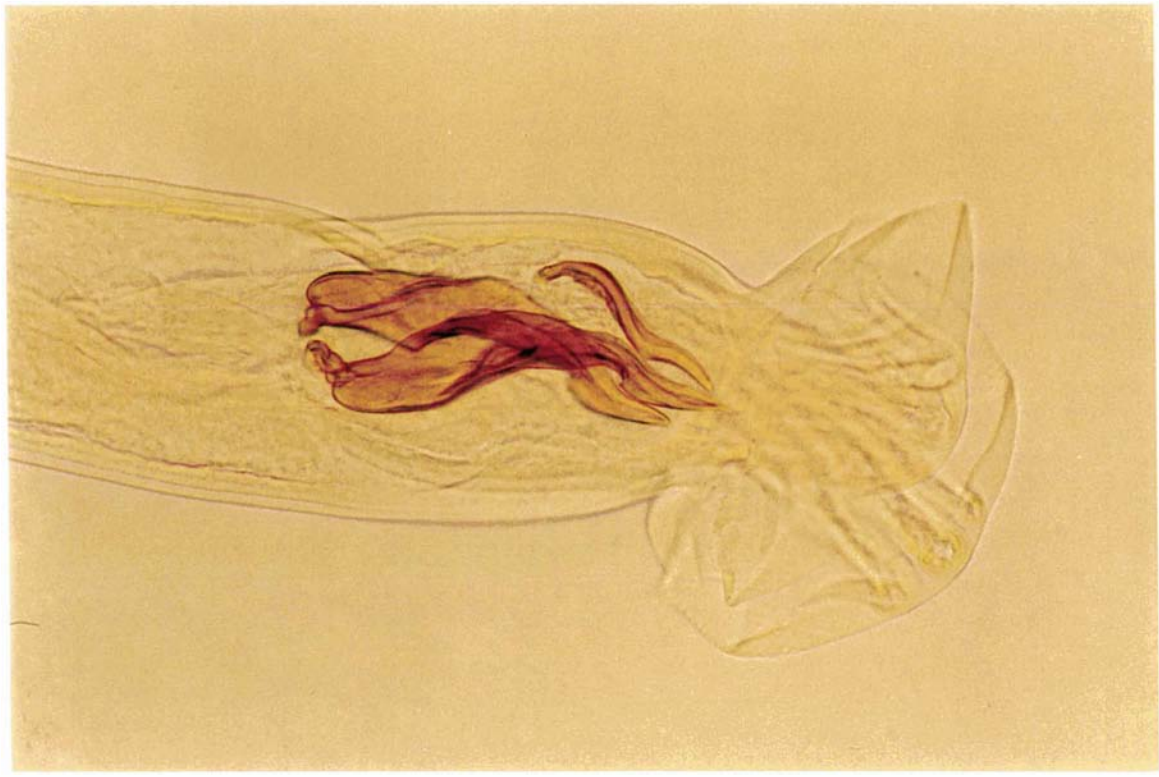
G.M.- Geometric mean

Table 3.2: Nematode worm counts and faecal egg counts in goats infected with ivermectin-resistant strain (89% *Trichostrongylus* spp. and 11% *Ostertagia* spp.) and left untreated or dosed with oral ivermectin at dose rate of 0.2 mg/kg b.wt.

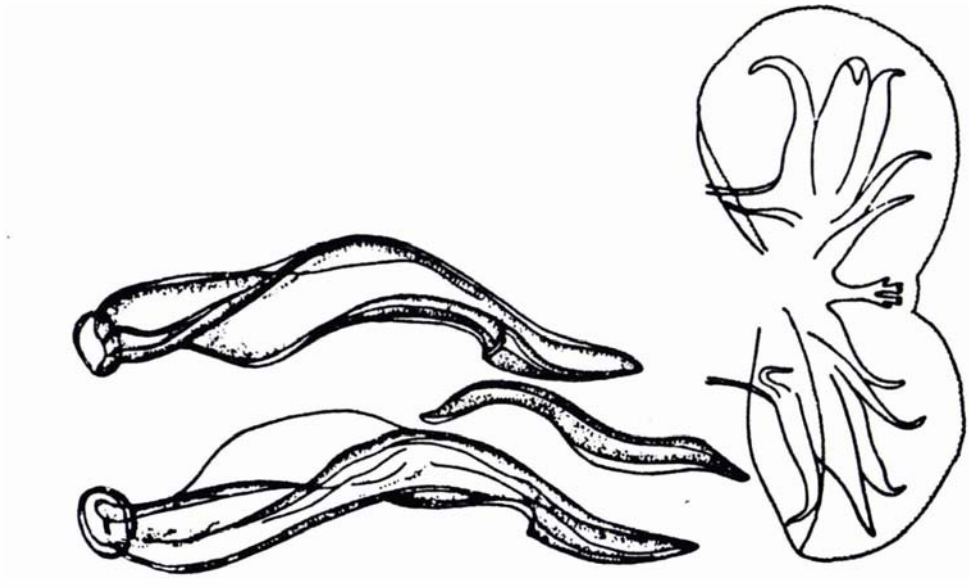
Treatment group	**Nematode worm count**		**Faecal egg count – days after treatment**	
	O. circumcincta	***T. colubriformis***	**Day 0**	**Day 7**
Control	180	1560	850	950
(Goats)	190	1860	650	1050
	130	2200	900	1250
	100	630	100	250
	210	1760	850	950
Arithmetic Mean (S.E.)	**162** (20.4)	**1602** (264.8)	**670** (148.8)	**890** (169.1)
Geometric Mean	**156.3**	**1479**	**531.1**	**784**
Ivermectin	150	1730	750	750
(Goats)	170	1160	850	850
	200	1310	950	700
	220	1880	750	700
	150	870	250	200
Arithmetic Mean (S.E.)	**178** (14.0)	**1390** (185.1)	**710** (120.8)	**640** (113.4)
Geometric Mean	**175.9**	**1338.7**	**647.1**	**574.3**
% Reduction (A.M.)	0	13		32
% Reduction (G.M.)	0	10		40

A.M.- Arithmetic mean

G.M.- Geometric mean



(a)



(b)

Plate 3.1: Comparison of ivermectin-resistant male *T. colubriformis* nematode (a) with published figure from Skrjabin (1954) (b), showing typical spicule morphology for the species.

Both *O. circumcincta* (80%) and *Ostertagia trifurcata* (20%) morphological types were identified in the lambs and goats with the proportion similar in both animal species. The spicule morphology (relatively long spicules but slightly unequal in length, with somewhat triangular projection at posterior end) of the male ivermectin-resistant *Trichostrongylus* nematodes confirmed the species as *T. colubriformis* (Plate 3.1). No nematodes were detected in the large intestines of any animal.

The results of the larval development assays for *T. colubriformis* are shown in Fig. 3.1 (see Appendices 3.3 to 3.10 for raw data). The LC_{50} values of the two control groups were relatively constant throughout the experiment ranging from 1.5 to 2.4 ng/ml with ivermectin and from 1.2 to 2.0 ng/ml with avermectin B_2. Prior to ivermectin treatment the LC_{50} values of the treated groups were similar and not significantly different ($p>0.05$) (see Appendix 3.1 for statistical analysis) from the control groups. The LC_{50} values of sheep nematodes were always slightly higher and significantly different ($p<0.05$) than those from goats for both analogues. Following ivermectin treatment, the LC_{50} values increased steadily until the animals were killed on day 35 post infection. When the two treatment groups were analysed in a 2-way ANOVA after treatment, the interaction between species and time was not significant ($p>0.05$) (see Appendix 3.1 for statistical analysis). The mean LC_{50} values of ivermectin and avermectin B_2 for the susceptible strain of *T. colubriformis* in sheep were found to be 0.517 and 0.636 ng/ml of agar, respectively (see Appendix 3.2). When the mean LC_{50} values of the resistant isolate of *T. colubriformis* in sheep prior to ivermectin treatment were compared with the mean LC_{50} values of the susceptible strain, the resistance ratios were found to be 3.8 and 2.7 with ivermectin and avermectin B_2 respectively. These ratios increased to 4.9 and 3.6 with ivermectin and avermectin B_2 respectively when similar comparison was made after ivermectin treatment in sheep. The LC_{50} values for *Ostertagia* spp. were not calculated because there were too few eggs to evaluate in the larval development assay.

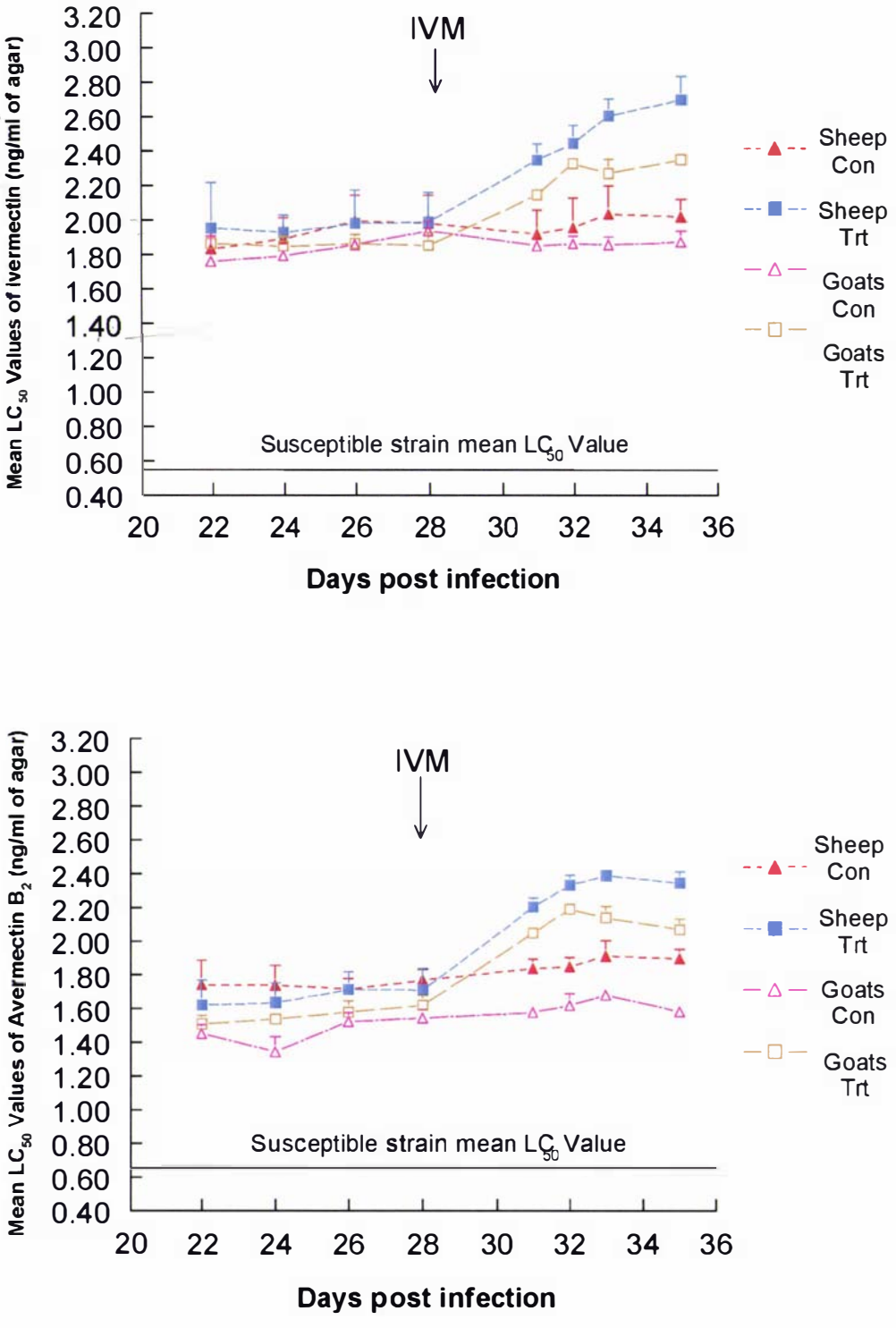


Figure 3.1: Mean LC_{50} values of ivermectin and avermectin B_2 in treatment (Trt; n=3) or control (Con; n=3) sheep and goats infected with 7000 ivermectin-resistant larvae of *T. colubriformis* on Day 0. On 28 days post infection both treatment groups were given 0.2mg/kg ivermectin (IVM) orally.

3.4. DISCUSSION

This is the first confirmed report of a field strain of *T. colubriformis* resistant to ivermectin. Previous confirmed cases of ivermectin resistance involved either isolates selected in laboratories (Giordano *et al.*, 1988; Shoop *et al.*, 1990) or detection of small proportions of *Trichostrongylus* in faecal larval cultures of ivermectin-treated animals (Varady *et al.*, 1993; McKenna *et al.*, 1990; Eddi *et al.* 1996; Echevarria *et al.*, 1996; Maciel *et al.*, 1996). In these reports the species of *Trichostrongylus* involved were not identified. Controlled efficacy tests *in vivo* have been considered the definitive means to determine whether resistance is present in the population (Conder and Campbell, 1995). Faecal egg counts may not be a reliable indicator of infection (Kingsbury, 1965; McKenna, 1981) although larval cultures can be used in conjunction with faecal egg count reduction tests to identify which parasites are resistant, highly fecund nematode species may mask species with low fecundity (West *et al.*, 1989) and species which have low mortality in culture may be overestimated relative to those that suffer high mortality in culture (Dobson *et al.*, 1992b).

The proportion of worms killed by ivermectin was higher in sheep than in goats. This might be due to differences in the efficacy of ivermectin in sheep and goats. Scott *et al.* (1990a) demonstrated that the clearance of ivermectin from the plasma of goats after oral administration of ivermectin was faster than from sheep. Similar studies have shown that levamisole (McKenna and Watson, 1987), albendazole (Hennessy *et al.*, 1993a) and oxfendazole (Bogan *et al.*, 1987; Sangster *et al.*, 1991) are required in higher doses for efficacy in goats than sheep. Against fully susceptible nematodes this may have little effect on efficacy but against in a population containing ivermectin-resistant nematodes it may result in lower efficacy of ivermectin in goats compared with sheep. Whether this has a role in the fact that this isolate was originally reported in goats and not sheep despite ivermectin being widely used in both animal species in New Zealand is not known. Ivermectin resistance in *O. circumcincta* has been found several times in goats but only reported recently in sheep in New Zealand (Mason *et al.*, 1999).

In the present study, arithmetic mean and geometrical mean faecal egg counts were used in calculating the percentage reduction of eggs in the treatment group as

compared to the control group (Table 3.1 and 3.2). Both arithmetic and geometric mean provided the same conclusions. The role of the arithmetic mean versus geometric means in the faecal egg count reduction test has been considered by Dash *et al.* (1988) and McKenna (1990). Dash *et al.* (1988) concluded that arithmetic means were the most appropriate in the calculation of faecal egg count reduction for detection of anthelmintic resistance. This was supported by McKenna (1990) who suggested that not only is the arithmetic mean simpler to calculate and better suited for comparative purposes, it is also likely to provide a more conservative and truer measure of anthelmintic efficacy.

Avermectins have been reported to suppress nematode fecundity in *H. contortus* (Le Jambre, 1993a), *O. circumcincta* (Jackson and Coop, 1995) and *Cooperia curticei* (McKellar *et al.*, 1988). The egg counts for ivermectin-resistant isolates of *H. contortus* (Le Jambre, 1993a) and *O. circumcincta* (Jackson and Coop, 1995) were zero up to 14 days following treatment. In contrast to these findings, in the present study, the egg counts were not suppressed up to the level as reported by these authors after 7 days of ivermectin treatment in both *T. colubriformis* and *O. circumcincta*. These observations are consistent with the work of Echevarria and Trindade (1989) who reported that for an ivermectin-resistant isolate of *H. contortus*, eggs continued to be present in faeces following treatment with ivermectin for at least 7 days after drenching. Thus it appears that all the ivermectin-resistant isolates do not show suppression of egg production following ivermectin treatment.

Following ivermectin treatment the LC_{50} values for *T. colubriformis* increased in the sheep and goats. This is probably due to elimination of the susceptible population of *T. colubriformis* and an increased proportion of resistant worms. The resistance ratios recorded from larval development assays with ivermectin and avermectin B_2 throughout the experiment were always small but are of a similar magnitude to that reported for ivermectin-resistant *O. circumcincta* in a larval development assay (Amarante *et al.*, 1997).

The choice of the avermectin analogue has been shown to influence the resulting resistant ratios in larval development assays with *H. contortus*. Gill *et al.* (1995) found relatively small resistance ratios with ivermectin-resistant field isolates of *H. contortus*

in a larval development assay using ivermectin but they reported that avermectin B_2 was the most sensitive probe for ivermectin-resistant status as it gave better discrimination between resistant and susceptible strains of *H. contortus*. However in the present study with *T. colubriformis*, the resistance ratios recorded with avermectin B_2 were smaller than that recorded with ivermectin. Therefore avermectin B_2 does not seem to be the drug of choice for diagnosis of ivermectin resistance in *T. colubriformis* with a larval development assay.

The LC_{50} values using ivermectin and avermectin B_2 were lower in goats as compared to sheep. This might be due to a difference in the immune status of the two host species. Host immune response has been thought to influence the LC_{50} values in a larval development assay in sheep as these values change with the age of infection and are reduced by treatment of sheep with high doses of corticosteroids (Hoza *et al.*, 1997). Because of the small resistance ratios and the variation of LC_{50} values with host, it would seem that a larval development assay with ivermectin or avermectin B_2 would probably struggle to accurately identify ivermectin resistance in *T. colubriformis* in the field.

The difference in efficacy between sheep and goats was not necessarily reflected in a relatively larger increase in LC_{50} values of sheep after treatment as the interaction between time and host in the 2-way ANOVA of LC_{50} values after treatment, was not significant (see Appendix 3.1 for statistical analysis). However, this may just reflect the insensitivity of ivermectin and avermectin B_2 to detect such a difference in a larval development assay.

The establishment rates of *Ostertagia* spp. and *T. colubriformis* in the control goats and sheep indicate that these isolates appear to be equally infective for sheep and goats under experimental conditions. These results are in agreement with the previous findings of Pomroy *et al.* (1992) who found that an ivermectin-resistant strain of *Ostertagia* spp. isolated from goats was also readily infective for sheep. The practical implications are that this isolate of ivermectin-resistant *T. colubriformis* could be readily transferred to sheep. Its apparent resistance to benzimidazoles further compounds the potential problem. Further Chapters will explore the genetic basis of

ivermectin resistance in this isolate named Massey Tc 97, its other biological characteristics and efficacy of moxidectin against this strain.

CHAPTER FOUR

RE-EMERGENCE OF IVERMECTIN-RESISTANT *OSTERTAGIA CIRCUMCINCTA* IN SHEEP GRAZED ON PASTURE CONTAMINATED WITH GOAT-DERVIED IVERMECTIN-RESISTANT *OSTERTAGIA* SPP.

4.1. INTRODUCTION

In New Zealand, goat farming often depends heavily on anthelmintics to control gastrointestinal nematode infections, because of the intensive grazing management systems used as well as the susceptibility of goats to parasites. There are number of reports of ivermectin-resistant *Ostertagia circumcincta* (Badger and McKenna, 1990; Pomroy *et al.*, 1992) and more recently *Trichostrongylus colubriformis* (see Chapter 3) in goats. However, until 1999 (Mason *et al.*, 1999) there had been no reports of ivermectin resistance in *Ostertagia* spp. in sheep despite mixed grazing of sheep and goats, which is a common practice in New Zealand. In the face of this practice, there are concerns that resistance to ivermectin in goats will emerge rapidly and the resistant worms will spread readily from goats to sheep. Experimentally these ivermectin-resistant isolates are infective for sheep (Pomroy *et al.*, 1992; see Chapter 3).

Ivermectin resistance in *O. circumcincta* was identified in goats on one unit of the Massey University research farm in 1991 (Pomroy *et al.*, 1992). Anthelmintics from other action families were used between 1991 and 1995. A faecal egg count reduction test conducted in 1995 revealed little evidence of ivermectin resistance in sheep. In 1995 a replicated trial was established (Pomroy *et al.*, 1997) to determine how long it would take for ivermectin resistance to re-emerge in separately-grazed young sheep and goats. All the animals were drenched 10 times with ivermectin during the two-year trial period. By late 1996 ivermectin resistance was apparent in the goats but not in the sheep despite the drenching frequency being the same for both sheep and goats (Pomroy *et al.*, 1997). A follow up crossover study was planned with the aim of investigating if sheep are at risk from grazing pasture contaminated with goat-derived ivermectin-resistant *Ostertagia* spp.

4.2. MATERIALS AND METHODS

Trial design

The trial was commenced in May 1997 and was completed in June 1998.

Twenty-four goats six months of age and sixteen sheep nine months of age were drenched with a double dose of ivermectin and a combination product of ricobendazole and levamisole to remove any existing worm burdens. Fourteen days after drenching, all the animals had negative worm egg counts. The goats were then randomly allocated into 4 groups of 6 animals each and the sheep into 4 groups of 4 animals each. Each group was setstocked separately on 0.5ha paddocks (Plate 4.1 and 4.2). Half of these paddocks had been previously grazed by goats harboring ivermectin-resistant *O. circumcincta* worms and half by sheep (see Fig. 4.1 for detailed allocation of animals into different paddocks). The difference in numbers of goats and sheep setstocked in each paddock was due to the difference in possible stocking rate of these ruminants. In November, 1997, all the sheep and in February, 1998 all the goats were replaced with younger animals of approximately three and half to four months of age to ensure worm burdens were sufficient to carry out faecal egg count reduction tests.

Rectal faecal samples were collected from all the animals every two weeks. Each host species was drenched with the recommended therapeutic dose of ivermectin (0.2mg/kg)(Ivomec for sheep and goats, MSD AgVet) when any paddock mean faecal egg count for that host species exceeded 500 eggs per gram. All the treatments were administered by syringe according to individual body weight.

Susceptible strain

Three lambs approximately 6 months of age were drenched effectively with a double dose of ivermectin and a combination product of ricobendazole and levamisole to remove any existing worm burdens and housed indoors. Fourteen days after drenching, they were infected twice weekly for 7 weeks with 2500 infective larvae of an ivermectin-susceptible strain of *O. circumcincta*. Faecal samples from these animals were collected on 28, 35 and 42 days after the first infection and were used

Plate 4.1: Experimental paddocks utilised for the cross-over grazing trial.

Plate 4.2: Goats grazing in the experimental paddocks previously grazed by goats.

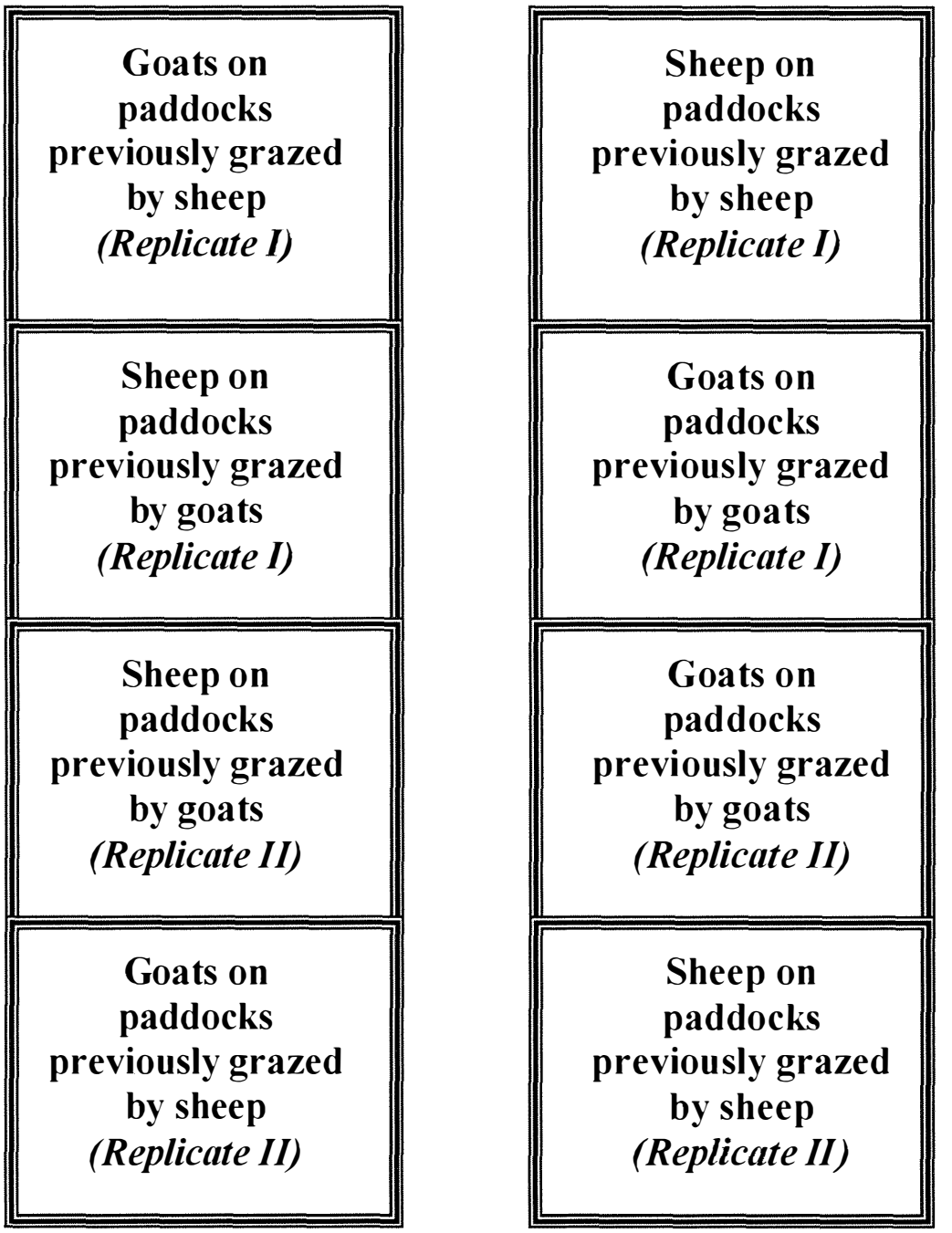


Figure 4.1: Layout of the trial design of the cross over study. Animals in right hand side paddocks were setstocked similarly as in previous trial (Pomroy *et al.*, 1997) and the animals on left-hand side paddocks were crossed over.

to determine the LC_{50} values of ivermectin and avermectin B_2 for susceptible *Ostertagia* spp. in the larval development assay.

Parasitological techniques

Ivermectin resistance was monitored using faecal egg count reduction tests, larval cultures pre- and post-treatment and larval development assays.

Faecal egg counts were performed with a modified McMaster method where each egg counted represented 50 eggs per gram of faeces (see Appendix I). The post-treatment faecal egg counts were available from the next sampling in two weeks. Bulked faecal samples from each group were also cultured at 27^{O}C to provide infective larvae for identification on each sampling occasion. The later results were used to provide estimates of the mean number of eggs of *Ostertagia* spp. present in each group, enabling reduction in faecal egg count of *Ostertagia* spp. to be calculated.

Larval development assays were carried out at each sampling depending on the availability of sufficient eggs. The assay was conducted as described previously in Section 2.2 (see Appendix VIII for detailed method) except that the eggs were recovered separately for each group from pooled faecal samples. Ivermectin and avermectin B_2 were utilised to assess the susceptibility of free-living stages and 200 µl of 2% agar was added to each drug concentration in a 96 well microtitre plate. The third stage larvae were differentiated by species on the basis of size (Gordon, 1933) and general morphology of the larvae (Lancaster and Hong, 1987).

Statistical analysis

The proportions of third stage larvae of *Ostertagia* spp. in the larval development assay were calculated by dividing the number of third stage *Ostertagia* spp. larvae in the test wells with the number of third stage *Ostertagia* spp. larvae in the control wells. The proportions were fitted to a sigmoid curve and the LC_{50} values were calculated using the software programme SlideWrite Version 3 (Advanced Graphics Software Inc., U.S.A.) and expressed as ng/ml in the agar phase. The resistance ratios were calculated for sheep by dividing the LC_{50} value for the resistant strain by the mean LC_{50} value for the susceptible strain.

4.3. RESULTS

GOAT GROUPS

Goats grazed on paddocks previously grazed by goats

In general results in both replicates of goats grazed on paddocks previously grazed by goats were similar. *Ostertagia* spp. in both replicates showed a high level of ivermectin resistance (Fig. 4.1 and see Appendices 4.1 & 4.2 for raw data). Initially, the percentage reduction in the mean faecal egg counts of *Ostertagia* spp. in replicate I was found to be 52% in the first faecal egg count reduction test where as in replicate II it was zero. In both replicates it subsequently fell to zero.

The percentage reduction in the mean faecal egg counts of *Ostertagia* spp. generally remained about zero to 25% throughout the experiment including in the new replacement goats in both replicates except on one occasion when it was 57% in replicate II. *Ostertagia* spp. dominated the pre-treatment faecal cultures in both replicates. The post-treatment faecal cultures consisted mainly of *Ostertagia* spp. and a small percentage of *Trichostrongylus* spp. larvae.

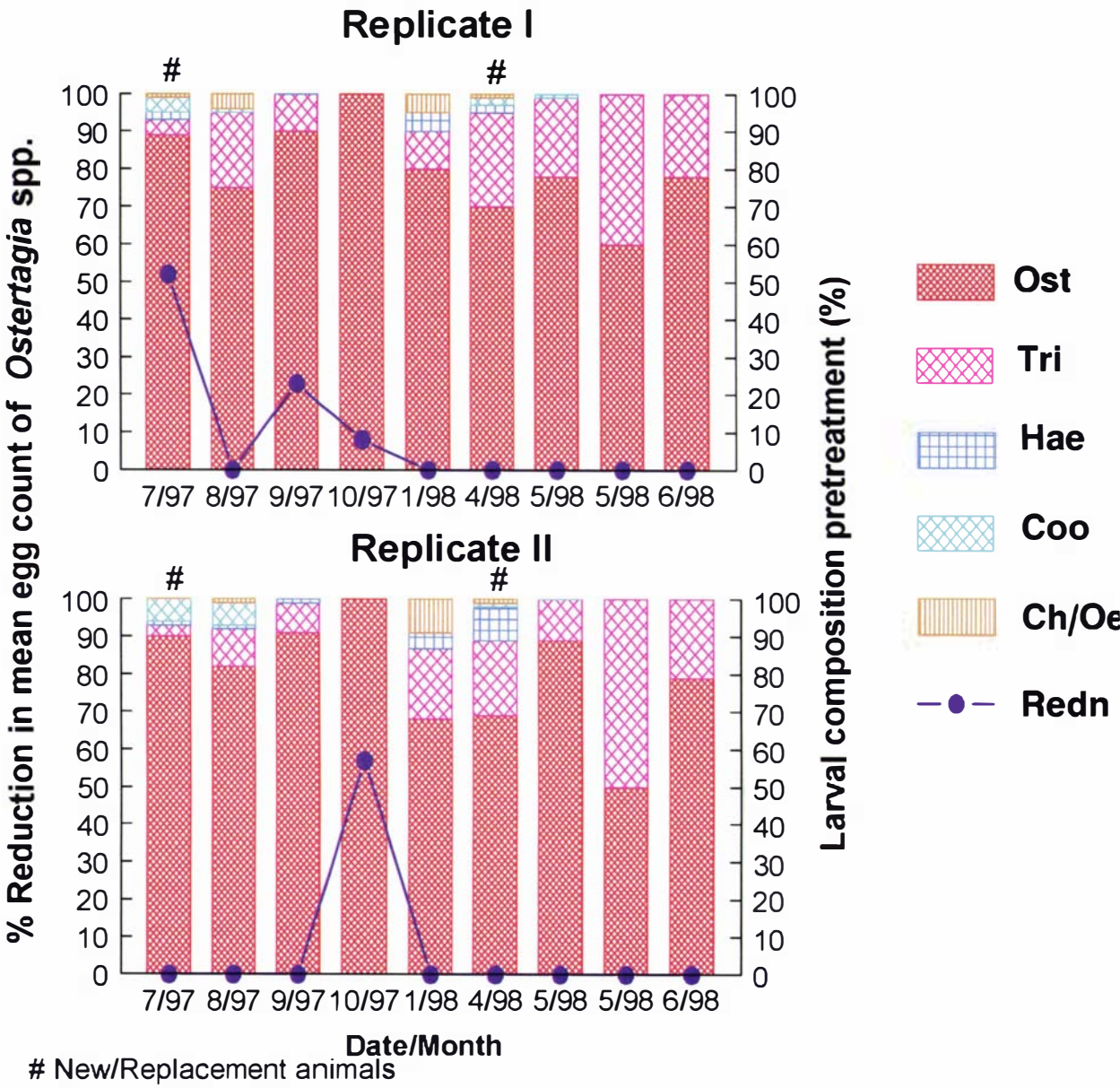


Figure 4.2: Mean faecal egg count reductions for *Ostertagia* spp. and pre-treatment larval composition in goats (n=6 in each replicate) grazed on paddocks previously grazed by goats. Goats were given 0.2 mg/kg b.wt. ivermectin orally and post-treatment faecal samples were collected after 2 weeks.

Goats grazed on paddocks previously grazed by sheep

The results in both replicates of goats grazed on paddocks previously grazed by sheep were similar. Both replicates revealed the presence of ivermectin resistance in *Ostertagia* spp. The percentage reduction in the mean faecal egg counts of *Ostertagia* spp. in replicate I was initially 61% in the first faecal egg count reduction test whereas in replicate II it was 57 and 27% in the first two faecal egg count reduction tests. In both replicates it fell to around zero in the subsequent faecal egg count reduction tests (Fig. 4.3, see Appendix 4.3 and 4.4 for raw data). When the new replacement goats were introduced, the reduction in mean faecal egg count for *Ostertagia* spp. increased to 70% and subsequently it fell to zero in replicate I where as in replicate II it was zero in the first three faecal egg count reduction tests. The last faecal egg count reduction test in replicate II showed a 21% reduction in the faecal egg counts of *Ostertagia* spp. *Ostertagia* spp. dominated the pre-treatment faecal cultures on most occasions. The post-treatment faecal cultures consisted mainly of *Ostertagia* spp. and a small percentage of *Trichostrongylus* spp. larvae.

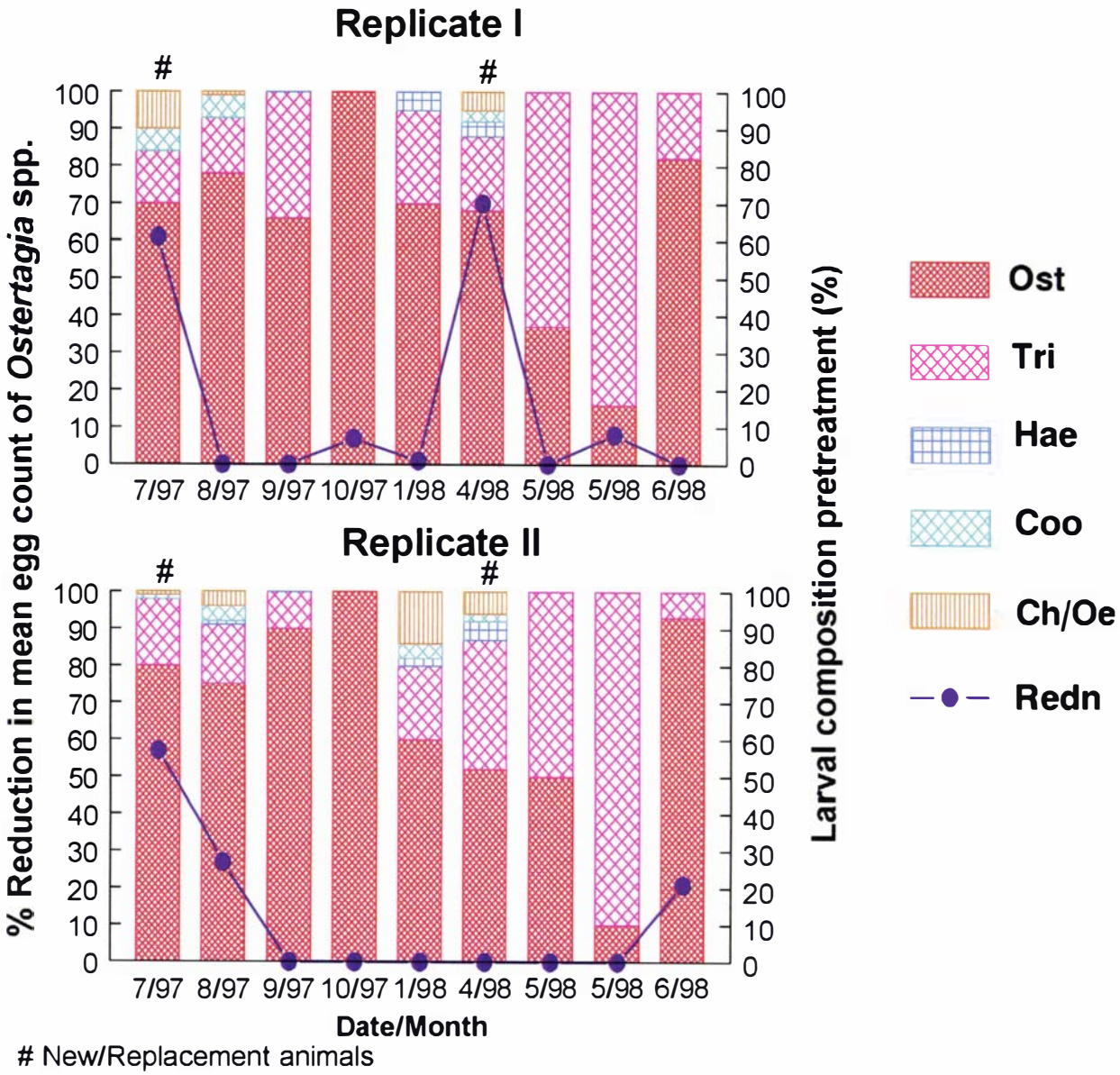


Figure 4.3: Mean faecal egg count reductions for *Ostertagia* spp. and pre-treatment larval composition in goats (n=6 in each replicate) grazed on paddocks previously grazed by sheep. Goats were given 0.2 mg/kg b.wt. ivermectin orally and post-treatment faecal samples were collected after 2 weeks.

SHEEP GROUPS

During the first six months, faecal egg count reduction tests and larval development assays could not be performed in all groups of sheep due to low faecal egg counts. In Nov. 1997 these older lambs were replaced with younger lambs of about three and a half months of age.

Sheep grazed on paddocks previously grazed by sheep

In general the results of both replicates of sheep grazed on the paddocks previously grazed by sheep were similar. In both replicates, the faecal egg count reductions of *Ostertagia* spp. remained close to 100% but declined to around 90% on occasions (Fig. 4.4 and see Appendix 4.5 for raw data).

The pre-treatment larval cultures in both replicates contained mixed genera with *Trichostrongylus* spp. being dominant on many occasions. *Ostertagia* was the only [illegible] present in the post-treatment cultures.

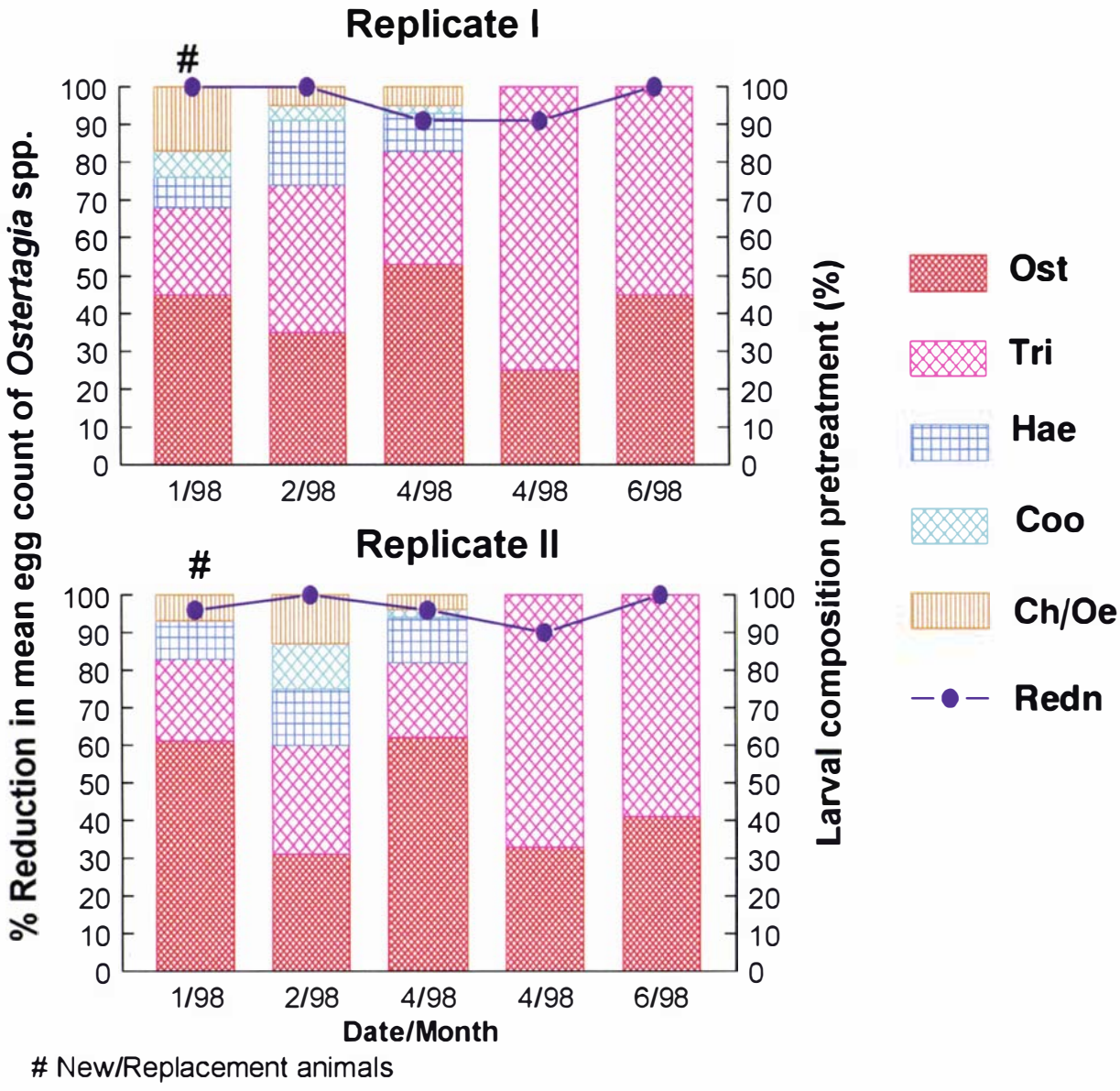


Figure 4.4: Mean faecal egg count reductions for *Ostertagia* spp. and pre-treatment larval composition in sheep (n=4 in each replicate) grazed on paddocks previously grazed by sheep. Sheep were given 0.2 mg/kg b.wt. ivermectin orally and post-treatment faecal samples were collected after 2 weeks.

Sheep grazed on paddocks previously grazed by goats

The results of both the replicates of sheep grazed on paddocks previously grazed by goats were very similar. Ivermectin resistant *Ostertagia* spp. appeared in replacement sheep after 5 months on paddocks grazed previously by goats. The faecal egg count reductions of *Ostertagia* spp. were around 100% in the first two faecal egg count reduction tests in both the replicates but these fell to between 48-65% in subsequent faecal egg count reduction tests (Fig. 4.5 and see Appendix 4.6 for raw data). Resistance remained apparent up to the end of the experiment. The pre-treatment larval cultures contained mixed genera with *Trichostrongylus* spp. being dominant on many occasions. *Ostertagia* spp. dominated the post-treatment cultures.

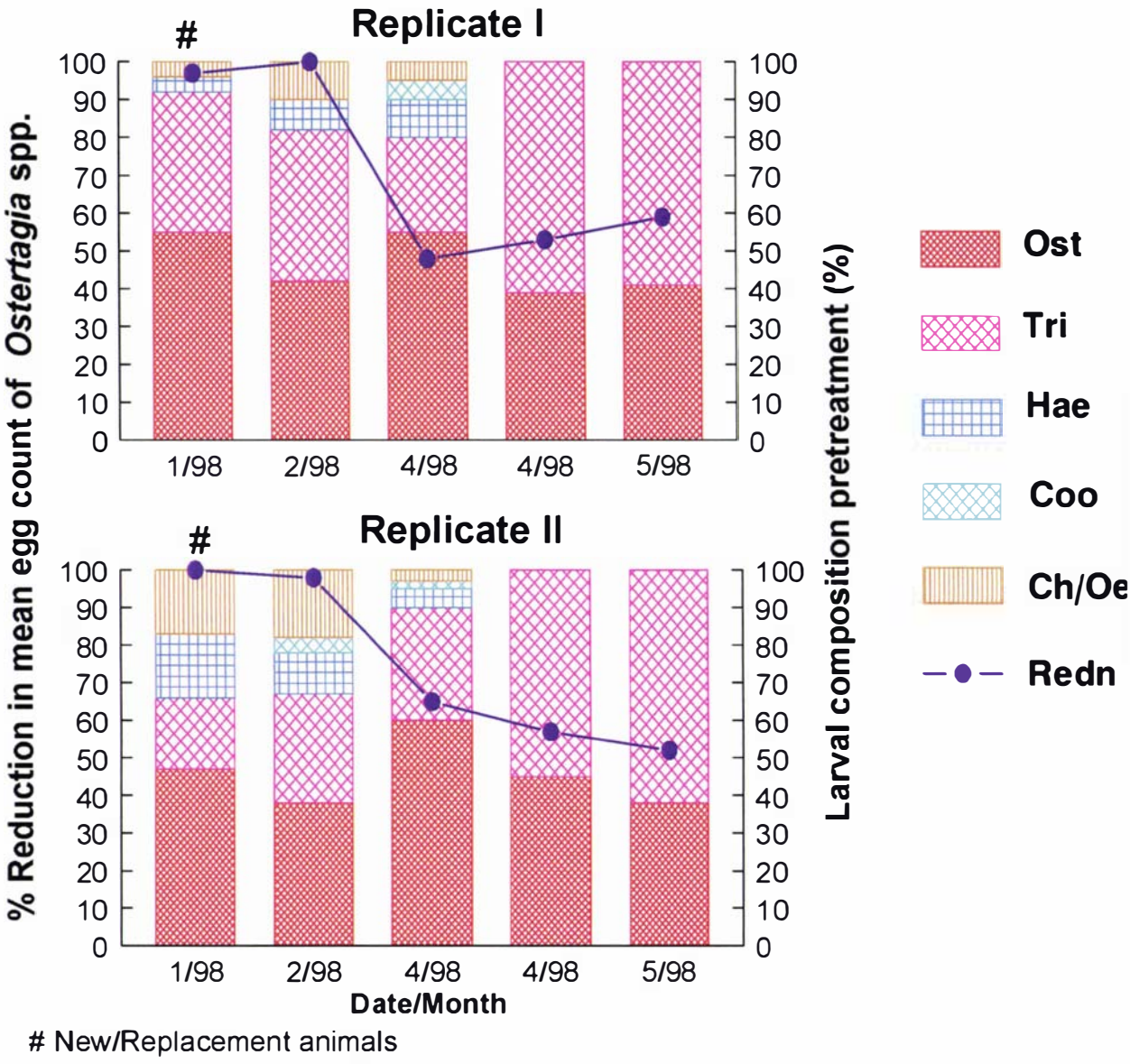


Figure 4.5: Mean faecal egg count reductions for *Ostertagia* spp. and pre-treatment larval composition in sheep (n=4 in each replicate) grazed on paddocks previously grazed by goats. Sheep were given 0.2 mg/kg b.wt. ivermectin orally and post-treatment faecal samples were collected after 2 weeks.

Larval development assay

Goat groups

The results of the larval development assay were similar in the replicates within each treatment (Fig 4.6). However, no statistical analysis was possible as each data point in each group consisted of a LC_{50} value from pooled faecal samples of six goats. The LC_{50} values of ivermectin for *Ostertagia* spp. in all the goat groups were higher than the LC_{50} values for the susceptible strain in sheep (Fig 4.6). The LC_{50} values of ivermectin for *Ostertagia* spp. in all the groups of goats were at the lowest point at the start of the experiment and after new replacement animals were introduced. The LC_{50} values rose further after ivermectin treatments and then declined again. However, when two ivermectin treatments followed each other in close succession, the LC_{50} values of ivermectin did not rise further after the second ivermectin treatment (see data points of 31/10 and 10/6 in Fig. 4.6).

The LC_{50} values of ivermectin in the first few larval development assays for goats grazed on paddocks previously grazed by sheep were slightly lower than the LC_{50} values of ivermectin for goats grazed on paddocks previously grazed by goats both at the start of the trial and after new replacement animals were introduced. However, subsequent larval development assays could not provide any conclusive evidence of a difference in the LC_{50} values of ivermectin of the different goat groups.

Avermectin B_2 showed similar patterns to ivermectin (Fig. 4.6) but the LC_{50} values of avermectin B_2 for *Ostertagia* spp. were always lower in all groups of goats as compared to the LC_{50} values of ivermectin.

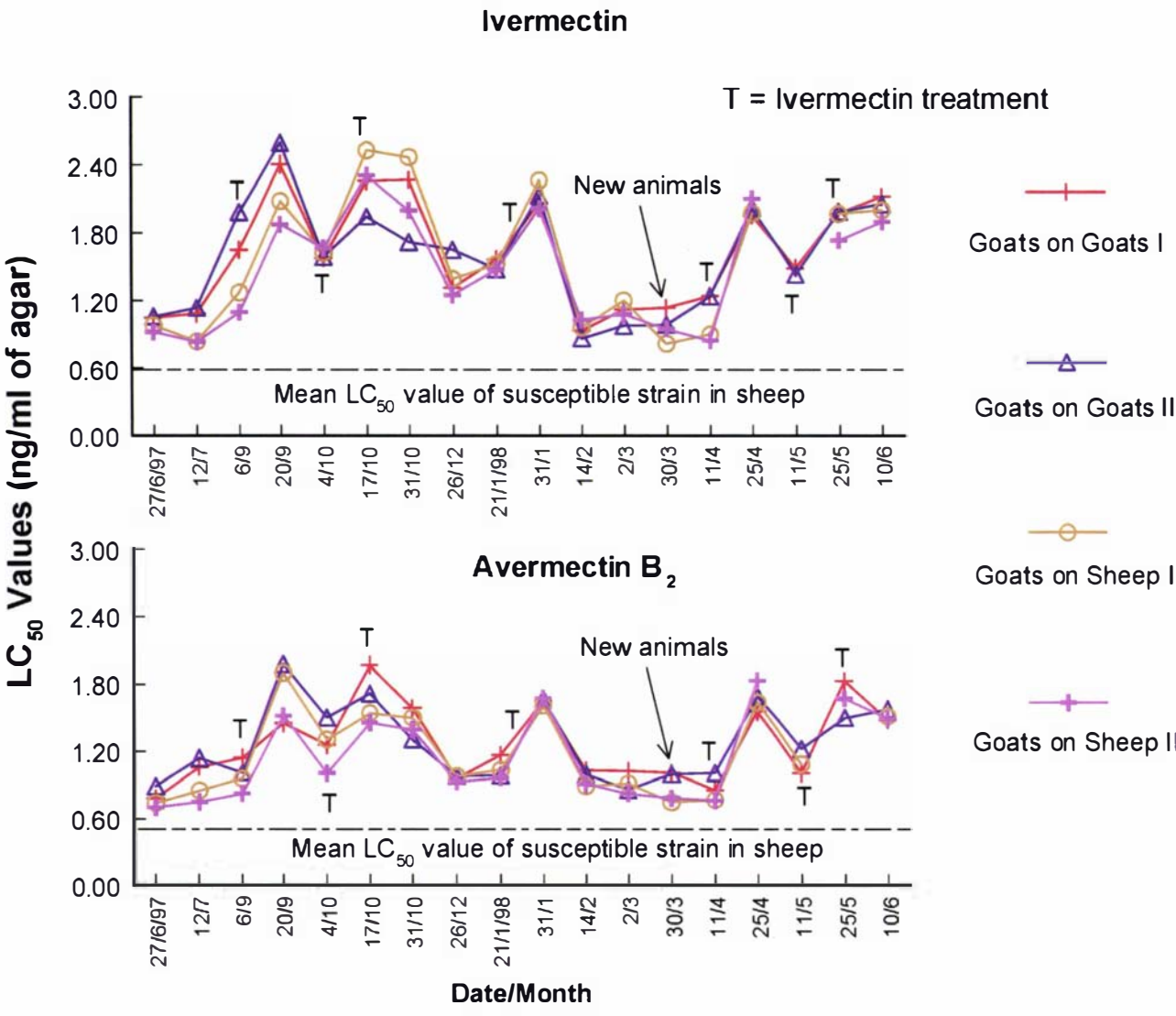


Figure 4.6: LC_{50} values of ivermectin and avermectin B_2 in goats grazed either on paddocks previously grazed by goats or sheep. Each data point represents the LC_{50} value from pooled faecal samples of six goats.

Sheep groups

The results of the larval development assay for the sheep groups were similar in the replicates within each treatment (Fig 4.7). However, no statistical analysis was possible as each data point in each group consisted of a LC_{50} value from pooled faecal samples of four sheep. The LC_{50} values of ivermectin for *Ostertagia* spp. in all the groups of sheep were higher than the LC_{50} values of the susceptible strain in sheep (Fig. 4.7 and see Appendix 4.7). The LC_{50} values were at the lowest point at the start of the experiment in all the sheep groups and rose further after the first ivermectin treatment in sheep grazed on the paddocks previously grazed by goats. Sufficient eggs were not available for the post-treatment larval development assays in sheep grazed on paddocks previously grazed by sheep. After the second ivermectin treatment in sheep grazed on the paddocks previously grazed by goats, which was given 2 weeks after the first treatment, the LC_{50} values did not rise further (see data point of 11/5 in Fig. 4.7). The LC_{50} values later declined to the original starting value four weeks after ivermectin treatment. The LC_{50} values after each ivermectin treatment in the sheep grazed on the paddocks previously grazed by goats were similar to the goat groups and did not elevate further.

The LC_{50} values of ivermectin for *Ostertagia* spp. in both replicates of sheep grazed on paddocks previously grazed by sheep were lower than the LC_{50} values of sheep grazed on paddocks previously grazed by goats before ivermectin treatment.

When the LC_{50} values of ivermectin for the resistant strain were compared with the mean LC_{50} value for the susceptible strain (see Appendices 4.7 and 4.8), the resistance ratios in sheep grazed on paddocks previously grazed by goats before ivermectin treatment varied from 1.6 to 2.4 and 1.5 to 2.4 in replicate I and II respectively whereas the resistance ratio in sheep grazed on paddocks previously grazed by sheep varied from 1.4 to 1.7 and 1.6 to 1.9 in replicate I and II respectively. The resistance ratios increased further to 3.2 to 3.8 and 3.6 to 3.8 after ivermectin treatment in replicate I and II of the sheep grazed on paddocks previously grazed by goats. Avermectin B_2 also showed similar trends (Fig. 4.7 and Appendix 4.7).

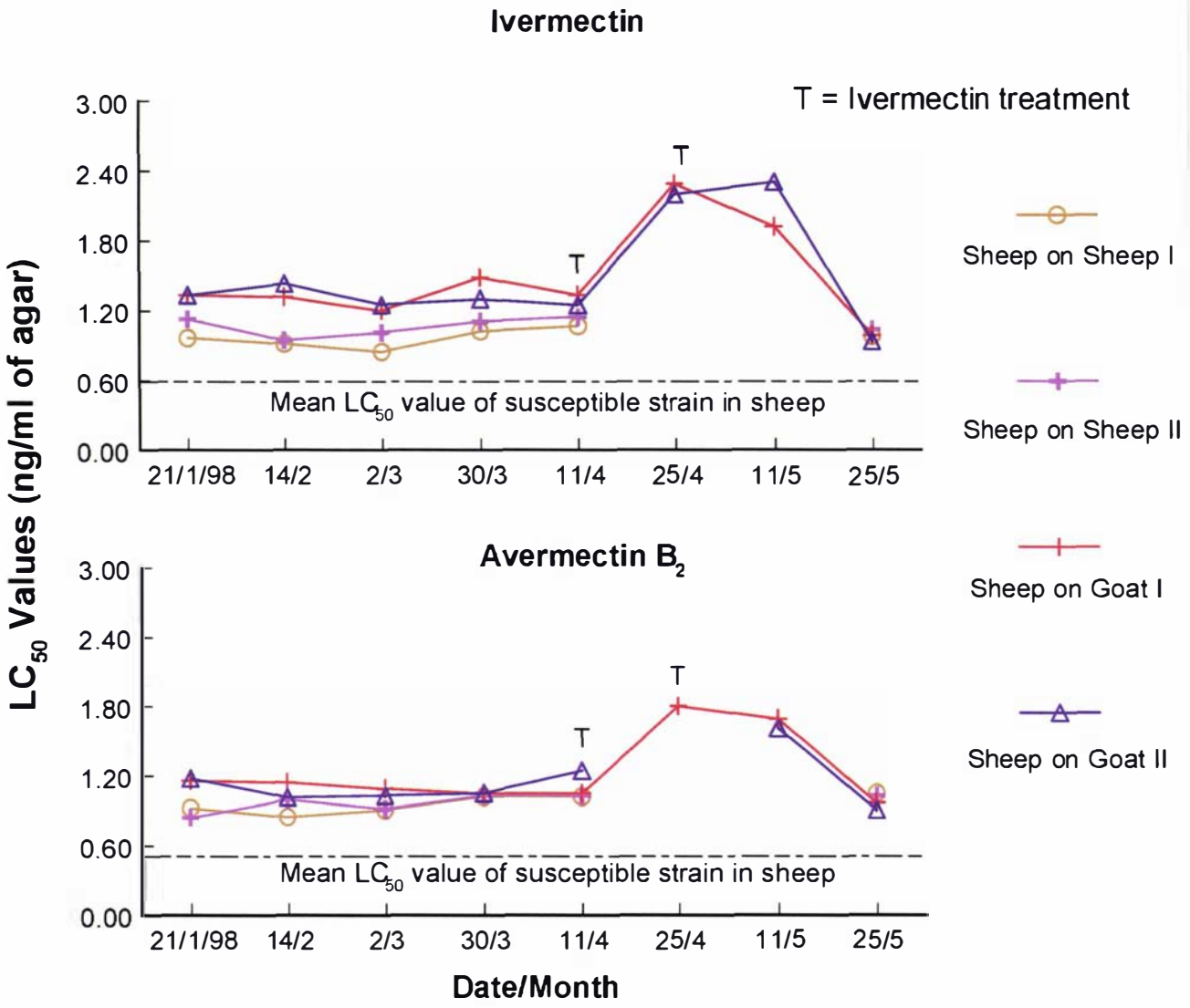


Figure 4.7: LC_{50} values of ivermectin and avermectin B_2 in sheep grazed either on paddocks previously grazed by goats or sheep. Each data point represents the LC_{50} value from pooled faecal samples of four sheep.

4.4. DISCUSSION

The protocol of drenching a particular host species with ivermectin, when any group mean faecal egg count for that host species exceeded 500 eggs per gram, was chosen because the faecal egg counts have been reported to be higher in goats as compared to sheep grazed on the same pasture (Pomroy *et al.*, 1986; Brunsdon, 1986). This is probably due to the fact that goats fail to display the level of resistance to nematode infections that is seen in sheep (Pomroy and Charleston, 1989). Therefore, if the protocol of drenching all the groups irrespective of species, when any group mean faecal egg count exceeded 500 eggs per gram, had been chosen, then the sheep groups would have been drenched each time along with the goat groups even when the sheep groups had lower faecal egg counts. Hence, it would have made difficult to detect resistance with faecal egg count reduction tests in sheep groups. This was seen as a problem in the earlier phase of this study.

During first six months of the trial, faecal egg count reduction tests and larval development assays could not be performed in the groups of sheep due to low faecal egg counts. The sheep available at the start of experiment were pasture reared 9 months old animals from the previous trial and were presumed to still be susceptible. However, the low faecal egg counts in these animals indicated the development of sufficient immune response which inhibited faecal egg output below the levels required to carry out faecal egg count reduction tests and larval development assays. Therefore, in Nov. 1997, at the earliest opportunity, these older lambs were replaced with younger lambs. The absence of eggs in all of the sheep groups during the first six months was unfortunate but has not affected the interpretation of the experiment.

All the goat groups, whether grazed on paddocks previously grazed by goats or sheep, showed a high level of ivermectin resistance in *Ostertagia* spp. during the very early stages of the trial. Further faecal egg count reduction tests in the goat groups including the newly replaced animals also indicated a high level of resistance as expected. During the first six months, none of the sheep groups were treated with ivermectin. As a result no further selection for ivermectin resistance took place and accordingly it is reasonable to compare the results of faecal egg count reduction tests and larval

development assay for the newly replaced lambs with the initial results of the goat groups.

The appearance of ivermectin resistance in the replacement lambs after five months grazing on paddocks previously grazed by goats indicates that the sheep can get infected with ivermectin-resistant *Ostertagia* spp. parasites if grazed on the pasture previously grazed by goats habouring ivermectin-resistant *Ostertagia* spp. However, the reductions in the faecal egg counts for *Ostertagia* spp. after ivermectin treatment were higher in sheep compared to goats grazed on the paddocks previously grazed by goats but the larval development assays indicated that the LC_{50} values of ivermectin and avermectin B_2 for *Ostertagia* spp. prior to ivermectin treatment in sheep and goats grazed on the paddocks previously grazed by goats were in the similar range (Figs. 4.6 and 4.7). These observations of the larval development assay probably reflected that the *Ostertagia* spp. parasitic populations with similar level of resistance were present in all the paddocks previously grazed by goats. Hence, the higher reductions in the faecal egg counts in sheep as compared to goats probably indicate a difference in the efficacy of ivermectin between sheep and goats against the ivermectin-resistant population of *Ostertagia* spp. These results support the previous findings of chapter 3, in which it was demonstrated that ivermectin is more effective in sheep as compared to goats against ivermectin-resistant *T. colubriformis*. This is also consistent with the findings that showed plasma concentration at 24 hours and area under the curve were lower in goats as compared to sheep after oral administration of ivermectin (Scott *et al.*, 1990a). They also demonstrated that the clearance of ivermectin from plasma was more rapid in goats than sheep.

The greater reduction in faecal egg counts of *Ostertagia* spp. for sheep as compared to goats was not reflected by higher post-ivermectin treatment LC_{50} values of ivermectin and avermectin B_2 for sheep than goats. This is possibly due to the lack of sensitivity of ivermectin and avermectin B_2 in differentiating different levels of resistance in *Ostertagia* spp. in larval development assay.

Another possible explanation for higher levels of resistance in *Ostertagia* spp. in goats as compared to sheep could be a difference in the dynamics of *Ostertagia* spp. between goats and sheep. Previous studies with *O. circumcincta* have indicated that a

function of the establishment of ingested L_3 is the gradual turnover of the existing worm burdens. A death rate of adult worms has been estimated as 4% per day in lambs (Paton *et al.*, 1984) and can be as high as 11% in adult ewes (Leathwick *et al.*, 1997). The rise in LC_{50} values after ivermectin treatments is presumably due to elimination of the susceptible population of *Ostertagia* spp. and thereafter decline in LC_{50} values of ivermectin is presumably due to establishment of the susceptible population of *Ostertagia* spp and dilution of the resistant population. These findings support the hypothesis that the population of worms is turning over. However, the LC_{50} values of ivermectin for *Ostertagia* spp. in goats grazed on the paddocks previously grazed by goats did not decline to the original starting values after 4 weeks of the first ivermectin treatment at the start of the trial and in newly replaced animals (see data points of 4/10 and 11/5 in Fig 4.6) whereas in sheep grazed on paddocks previously grazed by goats, the LC_{50} values declined to the original starting levels after 4 weeks of the second ivermectin treatment (see data point 25/5 in Fig. 4.7). Therefore, it is possible that ivermectin-resistant *Ostertagia* spp. survive a longer time in goats than in sheep and the turn over of the ivermectin survivor worms is slower in goats than sheep. This difference in the dynamics may lead to a greater accumulation of ivermectin-resistant *Ostertagia* spp. worm burdens in goats than in sheep.

Ivermectin resistance is considered to be inherited as a dominant trait in *H. contortus* (Dobson *et al.*, 1996; Le Jambre *et al.*, 2000) and *O. circumcincta* (Coles, 1997) in sheep. Ivermectin resistance in a goat-isolated strain of *T. colubriformis* is inherited as an incompletely dominant trait (see Chapter 5). It is reasonable to assume from the these reports and from the prevalence of widespread ivermectin resistance in *Ostertagia* spp. in goats that there is also some degree of dominance associated with the nature of inheritance of ivermectin resistance in *Ostertagia* spp. in goats.

The three factors mentioned above i.e. lower efficacy of ivermectin in goats against ivermectin-resistant *Ostertagia* spp., possibly extended survival time of ivermectin-resistant *Ostertagia* spp. in goats as compared to sheep and possibly some degree of dominance associated with inheritance of this resistance may lead to the rapid spread of ivermectin-resistant *Ostertagia* spp. in goats and from goats to sheep.

Ivermectin resistance was not consistently identified on the basis of faecal egg count reduction tests in either of the replicates of sheep grazed on paddocks previously grazed by sheep, although on two occasions in replicate I and one occasion in replicate II, faecal egg count reductions of *Ostertagia* spp. fell to around 90%. However, the LC_{50} values of ivermectin and avermectin B_2 in larval development assays in both the replicates of sheep grazed on paddocks previously grazed by sheep were always higher than the susceptible strain. The resistance ratios ranged from 1.4 to 2.0 in both replicates. These findings might indicate that ivermectin resistance was also emerging in the sheep grazed on the paddocks previously grazed by sheep. In the previous trial (Pomroy *et al.*, 1997) faecal egg count reductions also fell to around 90% on occasions in the sheep groups. It is possible, that if the trial had been extended, then ivermectin-resistant *Ostertagia* spp. would have emerged in sheep grazed on paddocks previously grazed by sheep.

In all the goat groups, even when pre-treatment faecal cultures were not dominated by *Ostertagia* spp., the faecal egg count reduction tests showed lower reductions in the total faecal egg counts with the exception of some initial faecal egg count reduction tests (see Appendices 4.1 to 4.4). On one occasion (10/97) in the second replicate of goats grazed on paddocks previously grazed by goats, the pre-treatment larvae culture showed 100% *Ostertagia* larvae but the faecal egg count reduction test revealed a 57% reduction in mean egg count, which was unexpected. This might have occurred due to the discrepancies in the faecal egg counting procedure. However, the higher pre-treatment egg counts does not support this hypothesis. Hence, It is difficult to explain the reason for this anomaly.

In New Zealand at least on one previous occasion, ivermectin-resistant *Ostertagia* spp. have been found in sheep grazing pasture contaminated by goats (Watson and Hosking, 1990). They reported a 93% reduction in *Ostertagia* worm counts after ivermectin treatment in lambs, which were grazed on a goat unit for 34 days in February/March 1988. In the present study, the reductions in faecal egg counts for *Ostertagia* spp. were lower (48-65%) after ivermectin treatment in sheep grazed on the pasture previously grazed by goats. The different results obtained in the present study and the study of Watson and Hosking (1990) might be due differences in the level of resistance in the two strains. In 1988 ivermectin resistance was an emerging problem

in goats and it was the first case of ivermectin resistance reported in New Zealand. Since then, ivermectin has been used widely for parasite control and ivermectin-resistant *Ostertagia* spp. have been reported more frequently in goats in New Zealand (Pomroy *et al.*, 1992; Watson *et al.*, 1996; see Chapter 3). Moreover, the present trial was a follow up study of a previous trial (Pomroy *et al.*, 1997).

In contrast to the findings of the present study, ivermectin resistance could not be detected by faecal egg count reduction tests in 12 month old hoggets and ewes which had grazed for about 6 weeks on pasture contaminated with ivermectin-resistant *Ostertagia* spp. derived from goats. *Ostertagia* spp. showed evidence ivermectin resistance only in one hogget (worm burden of 2300 after ivermectin treatment) out of 10 hoggets and 10 ewes (Pomroy *et al.*, 1992). It was the same farm but different paddocks where the previous trial (Pomroy *et al.*, 1997) and the present follow up study was undertaken. In the present study, the first two faecal egg count reduction tests in both replicates of sheep grazed on goat-contaminated pasture showed no evidence of ivermectin resistance but resistance emerged in the third faecal egg count reduction test (after 5 months of grazing) and remained apparent up to the end of the experiment. The goats and sheep were treated ten times with ivermectin in the previous two years (Pomroy *et al.*, 1997). Therefore, the additional selection pressure and the time to recycle the parasites seem to be an important factor in the emergence of ivermectin resistance in sheep grazed on goat contaminated pastures.

The LC_{50} values of ivermectin in both the replicates of goats grazed on paddocks previously grazed by sheep were lower in the first few larval development assays than those from the goats grazed on paddocks previously grazed by goats. The new replacement animals also showed the same trend. However, subsequent larval development assays could not differentiate conclusively between the LC_{50} values from the different groups of goats. Although ivermectin-resistant *Ostertagia* spp. populations were present in all the goat paddocks but the differences observed in the initial few larval development assays might suggest that the population harboured by the goats grazed on paddocks previously grazed by goats was slightly more resistant than the population haboured by goats grazed on paddocks previously grazed by sheep. Hence, the less resistant population of worms in goats grazed on paddocks previously grazed by sheep was

reflected as resistant in the faecal egg count reduction tests probably due to the lower efficacy of ivermectin in these goats.

The LC_{50} values of ivermectin in both the replicates of sheep grazed on paddocks previously grazed by sheep prior to ivermectin treatment were lower than for the sheep grazed on paddocks previously grazed by goats. These results might suggest that the populations of ivermectin-resistant *Ostertagia* spp. were present in all paddocks but the population harboured by sheep grazed on paddocks previously grazed by goats were slightly more resistant than the population harboured by sheep grazed on paddocks previously grazed by sheep. The less resistant population of worms in sheep grazed on paddocks previously grazed by sheep was reflected as susceptible population in faecal egg count reduction tests probably due to higher efficacy of ivermectin in sheep than in goats. The more resistant population of worms in the sheep grazed on paddocks previously grazed by goats were also reflected as resistant population in the faecal egg count reduction tests in sheep but there were higher reductions in faecal egg counts for *Ostertagia* spp. in sheep grazed on paddocks previously grazed by goats than goats grazed on paddocks previously grazed by goats. This probably again indicated that the ivermectin was more effective in sheep than in goats.

The resistance ratios recorded with ivermectin in the larval development assay for the sheep were always small even after treatment (range 1.4 to 3.8) and these were of similar magnitude (range 1.3 to 5.6) to that for ivermectin-resistant *O. circumcincta* (Amarante *et al.*, 1997). The choice of avermectin analogue has been shown to influence the resulting resistance ratios in larval development assays with *H. contortus*. Gill *et al.* (1995) reported that avermectin B_2 is the most sensitive probe for diagnosis of ivermectin resistance as it gave better differentiation between the resistant and susceptible strain of *H. contortus*. However in the present study with *Ostertagia* spp., the resistance ratios recorded with avermectin B_2 were similar to ivermectin. Therefore, avermectin B_2 does not seem to be the drug of choice for diagnosis of ivermectin resistance in *Ostertagia* spp. with a larval development assay. Therefore, it would seem that a larval development assay using ivermectin or avermectin B_2 would struggle to diagnose ivermectin resistance in *O. circumcincta* in the field. Further studies, investigating the usefulness of other macrocyclic analogues, for example,

ivermectin monosaccharide (G.C. Coles, personal communication) will be necessary to achieve better discrimination between resistant and susceptible strains of *Ostertagia* and to differentiate conclusively between various groups of goats and sheep.

In conclusion, the ivermectin-resistant *Ostertagia* spp. infective larvae were present in all the paddocks whether previously grazed by sheep or goats although they varied slightly in the level of resistance. It was the difference in the efficacy of ivermectin when administered to sheep or goats, which was, reflected by higher reductions in faecal egg counts in sheep compared with goats grazed on paddocks previously grazed by goats. Nevertheless, sheep are at risk from grazing pasture contaminated with goat-derived ivermectin-resistant *Ostertagia* spp. These resistant parasites may pose a potential threat to the sheep industry. The larval development assay requires the use of a macrocyclic lactone other than ivermectin and avermectin B_2 to be useful in detecting ivermectin resistance in *O. circumcincta* and to clearly differentiate between various levels of resistance.

CHAPTER FIVE

INHERITANCE OF IVERMECTIN RESISTANCE IN *TRICHOSTRONGYLUS COLUBRIFORMIS*

5.1. INTRODUCTION

The development of parasites exhibiting resistance to ivermectin makes it difficult for farmers and scientists to formulate effective worm control strategies. An understanding of the genetic mechanisms of ivermectin resistance is important, as the differences in the way ivermectin resistance is inherited are expected to affect the rate at which resistance develops. If resistance results from a completely dominant trait, then depending upon the selection intensity, at recommended dose rates, first generation (F_1) worms would contribute resistance (R) genes to the next generation. Generally, the rate of increase in resistance within a population will be greater if resistance is controlled by a dominant trait as compared to a recessive trait. This occurs because most recessive alleles are present typically in heterozygotes and the worms will be removed with treatment (Georghiou, 1972; Wood and Mani, 1981). Similarly, resistance will develop more rapidly if it is determined by a single gene than if determined by two or more genes. This occurs because, when resistance is polygenic there are more genotypes containing susceptible (S) alleles that are not completely removed by anthelmintic treatment and that can contribute those S alleles to future generations (Barnes *et al.*, 1985).

Genetic studies of ivermectin-resistant field isolates of *Haemonchus contortus* (Dobson *et al.*, 1996; Le Jambre *et al.*, 2000) have shown that ivermectin resistance is largely inherited as a dominant trait in this genera. Similarly, on the basis of limited data, Coles (1997) suggested that ivermectin resistance is also inherited as a dominant trait in *Ostertagia circumcincta*. It has also been suggested that ivermectin resistance is inherited as an incompletely dominant trait in a laboratory selected ivermectin-resistant isolate of *T. colubriformis* (Gill *et al.*, unpublished in Gill and Lacey, 1998).

The present trial was undertaken to investigate the nature of inheritance of ivermectin resistance in the field isolate of *T. colubriformis* originally obtained from goats (see Chapter 3).

5.2. MATERIALS AND METHODS

TRIAL I

Parasite isolates

The ivermectin-resistant strain of *T. colubriformis* had been isolated from a goat farm in Northland, New Zealand in 1997. Since its isolation (see Chapter 3), the strain had been passaged a further three times through goats, each time selected with ivermectin, once with 200 μg/kg body weight and twice with 400 μg/kg body weight. This further selection with ivermectin was performed to make the strain more homozygous for resistance.

The ivermectin-susceptible isolate used for cross mating was a laboratory strain, isolated from sheep prior to the introduction of ivermectin in New Zealand. Since then, this strain had been passaged through sheep many times and had never been exposed to anthelmintics.

Sheep

Nine Romney lambs aged about 3 months old were weighed and housed indoors following effective drenching with a double dose of ivermectin and a double dose of a combination product of ricobendazole and levamisole to remove any existing worm burdens. Lambs were fed a ration of lucerne and barley-based nuts and given access to hay and water *ad-libitum*.

Design

Six lambs were randomly allocated into two groups of three animals each. The lambs in group I were infected with 25,000 infective larvae of the ivermectin-resistant isolate consisting of 83% *T. colubriformis* and 17% *Ostertagia* spp. larvae. A mixed challenge was given because a smaller proportion of ivermectin-resistant *Ostertagia* spp. larvae were also present in the faecal culture obtained from the goats from which

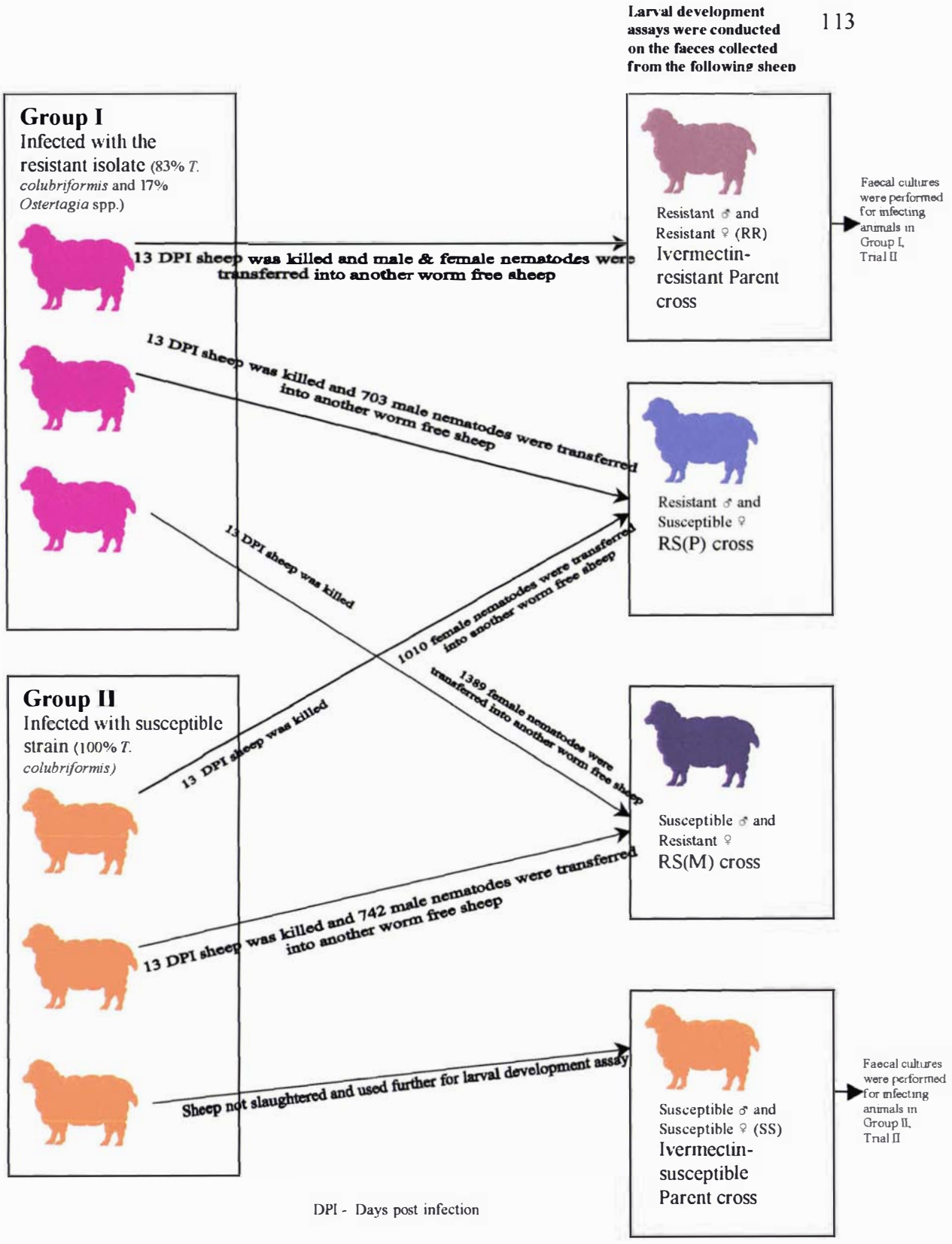


Figure 5.1: Diagrammatic representation of Trial I in which 13-day old *T. colubriformis* nematodes were transferred surgically into three worm free lambs to obtain ivermectin-resistant parent (RR) cross and two first generation (F_1) reciprocal crosses (RS(P) and RS(M)) of ivermectin-resistant and ivermectin-susceptible parents. The sheep which was not slaughtered in Group II was infected with ivermectin-susceptible parent (SS) cross.

this ivermectin-resistant strain of *T. colubriformis* was originally isolated (see Chapter 3) and it had not been passaged surgically to remove the ivermectin-resistant *Ostertagia* spp. The lambs in group II were infected with 25,000 infective larvae of the ivermectin-susceptible strain of *T. colubriformis*.

Parasitological techniques

Thirteen days post infection (DPI), all the lambs except one animal from group II, were killed humanely by captive bolt stunning and exsanguination. One animal from group II that was infected with the ivermectin-susceptible *T. colubriformis* strain was retained. Immediately after slaughter, the small intestines were removed and the contents were collected in Hanks solution (0.98g Hanks Balanced Salt Solution-Life Technologies, U.S.A. + 0.35g Sodium bicarbonate + 1g glucose/litre of distilled water) maintained at 37^{O}C in a water bath. The live male and female *T. colubriformis* nematodes were separated with the aid of a dissecting microscope at 15x magnification and maintained in Hanks solution at 37$^{\bullet}$C to ensure viability of nematodes. Later on the same day, these nematodes were surgically transferred into the duodenum of another three worm-free lambs as shown in Fig. 5.1.

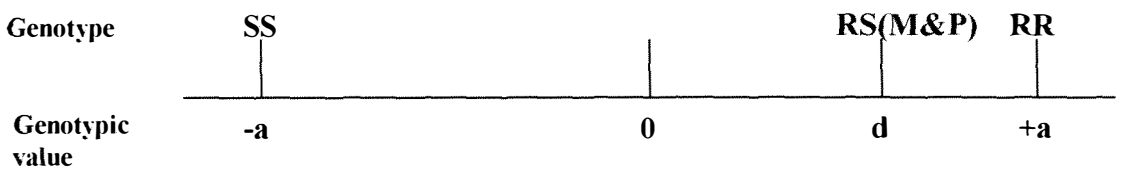
Larval development assay

The larval development assay was performed as previously described in Section 2.2 except that the assay was conducted using ivermectin at 4 day intervals from 23 to 35 days post infection (10-22 days post worm transplantation) and each concentration of ivermectin was mixed with 150 µl of 2% agar in a 96 well microtitre plate. Ivermectin aglycone, which is the analogue of choice for diagnosis of ivermectin resistance in *T. colubriformis* (see Chapter 7), was not available at the time of this trial. The detailed procedure of the assay is also presented in Appendix VIII.

Statistical analysis

The LC_{50} values from the larval development assays were calculated as in section 3.2. The proportion of eggs developing into infective third stage larvae was calculated from the dose response curves, using the software programme SlideWrite Version 3.0 (Advanced Graphics Software Inc., U.S.A.).

The degree of dominance was assessed by estimating genotypic values (a and d) of the following scale from the mean LC_{50} values of the days tested (Falconer, 1981). The genotypic values of the ivermectin-susceptible parent (SS) cross, ivermectin-resistant parent (RR) cross and first generation (F_1) reciprocal crosses (RS(M and P)) were called as -a, +a and d respectively (Falconer, 1981).



$$\text{Where } a = \frac{\text{Mean } LC_{50} \text{ value * for RR cross - Mean } LC_{50} \text{ value * for SS cross}}{2}$$

0 (Point of Zero) = Mid way mean LC_{50} value* for ivermectin-resistant and ivermectin-susceptible parent crosses

d = Mean LC_{50} values* for the F_1 reciprocal crosses - Mid way mean LC_{50} value* between ivermectin-resistant and ivermectin-susceptible parent crosses

* Mean LC_{50} values on 23 to 35 days post infection

TRIAL II

It was not possible to replicate Trial I because of the time involved in differentiating 13-day old nematodes by sex and the need to transplant the nematodes surgically soon after collection to ensure they remained viable. Therefore, each genetic cross of susceptible and resistant nematodes was represented in only one animal. It was decided to repeat the trial to improve the validity of results, as it is possible that the host may influence the larval development assay in some way.

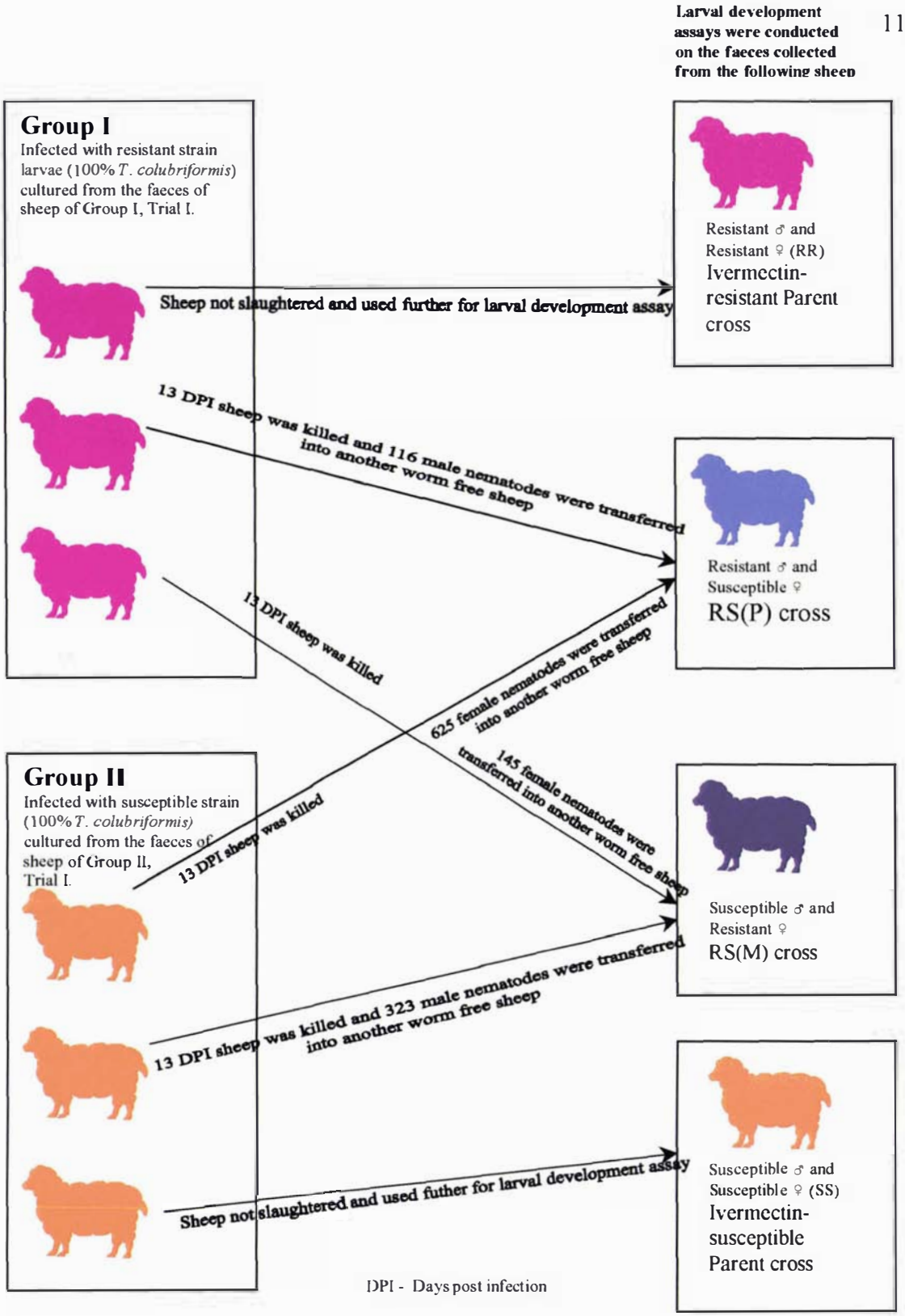


Figure 5.2: Diagrammatic representation of Trial II in which 13-day old *T. colubriformis* nematodes were transferred surgically into two worm free lambs to obtain two F_1 reciprocal crosses (RS(P) and RS(M)) of ivermectin-resistant and ivermectin-susceptible parents. The sheep which were not killed in Group I and II were infected with ivermectin-resistant parent cross (RR) and ivermectin-susceptible parent cross (SS) respectively.

A similar trial design was used except that the lambs in group I were infected with the larvae collected from faeces of the lamb infected with the ivermectin-resistant strain in trial I. The lambs in group II were infected with the larvae collected from the faeces of the lamb infected with the ivermectin-susceptible strain in trial I. Four of the six lambs were slaughtered (Fig. 5.2). One lamb from group I was retained because the ivermectin-resistant strain of *T. colubriformis* used to infect the lambs in group I was now a pure culture. Therefore this lamb harboured ivermectin-resistant parent cross of *T. colubriformis* nematodes. Similarly, one lamb from group II was retained because it harboured ivermectin-susceptible parent cross of *T. colubriformis* nematodes. The surgery was performed in two lambs to obtain two F_1 reciprocal crosses of the resistant and susceptible nematodes. The details of nematodes, which were surgically transferred into the duodenum of two worm-free lambs, are shown in Fig. 5.2. The larval development assays were carried out at weekly intervals from 23 to 79 DPI (10-66 days post worm transplantation) to observe whether the LC_{50} values change with the age of infection or not.

5.3. RESULTS

The LC_{50} values of ivermectin for the F_1 crosses between resistant female and susceptible male nematodes (RS(M)) and the crosses of resistant male and susceptible female nematodes (RS(P)) were consistently lower than the LC_{50} values for the ivermectin-resistant parent nematodes but greater than the LC_{50} values for the ivermectin-susceptible parent nematodes in both trials (Fig. 5.3 and 5.4). The LC_{50} values were relatively constant in each animal from 23 to 35 days post infection.

The LC_{50} values of ivermectin for the cross between resistant female and susceptible male nematodes were marginally greater than the corresponding values for the cross of resistant male and susceptible female nematodes in Trial I. This trend was not apparent in Trial II where there was no practical difference in the LC_{50} values between RS(P) and RS(M) crosses.

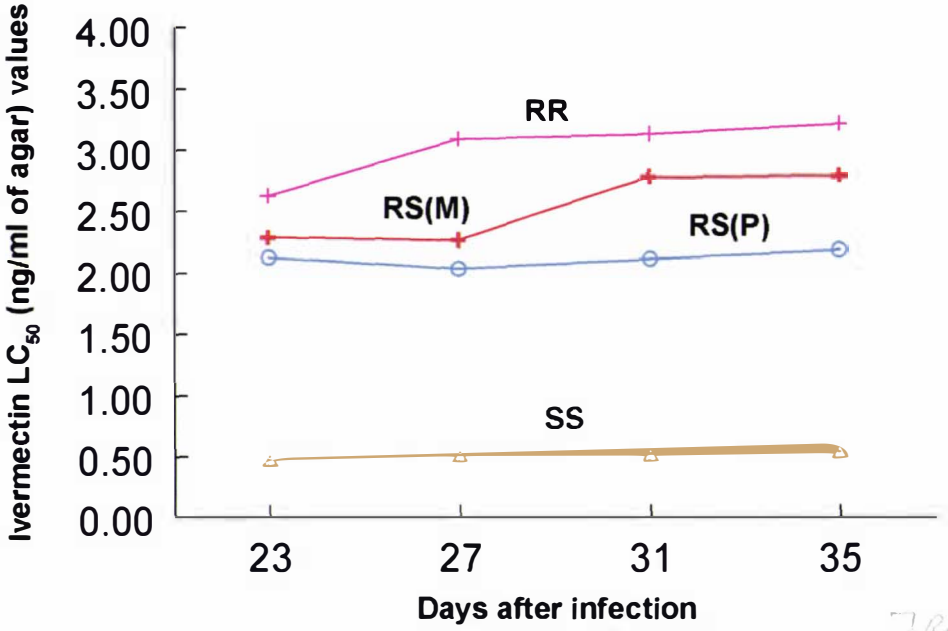


Figure 5.3: LC_{50} values of ivermectin for ivermectin-resistant parents (RR), F_1 cross of resistant female and susceptible male (RS(M)), F_1 cross of resistant male and susceptible female (RS(P)) and ivermectin-susceptible parents (SS) of *T. colubriformis* nematodes in trial I at days 23 to 35 post infection. Note there is one animal per group.

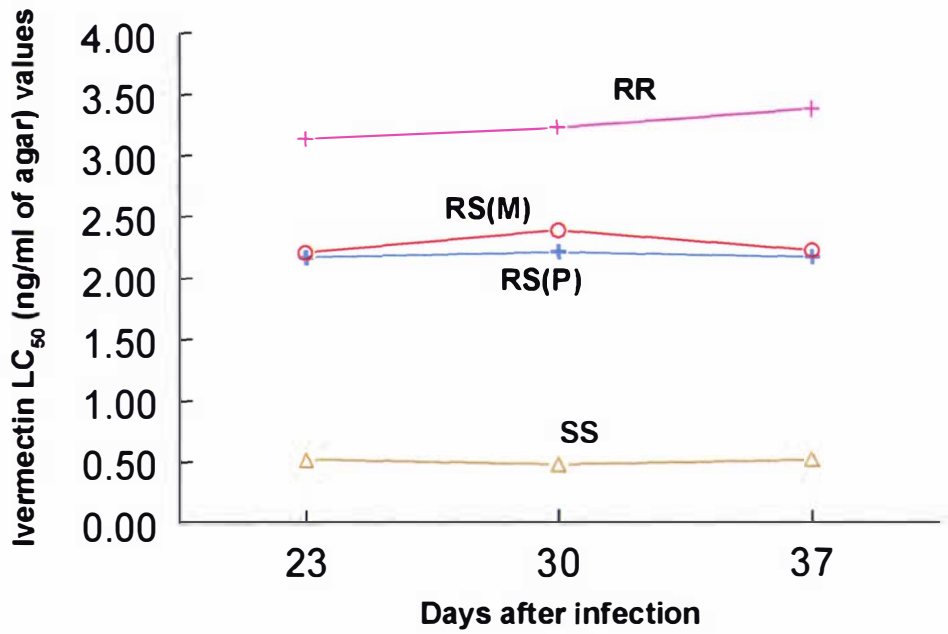
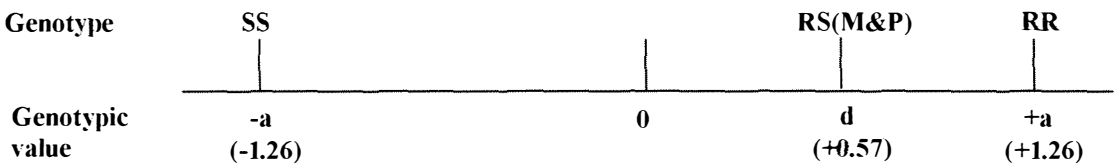


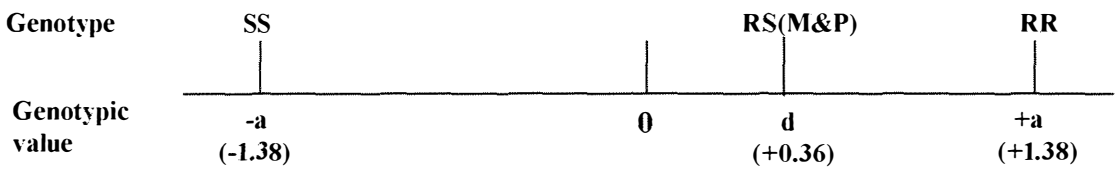
Figure 5.4: LC_{50} values of ivermectin for ivermectin-resistant parents (RR), F_1 cross of resistant female and susceptible male (RS(M)), F_1 cross of resistant male and susceptible female (RS(P)) and ivermectin-susceptible parents (SS) of *T. colubriformis* nematodes in trial II at days 23 to 37 post infection. Note there is one animal per group.

The values of a and d for Trials I and II were calculated (see Appendix 5.1 and 5.2 for details) and are shown below. The estimated d value lay between mid zero point and +a in both the trials. The estimated d value was slighter greater in trial I than in trial II.

Trial I



Trial II



In trial II the lambs were monitored using the larval development assay for a longer period to determine if the LC_{50} values varied as the parasite burden aged. The LC_{50} values were relatively constant for each animal at 23-37 days post infection, then rose to a peak for each animal between 51 to 58 days post infection followed by a decline close to the original starting values by 72 days post infection for all the animals (Fig. 5.5).

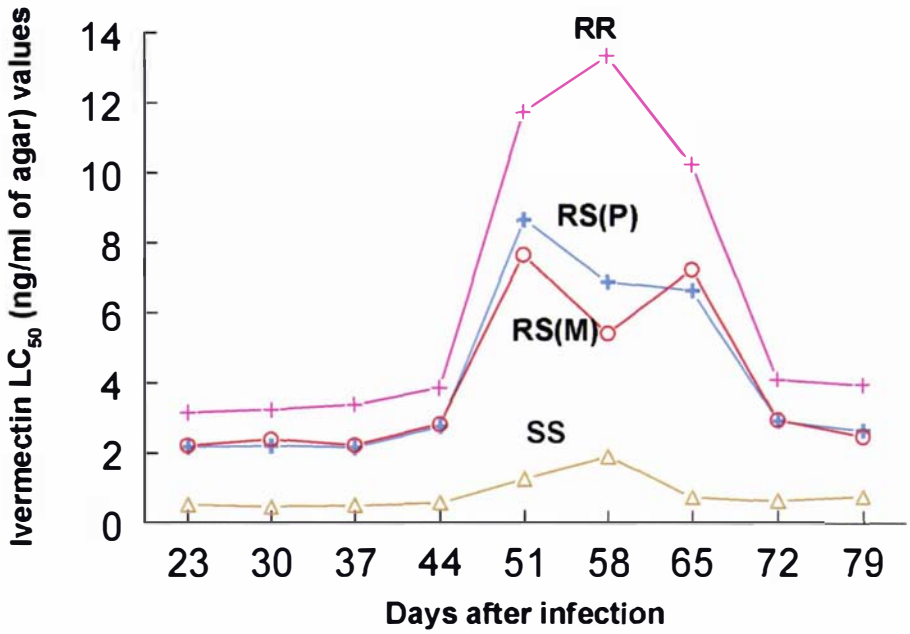


Figure 5.5: LC_{50} values of ivermectin for ivermectin-resistant parents (RR), F_1 cross of resistant female and susceptible male (RS(M)), F_1 cross of resistant male and susceptible female (RS(P)) and ivermectin-susceptible parents (SS) of *T. colubriformis* nematodes in trial II at days 23 to 79 post infection. Note there is one animal per group.

5.4. DISCUSSION

Genetic studies of anthelmintic resistance have been made easier with the development of *in vitro* assays such as those which monitor inhibition of development in the free living larval stages of the parasites (Gill *et al.*, 1995). These assays are cheaper and easier to perform than *in vivo* assays, which require large numbers of animals.

From the results of this study, it is apparent that ivermectin resistance in this isolate of *T. colubriformis* is inherited as an incompletely dominant trait and there was no evidence of sex linkage. The LC_{50} values of ivermectin for the two F_1 reciprocal crosses of ivermectin-resistant and ivermectin-susceptible parents (Resistant males × Susceptible females and Resistant females × Susceptible males worms) were lower than the ivermectin-resistant parent nematodes but much greater than the ivermectin-susceptible parent nematodes. The small difference observed in Trial I in the LC_{50} values for the crosses between resistant female and susceptible male nematodes and

the crosses between resistant male and susceptible female nematodes may reflect a difference between the immune response of the two animals rather than the influence of sex on inheritance of ivermectin resistance in *T. colubriformis*. However, the results of Trial II were similar to the first trial but no differences in the LC_{50} values were observed between the crosses of resistant female and susceptible male nematodes and the crosses of resistant male and susceptible female nematodes. This supports the view that the differences recorded in Trial I between the LC_{50} values for the two heterozygous crosses were influenced by the immune status of the animals. Hoza *et al.* (1997) also showed that the host immune response influenced the LC_{50} values of ivermectin using a larval development assay in sheep.

The estimated d value, which effects the degree of dominance also supports the inheritance of ivermectin resistance as an incompletely dominant trait. The estimated d value lay between mid zero point and +a in both the trials although there was some evidence of more dominance in trial I than in trial II. There is no dominance when the d value is zero. Resistance is completely dominant when the d value is equal to +a and the resistance is overdominant when the d value is greater than +a (Falconer, 1981). The slightly greater dominance (higher d value) in Trial I as compared to Trial II is most likely influenced by the difference in the host immune response.

The difficulties involved with these types of genetic studies are the time involved in separating the immature worms into males and females and the requirement for surgical transplantation of these worms as quickly as possible. Therefore, only one animal per genetic cross was feasible at a time and investigation of individual animal variation was not possible. Previous genetic studies with *H. contortus* (Dobson *et al.*, 1996; Le Jambre *et al.*, 2000) and *O. circumcincta* (Coles, 1997) also utilised one animal per genetic cross. However, in the present study, the trial was repeated to help establish the validity of the results.

In contrast to the findings of the present study, ivermectin resistance in field isolates of *H. contortus* (Dobson *et al.*, 1996; Le Jambre *et al.*, 2000) and *O. circumcincta* (Coles, 1997) is considered to be inherited as a completely dominant trait. In these studies, the level of ivermectin resistance of F_1 crosses was indistinguishable from that of avermectin-resistant parents in a larval development assay. The genetic analysis of

avermectin resistance in a field isolate of *H. contortus* showed that a single gene largely controls avermectin resistance. The dose response curves obtained for F_2 crosses indicated the presence of 25% ivermectin-susceptible individuals, suggesting that a single gene controls the ivermectin resistance in *H. contortus* (Le Jambre *et al.*, 2000). In the present study, no F_2 reciprocal crosses were obtained and hence the involvement of single or multiple genes in inheritance of ivermectin resistance in *T. colubriformis* could not be determined. Further work is needed to explore this aspect.

The findings of the present study are also in contrast to the genetic control of resistance to the benzimidazoles (Roos *et al.*, 1993; Grant, 1994) and levamisole (Martin and McKenzie, 1990), which were shown to be recessive traits. Ivermectin resistance in insects has also been found to be recessive. Argentine and Marshall Clark (1990) found that ivermectin resistance in Colorado potato beetle, *Leptinotarsa decemlineata* was inherited autosomally and was recessive. Similarly, ivermectin resistance in the housefly was found to be inherited as an autosomal recessive trait (Konno and Scott, 1991). The different nature of inheritance of ivermectin resistance in insects and ivermectin-resistant nematodes is probably due to the different mechanism of action of ivermectin in insects and nematodes (see Section 1.2.1.3).

The degree of dominance of a resistance trait will determine the number of surviving adults after treatment and consequently how rapidly resistance may emerge. For example, in the present study, when the ivermectin concentration in agar was 3.16 ng/ml then on average 46% and 50% of eggs from two F_1 reciprocal crosses (possibly heterozygotes) and ivermectin-resistant parent (possibly homozygous) nematodes survived and developed to third stage larvae in the larval development assay whereas the survival and the development rate was only 15% for eggs from the ivermectin-susceptible parent (possibly homozygous) nematodes (see Appendix 5.3). In the early stage of the development of resistance the R allele(s) frequency is low and the majority of resistance alleles are present in heterozygous nematodes and the numbers of homozygous resistant nematodes are very low. When the resistance is a completely dominant trait, the anthelmintic is not effective against heterozygous nematodes and will leave behind the resistant nematodes carrying R alleles. As the susceptible nematodes are removed by the anthelmintic, the R allele frequency in the population will increase. Similarly, when the resistance is an incompletely dominant trait, the

anthelmintic is partially effective against heterozygous nematodes and will leave behind some of the resistant nematodes carrying R alleles. As the susceptible nematodes are removed by the anthelmintic the R allele frequency in the population will increase, but at a slower rate, compared to a dominant trait. When the resistance is inherited as a recessive trait, the anthelmintic is highly effective against heterozygous nematodes and will remove most of resistant alleles. Selection for resistance will be slower because the number of the resistant genes is diminished at almost the same rate as the susceptible gene pool. It has also been suggested on the basis of a model that if resistance is inherited as a dominant trait, it will develop more rapidly than if resistance is inherited as an incompletely dominant or recessive trait (Barnes *et al.*, 1995). Hence it is expected that ivermectin resistance in *H. contortus* and *O. circumcincta* will develop more quickly than ivermectin resistance in *T. colubriformis*. This could explain why ivermectin resistance in *H. contortus* and *O. circumcincta* has been reported in a number of countries (see Section 1.5) whereas ivermectin resistance in *T. colubriformis* has not. However, ivermectin resistance in *H. contortus* has not yet been reported in New Zealand. The reasons for this are unknown.

McKenzie (1985) has argued that selection with suboptimal doses of an anthelmintic are more likely to produce polygenic resistances whereas selection using recommended doses will select for monogenic resistances. This argument was later supported by Gill *et al.* (1998) who showed that selection with suboptimal doses of ivermectin (Laboratory selection) produced a different mechanism of ivermectin resistance to that produced by selection with the recommended dose (Field selection) in *H. contortus*. Ivermectin is known to affect both pharyngeal pumping (i.e. development) and motility in parasitic nematodes. In their study, avermectin resistance in two laboratory isolates was not associated with a decreased sensitivity to avermectin inhibition in a larval development assay or in a L_3 motility assay whereas a field isolate showed markedly reduced sensitivity to avermectin inhibition of both larval development and L_3 motility. The nature of inheritance of avermectin resistance was found to be a completely dominant, monogenic trait in the field isolate but the nature of inheritance in laboratory isolates is still unknown (Gill *et al.*, 1998). In contrast to these findings, the field-isolated ivermectin-resistant strain of *T. colubriformis* in the present study was found to be have a similar mode of inheritance as found with the

laboratory-selected strain of *T. colubriformis* (Gill *et al.* unpublished in Gill *et al.*, 1998). A possible explanation could be that the isolate used in the present study was originally recovered from goats where suboptimal dosing might have occurred due to differences in the pharmacokinetics of ivermectin between sheep and goats (Scott *et al.*, 1990a). This may have resulted in a similar mode of inheritance of ivermectin resistance in this goat isolate and the laboratory-selected isolate of *T. colubriformis*.

The rise and fall in the LC_{50} values of ivermectin in the larval development assay in all the animals in trial II might be due to the involvement of host immune response and/or might be related to the age of parasites, with nematodes of a certain age being more resilient to the effects of anthelmintic. However, the exact reason for this rise and fall in LC_{50} values has not yet been determined. These findings are in agreement with the similar rise and fall reported using ivermectin with *T. colubriformis* (Hoza, 1998) and *O. circumcincta* (Amarante *et al.*, 1997). Borgsteede and Couwenberg (1987) also reported a remarkably similar pattern for *H. contortus* and thiabendazole to the extent that the overall trends, the relative size of the increase and the time to reach peak LC_{50} values were similar to those reported in the present study. In contrast to these trends, Scott *et al.* (1989) found no obvious pattern to the changes in LC_{50} values in an egg hatch assay with a benzimidazole-resistant isolate of *O. circumcincta*. Rather, they reported an erratic pattern with higher LC_{50} values in the early part of infection, falling to a lower level later.

The larval development assay always differentiated between ivermectin-resistant parents, ivermectin-susceptible parents and two reciprocal F_1 crosses when the comparisons were made on the same day. However, taking the extreme values (high or low) associated with the rise and fall in LC_{50} values with the age of parasite, ivermectin in a larval development assay would be just able to differentiate between ivermectin-resistant parent and ivermectin-susceptible parents but probably not ivermectin-susceptible parents from reciprocal F_1 crosses. The overlaps of these LC_{50} values for ivermectin when compared at different times of infection may have important implications for the use of this type of assay in field where a range of parasite age would be expected. However, when ivermectin aglycone is used as an analogue in the larval development assay, resistance can be detected under both field and experimental conditions (see Chapter 7).

This Chapter has shown how ivermectin resistance is inherited in an ivermectin-resistant strain of *T. colubriformis*. The next Chapter will deal with the fitness characteristics of this strain in comparison with the two ivermectin-susceptible field isolates of *T. colubriformis* to determine whether reversion to susceptibility is likely to occur in this strain or not.

CHAPTER SIX

FITNESS STUDIES WITH AN IVERMECTIN-RESISTANT FIELD ISOLATE OF *TRICHOSTRONGYLUS COLUBRIFORMIS*

6.1. INTRODUCTION

For long-term rotation of anthelmintics to be effective and to avoid multiple resistance, it is necessary for the nematodes, which have become resistant to one anthelmintic, to revert to being susceptible again during the time that a second anthelmintic is being used. Reversion requires that the susceptible phenotype has a greater biological fitness than the resistant phenotype in the absence of anthelmintic treatment. Studies with benzimidazole-resistant *Haemonchus contortus* (Kelly *et al.*, 1978) and a phenothiazine-resistant strain of *H. contortus* (Drudge, 1957b) suggest that the resistant genome is more fit than the susceptible genome. Recently, a study utilising PCR technique to investigate the fitness status of benzimidazole-resistant *Ostertagia circumcincta* suggested that the resistant genotypes (RR and RS) were equally as fit as the susceptible genotype (SS) within the same strain (Elard *et al.*, 1998). In contrast to these findings, Maingi *et al.* (1990) demonstrated that a thiabendazole-resistant strain of *H. contortus* was less fit than a thiabendazole-susceptible strain of *H. contortus*. However, further selection of the thiabendazole-resistant strain for four generations produced similar fitness characteristics comparable to that of the susceptible strain. Trials aimed to investigate the potential for benzimidazole-resistant strains of *H. contortus* (Hall *et al.*, 1982), *O. circumcincta* (Martin *et al.*, 1988a) and *T. colubriformis* (Hall *et al.*, 1982) to revert towards anthelmintic susceptibility found no reversion to susceptibility in these nematode species. Therefore, resistance will be stable and reversion will be very slow to occur, if it occurs at all.

There are reports of populations of *H. contortus* (Simpkin and Coles, 1978; Kelly and Hall, 1979), *O. circumcincta* (Donald *et al.*, 1980; Waller *et al.*, 1983) and *T. colubriformis* (Simpkin and Coles, 1978; Waller *et al.*, 1989), previously resistant to benzimidazoles, showing some reversion towards susceptibility. However, these

studies could not demonstrate clearly the extent of reversion to susceptibility, which occurred in these nematode species.

Most of the studies have examined the fitness status or reversion to susceptibility in benzimidazole-resistant strains of nematodes. No study has examined the fitness characteristics of ivermectin-resistant strains of nematodes. Therefore, the present work was undertaken to investigate the biological fitness of the ivermectin-resistant strain of *T. colubriformis* by comparing it with two normal ivermectin-susceptible field isolates of same parasite. Parameters measured were infectivity, survivability of worms in sheep, fecundity, efficiency of development of eggs to infective larvae on pasture and in laboratory conditions and the survival of infective larvae at different temperatures under laboratory conditions.

7.2. MATERIALS AND METHODS

Sheep

Forty-five Romney lambs about 5 months old were weighed, effectively drenched with a double dose of ivermectin and a double dose of a combination product of ricobendazole and levamisole to remove any existing worm burdens and housed. Lambs were fed a ration of lucerne and barley based nuts and given access to hay and water *ad-libitum*.

Parasite isolates

The larvae cultured from the faeces of a sheep infected with the ivermectin-resistant strain of *T. colubriformis* (Massey Tc 97) in trial II of chapter 5 were used to infect the sheep in this trial.

The ivermectin-susceptible strain I of *T. colubriformis* (Massey Tc 98) was isolated from sheep on a Massey University farm, Manawatu district. Two sheep were randomly selected and killed humanely by captive bolt stunning and exsanguination. The small intestines were removed and the contents were collected in Hanks solution (0.98 gms Hanks Balanced Salt Solution-Life Technologies, U.S.A. + 0.35g Sodium bicarbonate + 1g glucose/litre of distilled water) maintained at 37^{O}C in water bath. The live male (447 in number) and female (789 in number) *T. colubriformis* worms

were collected with the aid of a dissecting microscope and maintained in the Hanks solution at 37^{O}C. These worms were surgically transferred within 7 hours after collection into the duodenum of another worm-free lamb. Faeces were subsequently collected and cultured from this lamb and used to reinfect the lamb to augment the level of infection. Further faecal samples were collected and cultured for fitness study trial.

The ivermectin susceptible strain II of *T. colubriformis* (Massey Tc 99) was isolated from two sheep obtained from the Taranaki region in the similar way as described above except a total of 467 (286 female and 181 male) worms were collected and transferred surgically into another worm-free lamb.

The larvae of the ivermectin-resistant strain, the ivermectin-susceptible strain I and the ivermectin-susceptible strain II to be used for the fitness study trial were stored separately in 100 ml deionised water at 10^{O}C for 20, 47 and 4 days after collection respectively.

The numbers of larvae of each strain were counted separately in five aliquots of 100 µl of larval suspension. Mean numbers of larvae were used separately to estimate the amount of suspension containing 15000 larvae, needed for the infection of lambs for each of the three strains.

Design

Forty-five lambs were randomly allocated into three groups of 15 animals each. Group I was infected with 15000 infective larvae of the ivermectin-resistant strain (Massey Tc 97 strain). The lambs in group II were infected with 15000 infective larvae of the ivermectin-susceptible strain I (Massey Tc 98) and the lambs in group III were infected with 15000 infective larvae of the ivermectin-susceptible strain II (Massey Tc 99).

Efficiency of development from eggs to larvae under natural conditions

Once the worms had matured and eggs were shed in the faeces, the efficiency of development from eggs to larvae on pasture was assessed. A pasture site, which had not been grazed by sheep or cattle for at least 10 years, was selected for this trial.

Faecal samples were collected from five animals selected at random from each of the three groups of sheep at 22 days post infection. Faecal egg counts were performed in triplicate and the mean faecal egg count was used to estimate the total number of eggs in 100g of faeces collected from each of these animals. On 24th April 1999 (22 days post infection), 100g of faeces from each animal were placed separately in the plastic pipes (20 cm diameter and 20 cm long), pushed half way into the ground so that the larvae could not escape from herbage and soil (see Plate 6.1 and 6.2). There were 2 replicates per animal (i.e. 10 replicates per *T. colubriformis* isolate). The grass was maintained at a height below the top of the pipes by clipping with scissors. The clippings remained within the pipes. The whole procedure was repeated 2 days later on 26th April 1999 (24 days post infection).

Twenty-one days after contamination of the plots with 100g of faeces, the pasture, the top 2-inches of soil and the remaining faeces were collected separately from each plot. The larvae were recovered from each sample for counting (see Appendix III, IV and V for methods).

In Chapter 2, the mean recovery rate of a known number of larvae from pasture was 60% and from soil was 11% (see Section 2.3 for details). This gives a correction factor for recovery rates of $\times$1.67 for pasture and $\times$8.97 for soil. These correction factors were applied to the reported larval counts in these studies.

Daily records of minimum, maximum temperature and rainfall near the site during the trial period were obtained from Mr. Richard Heerdegen of the Geography department of Massey University.

Plate 6.1: Experimental plots showing plastic pipes (20 cm diameter and 20 cm long), pushed half way into the ground used for the development of eggs to larvae under natural conditions.

Plate 6.2: Experimental plot showing a plastic pipe containing 100g of faeces used in the trials for development of eggs to larvae under natural conditions.

Survival of Larvae at different temperatures under laboratory conditions

Faeces were collected from five animals selected at random from each of the three groups of lambs at 24 days post infection and larvae were cultured separately for each animal in the laboratory. Approximately 2000 larvae in 15 ml distilled water were placed in 50 ml tissue culture bottles and held at different temperatures $10^{O}C$, $20^{O}C$, $25^{O}C$ and $30^{O}C$. There were two replicates for each animal at each temperature. The survival of larvae was monitored by determining the percentage of live larvae at weekly intervals for 17 weeks and then at four weekly intervals for a further 20 weeks (see Appendix VII for method).

Development of eggs to larvae under laboratory conditions, infectivity, survivability of adult worms in sheep, length of male and female worms and egg laying ability of adult worms

The three groups of 15 lambs were slaughtered in three lots (5 from each group) on days 28, 42 and 84 post infection. At each sampling, faecal cultures from individual animals in triplicate (see Appendix VI for method), 10% worm counts of intestinal contents and digest (see Appendix II for method) were performed. In addition, 20 female and 20 male worms per animal were collected at slaughter and the lengths of male and female worms were measured using a computer-aided worm length measuring device, Sigma Scan (Jandel Scientific, U.S.A.). The number of eggs present in each female uterus was counted as a measure of fecundity. Faecal egg counts (see Appendix I for method) were also monitored at weekly intervals from days 21 to 84 post infection.

Larval development assay

Larval development assays were performed on faeces from three animals selected randomly from each group on days 73 and 76 post infection, using ivermectin, levamisole and thiabendazole (see Section 2.2 and Appendix VIII for detailed method). Each concentration of the drug was diluted with 150 µl of 2% agar in a 96-well microtitre plate. Ivermectin was used to confirm that the ivermectin-resistant strain was resistant and the other two field isolates were susceptible to ivermectin. Although the use of levamisole and thiabendazole in the larval development assay was

not necessary for the purpose of this trial, these were used to determine the susceptibility of the ivermectin-resistant and the other two field strains against these drugs. The LC_{50} values for the known susceptible laboratory strain at 24 days post infection (see Chapter 7) were used for comparative purposes to obtain the resistance ratios.

Statistical analysis

The statistical methods utilised to compare the faecal egg counts, worm counts, development of eggs to larvae under natural and laboratory conditions, worm length, and the number of eggs in female worms for the three strains are listed in Table 6.1 (see Appendix 6.1 for detailed statistical analysis). The animals were serially slaughtered at 28, 42 and 84 days post infection. Therefore, there was a declining number of animals in each group at these times. Thus an unbalanced analysis of variance was performed to analyse the faecal egg counts and development of eggs to larvae under laboratory conditions.

The data were transformed when necessary to normalise the data as determined by the random scatter pattern observed when residual *vs* predicted values were plotted. The statistical analysis was performed using the software programme SAS (SAS, U.S.A.).

The LC_{50} values from the larval development assays were calculated using the software programme SlideWrite Version 3.0 (Advanced Graphics Software Inc., U.S.A.).

Table 6.1: Statistical methods used for analysing the fitness study data using software program SAS.

S. NO.	TYPE OF DATA ANALYSED	TRANSFORMATI -ON	STATISTICAL METHOD	SAS PROCEDURE
1.	Egg counts for animals	Log	Unbalanced two-way ANOVA	Proc glm
2	Worm counts for animals killed on 28, 42 and 84 days post infection	Log	One-way ANOVA	Proc glm
3.	Development of larvae from eggs under natural conditions	Nil	Multivariate analysis of variance (MANOVA)	Proc glm
4.	Development of larvae from eggs under laboratory conditions at 28, 42 and 84 days post infection	Log	Unbalanced two-way ANOVA	Proc glm
5.	Worm lengths of female worms for animals killed on 28, 42 and 84 days post infection	Log	Nested Design	Proc Nested
6.	Eggs in female worms for animals killed on 28, 42 and 84 days post infection (DPI)	28 and 42 DPI - Log 84 DPI - Log + 1	Nested Design	Proc nested
7.	Worm lengths of male worms for animals killed on 28, 42 and 84 days post infection	Log	Nested Design	Proc nested

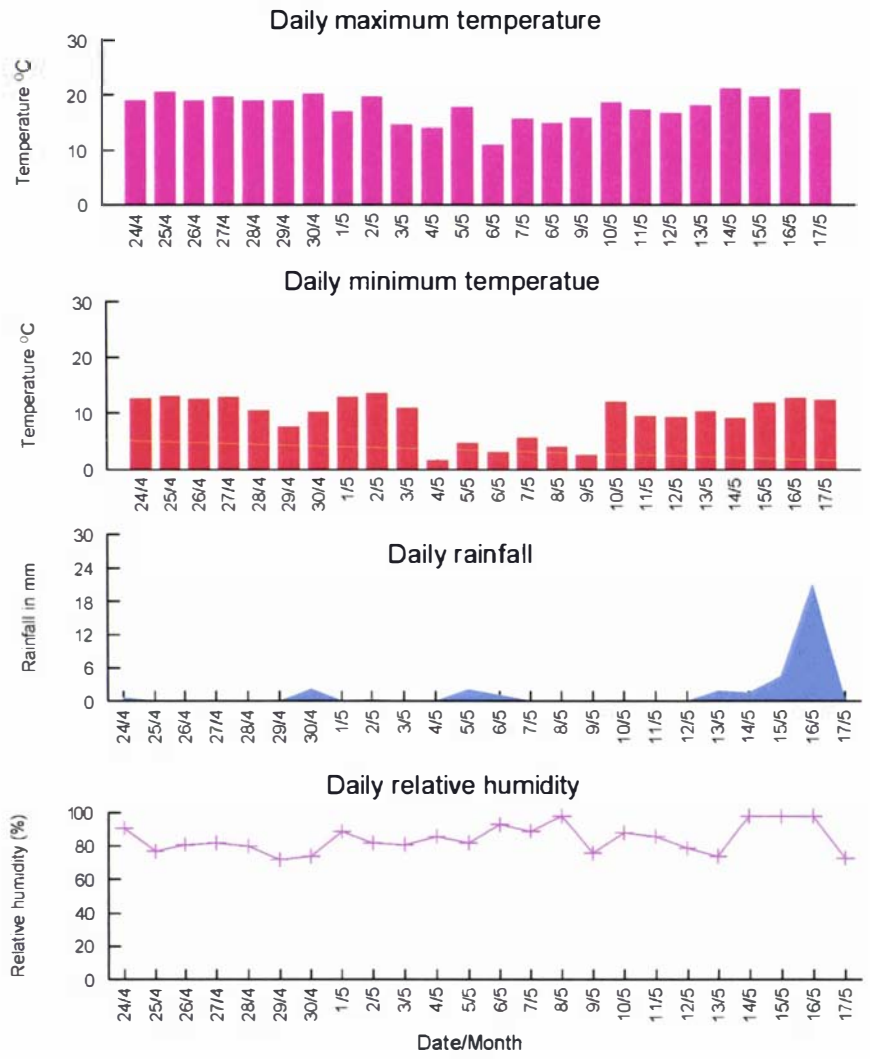


Figure 6.1: Daily maximum temperature, minimum temperature, rainfall and relative humidity during the experimental period of development of eggs to larvae for the ivermectin-resistant strain, the ivermectin-susceptible strains of *T. colubriformis* on pasture.

6.3. RESULTS

Weather parameters

The weather parameters recorded during the course of the trial are shown in Fig. 6.1. The average maximum temperature, minimum temperature and relative humidity recorded during the trial period were 17.8^{O}C, 9.4^{O}C and 84% respectively. These environmental conditions were conducive for the development of larvae from eggs during the trial period. The rainfall was not distributed evenly throughout the study period and most of the rain (21mm) fell on the day before the final collection of remaining faeces, pasture and soil from the experimental plots contaminated on 26th of April.

Infectivity and survivability of worms in sheep

The mean faecal egg counts from the sheep infected with either the ivermectin-resistant strain or the two ivermectin-susceptible strains were not significantly different from each other (p value = 0.3400)(Fig. 6.2, see Appendices 6.2, 6.3 and 6.4 for raw data, see Appendix 6.1 for statistical analysis). There was a significant difference between the time of collection of the faecal samples (p value = 0.0066) with highest mean faecal egg count at 42 days post infection. The interaction between strain and time of collection of faecal sample was always non-significant (p value = 0.5622).

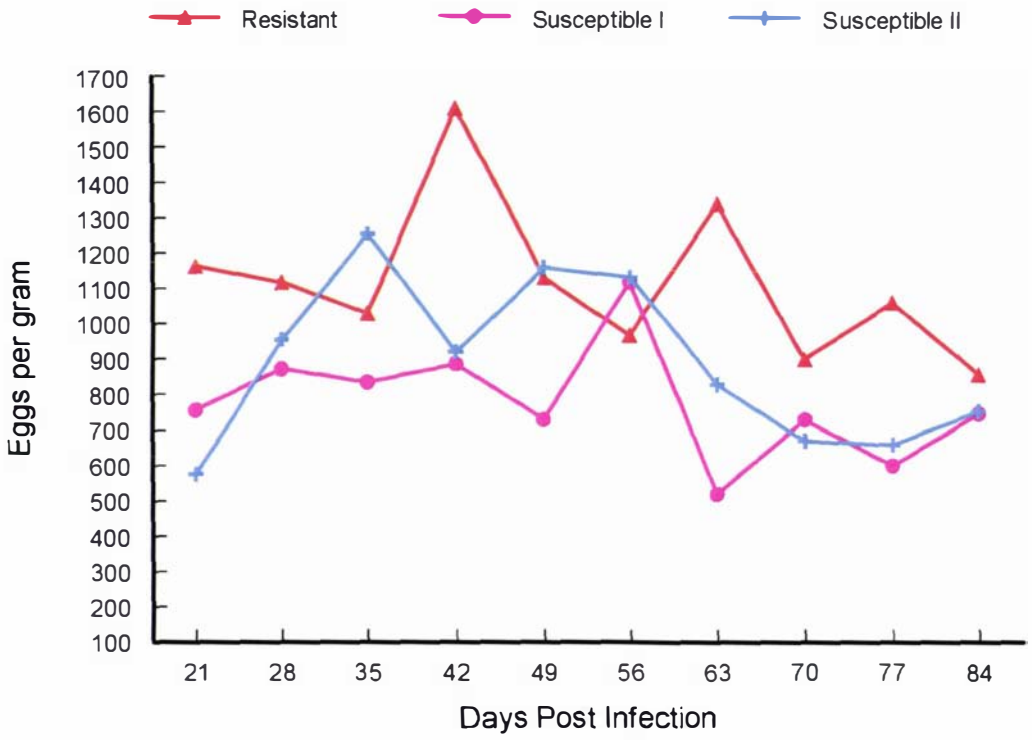


Figure 6.2: Mean faecal egg counts from lambs infected with either the ivermectin-resistant or the ivermectin-susceptible strain I or the ivermectin-susceptible strain II of *T. colubriformis*. Up to 28 days post infection (DPI) - mean faecal egg count (EPG) for 15 animals in each group, From 35 to 42 DPI - mean EPG for 10 animals in each group, From 49 to 84 DPI - mean EPG for 5 animals in each group.

In general, the mean establishment success of larvae in sheep was around 50% for each of the three strains. The worm burdens in sheep showed a similar pattern over the three slaughter times for all the three strains with the exception of one animal in the resistant strain group, which had a low worm burden of only 750 at 84 days post infection.

The arithmetic mean worm burdens at 28 days post infection in sheep infected with the ivermectin-resistant strain, the ivermectin-susceptible strain I and the ivermectin-susceptible strain II were 7972, 7104 and 7648, respectively and were not significantly different from each other (p value = 0.3438) (Fig. 6.3, see Appendices 6.5 to 6.7 for raw data, see Appendix 6.1 for statistical analysis). Similarly, the arithmetic mean worm burdens at 42 days post infection in sheep infected with the ivermectin-resistant strain, the ivermectin-susceptible strain I and the ivermectin-susceptible strain II were 8610, 7270 and 7790 respectively and were not significantly different from each other (p value = 0.5145). The mean worm burdens at 84 days post infection in sheep infected with the ivermectin-resistant strain, the ivermectin-susceptible strain I and the ivermectin-susceptible strain II were 7416, 7736 and 8632, respectively and were not significantly different from each other (p value = 0.5899).

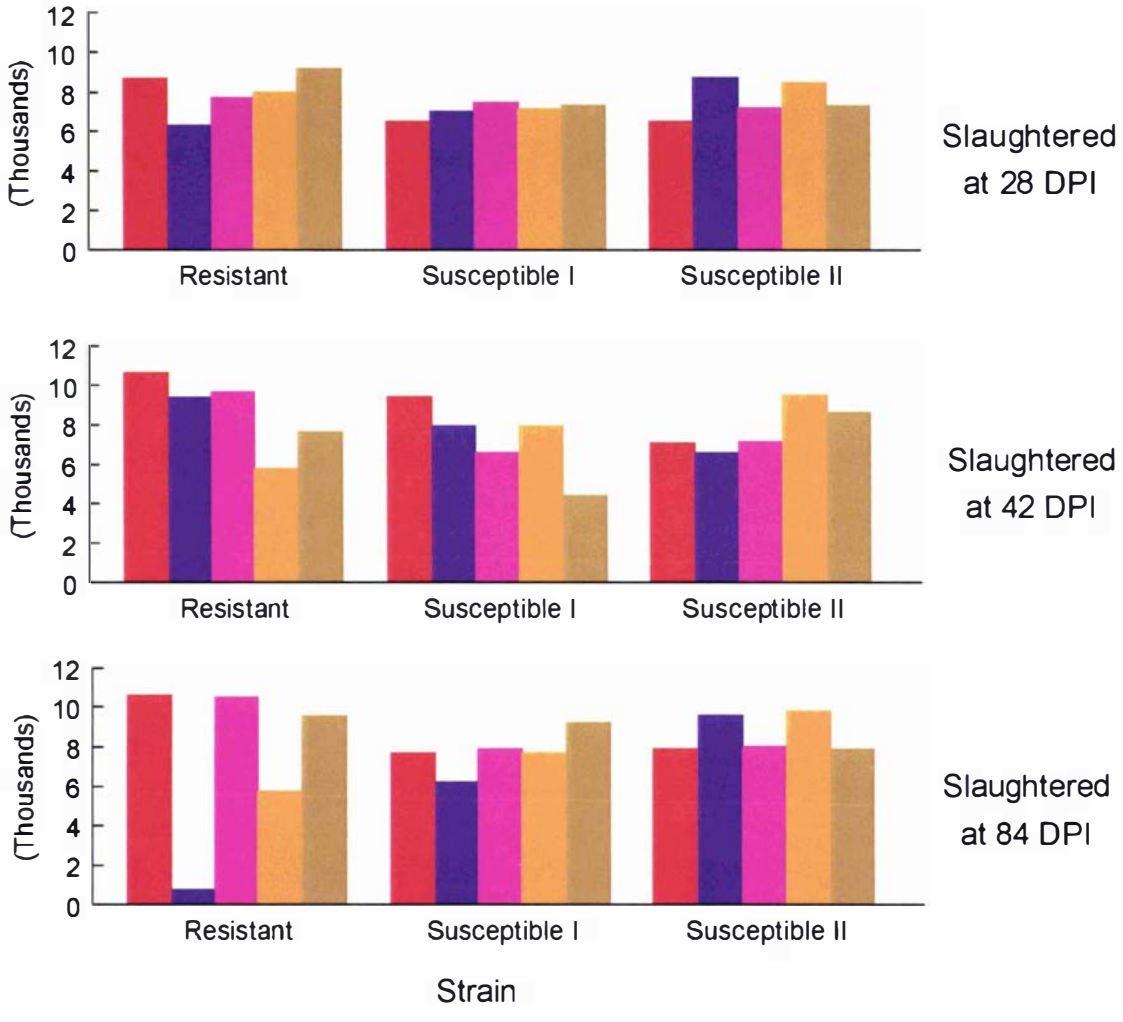


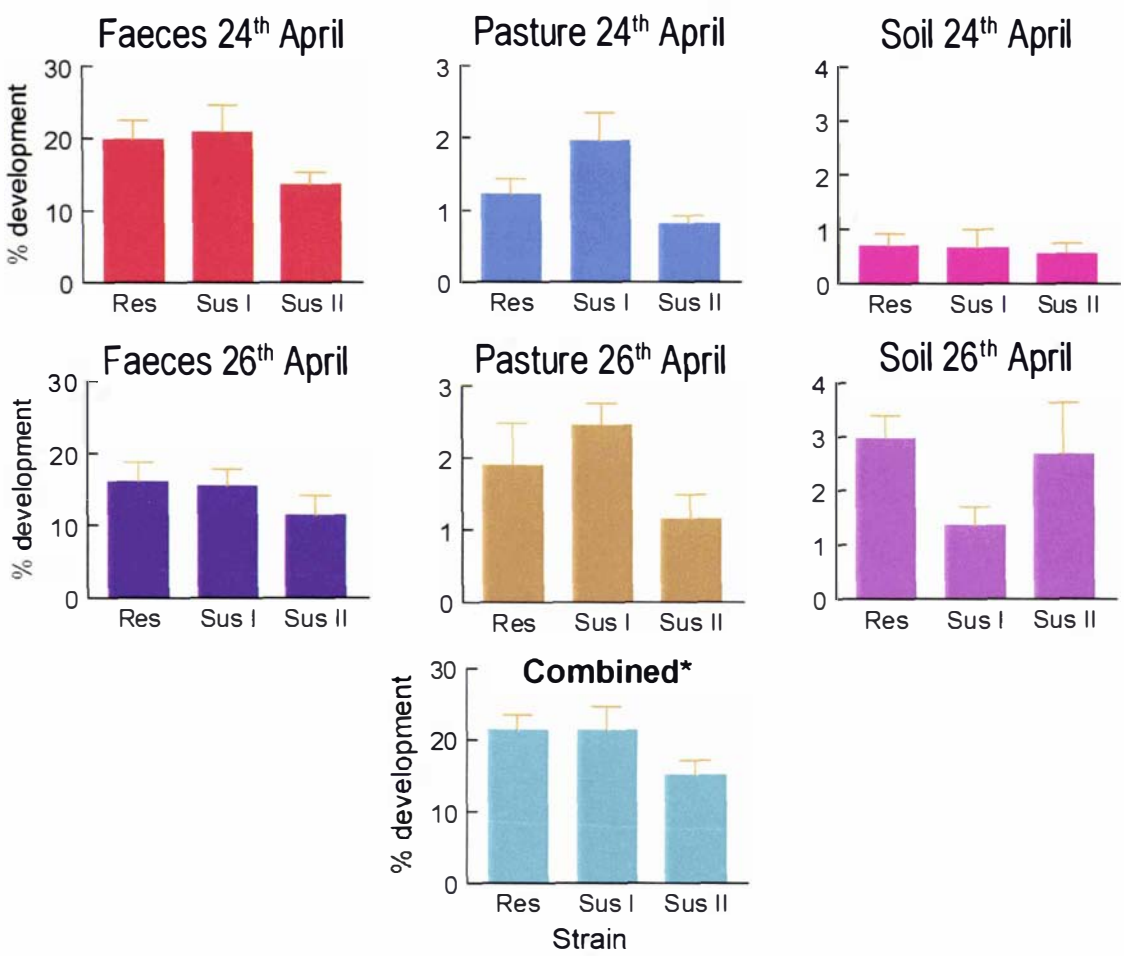
Figure 6.3: Individual worm counts for sheep infected with either the ivermectin-resistant strain or the ivermectin-susceptible strain I or the ivermectin-susceptible strain II of *T. colubriformis* (n=5 in each group). Lambs were slaughtered at either 24 or 42 or 84 days post infection (DPI).

Efficiency of development of larvae from eggs under natural conditions

The percentages of larvae that developed under natural conditions and were recovered from the faeces, pasture and soil are shown in Fig. 6.4, Appendix 6.8 and Appendix 6.9. In general, the development rates of larvae were high.

There was no significant difference (p value = 0.0624) between the resistant strain and the two susceptible strains in the combined percentage recovery of larvae from the faeces, pasture and soil when combined data for the plots contaminated on 24th April and 26th April was compared. There was also no significant difference in the percentage recovery of larvae from faeces (p value =0.0803) and soil (p value = 0.2337) between the three strains but there was a significant difference (p value = 0.0082) in the percentage recovery of larvae from the pasture between the three strains with the resistant strain being intermediate between the two susceptible strains (see Appendix 6.1).

There was a significant difference (p value = 0.0001) in the percentage recovery of larvae between the time of contamination of plots (24th April or 26th April) with lower recovery from faeces and higher recovery from pasture and soil for the plots contaminated on 26th April compared to the plots of 24th April. Twenty-one mm of rain fell on the day before the final collection of the remaining faeces, pasture and soil samples from the plots contaminated on 26th April which is believed to have influenced the migration and recovery of larvae.



* Mean percentage of development in the faeces, pasture and soil samples of 24th and 26th April

Figure 6.4: Mean percentage of larvae that developed from eggs under natural conditions from faeces collected (n=5 animals per group and two replicate per animal) at either 22 or 24 days after the infection of lambs with either the ivermectin-resistant strain or ivermectin-susceptible strain I or ivermectin-susceptible strain II of *T. colubriformis*. The faecal samples were placed on herbage at either 24th April or 26th April. Twenty-one days later remaining faeces, pasture and soil were collected for recovery of larvae.

Efficiency of development of larvae from eggs under laboratory conditions

Faecal cultures performed under laboratory conditions showed good development of larvae from eggs. The mean recovery rate of larvae for the three strains at 28, 42 and 84 days post infection ranged from 31 to 62%. There was a variation in the percentage of larvae recovered from the faeces of different animals (Fig. 6.5, see Appendices 6.10 to 6.12 for raw data).

The percentage of larvae that developed under laboratory conditions and were recovered from faeces for the ivermectin-resistant strain and the two susceptible strains was not significantly different (p value = 0.5986)(see Appendix 6.1 for statistical analysis). There was also no significant difference (p value = 0.8645) in the time of faecal culture (28 or 42 or 84 days post infection). The interaction between time of faecal culture and strain was also non-significant (p value = 0.1999).

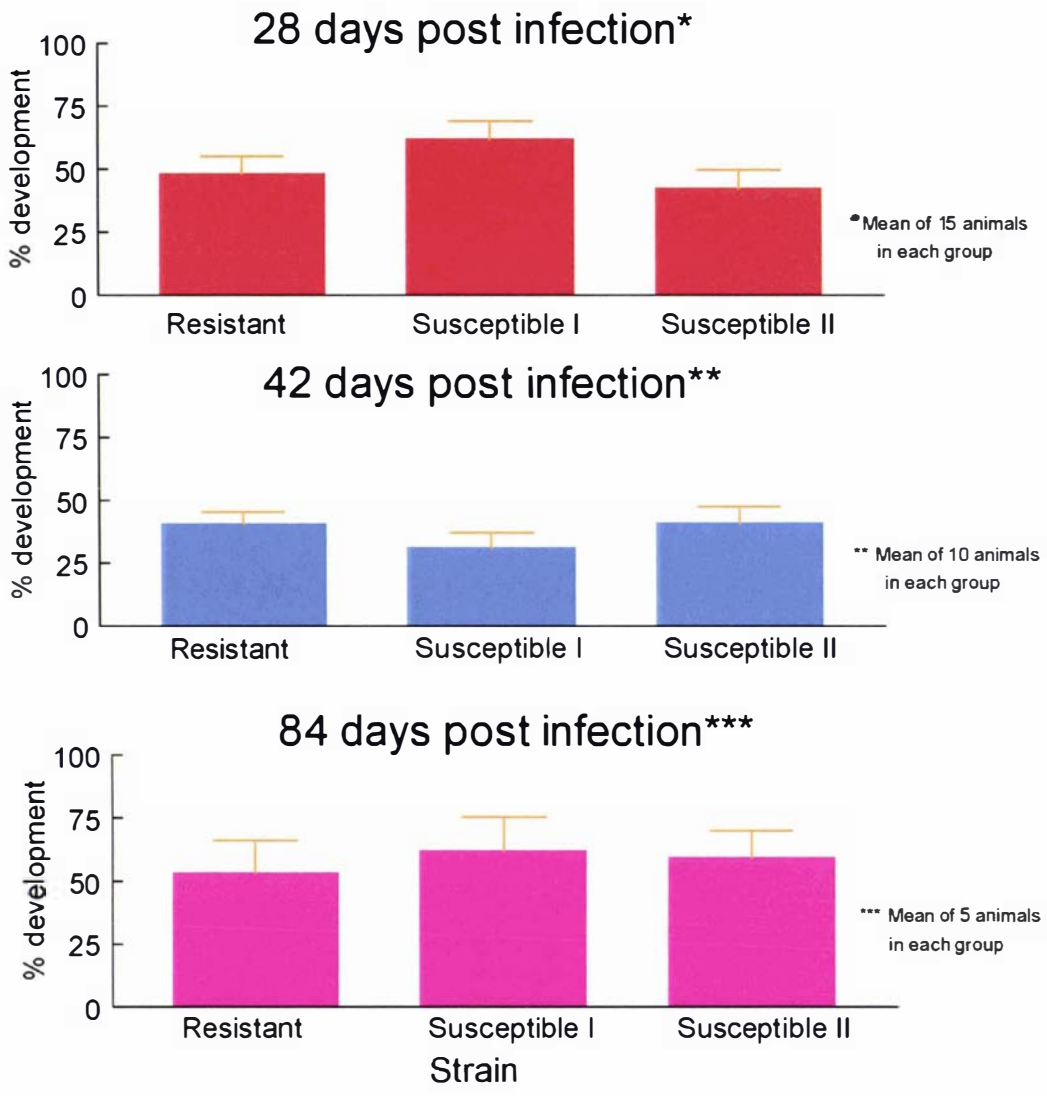


Figure 6.5: Mean percentage of larvae that developed from eggs to larvae under laboratory conditions from faeces (n=5 animals in each group and three replicates per animal) of sheep infected either with the ivermectin-resistant strain or the ivermectin-susceptible strain I or the ivermectin susceptible strain II of *T. colubriformis* and slaughtered at either 28, 42 or 84 days post infection.

Egg laying ability and lengths of adult worms

There were no significant differences between the ivermectin-resistant strain and the two ivermectin susceptible strains in the number of eggs in each worm at either 28 (p value = 0.4776) or 42 (p value = 0.4646) or 84 (p value = 0.8507) days post infection (Table 6.2, see Appendices 6.13 to 6.15 for raw data and Appendix 6.1 for statistical analysis).

Table 6.2: Mean female worm length and mean number of eggs/female for *T. colubriformis* collected at slaughter at 28 or 42 or 84 days post infection from lambs infected with the ivermectin-resistant strain or the ivermectin susceptible strain I or the ivermectin-susceptible strain II.

MEAN FEMALE WORM LENGTH IN MM (S.D.)			
Days Post Infection	Resistant strain	Susceptible strain I	Susceptible strain II
28	6.27 (0.478)	6.09 (0.513)	6.03 (0.606)
42	6.82 (0.545)	6.79 (0.878)	5.95 (0.878)
84	6.15 (0.832)	5.95 (0.630)	6.12 (0.732)
NUMBER OF EGGS/FEMALE WORM (S.D.)			
Days Post Infection	Resistant strain	Susceptible strain I	Susceptible strain II
28	14.90 (3.492)	14.40 (3.40)	14.02 (4.060)
42	15.06 (5.039)	14.50 (4.28)	12.39 (3.864)
84	8.87 (6.890)	7.72 (6.032)	9.75 (5.720)

There were no significant differences between the ivermectin-resistant strain and the two ivermectin susceptible strains in the length of female worms at either 28 (p value = 0.5800) or 84 (p value = 0.8810) days post infection (Table 6.2, see Appendices 6.13 to 6.15 for raw data and Appendix 6.1 for statistical analysis). However, there was a significant difference (p value = 0.0019) in the length of female worms between the three strains at 42 days post infection with the ivermectin-resistant strain females being longer than females of the ivermectin-susceptible strains.

Similarly, there were no significant differences between the ivermectin-resistant strain and the two ivermectin-susceptible strains in the length of male worms at either 28 (p

value = 1807) or 84 (p value = 0.9675) days post infection (Table 6.3, see Appendices 6.16 to 6.18 for raw data and Appendix 6.1 for statistical analysis). However, there was a significant difference (p value = 0.0019) in the length of male worms between the three strains at 42 days post infection with the ivermectin-resistant strain males being longer than males of the ivermectin-susceptible strains.

Table 6.3: Mean length of male *T. colubriformis* worms collected at slaughter at 28 or 42 or 84 days post infection from lambs infected with ivermectin-resistant strain or ivermectin susceptible strain I or ivermectin-susceptible strain II.

MEAN MALE WORM LENGTH IN MM (S.D.)			
Days Post Infection	Resistant Strain	Ivermectin-susceptible strain I	Ivermectin-susceptible strain II
28	5.21 (0.693)	4.88 (0.539)	4.70 (0.615)
42	5.07 (0.551)	4.18 (0.603)	4.09 (0.456)
84	4.64 (0.494)	4.59 (0.812)	4.61 (0.615)

Survival of Larvae at different temperatures under laboratory conditions

The survival of larvae decreased with the increase in temperature of storage for all the strains. The range of temperatures generally affected all the strains in a similar fashion; the strain surviving best at 30^{O}C also had the longer survival time at 25^{O}C, 20^{O}C and 10^{O}C.

At 10^{O}C, more than 80% of larvae were still alive at 37 weeks for the ivermectin-resistant strain and the ivermectin-susceptible strain I whereas only about 50% of larvae were live for the ivermectin-susceptible strain II at the same time (Fig. 6.6). All the larvae of the ivermectin-resistant strain and the ivermectin-susceptible strain I were dead at week 37 when held at 20^{O}C whereas 40% of larvae of the ivermectin-susceptible strain II were still alive (Fig. 6.7). Similarly, all the larvae of the ivermectin-resistant strain and the ivermectin-susceptible strain I were dead at week 25 when held at 25^{O}C whereas 23% larvae of the ivermectin-susceptible strain II were still alive (Fig. 6.8). All the larvae of the ivermectin-resistant strain and the ivermectin-susceptible strain I were dead at week 14 when held at 30^{O}C whereas 22%

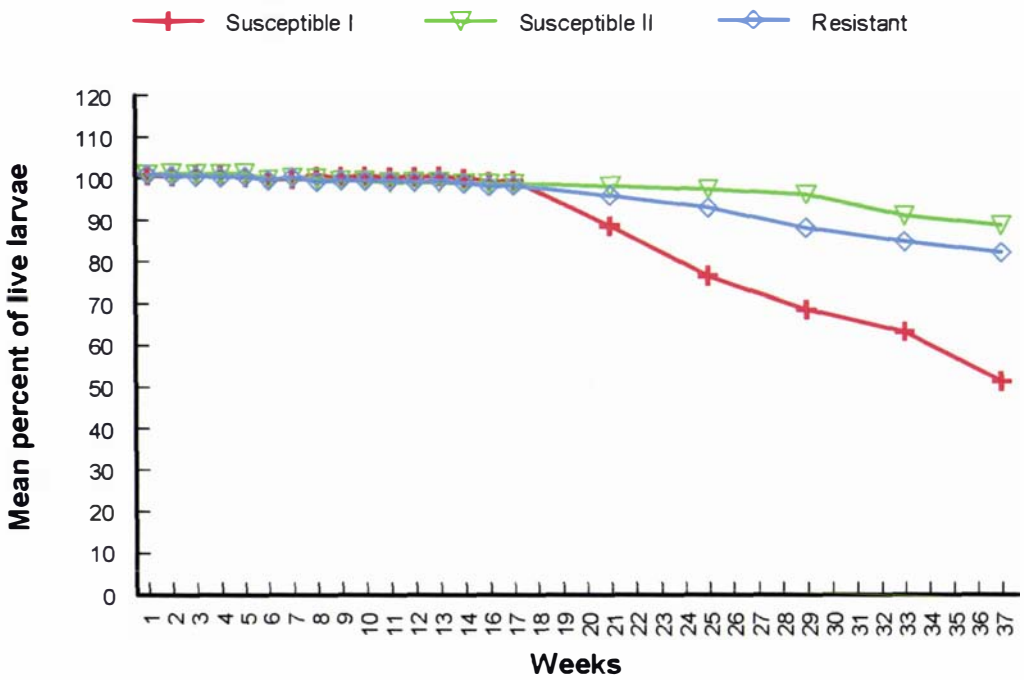


Figure 6.6: The mean percentage survival of larvae of *T. colubriformis* incubated at 10^{O}C under laboratory conditions from lambs infected with either ivermectin-resistant strain or ivermectin-susceptible strain I or ivermectin-susceptible strain II (n=5 animals per group and two replicate per animal).

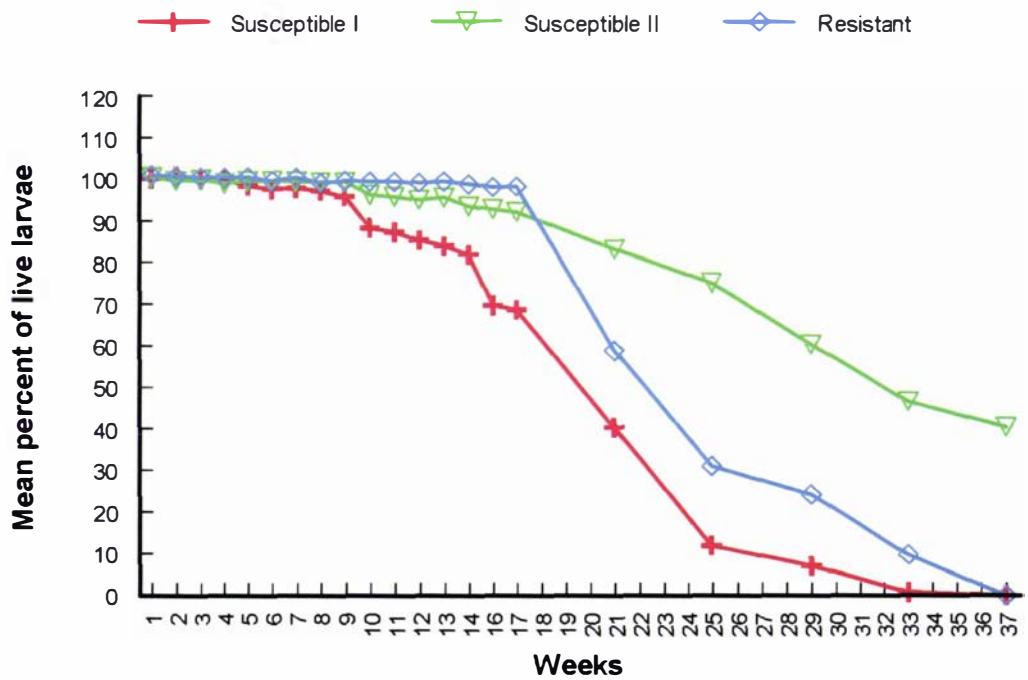


Figure 6.7: The mean percentage survival of larvae of *T. colubriformis* incubated at 20^{O}C under laboratory conditions from lambs infected with either ivermectin-resistant strain or ivermectin-susceptible strain I or ivermectin-susceptible strain II (n=5 animals per group and two replicate per animal).

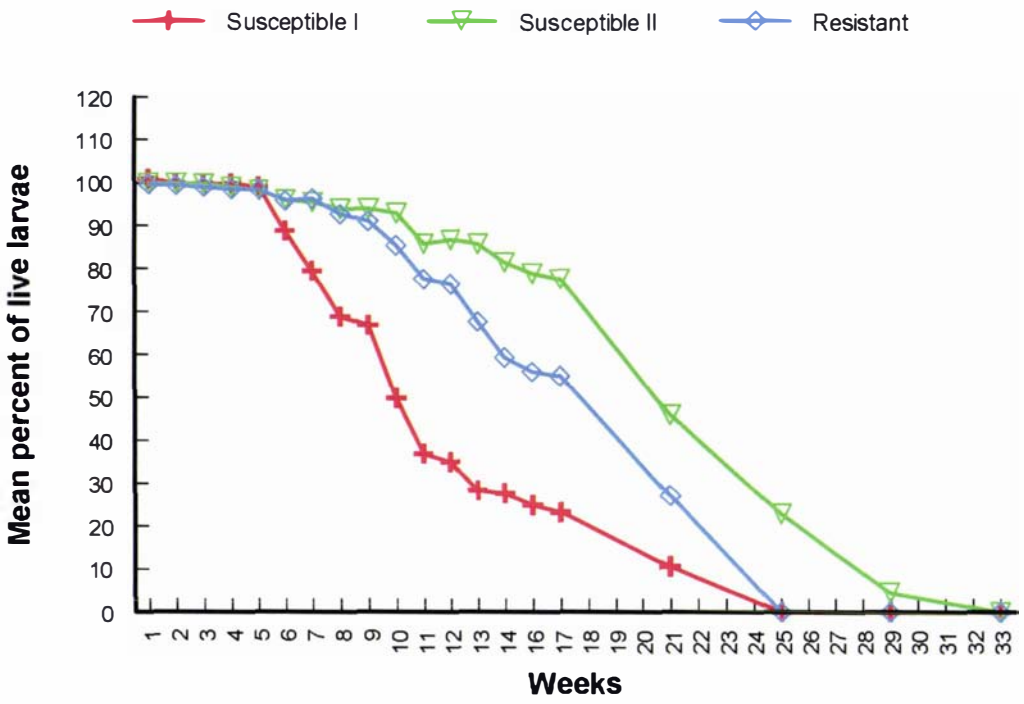


Figure 6.8: The mean percentage survival of larvae of *T. colubriformis* incubated at 25^{O}C under laboratory conditions from lambs infected with either ivermectin-resistant strain or ivermectin-susceptible strain I or ivermectin-susceptible strain II (n=5 animals per group and two replicate per animal).

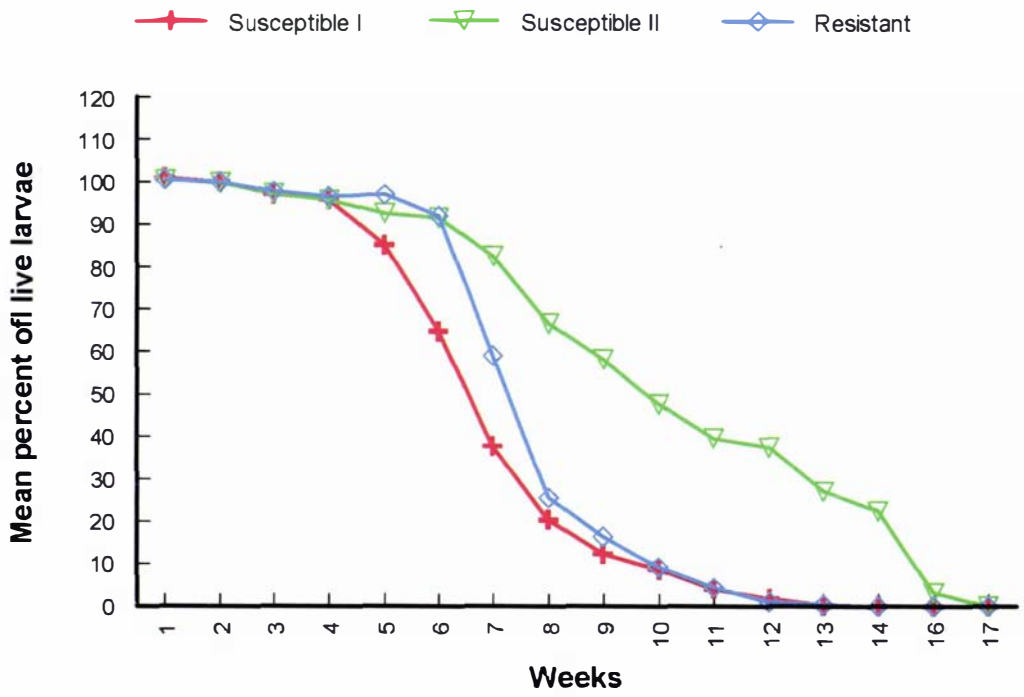


Figure 6.9: The mean percentage survival of larvae of *T. colubriformis* incubated at 30^{O}C under laboratory conditions from lambs infected with either ivermectin-resistant strain or ivermectin-susceptible strain I or ivermectin-susceptible strain II (n=5 animals per group and two replicate per animal).

larvae of the ivermectin-susceptible strain II were still alive (Fig. 6.9).

The survival of larvae of the ivermectin-resistant strain of *T. colubriformis* is intermediate between that of the two ivermectin-susceptible strains.

Larval development assay

The LC_{50} values of ivermectin, thiabendazole and levamisole for the ivermectin-resistant strain, the two ivermectin-susceptible strains are shown in Table 6.4. The LC_{50} values of ivermectin for the ivermectin-susceptible strains were similar to the LC_{50} values of a known ivermectin-susceptible laboratory strain of *T. colubriformis* (Table 6.4 and see Appendix 6.19 for raw data). The LC_{50} values of ivermectin for the ivermectin-resistant strain were approximately 4.6 times higher than the LC_{50} values of the ivermectin-susceptible strains. Similarly, the LC_{50} values of thiabendazole for the ivermectin-resistant strain were approximately 13 times higher than the LC_{50} values of the ivermectin-susceptible strains. The LC_{50} values of thiabendazole for the ivermectin-susceptible strains were similar to the LC_{50} values of a known ivermectin-susceptible laboratory strain. The LC_{50} values of levamisole for the ivermectin-susceptible strain I were 3.72 and 4.88 times higher than the ivermectin-resistant and the ivermectin-susceptible strain II respectively. The LC_{50} values of levamisole for the ivermectin-resistant strain were approximately 1.3 times higher than the ivermectin-susceptible strain II and a known ivermectin-susceptible laboratory strain.

Table 6.4: Mean LC_{50} values of ivermectin, thiabendazole and levamisole for the ivermectin-resistant strain, ivermectin-susceptible strain I and ivermectin-susceptible strain II of *T. colubriformis*.

MEAN LC_{50} VALUES (NG/ML OF AGAR)			
Strain	Ivermectin	Thiabendazole	Levamisole
Ivermectin-resistant strain*	3.04	203.2	346.6
Ivermectin-susceptible strain I*	0.66	15.65	1289.7
Ivermectin-susceptible strain II*	0.66	14.65	264.5
Known susceptible strain** (See chapter 7)	0.58	14.27	245.1

*Mean values of three animals at 73 and 76 days post infection
**Mean value of three animals at 24 days post infection

6.4. DISCUSSION

Infectivity, Survivability of adult worms in sheep, fecundity and worm lengths

Similar worm counts at 28 and 42 days after infection with *T. colubriformis* larvae indicate that the ivermectin-resistant strain of *T. colubriformis* establishes equally well in the host, as do the two susceptible field strains. This also indicates that storage of larvae at 10^{O}C for up to 47 days prior to infection did not influence their infectivity.

The results of worm counts at 84 days post infection suggest that there is no significant difference in survivability of adult worms of *T. colubriformis* in sheep up to 84 days post infection between the ivermectin-resistant strain and the two ivermectin-susceptible field strains.

The response of one sheep (No. 20) infected with the ivermectin-resistant strain of *T. colubriformis* is interesting. This sheep exhibited an apparent resistance to *T. colubriformis* as its faecal egg count was consistently lower and after 84 days post infection this sheep had the lowest worm burden in this experiment. Genetic variations has been observed in the response to gastrointestinal nematodes in sheep, and specific breeding programs developed to select for resistant animals (Woolaston, 1990).

Similar numbers of eggs per female, similar faecal egg counts and similar female worm length suggests that there is no difference in the fecundity of the ivermectin-resistant strain and two ivermectin-susceptible strains.

The statistical differences observed at 42 days post infection in the male as well as in the female *T. colubriformis* worm length for the three strains probably indicate the genetic variations in male and female worms of the three strains. This might not indicate the difference in the inhibition of larvae between the three strains, as the mean length of male and female worms of ivermectin-resistant strain were consistently higher throughout the trial than the two ivermectin-susceptible strains.

Efficiency of development from eggs to larvae under natural and laboratory conditions

The present study demonstrated that the number of infective larvae produced from eggs seeded onto pasture were similar for the ivermectin-resistant strain and the two susceptible field strains. This result is in agreement with the observation that faecal cultures of sheep infected with ivermectin-resistant *T. colubriformis* produced equal numbers of infective larvae as compared to the two susceptible field strains in the laboratory. However, more larvae of the susceptible strain I reached pasture followed by the ivermectin-resistant strain and the susceptible strain II. These differences might have occurred due to experimental error. Extreme care was taken while separating the pasture from the soil but inevitably some portions of pasture could not be separated from the soil. Higher mean recovery rates of larvae from soil for the ivermectin-resistant strain and the ivermectin-susceptible strain II than for the ivermectin-susceptible strain I support this hypothesis. Nevertheless, the mean recovery rate of larvae from pasture for the ivermectin-resistant strain was intermediate between the two ivermectin-susceptible strains.

More infective larvae of both the ivermectin-resistant strain and the susceptible strains of *T. colubriformis* were recovered from pasture and soil from plots contaminated on the 26th April as compared to 24th April. This difference is possibly due to the rain, which occurred before the final collection of samples of from the plots contaminated on 26th April (Fig. 6.4). It is possible that the faeces acted as reservoir for infective larvae and rain helped in the migration of the larvae from the faeces to pasture and soil. These results are in agreement of the findings of Gibson and Everett (1972) and Levine and Andersen (1973) who suggested that faecal pellets can act as a store of infective larvae and can be responsible for maintaining larval population on the herbage at steady levels for longer periods. Similarly, Callinan (1978) and Niezen *et al.* (1998) have also indicated migration of larvae from faeces persist as long as the faecal pellets persist. The role of faeces as a reservoir of larvae of cattle nematodes has also been well established (Anderson *et al.*, 1983; Barger *et al.*, 1984).

The range of maximum yields of larvae of *T. colubriformis* varied from 0.38 to 3.35% (0.23 to 2.0% uncorrected) and 0.43 to 5.44% (0.26 to 3.26% uncorrected) from

pasture samples from the plots contaminated on 24th April and 26th April, respectively. In contrast to the findings of the present study, maximum yield of larvae of *O. circumcincta* up to 16% from pasture has been reported (Callinan, 1978). Similar to these findings, high yields (20%) of *Trichostrongylus* spp. were also recorded by Donald (1968). It is possible that if the present trial had been extended, more larvae from pasture may have been recovered. However, other workers including Boag and Thomas (1975) have estimated that less than 1% of eggs ever become available as L_3 on herbage. In the present study, a higher rate of development of the eggs was achieved, possibly because the faeces were put on pasture in the last week of April and temperatures at that time were conducive for development of larvae (Fig. 6.4). The air temperature recorded during the course of study (late autumn) might have been optimum for larval development and survival because the temperature in the faeces is usually higher than the air temperature (Familton and McAnulty, 1995). Similarly, a study investigating the seasonal pattern of larvae on pasture suggest that the month of April, in New Zealand is the appropriate time for development of larvae of *T. colubriformis* under natural conditions because temperature and humidity levels are usually optimum for larval development in the autumn months (Vlassoff, 1973). Larval recoveries from pasture in New Zealand has a fairly regular pattern, with a smaller peak of larvae in the spring and a larger peak in the autumn (Vlassoff, 1973). The autumn larval peak in this report was numerically dominated by *Trichostrongylus* spp.

In the present study, the remaining faeces, pasture and soil samples were collected three weeks after the initial deposition of faeces. It appears from the higher recovery rates that the three weeks was the appropriate time for development of eggs to L_3. Similarly, Callinan (1978) suggested that L_3 began to appear on herbage within 14 days of egg deposition when the developmental conditions were optimal in western Victoria, Australia. Similarly, a study using tracer sheep, found that L_3 of *O. circumcincta* in northern New South Wales, were available within 2 weeks of egg deposition during favourable conditions (Southcott *et al.*, 1976). However, it is impossible to be very precise about development times and their relationship to time of egg deposition.

Survival of larvae at different temperatures under laboratory conditions

It appears from the results of the present study that the ivermectin-susceptible strain II of *T. colubriformis* is better adapted to the range of temperatures used followed by the ivermectin-resistant strain and the ivermectin-susceptible strain I respectively. This probably indicates that the larvae of the ivermectin-resistant strain are not at a disadvantage in survival compared with the two ivermectin-susceptible field isolates because wide differences in the survival time amongst different strains of *T. colubriformis* have been reported. Similarly, Andersen *et al.* (1966) reported similar ranges of survival times for a strain of *T. colubriformis* as found with the three strains in the present study at $20^{O}C$ and $30^{O}C$. It has also been demonstrated in related nematode species, *O. circumcincta* that the survival of infective larvae varies between isolates and depends on the geographical location of the isolate (Pandey *et al.*, 1993).

Larval development assay

Although the LC_{50} values change with the age of infection (see Section 5.3), the LC_{50} values recorded on 73 and 76 days post infection in the present trial were compared with the LC_{50} values recorded on 24 days post infection in the experiments conducted with the known ivermectin-susceptible laboratory strain in Chapter 7. These comparisons were possible because the LC_{50} values after 72 days post infection were similar to those recorded during 23 to 37 days post infection in Chapter 5.

The results of the larval development assay using ivermectin confirmed that the two field strains were susceptible to ivermectin but the resistant strain was not. The results also show that the ivermectin-resistant strain is resistant to thiabendazole. This supports the initial findings of a faecal egg count reduction test, performed by field veterinarians at the goat farm where this ivermectin-resistant strain was originally isolated. The albendazole treatment reduced the faecal egg counts by 42% and *Trichostrongylus* was the predominant genus in a post treatment faecal culture (see Section 3.1). The results of the larval development assay also suggest that the ivermectin-susceptible strain II is also susceptible to levamisole whereas the ivermectin-susceptible strain I is resistant to levamisole. The resistance ratio of 1.3 recorded for ivermectin-resistant strain suggests that this strain is also slightly resistant to levamisole.

The above fitness factors studied suggest that the ivermectin-resistant strain of *T. colubriformis* is equally fit as the two ivermectin-susceptible field isolates. In contrast to these findings, a significantly higher establishment rate, higher faecal egg output and higher development of larvae on pasture for benzimidazole-resistant strain of *H. contortus* compared with a benzimidazole-susceptible strain have been reported in sheep (Kelly *et al.*, 1978). The contrasting results of present study and study of Kelly *et al*, (1978) probably reflects the difference in the selection pressure applied to the two strains before their fitness characteristics were studied. After isolation from field, the benzimidazole-resistant strain of *H. contortus* was selected for 10 generations with 50 mg/kg thiabendazole and for 9 generations with 100 mg/kg thiabendazole (Kelly *et al.*, 1978). Such an intense selection might have resulted in selection of other alleles that might have produced a reorganised genome and provided better biological advantage for this benzimidazole-resistant strain of *H. contortus*.

The findings of the present study are also in contrast to the findings of Maingi *et al.* (1990) who demonstrated lower establishment rate and lower faecal egg output for a thiabendazole-resistant strain of *H. contortus* compared to a thiabendazole-susceptible strain. However, further selection of the thiabendazole-resistant strain for four generations resulted in similar establishment rate and faecal egg output for resistant and susceptible strains.

The finding of the present study has consequences for grazing sheep farmers as it appears that once this ivermectin-resistant strain of *T. colubriformis* is established on a farm, reversion to susceptibility will be slow, or will not occur at all, even if there is no further use of ivermectin. Several studies have suggested rotating the use of anthelmintics from different groups for the management of anthelmintic resistance (Coles and Roush, 1992). However, such a strategy may not result in a reversion to susceptibility if the fitness of the resistant worms is similar to the susceptible worms.

This Chapter has demonstrated the fitness characteristics of the ivermectin-resistant strain of *T. colubriformis* compared with the two ivermectin-susceptible field isolates. The next Chapter will demonstrate the efficacy of moxidectin oral and injectable formulations against the ivermectin-resistant strain of *T. colubriformis*.

CHAPTER SEVEN

EFFICACY OF IVERMECTIN ORAL, MOXIDECTIN ORAL AND MOXIDECTIN INJECTABLE FORMULATIONS AGAINST AN IVERMECTIN-RESISTANT STRAIN OF *TRICHOSTRONGYLUS COLUBRIFORMIS* IN SHEEP

7.1. INTRODUCTION

Anthelmintic resistance is becoming an increasing problem in the sheep and goat rearing areas of the world and is of concern to all involved with grazing livestock. Resistance has now been recorded in the field to every class of anthelmintic (see Section 1.5).

The macrocyclic lactone group of anthelmintics is the most recent broad-spectrum action family available for the control of parasites in sheep and goats. All members of this group are believed to share the same general mode of action (Conder *et al.*, 1993; Shoop *et al.*, 1995). Since ivermectin was introduced in New Zealand in the early 1980's, ivermectin resistance has been reported most frequently in *Ostertagia* species. Initially this was in goats (Badger and McKenna, 1990; Watson and Hosking, 1990; Pomroy *et al.*, 1992) but now has been recorded in sheep as well (Mason *et al.*, 1999). An ivermectin-resistant isolate of *T. colubriformis* was isolated from a goat farm in Northland, New Zealand in 1997. This isolate was found to be equally infective for sheep and ivermectin resistance was confirmed in sheep (see Chapter 3).

Moxidectin is also a macrocyclic lactone endectocide which is highly effective against nematodes of sheep in both artificial and natural infection (Bauer and Conraths, 1994; Coles *et al.*, 1994). Both oral and injectable formulations of moxidectin have been shown to have excellent efficacy against natural infections of *T. colubriformis*, *Ostertagia* species and *Haemonchus contortus* (Dorchies *et al.*, 1996). Moxidectin also provides persistent activity against the common gastrointestinal nematodes of sheep. However, moxidectin has less persistent activity against *T. colubriformis* as compared to the other common nematodes such as *O. circumcincta* and *H. contortus*

(Bairden *et al.*, 1995; Kerboeuf *et al.*, 1995). In addition injectable moxidectin has a longer period of persistent activity than moxidectin given orally (Bairden *et al.*, 1995; Kerboeuf *et al.*, 1995; Alvinerie *et al.*, 1998).

The moxidectin oral formulation has been successfully used to control *H. contortus* and *O. circumcincta* that are resistant to ivermectin in sheep (Craig *et al.*, 1992; Pankavich *et al.*, 1992; Pomroy *et al.*, 1992; Oosthiuzen and Erasmus, 1993; Pomroy and Whelan, 1993; Besier, 1996). However, side resistance to the moxidectin oral formulation has been reported in sheep against an ivermectin-resistant strain of *T. colubriformis* selected in the laboratory (Shoop *et al.*, 1993) and in goats against an ivermectin-resistant strain of *O. circumcincta* (Leathwick, 1995) although the efficacy of moxidectin oral formulation was higher than the ivermectin oral formulation in these studies. Similarly in cattle, side resistance to moxidectin injectable formulation has also been reported in an ivermectin-resistant *Cooperia onchophora* isolate (Vermunt *et al.*, 1996). The comparison of the therapeutic efficacy of injectable and oral formulations of moxidectin against ivermectin-resistant strains has not been determined. The purpose of the present study was to evaluate the activity of both oral and injectable formulations of moxidectin against a field isolate of ivermectin-resistant *T. colubriformis*.

7.2. MATERIALS AND METHODS

Sheep

Twenty-seven mixed breed wether lambs 8-10 weeks of age were used. The lambs were effectively drenched with a double dose of ivermectin and a double dose of a combination product of ricobendazole and levamisole to remove any existing worm burdens. The lambs were housed throughout the experiment and fed a ration of lucerne and barley based nuts and given free access to aged hay and water.

Parasite strains

The ivermectin-resistant strain of *T. colubriformis* (Massey Tc 97) had been isolated from a goat farm in Northland, New Zealand in 1997. Since its isolation, the strain had been passaged three times through goats, each time selected with ivermectin, once

with 200 μg/kg body weight and twice with 400 μg/kg body weight. This isolate was again passaged three times through sheep without further ivermectin selection to provide infective larvae for this trial.

The ivermectin-susceptible strain had been isolated from sheep before the introduction of ivermectin and since then had been passaged through sheep many times without exposure to any anthelmintic.

Experimental design

Seven days after drenching, 24 lambs were infected with 15000 infective larvae of the ivermectin-resistant strain of *T. colubriformis*. Twenty-six days post infection, the lambs infected with the ivermectin-resistant strain were ranked on the basis of their faecal egg counts and randomly divided into four groups of six animals each, so that each group had similar faecal egg counts. These groups were randomly allocated into four treatment groups as follows; the first group was left untreated and acted as a control, the second group was drenched with ivermectin (Ivomec liquid for sheep and goats, MSD AgVet - Batch Number V8250, Expiry date 7/2000) at 0.2mg/kg b.wt., the third group was drenched with moxidectin (Cydectin oral drench for sheep, Cyanamid - Batch number B/N V02589/3, Expiry date 6/2001) at 0.2 mg/kg b.wt. and the fourth group was injected with moxidectin (Vetdectin for cattle and sheep, Cyanamid - Batch number 5440/A, Expiry date 6/2001) at 0.2 mg/kg b.wt. subcutaneously. Twelve days after treatment all of the lambs were killed and their small intestines were removed for worm counting.

The remaining three lambs were infected with 15000 infective larvae of an ivermectin-susceptible strain of *T. colubriformis* to provide eggs for larval development assays for comparison purposes with the resistant strain. To confirm the efficacy of the batch of moxidectin injection, these three animals were injected with moxidectin at 0.2 mg/kg b.wt. subcutaneously at day 31 post infection. Seven days after treatment these lambs were killed and their small intestines were removed for worm counting.

Parasitological procedures

Faecal samples were collected from all the lambs at 7, 14, 21, 24 and 26 days after infection and at 3, 5 and 7 days after treatment. Faecal samples were also collected from the lambs infected with the resistant strain at 10 days after treatment. The egg counts were estimated by a modified McMaster procedure (see Appendix I for method) where each egg counted represents 50 eggs per gram.

Ten- percent aliquots of the small intestine contents and pepsin digests (see Appendix II for method) were sieved through a 53 μm sieve and the nematodes retained were counted.

Susceptibility of the free-living stages of *T. colubriformis* was assessed at 24 days post infection in a larval development assay using eggs from the three animals with the highest faecal egg counts from the lambs infected with the ivermectin-resistant strain and eggs from three animals infected with the ivermectin-susceptible strain. This assay is largely as described in Appendix VIII. Briefly, stock solutions of ivermectin (24 μg/ml), avermectin B_2 (43.75 μg/ml), ivermectin aglycone (141.75 μg/ml), moxidectin (33.33 μg/ml), thiabendazole (258 μg/ml) and levamisole (129 μg/ml) were prepared in dimethlyl sulphoxide (DMSO) and were serially diluted 1:2 with DMSO to give 16 concentrations for ivermectin, avermectin B_2, moxidectin, thiabendazole and levamisole and 25 concentrations for ivermectin aglycone. The ivermectin, moxidectin and levamisole were diluted from standard sheep oral drench formulations. The thiabendazole, avermectin B_2 and ivermectin aglycone were powdered active ingredients. The thiabendazole and avermectin B_2 were a gift from Merial, New Zealand and ivermectin aglycone was a gift from Virbac Laboratories Ltd., New Zealand. Two μl of each drug concentration, or DMSO only for controls, were dispensed into wells of 96-well microtitre plates and diluted with 150 μl of 2% agar (Bacto-agar, DIFCO). Each concentration was run in duplicate and the controls were run in triplicate. One hundred μl of egg suspension consisting of 50 eggs and 40 μl nutritive medium was added in each well. The plates were then incubated and larvae counted as described in Section 2.2 (see Appendix VIII for detailed method). Data were corrected for the mean number of eggs not developed in the three control wells. The

LC_{50} values were calculated using the software program SlideWrite version 5.01 (Advanced Software Inc., U.S.A.) and expressed as ng/ml in the agar phase.

Statistical analysis

Egg and worm counts were transformed to $\log_{10}$ (count + 1) to calculate geometric means. The percent reductions in the faecal egg count and worm counts were calculated as described in Section 3.2 using both arithmetic and geometric means.

Multiple comparisons of faecal egg counts on each day, or worm counts at slaughter were made on $\log_{10}$ (count + 1) data by the Tukey's test using the software program SAS (SAS, U.S.A.).

The resistance ratios for each drug tested in the larval development assay were calculated by dividing the mean LC_{50} value for the resistant strain with the mean LC_{50} value for the susceptible strain.

7.3. RESULTS

The results of faecal egg counts of sheep infected with the ivermectin-resistant strain at the time of treatment and 3, 5, 7 and 10 days after treatment clearly show a difference in efficacy between the ivermectin oral, moxidectin oral and moxidectin injectable formulations (Table 7.1).

The moxidectin oral formulation reduced faecal egg counts by 98 - 100% on all occasions. By comparison, the faecal egg counts in the moxidectin injection group were never significantly ($p>0.05$)(see Appendix 7.1 for statistical analysis) different from the control group. The reduction in mean faecal egg count in the ivermectin oral group was intermediate between moxidectin oral and moxidectin injectable groups being significantly lower ($p<0.05$) than that of the moxidectin oral formulation on all occasions but significantly higher than ($p<0.05$) the moxidectin injectable group (Table 7.1).

Ivermectin oral, moxidectin oral and moxidectin injection groups achieved a 62%, 98% and 4% reduction in arithmetic mean worm burdens respectively (Table 7.1) indicating the moxidectin oral formulation was highly effective but the moxidectin

injectable formulation was ineffective against this ivermectin-resistant strain of *T. colubriformis*. In contrast moxidectin injectable formulation was effective against an ivermectin-susceptible strain of *T. colubriformis* (Table 7.1). The efficacy of the ivermectin oral formulation against an ivermectin-resistant strain of *T. colubriformis* was intermediate between and significantly different ($p<0.05$) to that of the moxidectin oral and moxidectin injectable formulations.

When the mean LC_{50} values of the resistant isolate of *T. colubriformis* were compared with the LC_{50} values of the susceptible strain, the highest resistance ratio of the macrocyclic lactones was achieved with ivermectin aglycone followed by ivermectin, avermectin B_2 and moxidectin (Table 7.2). The resistance ratio was quite high for thiabendazole and marginally elevated for levamisole.

Table 7.1: Nematode worm counts and faecal egg counts in lambs infected with either ivermectin-resistant or susceptible strains of *T. colubriformis* and left untreated or dosed with either ivermectin oral, moxidectin oral or moxidectin injectable formulation.

Treatment group	Nematode worm count	Faecal egg count – days after treatment				
	T. colubriformis	Day 0	Day 3	Day 5	Day 7	Day 10
Control	7320	900	1500	1850	200	650
(Lambs infected	7120	450	800	850	700	650
with resistant	7460	1500	1100	1500	550	1250
strain)	7430	1350	1650	1950	1150	1050
	8260	1400	1450	1600	1850	1050
	7500	850	1400	1350	1300	1300
Arithmetic Mean (S.E.)	**7515[a] (159)**	**1075[a] (167)**	**1317[a] (127)**	**1517[a] (161)**	**958[a] (242)**	**992[a] (116)**
Ivermectin	6320	1150	500	350	650	600
(Lambs infected	2200	550	300	300	350	350
with resistant	840	1050	50	50	150	100
strain)	2760	1200	100	200	400	400
	4200	1200	250	450	500	500
	780	700	0	50	100	100
Arithmetic Mean (S.E.)	**2850[b] (868)**	**975[a] (115)**	**200[b] (76)**	**233[b] (67)**	**358[a] (85)**	**342[b] (84)**
% Reduction (A.M.)	***62***		***83***	***83***	***59***	***62***
% Reduction(G.M.)	***71***		***94***	***88***	***59***	***69***
Moxidectin	100	500	0	50	0	0
(Oral)	40	250	0	0	0	0
(Lambs infected	720	1350	50	100	0	0
with resistant	60	650	0	0	0	0
strain)	40	900	0	0	0	0
	40	1050	0	0	0	0
Arithmetic Mean (S.E.)	**167[c] (111)**	**783[a] (162)**	**8[c] (8)**	**25[c] (17)**	**0[b] (0)**	**0[c] (0)**
% Reduction (A.M.)	***98***		***99.9***	***98***	***100***	***100***
% Reduction (G.M.)	***99***		***99.8***	***99.6***	***100***	***100***
Moxidectin	7660	1250	1550	1150	250	1150
(Injection)	8460	1500	1550	1500	900	2200
(Lambs infected	7660	900	850	900	550	650
with resistant	4540	850	550	950	650	600
strain)	8240	600	1650	1450	950	1200
	6540	700	800	1200	950	600
Arithmetic Mean (S.E.)	**7183[a] (694)**	**967[a] (140)**	**1158[a] (195)**	**1192[ab] (101)**	**708[a] (114)**	**1067[a] (253)**
% Reduction (A.M.)	***4***		***2***	***13***	***18***	***0***
% Reduction (G.M.)	***6***		***10***	***14***	***9***	***0***
Moxidectin	0	2400	0	0	0	-
(Injection)	0	850	0	0	0	-
(Lambs infected	60	1700	0	0	0	-
with susceptible strain)						
Arithmetic Mean (S.E.)	**20 (6.7)**	**1650 (448.1)**	**0 (0)**	**0 (0)**	**0 (0)**	-

[a, b, c] Results followed by different superscript letters in the same column are significantly different ($p<0.05$) and were calculated from $\log_{10}(X+1)$ transformed data.
A.M.- Arithmetic mean; G.M.- Geometric mean

Table 7.2: Mean LC_{50} values and resistance ratios obtained from a larval development assay in lambs infected with ivermectin-resistant or susceptible strain of *T. colubriformis*.

Drug tested	Mean LC_{50} values (ng/ml of agar)		Resistance Ratio
	Resistant strain	*Susceptible strain*	
Ivermectin	2.290	0.579	**3.95**
Avermectin B_2	1.745	0.636	**2.74**
Ivermectin aglycone	15.331	0.411	**37.32**
Moxidectin	2.727	1.915	**1.42**
Thiabendazole	209.070	14.272	**14.65**
Levamisole	443.710	245.090	**1.81**

7.4. DISCUSSION

The present study is the first in which the efficacy of ivermectin oral, moxidectin oral and moxidectin injectable formulations against an ivermectin-resistant field strain of *T. colubriformis* has been compared.

The results of faecal egg counts, worm counts and larval development assays obtained in this experiment clearly confirm the presence of ivermectin resistance in the Massey Tc 97 isolate of *T. colubriformis*. Interestingly, the percentage reduction in worm counts with the ivermectin oral formulation is similar to Chapter 3 despite the increased selection pressure applied, suggesting that the full genetic potential of ivermectin resistance may have been reached in this strain.

It is evident from the faecal egg count and worm count reduction tests that the moxidectin oral formulation at therapeutic dose levels is highly effective against this strain of ivermectin-resistant *T. colubriformis* but the moxidectin injectable formulation is not. Similar results with the moxidectin oral formulation were achieved against ivermectin-resistant strains of *H. contortus* (Craig *et al.*, 1992; Pankavich *et al.*, 1992; Oosthiuzen and Erasmus, 1993) and *O. circumcincta* (Pomroy and Whelan, 1993). Mutual resistance between ivermectin and moxidectin oral formulation has been reported against laboratory selected ivermectin-resistant strains of *T. colubriformis* (Shoop *et al.*, 1993) and *Ostertagia* species (Leathwick, 1995; Sutherland *et al.*, 1999) in sheep and goats but the efficacy of the moxidectin oral formulation was always higher than with the ivermectin oral formulation at the recommended dose rate. The higher efficacy of the moxidectin oral formulation

achieved in the present study as compared to the ivermectin oral formulation may in part relate to differences between the two molecules but may also be affected by pharmacokinetics (Shoop *et al.*, 1995). However, in cattle a similar level of efficacy was achieved by ivermectin and moxidectin with the same route of administration against an ivermectin-resistant strain of *Cooperia* spp. (Vermunt *et al.*, 1996; Timms *et al.*, 1997). Therefore, the superior efficacy of moxidectin is not a universal feature. Nevertheless, the findings of the present study clearly demonstrate that the moxidectin oral formulation provided a high degree of control against an ivermectin-resistant strain of *T. colubriformis*.

It is also acknowledged that different formulations of the same macrocyclic lactone analogue can also effect the range of efficacy (Timms *et al.*, 1997). The greater efficacy of the oral formulation achieved in the present study may relate to higher peak plasma levels, hence presumably higher tissue/mucous levels in the small intestine at a level sufficient to be lethal for ivermectin-resistant *T. colubriformis* which is apparently not reached with the injectable formulation. Alvinerie *et al.* (1998) has shown in sheep that whilst the injectable formulation is more persistent than the oral formulation, the peak plasma concentration with the oral formulation was 3.4 times higher than the injectable formulation. Clearly, there is a complex interaction between efficacy of different analogues of macrocyclic lactones and the effect of formulation on pharmacokinetics and hence the efficacy of these different formulations. The existence of resistant strains such as in this experiment has allowed this interaction to be demonstrated.

Moxidectin is more persistent than ivermectin given by the same route (Lanusse *et al.*, 1997) and the moxidectin injectable formulation is more persistent than the moxidectin oral formulation (Bairden *et al.*, 1995; Kerboeuf *et al.*, 1995; Alvinerie *et al.*, 1998). It has been suggested that more persistent drugs would remove a larger proportion of resistant adult phenotypes because of the longer exposure of the drug to the nematodes (Hennessy, 1994). This may occur for benzimidazole anthelmintics (Hennessy and Prichard, 1981; Sangster *et al.*, 1991) but the results presented here indicate this is not the case with moxidectin injectable formulation against ivermectin-resistant *T. colubriformis*. The comparison of faecal egg counts does not support a gradual decline in adult parasite number, as the faecal egg counts of the moxidectin

injection group did not vary significantly throughout the trial. This is not universal as ivermectin SR bolus, which releases low levels of ivermectin, will kill ivermectin-resistant *Cooperia* spp. in cattle (Vermunt *et al.*, 1996; Rehbein *et al.*, 1998).

Recently, it has been shown that the moxidectin oral formulation provides reduced persistent protection against incoming larvae of ivermectin-resistant strains of *O. circumcincta* (Sutherland *et al.*, 1997) and *H. contortus* (Chick *et al.*, 1998). Interestingly, the persistent activity of moxidectin against incoming infective larvae is of lesser duration (2 weeks only) against susceptible *T. colubriformis* as compared to up to 5 weeks against susceptible *H. contortus* and *O. circumcincta* (Bairden *et al.*, 1995). These findings suggest that more active drug concentration is required at the site of action to kill incoming larvae of susceptible *T. colubriformis* than required for susceptible *H. contortus* and *O. circumcincta* larvae. Therefore, it is likely that moxidectin will provide less protection against incoming larvae of this ivermectin-resistant strain of *T. colubriformis*.

No single macrocyclic lactone analogue appears to be effective in diagnosing macrocyclic lactone resistance in all strongylid nematodes using a larval development assay. In this study, ivermectin aglycone achieved slightly more than a 10-fold increase in the resistance ratio with an ivermectin-resistant field isolate of *T. colubriformis* compared to ivermectin and avermectin B_2. Therefore, of those analogues compared, ivermectin aglycone seems to be the drug of choice for the diagnosis of ivermectin resistance in *T. colubriformis*. Similar results were achieved with a laboratory-selected ivermectin-resistant strain of *T. colubriformis* (Gill amd Lacey 1998). However, avermectin B_2 discriminates better than ivermectin aglycone between ivermectin-resistant and ivermectin-susceptible strains of *H. contortus* (Gill *et al.*, 1995) whilst ivermectin monosaccharide appears to be more effective than ivermectin, avermectin B_2 and ivermectin aglycone in discriminating between resistant and susceptible *O. circumcincta* (G.C. Coles, personal communication). Similarly, the resistance ratios using ivermectin aglycone were only slightly better than ivermectin in a larval development assay with cyathostomine nematodes in horses (Islam *et al.*, 1999). These results suggest it is necessary to use several different analogues when conducting a field survey to detect ivermectin resistance.

Resistance ratios recorded in the present study with thiabendazole were also very high, suggesting that this strain is also resistant to benzimidazoles. This supports the initial findings of field veterinarians who achieved a 42% reduction in faecal egg counts with albendazole in goats on the farm where this strain was originally isolated. The resistance ratio of 1.81 with levamisole suggests limited, if any, resistance to this compound.

This study has demonstrated that the moxidectin oral formulation has markedly different efficacy against ivermectin-resistant *T. colubriformis* as compared to the moxidectin injectable formulation in sheep. Therefore, the moxidectin oral formulation may be used to control ivermectin-resistant populations of *T. colubriformis* in sheep, at least in the short-term. On the other hand, treatment of animals harbouring ivermectin-resistant *T. colubriformis* worms with the moxidectin injectable formulation will allow survival of these resistant worms in sheep and hence could promote development of an ivermectin-resistant *T. colubriformis* population on the farm. Moxidectin oral formulation would therefore be a better choice as a quarantine drench to avoid the introduction of ivermectin-resistant parasites onto a farm.

CHAPTER EIGHT

GENERAL DISCUSSION

At the commencement of this study, there was no confirmed report of isolation of a field strain of ivermectin-resistant *T. colubriformis*. The initial aim was to confirm the presence of ivermectin resistance in a goat-originated strain of *T. colubriformis* in goats and sheep by slaughter study. Further experiments were designed to investigate the mechanism of inheritance of ivermectin resistance in this strain, the fitness status of this strain compared with two ivermectin-susceptible field isolates and the efficacy of moxidectin oral and injectable formulations against this strain. Ivermectin resistance in *Ostertagia* spp. was diagnosed in New Zealand in 1990 (Badger and McKenna, 1990). Since then it has been reported a number of times. Although no surveys have been conducted, ivermectin-resistant *Ostertagia* spp. is considered to be common in goats. On at least one occasion ivermectin-resistant *Ostertagia* spp. was found to be experimentally infective for sheep (Pomroy *et al.*, 1992). However, until 1999 there had been no reports of ivermectin-resistant *Ostertagia* spp. in sheep despite the two ruminant animals being farmed together in many cases. Therefore, a cross-over grazing trial was conducted to investigate if sheep are at risk from grazing pasture contaminated with goat-derived ivermectin-resistant *Ostertagia* spp.

The results of the present work confirmed ivermectin resistance in field isolates of *T. colubriformis* and *O. circumcincta* in goats and sheep. There were indications of these isolates being equally infective for sheep. This was the first confirmed report of isolation of ivermectin resistance in *T. colubriformis* from the field worldwide.

Interestingly, the reduction in egg and worm counts after ivermectin treatment was higher in sheep than in goats artificially infected with the same isolates (Chapter 3) which suggests a difference in efficacy of ivermectin in goats and sheep against these ivermectin-resistant strains of *T. colubriformis* and *O. circumcincta*. These findings were supported by the findings of the cross-over grazing trial (Chapter 4) in which higher reductions in faecal egg counts after ivermectin treatment were observed in sheep as compared to goats grazed on the paddocks previously grazed by goats habouring ivermectin-resistant *Ostertagia* spp. worms. Similarly, higher reductions in faecal egg counts after ivermectin treatment were observed in sheep grazed on the

paddocks previously grazed by sheep whereas the results of faecal egg count reduction tests showed lower reductions in faecal egg counts for the goats grazed on paddocks previously grazed by sheep. Ivermectin may be fully effective against ivermectin-susceptible nematodes in both goats and sheep but emergence of ivermectin-resistant *T. colubriformis* and *O. circumcincta* (Chapter 3) and the cross-over grazing study with the ivermectin-resistant strain of *Ostertagia* spp. (Chapter 4) have demonstrated the different efficacy of ivermectin in sheep and goats against these isolates. This is consistent with a pharmacokinetic study conducted with ivermectin in goats (Scott *et al.*, 1990a) which showed a rapid excretion of the drug in goats as compared to sheep. Goats also appear to metabolise other anthelmintics more rapidly than sheep. Efficacy studies conducted with levamisole (McKenna and Watson, 1987), albendazole (Hennessy *et al.*, 1993a) and oxfendazole (Bogan *et al.*, 1987; Sangster *et al.*, 1991) suggest that these drugs are required in higher doses in goats than in sheep. Therefore, the results of the present study confirm the need to evaluate anthelmintic drugs in goats and sheep independently and not to assume that data from sheep apply to goats.

Underdosing with anthelmintic is considered to be an important predisposing risk factor for the emergence of anthelmintic resistance because the partly-resistant (heterozygous) nematodes which might be killed with a full therapeutic dose are allowed to survive. This provides an excellent opportunity for heterozygous nematodes to breed and provide the next generation, which is likely to include at least some resistant nematodes. The number of resistant nematodes in the next generation is dependent on the genetics of anthelmintic resistance for the nematode and anthelmintic in question. Hence, continuous treatment of goats with ivermectin at the recommended therapeutic dose rate for sheep may lead to an increase in frequency of genes regulating resistance in the nematode population as heterozygous-resistant nematodes are more likely to survive in goats than in sheep. Therefore, mixed grazing of sheep and goats may allow the spread of resistant worms from goats to sheep. This hypothesis was confirmed in the cross-over grazing trial (see Chapter 4) involving ivermectin-resistant *Ostertagia* spp. It was evident that ivermectin resistance appeared in *Ostertagia* spp. in sheep grazed on the pasture previously grazed by goats harbouring ivermectin-resistant *Ostertagia* spp. worms but sheep grazed on the pasture previously grazed by sheep showed very little evidence of resistance. On the other

hand goats grazed on the pasture previously grazed by goats or sheep showed a high level of ivermectin resistance against *Ostertagia* spp.

The results of the inheritance study (Chapter 5) suggest that ivermectin resistance in *T. colubriformis* is inherited as an incompletely dominant trait. These findings are in agreement with the results of an inheritance study conducted in a laboratory-selected ivermectin-resistant strain of *T. colubriformis* (Gill *et al.* unpublished in Gill and Lacey, 1998). However, ivermectin resistance in *H. contortus* (Dobson *et al.*, 1996; Le Jambre *et al.*, 2000) and *O. circumcincta* (Coles, 1997) is believed to be inherited as a completely dominant trait and is controlled by a single gene. Therefore, it appears that ivermectin resistance in *T. colubriformis* is likely to spread less rapidly in the field compared with ivermectin resistance in *H. contortus* and *O. circumcincta*. On the other hand, ivermectin resistance in *T. colubriformis* will spread more rapidly than benzimidazole resistance in *T. colubriformis* and *H. contortus* (Roos *et al.*, 1993; Grant, 1994) and levamisole resistance in *H. contortus* (Dobson *et al.*, 1996), which are considered to be inherited as recessive traits.

The fitness status of this ivermectin-resistant strain of *T. colubriformis* was compared with the two ivermectin-susceptible field isolates of *T. colubriformis*. No difference in the establishment rate, longevity of infection, fecundity, development of eggs to larvae under natural conditions or laboratory conditions was observed between the three strains (Chapter 6). The ivermectin-resistant strain was intermediate in survival between the two susceptible strains at 10^{O}C, 20^{O}C, 25^{O}C and 30^{O}C under laboratory conditions. Hence, ivermectin resistance in this strain of *T. colubriformis* will be stable and reversion to susceptibility, if any, will be slow to occur, even if ivermectin is not used.

It has been suggested that moxidectin as an oral formulation is highly effective against ivermectin-resistant strains of *H. contortus* and *O. circumcincta* in sheep (Craig *et al.*, 1992; Pankavich *et al.*, 1992; Oosthiuzen and Erasmus, 1993; Pomroy and Whelan, 1993). In some instances mutual resistance between ivermectin and moxidectin has been reported in an ivermectin-resistant strain of *O. circumcincta* (Leathwick, 1995; Sutherland *et al.*, 1999) and in a laboratory-selected strain of *T. colubriformis* (Shoop *et al.*, 1993) in sheep and goats. However, in these reports the efficacy of the

moxidectin oral formulation was always substantially higher than the ivermectin oral formulation at recommended therapeutic dose rates. The results of the efficacy study (Chapter 7) support the view that the moxidectin oral formulation provides a high degree of efficacy against the ivermectin-resistant strain of *T. colubriformis* in sheep. In contrast, the moxidectin injectable formulation was found to ineffective, probably due to a difference in pharmacokinetic behaviour of the two formulations of moxidectin in sheep. Therefore, continued use of the moxidectin injectable formulation will allow the survival of ivermectin-resistant *T. colubriformis* populations and could promote the development of ivermectin resistance in *T. colubriformis* in sheep. On the other hand the moxidectin oral formulation could be used to control ivermectin-resistant *T. colubriformis* populations in sheep, at least in the short term. As the pharmacokinetics of ivermectin appear to be different in sheep and goats (Chapters 3 and 4; Scott *et al.*, 1990a), further work on the efficacy of moxidectin oral and injectable formulations in goats against ivermectin-resistant *T. colubriformis* will be of interest.

In a series of experiments, the larval development assay was utilised successfully to diagnose ivermectin resistance in *T. colubriformis* (Chapters 3, 5, 6 and 7) and *Ostertagia* spp. (Chapter 4). The ivermectin-resistant strain of *T. colubriformis* was also found to be resistant to benzimidazoles (Chapter 6 and 7). The present work has demonstrated that ivermectin aglycone is the drug of choice for diagnosis of ivermectin resistance in *T. colubriformis* in a larval development assay which is in agreement with the study of Gill and Lacey (1998). Due to the ivermectin aglycone being unavailable during the early experiments, these were conducted using ivermectin and avermectin B_2, which resulted in smaller resistance ratios with both ivermectin and avermectin B_2 for *T. colubriformis*. Hence, it appears that the larval development assay using ivermectin and avermectin B_2 may not be able to detect ivermectin resistance in *T. colubriformis* in the field. However, both ivermectin and avermectin B_2 were still able to discriminate between resistant and susceptible *T. colubriformis* under controlled experimental conditions. Another important factor reducing the suitability of this assay using ivermectin under field conditions would be the variability of LC_{50} values with the time after infection. The resistance ratios for *O. circumcincta* were also very small when ivermectin and avermectin B_2 were used in

the larval development assay but these resistance ratios were of a similar magnitude to those recorded with ivermectin against ivermectin-resistant *O. circumcincta* by Amarante *et al.* (1997). Nevertheless, avermectin B_2 has been found to discriminate better between the ivermectin-resistant and susceptible strains of *H. contortus* (Gill *et al.*, 1995) while ivermectin monosaccharide is more effective in the diagnosis of ivermectin resistance in *O. circumcincta* (G.C. Coles, personal communication). Therefore, it may be necessary to use several different analogues of macrocyclic lactones when conducting a field survey to diagnose ivermectin resistance in nematodes.

These studies have isolated an ivermectin-resistant strain of *T. colubriformis* for the first time from goats and demonstrated that this strain might be equally infective for sheep. The ivermectin oral formulation when administered to sheep is more effective against the ivermectin-resistant strains of *T. colubriformis* and *O. circumcincta* than when administered to goats infected with the same isolates. Ivermectin resistance in *T. colubriformis* is inherited as an incompletely dominant trait and once this strain is introduced onto a farm, resistance is likely to spread readily between goats and sheep. The ivermectin resistance in this strain of *T. colubriformis* is stable and no reversion to susceptibility is expected, even if there is no further use of ivermectin. The moxidectin oral formulation is highly effective against this ivermectin-resistant strain of *T. colubriformis* in sheep and it could be used as a quarantine drench to help prevent the introduction of this resistant strain onto the farm, at least in the short term. By contrast, the treatment of animals with the moxidectin injectable formulation could assist the development of macrocyclic resistance. The work has also demonstrated that sheep are at risk from grazing pasture contaminated by goat derived ivermectin-resistant *Ostertagia* spp.

Appendices for the Materials and methods

APPENDIX I

Modified McMaster technique for counting of eggs

Equipment/Chemical

Bowl

Coarse Sieve (aperture approximately 0.85 mm)

Electronic balance (Delta Range^{R} U.S.A.) 0.1g

Laboratory counter (Clay Adams)

Light microscope

McMaster counting slides (Paracount-EPG, Olympic Equine Products, Issaquah, U.S.A.)

Pasteur pipette

Saturated sodium chloride solution (specific gravity 1.2)

Spoon

Procedure

1. Bowl, sieve and spoon were placed on the balance and their weight adjusted to zero.
2. Two grams of faeces were weighed.
3. Twenty-eight ml of saturated salt solution was added into sieve and faeces worked thoroughly and then residues in the sieve were discarded.
4. While stirring the content to and fro in bowl, two samples were removed with a Pasteur pipette and both chambers of McMaster counting slide (0.15 ml each side) were filled.
5. Slide was left for 2 minutes before counting to allow the eggs to float.
6. The slide was placed on microscope and examined at 100x for presence of eggs
7. The number of eggs was multiplied with 50 to get eggs per gram.

APPENDIX II

Procedure for worm counting

Equipment/Chemical

Agee Jars - 1 litre size

Beakers - 2 litre and 250 ml size

Concentrated Hydrochloric acid (11.65M, BDH)

Gut scissors

Laboratory counter (Clay Adams)

Pepsin (70 FIP-U/g, Riedel-de Haen, Germany)

Plastic bucket 10 litre capacity marked at 2L and 4L

Running water

Scored petri dish

Sieve 53 µm

Stero Microscope

Water bath

Wooden stirrer - flat in section 40 cm ×2 cm×0.5 cm

Procedure

(a) Contents

1. The abomasum/small intestine kept out of the deep freezer at room temperature overnight.
2. Abomasum/small intestine was placed in bucket and opened along its length. The opened organ was pulled between fingers under a light stream of water.
3. The contents of the bucket were made to 2 or 4 litres with water and mixed vigorously by stirring to and fro and at the same time samples were taken into a beaker until one tenth of the total volume was removed. Another one-tenth sample was taken to an Agee jar and 10% by volume of 40% formalin was added for preservation as a spare sample.
4. The sample was poured from beaker into 53 µm sieve and washed gently with a steady flow of water until clear. The sieved material was washed back into beaker.

5. The sample was examined under dissecting microscope by means of scored petri dish.

(b) Pepsin digest

1. The washed abomasum /small intestine was added to the following solution
   Pepsin 20g + Water 600 ml + Concentrated hydrochloric acid 10 ml
2. 150 ml of solution per metre length of small intestine or 610 ml solution for whole abomasum was allowed.
3. The abomasum/small intestine was incubated for 2 hours at 37^{O}C in a water bath.
4. The digested abomasum/small intestine was placed in a bucket and the organ was washed between the fingers under a light stream of water.
5. The contents of the bucket were made to 2 litres with water and mixed vigorously by stirring to and fro and at the same time samples were taken into a beaker until one tenth of the total volume was removed. Another one-tenth sample was removed to an Agree jar and 10% by volume of 40% formalin was added for preservation as a spare sample.
6. The 10% sample was poured from the beaker into 53 µm sieve and washed gently with a steady flow of water until clear. The sieved material was then washed back into the beaker.
7. The sample was examined under a stereo microscope at 15x in a scored petri dish.

APPENDIX III

Collection of larvae from faeces collected from plots in Fitness study

Equipment/Chemical

Beaker 250 ml

Counting slide - 2 ml capacity (Fig. III)

Cover slips

Deionised water

Eppendorp pipette 200 µl

Faeces

Glass funnel (25 cm diameter) with rubber tubing attached and closed by a clip

Graduated cylinder - 1 litre

Laboratory counter (Clay Adams)

Light microscope

Lugol's iodine (5% iodine and 10% potassium iodide in water)

Spoon

Stand for funnel

Tissue culture bottles 250 ml

Tissue paper

Wire mesh sieve (2 mm aperture, 20 cm diameter)

Vacuum pump

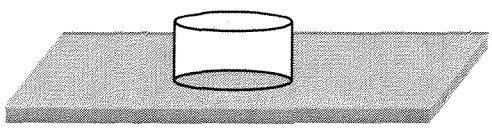


Fig. III: Counting slide.

Procedure

1. Faeces were broken up and the larvae were recovered by the Baermann technique as follows:

i. The clip of the rubber tubing attached to funnel was closed.
ii. The funnel was filled to within 2 cm of the top with deionised water.
iii. Faeces were placed on wire mesh sieve with a layer of tissue underneath the faeces.
iv. The wire mesh sieve was placed in the funnel so that faeces submerged in water.
v. It was left to stand for 24 hours.
vi. The clip was carefully released and 150 ml of water containing larvae was tapped off from rubber tubing into a graduated cylinder.

2. The cylinder was filled up with water to 1000 ml mark. and was allowed to stand for 24 hours so that larvae could settled at the bottom.
3. The supernatant was sucked off until 100 ml of contents remained in cylinder.
4. The contents were collected in a tissue culture bottle.
5. The content were transferred into a beaker and mixed to and fro with spoon.
6. While mixing, 200 µl of contents with Eppendorp pipette were taken and put them in the counting slide.
7. Few drops of Lugol's iodine were added in the counting slide.
8. The water was added into counting slide until it was filled to a shallow meniscus.
9. Two minutes time was given for larvae to settle at the bottom of counting slide.
10. The counting slide was covered with cover slip.
11. The counting slide was put under microscope and the larvae counted under 100x magnification.
12. Five counts per sample were done.
13. Mean of five samples was taken and multiplied by 500 to get the total number of larvae.
14. Remaining sample in tissue culture bottle was kept at 10^{O}C.

APPENDIX IV

Collection of larvae from pasture

Equipment/Chemical

Beaker - 250 ml

Cold and hot water

Counting slide - 2 ml capacity (Fig. III)

Cover slips

Eppendorp pipette 500 µl

Graduated Cylinder - 500 ml

Laboratory counter (Clay Adams)

Light microscope

Lugol's iodine (5% iodine and 10% potassium iodide in water)

Non-ionic detergent (Tween 20, BDH)

Pasture

Plastic centrifuge tube (Falcon) - 50 ml

Plastic funnel with rubber tubing attached and closed by a clip (Fig. IV)

Small glass funnel (15 cm diameter) with rubber tubing attached and closed by a clip (Plate VIb)

Small wire mesh sieve (1 mm aperture, 10 cm diameter)

Stand for small glass funnel

Stand for plastic funnel

Thermometer

Tissue paper

Vacuum pump

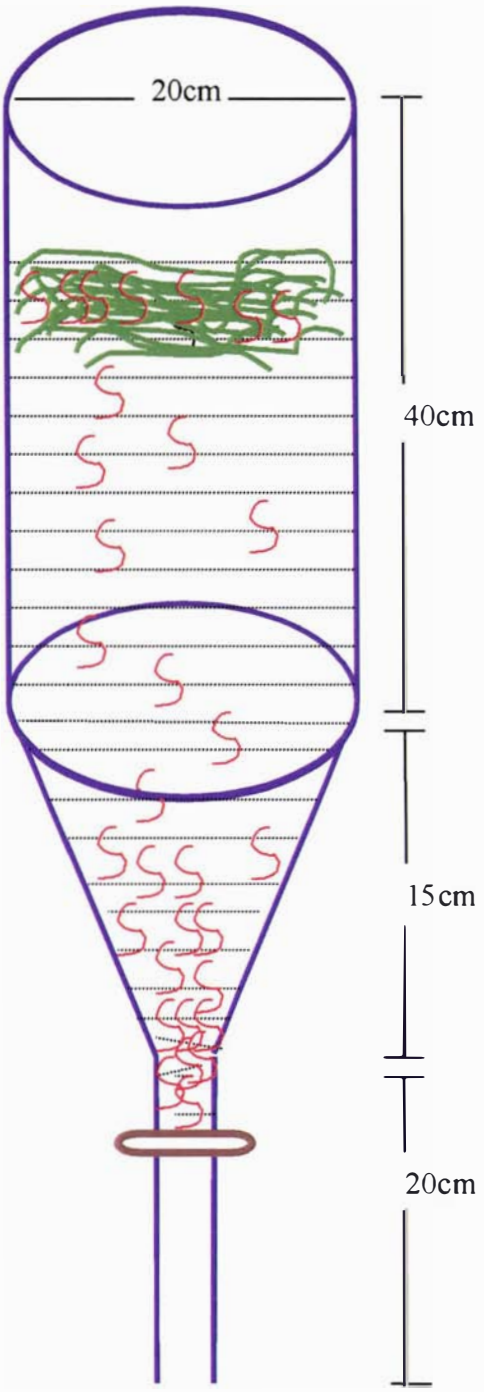


Fig. IV: Plastic funnel used for collection of larvae from pasture.

Procedure:

1. The clip of the rubber tubing attached to the funnel was closed.
2. The plastic funnel was filled with warm water (approximately $27^{O}C$) within 10 cm of the top.
3. A drop of non-ionic detergent was added in water.
4. The herbage (approximately 50g) was put in the water and mixed thoroughly.
5. It was left to stand for 24 hours.
6. The clip was released carefully and 150 ml of water containing larvae was tapped off from funnel into a beaker.
7. The larvae were cleaned by Baermann technique as follows:
   (i) The clip on the rubber tubing of small glass funnel was closed.
   (ii) The funnel was filled with approximately 100 ml of water.
   (iii) The contents from beaker were poured into small glass funnel through a small wire mesh sieve with a layer of tissue.
   (iv) It was allowed to stand for 24 hours
   (v) The clip was carefully released and 50 ml of water containing larvae was tapped off in to 50 ml plastic centrifuge tubes.
8. The contents in 50 ml plastic centrifuge tube were allowed to stand for 12 hours.
9. The supernatant was sucked off up to 20 ml mark.
10. The tube was shaken thoroughly and 0.5 ml contents were sucked with Eppendorp pipette and put in counting slide.
11. Few drops of Lugol's iodine were added in the counting slide.
12. The water was added until the counting slide was filled.
13. Two minutes were allowed for the larvae to settle at the bottom of counting slide.
14. The counting slide was covered with cover slip.
15. The counting slide was put under microscope and the larvae counted under 100x magnification.
16. Two counts per sample were done.
17. The mean of two samples was taken and multiplied with 40 to get the total number of larvae.

APPENDIX V

Collection of larvae from soil

Equipment/Chemical

Agee jars - 500 ml

Buckets 6 litre

Counting slide - 2 ml capacity (Fig. III)

Cover slips

Electronic balance (Delta Range^{R} U.S.A.) 0.1g

Eppendorp pipette 1 ml

Laboratory counter (Clay Adams)

Light microscope

Lugol's iodine (5% iodine and 10% potassium iodide in water)

Plastic centrifuge tube (Falcon) - 50 ml

Plastic trays (40 cm×27 cm×6 cm) with fiberglass mesh (2 mm aperture) at the bottom

Plastic trays (40 cm×27 cm×6 cm) with intact bottom

Small glass funnel (15 cm diameter) with rubber tubing attached and closed by a clip (Plate VIb)

Small wire mesh sieve (1 mm aperture, 10 cm diameter)

Small wooden pieces

Soil

Stand for small glass funnel

Tissue paper

Vacuum pump

Procedure

1. Approximately one litre of water was added into the plastic tray with the intact bottom.
2. The plastic tray with fiberglass mesh was placed over the plastic tray with intact bottom.
3. Eight hundred g of soil was placed on fiberglass mesh tray with a layer of tissue underneath the soil.

4. Water was added until the soil submerged and it was left to stand for 24 hours so that the larvae could migrate into the water at the bottom of two trays.
5. The fiberglass mesh tray containing soil was lifted with the help of small wooden blocks from both sides and the soil was allowed to drain for 5 hours in the plastic tray with intact bottom.
6. The soil sample was discarded and the water containing larvae collected in 6 litre bucket.
7. The bucket was allowed to stand for 24 hours.
8. The supernatant was sucked off up to 150 ml mark.
9. The contents were poured into 500 ml Agee jars.
10. The larvae were then cleaned by Baermann technique as follows:
    (i) The clip on the rubber tubing of small glass funnel was closed.
    (ii) The funnel was filled with approximately 100 ml of water.
    (iii) The contents from jar were poured into small glass funnel through a small wire mesh sieve with a layer of tissue.
    (iv) It was allowed to stand for 24 hours
    (v) The clip was carefully released and 50 ml of water containing larvae was tapped off into 50 ml plastic centrifuge tubes.
    (vi) If the contents were still dirty then the Baermann procedure was repeated through the small glass funnel.
    (vii) The centrifuge tube was allowed to stand for 24 hours.
    (viii) The supernatant was discarded up to 20 ml mark.
11. The tube was shaken thoroughly and 2 ml contents were sucked with Eppendorp pipette and put in counting slide.
12. Few drops of Lugol's iodine were added in the counting slide.
13. Water was added until counting slide filled.
13. The larvae were allowed to settle at the bottom of counting slide for two minutes.
14. The counting slide was covered with cover slip.
15. The counting slide was put under microscope and larvae counted under 100x magnification.
16. Two counts per sample were done.
17. The mean of two samples was taken and multiplied with 10 to get the total number of larvae.

APPENDIX VI

Culture and collection of larvae from faeces under laboratory conditions in experiments described in Chapter 6

Equipment/Chemical

Counting slide - 2 ml capacity (Fig. III)

Cover slips

Deionised water

Electronic balance (Delta Range^{R} U.S.A.) 0.1g

Eppendorp pipette 1 ml

Faeces

Incubator 25^{O}C

Laboratory counter (Clay Adams)

Large Petri dish (Falcon, U.S.A.) 90 mm×15 mm

Light microscope

Lugol's iodine (5% iodine and 10% potassium iodide in water)

Plastic centrifuge tube (Falcon) - 50 ml

Small glass funnel (15cm diameter) with rubber tubing attached and closed by a clip (Plate VIb)

Small Petri dish (Falcon, U.S.A.) 60 mm×15 mm

Small wire mesh sieve (1 mm aperture, 10 cm diameter)

Squeeze bottle - 250 ml

Stand for small glass funnel

Tissue paper

Vacuum pump

Procedure

1. Two gram, three egg counts (see Appendix I) were performed and mean egg count was calculated.
2. Ten gram of faeces were weighed and these were placed into the small petri dish without a lid.

3. The small petri dish was placed in the large petri dish and distilled water was put around small petri dish (Plate VIa). Large petri dish was covered with the lid and incubated at 25^{O}C for 10 days.
4. Distilled water was added around the small petri dish if needed to ensure the culture was not dry.
5. The larvae were recovered by the Baermann technique as follows:
   (i) The clip on the rubber tubing of the small glass funnel was closed.
   (ii) The funnel was filled to within 2 cm of the top with deionised water.
   (iii) The faeces were placed on the wire mesh sieve with a layer of tissue underneath the faeces.
   (iv) The wire mesh sieve was placed in the funnel and more water was added so that the faeces were submerged.
   (v) It was left to stand for 24 hours.
   (vi) The clip was released carefully and 50 ml of water containing larvae was tapped off from the rubber tubing into a 50 ml plastic centrifuge tube.
   (vii) The larvae were allowed to settle at the bottom for 24 hours.
   (viii) The supernatant was sucked off up to the 20 ml mark.
6. While mixing thoroughly, 500 µl of contents were taken using the Eppendorp pipette and put into a counting slide.
7. Few drops of Lugol's iodine were added in the counting slide.
8. The water was added until counting slide filled.
9. The larvae were allowed to settle at the bottom of counting slide for two minutes.
10. It was later covered with cover slip.
11. The counting slide was put under microscope and the larvae counted under 100x magnification.
12. Five counts per sample were done.
13. Mean of two samples was taken and multiplied it with 40 to get the total number of larvae.

Plate VIa: Faeces in a small petri dish enclosed within a large petri dish containing water as used in the method for development of larvae under laboratory conditions.

Plate VIb: Small glass funnels used in the Baermann's technique for collection of larvae.

APPENDIX VII

Procedure for counting the larvae kept at different temperatures in experiments described in Chapter 6

Equipment

Beaker 250 ml

Counting slide (Fig. III)

Cover slips

Deionised water

Dissecting microscope

Eppendorp pipette 100 µl to 50 ml

Incubators $10^{O}C$, $20^{O}C$, $25^{O}C$ and $30^{O}C$

Laboratory counter (Clay Adams)

Light microscope

Lugol's Iodine

Small Petri dish (Falcon, U.S.A.) 60 mm×15 mm

Spoon

Squeeze bottle - 250 ml

Tissue culture bottles (Falcon, U.S.A.) 50 ml

Vacuum pump

Procedure

1. The larval solution was poured into a 250 ml beaker and it was mixed to and fro with spoon.
2. While mixing, 100 µl of contents were taken using the Eppendorp pipette and put into the counting slide.
3. Few drops of Lugol's iodine were added in the counting slide.
4. The water was added until the counting slide was filled.
5. The larvae were allowed to settle at the bottom of counting slide for two minutes.
6. It was later covered with cover slip.
7. The counting slide was put under microscope and the larvae counted under 100x magnification.

8. Five counts per sample were done. The mean number was used to estimate the amount of larval solution that contained 2000 larvae.
15. Approximately 2000 larvae were dispensed into tissue culture bottles. The volume was adjusted with deionised water to 15 ml.
16. The tissue culture bottles were placed at $10^{O}C$, $20^{O}C$, $25^{O}C$ and $30^{O}C$ and the caps were kept slightly loose.
17. Every week tissue culture bottles were taken out from incubators and allowed to stay at room temperature for 1 hour before counting.
18. The volumes in the tissue culture bottles were adjusted to 15 ml by adding or withdrawing deionised water before counting.
19. Tissue culture bottles were shaken vigorously and 800 µl of contents were placed in the center of petri dish and live larvae counted under dissecting microscope.
20. Two counts per sample were done
21. After counting the contents of the petri dish were washed back into the tissue culture bottle and the tissue culture bottles placed back in respective incubators.
22. The mean of the two values was taken and multiplied by 18.75 to get the total number of live larvae.

APPENDIX VIII

Procedure for Larval development assay

Equipment

96-well (Nunclon^{MT}) round-bottomed microtitre plates - volume of each well 300 µl

Beakers - volume 50 ml to 3000 ml

Centrifuge (ILEC Centra-8 International Equipment Company)

Cover slips

Deionised water

Desiccator (To incubate plates)

Distilled water

Electronic balance (Delta Range^{R} U.S.A.) 0.1g

Eppendorp pipette - 2 µl, 20 µl, 200 µl and 1000 µl pipettes

Heating Plate (Heidolph, Germany)

Incubator - 25^{O}C

Laboratories counter (Clay Adams)

Measuring cylinders - volume 250 ml to 1000 ml

Microscope (Olympus)

Microwave (Panasonic)

Plastic centrifuge tubes (Falcon) - volume 50 ml

Plastic spoon

Scored glass sides

Sieves - 1 mm aperture, diameter 12 cm; 20 µm aperture, diameter 10.2 cm; 60 µm aperture, diameter 7.5 cm; 100 µm aperture diameter 10.2 cm.

Squeeze bottle - volume 250 ml

Vacuum pump

Chemicals

Anthelmintics

(a) Ivermectin

Ivermectin (0.08%) (Ivomec oral drench for sheep and goats, MSD AgVet, New Zealand Ltd.)

0.08% ivermectin drench = 0.8 g/litre i.e. 0.8 mg/ml i.e. 800 µg/ml

Therefore 1 ml of 800 µg/ml of ivermectin + 33.3 ml Dimethyl suphoxide (DMSO) gave 24 µg/ml stock solution.

When 200 µl of agar was used

The final concentration is shown when 200 µl of agar was added to 2 µl of each ivermectin dilution

1. 2 µl of stock solution (24 µg/ml) in 200 µl of agar = 0.24 µg/ml in the agar phase
2. 1 ml of 24 µg/ml + 1 ml of DMSO = 12 µg/ml = 0.12 µg/ml in the agar phase
3. 1 ml of 12 µg/ml + 1 ml of DMSO = 6 µg/ml = 0.06 µg/ml in the agar phase
4. 1 ml of 6 µg/ml + 1 ml of DMSO = 3 µg/ml = 0.03 µg/ml in the agar phase
5. 1 ml of 3 µg/ml + 1 ml of DMSO = 1.5 µg/ml = 0.015 µg/ml in the agar phase
6. 1 ml of 1.5 µg/ml + 1 ml of DMSO = 0.75 µg/ml = 0.0075 µg/ml in the agar phase
7. 1 ml of 0.75 µg/ml + 1 ml of DMSO = 0.375 µg/ml = 0.00375 µg/ml in the agar phase
8. 1 ml of 0.375 µg/ml + 1 ml of DMSO = 0.1875 µg/ml = 0.001875 µg/ml in the agar phase
9. 1 ml of 0.1875 µg/ml + 1 ml of DMSO = 0.09375 µg/ml = 0.00094 µg/ ml in the agar phase
10. 1 ml of 0.09375 µg/ml + 1 ml of DMSO = 0.046875 µg/ml = 0.00047 µg/ml in the agar phase
11. 1 ml of 0.046875 µg/ml + 1 ml of DMSO = 0.02343 µg/ml = 0.00023 µg/ml in the agar phase
12. 1 ml of 0.02343 µg/ml + 1 ml of DMSO = 0.01172 µg/ml = 0.00012 µg/ml in the agar phase
13. 1 ml of 0.01172 µg/ml + 1 ml of DMSO = 0.005859 µg/ml = 0.00006 µg/ml in the agar phase
14. 1 ml of 0.005859 µg/ml + 1 ml of DMSO = 0.002929 µg/ml = 0.00003 µg/ml in the agar phase
15. 1 ml of 0.002929 µg/ml + 1 ml of DMSO = 0.001465 µg/ml = 0.000015 µg/ml in the agar phase

16. 1 ml of 0.001465 μg/ml + 1 ml of DMSO = 0.0007325 μg/ml = 0.000007 μg/ml in the agar phase

When 150 μl of agar was used

The final concentration is shown when 150 μl of agar was added to 2 μl of each ivermectin dilution

1. 2 μl of stock solution (24 μg/ml) in 150 μl of agar = 0.32 μg/ml in the agar phase
2. 1 ml of 24 μg/ml + 1 ml of DMSO = 12 μg/ml = 0.16 μg/ml in the agar phase
3. 1 ml of 12 μg/ml + 1 ml of DMSO = 6 μg/ml = 0.08 μg/ml in the agar phase
4. 1 ml of 6 μg/ml + 1 ml of DMSO = 3 μg/ml = 0.04 μg/ml in the agar phase
5. 1 ml of 3 μg/ml + 1 ml of DMSO = 1.5 μg/ml = 0.02 μg/ml in the agar phase
6. 1 ml of 1.5 μg/ml + 1 ml of DMSO = 0.75 μg/ml = 0.01 μg/ml in the agar phase
7. 1 ml of 0.75 μg/ml + 1 ml of DMSO = 0.375 μg/ml = 0.005 μg/ml in the agar phase
8. 1 ml of 0.375 μg/ml + 1 ml of DMSO = 0.1875 μg/ml = 0.0025 μg/ml in the agar phase
9. 1 ml of 0.1875 μg/ml + 1 ml of DMSO = 0.09375 μg/ml = 0.00125 μg/ml in the agar phase
10. 1 ml of 0.09375 μg/ml + 1 ml of DMSO = 0.046875 μg/ml = 0.00063 μg/ml in the agar phase
11. 1 ml of 0.046875 μg/ml + 1 ml of DMSO = 0.02343 μg/ml = 0.00031 μg/ml in the agar phase
12. 1 ml of 0.02343 μg/ml + 1 ml of DMSO = 0.01172 μg/ml = 0.00016 μg/ml in the agar phase
13. 1 ml of 0.01172 μg/ml + 1 ml of DMSO = 0.005859 μg/ml = 0.00008 μg/ml in the agar phase
14. 1 ml of 0.005859 μg/ml + 1 ml of DMSO = 0.002929 μg/ml = 0.00004 μg/ml in the agar phase
15. 1 ml of 0.002929 μg/ml + 1 ml of DMSO = 0.001465 μg/ml = 0.00002 μg/ml in the agar phase
16. 1 ml of 0.001465 μg/ml + 1 ml of DMSO = 0.0007325 μg/ml = 0.00001 μg/ml in the agar phase

(b) Avermectin B_2 (Kindly donated by MSD AgVet, New Zealand Ltd.)

Molecular weight of Avermectin B_2 = 875 g/mol

1 molar = 875g in 1000 ml i.e. 875 μg × 10^6 in 1000 ml i.e. 875 μg × 10^3 in 1 ml

So 1 μM = 0.875 μg/ml i.e. 50 μM = 0.875 × 50 = 43.75 μg/ml

Therefore 1 ml contains 43.75 μg/ml avermectin B_2

So 50 ml contain = 2187.5 μg = 2.19 mg

Therefore, 2.19 mg powder diluted in 50 ml of DMSO = 43.75 μg/ml

When 200 μl of agar was used

The final concentration is shown when 200 μl of agar was added to 2 μl of each avermectin B_2 dilution

1. 2 μl of stock solution (43.75 μg/ml) in 200 μl of agar = 0.4375 μg/ml in the agar phase
2. 1 ml of 0.4375 μg/ml + 1 ml of DMSO = 21.9 μg/ml = 0.219 μg/ml in the agar phase
3. 1 ml of 21.9 μg/ml + 1 ml of DMSO = 10.95 μg/ml = 0.1095 μg/ml in the agar phase
4. 1 ml of 10.95 μg/ml + 1 ml of DMSO = 5.475 μg/ml = 0.0548 μg/ml in the agar phase
5. 1 ml of 5.475 μg/ml + 1 ml of DMSO = 2.7375 μg/ml = 0.0274 μg/ml in the agar phase
6. 1 ml of 2.7375 μg/ml + 1 ml of DMSO = 1.3688 μg/ml = 0.0137 μg/ml in the agar phase
7. 1 ml of 1.3688 μg/ml + 1 ml of DMSO = 0.6844 μg/ml = 0.0068 μg/ml in the agar phase
8. 1 ml of 0.6844 μg/ml + 1 ml of DMSO = 0.3422 μg/ml = 0.0034 μg/ml in the agar phase
9. 1 ml of 0.3422 μg/ml + 1 ml of DMSO = 0.1711 μg/ml = 0.0017 μg/ml in the agar phase
10. 1 ml of 0.1711 μg/ml + 1 ml of DMSO = 0.0855 μg/ml = 0.00085 μg/ml in the agar phase

11. 1 ml of 0.0855 μg/ml + 1 ml of DMSO = 0.0428 μg/ml = 0.00043 μg/ml in the agar phase
12. 1 ml of 0.0428 μg/ml + 1 ml of DMSO = 0.0214 μg/ml = 0.00021 μg/ml in the agar phase
13. 1 ml of 0.0214 μg/ml + 1 ml of DMSO = 0.0107 μg/ml = 0.00011 μg/ml in the agar phase
14. 1 ml of 0.0107 μg/ml + 1 ml of DMSO = 0.00534 μg/ml = 0.00005 μg/ml in the agar phase
15. 1 ml of 0.00535 μg/ml + 1 ml of DMSO = 0.00267 μg/ml = 0.000027 μg/ml in the agar phase
16. 1 ml of 0.00267 μg/ml + 1 ml of DMSO = 0.00134 μg/ml = 0.0000134 μg/ml in the agar phase

When 150 μl of agar was used

The final concentration is shown when 150 μl of agar was added to 2 μl of each avermectin B_2 dilution

1. 2 μl of stock solution (43.75 μg/ml) in 150 μl of agar = 0.5833 μg/ml in the agar phase
2. 1 ml of 0.4375 μg/ml + 1 ml of DMSO = 21.9 μg/ml = 0.2917 μg/ml in the agar phase
3. 1 ml of 21.9 μg/ml + 1 ml of DMSO = 10.95 μg/ml = 0.1458 μg/ml in the agar phase
4. 1 ml of 10.95 μg/ml + 1 ml of DMSO = 5.475 μg/ml = 0.0729 μg/ml in the agar phase
5. 1 ml of 5.475 μg/ml + 1 ml of DMSO = 2.7375 μg/ml = 0.0365 μg/ml in the agar phase
6. 1 ml of 2.7375 μg/ml + 1 ml of DMSO = 1.3688 μg/ml = 0.0182 μg/ml in the agar phase
7. 1 ml of 1.3688 μg/ml + 1 ml of DMSO = 0.6844 μg/ml = 0.0091 μg/ml in the agar phase
8. 1 ml of 0.6844 μg/ml + 1 ml of DMSO = 0.3422 μg/ml = 0.00455 μg/ml in the agar phase

9. 1 ml of 0.3422 µg/ml + 1 ml of DMSO = 0.1711 µg/ml = 0.0023 µg/ml in the agar phase
10. 1 ml of 0.1711 µg/ml + 1 ml of DMSO = 0.0855 µg/ml = 0.00114 µg/ml in the agar phase
11. 1 ml of 0.0855 µg/ml + 1 ml of DMSO = 0.0428 µg/ml = 0.00057 µg/ml in the agar phase
12. 1 ml of 0.0428 µg/ml + 1 ml of DMSO = 0.0214 µg/ml = 0.00028 µg/ml in the agar phase
13. 1 ml of 0.0214 µg/ml + 1 ml of DMSO = 0.0107 µg/ml = 0.00014 µg/ml in the agar phase
14. 1 ml of 0.0107 µg/ml + 1 ml of DMSO = 0.00534 µg/ml = 0.00007 µg/ml in the agar phase
15. 1 ml of 0.00535 µg/ml + 1 ml of DMSO = 0.00267 µg/ml = 0.000036 µg/ml in the agar phase
16. 1 ml of 0.00267 µg/ml + 1 ml of DMSO = 0.00134 µg/ml = 0.000018 µg/ml in the agar phase

(c) Ivermectin aglycone (99.2% powder, kindly donated by Virbac Laboratories Ltd., New Zealand)

Molecular weight of Ivermectin aglycone = 566.73 g/mol

1 molar = 566.73g in 1000 ml i.e. 566.73 µg × 10^6 in 1000 ml i.e. 566.73 µg × 10^3 in 1 ml

So 1 µM = 0.567 µg/ml i.e. 250 µM = 0.567 × 250 = 141.75 µg/ml

Therefore 1 ml contains 141.75 µg/ml ivermectin aglycone

So 50 ml contain = 7087.5 µg = 7.0875 mg

Therefore, 7.0875 mg powder diluted in 50 ml of DMSO = 141.75 µg/ml

The following dilutions were used for ivermectin aglycone. The final concentration is shown when 150 µl of agar was added to 2 µl of each ivermectin aglycone dilution

1. 2 µl of stock solution (141.75 µg/ml) in 150 µl of agar = 1.89 µg/ml in the agar phase

2. 1 ml of 141.75 μg/ml + 1 ml of DMSO = 70.875 μg/ml = 0.945 μg/ml in the agar phase
3. 1 ml of 70.875 μg/ml + 1 ml of DMSO = 35.437 μg/ml = 0.4725 μg/ml in the agar phase
4. 1 ml of 35.437 μg/ml + 1 ml of DMSO = 17.719 μg/ml = 0.236 μg/ml in the agar phase
5. 1 ml of 17.719 μg/ml + 1 ml of DMSO = 8.86 μg/ml = 0.118 μg/ml in the agar phase
6. 1 ml of 8.86 μg/ml + 1 ml of DMSO = 4.43 μg/ml = 0.059 μg/ml in the agar phase
7. 1 ml of 4.43 μg/ml + 1 ml of DMSO = 2.21 μg/ml = 0.0295 μg/ml in the agar phase
8. 1 ml of 2.21 μg/ml + 1 ml of DMSO = 1.11 μg/ml = 0.01477 μg/ml in the agar phase
9. 1 ml of 1.11 μg/ml + 1 ml of DMSO = 0.5537 μg/ml = 0.00738 μg/ml in the agar phase
10. 1 ml of 0.5537 μg/ml + 1 ml of DMSO = 0.277 μg/ml = 0.00369 μg/ml in the agar phase
11. 1 ml of 0.277 μg/ml + 1 ml of DMSO = 0.138 μg/ml = 0.0018 μg/ml in the agar phase
12. 1 ml of 0.138 μg/ml + 1 ml of DMSO = 0.069 μg/ml = 0.0009 μg/ml in the agar phase
13. 1 ml of 0.069 μg/ml + 1 ml of DMSO = 0.0346 μg/ml = 0.00046 μg/ml in the agar phase
14. 1 ml of 0.0346 μg/ml + 1 ml of DMSO = 0.017 μg/ml = 0.00023 μg/ml in the agar phase
15. 1 ml of 0.017 μg/ml + 1 ml of DMSO = 0.00865 μg/ml = 0.000115 μg/ml in the agar phase
16. 1 ml of 0.00865 μg/ml + 1 ml of DMSO = 0.0043 μg/ml = 0.0000577 μg/ml in the agar phase
17. 1 ml of 0.0043 μg/ml + 1 ml of DMSO = 0.00216 μg/ml = 0.0000288 μg/ml in the agar phase

18. 1 ml of 0.00216 μg/ml + 1 ml of DMSO = 0.00108 μg/ml = 0.0000144 μg/ml in the agar phase
19. 1 ml of 0.00108 μg/ml + 1 ml of DMSO = 0.00054 μg/ml = 0.000007 μg/ml in the agar phase
20. 1 ml of 0.00054 μg/ml + 1 ml of DMSO = 0.00027 μg/ml = 0.0000036 μg/ml in the agar phase
21. 1 ml of 0.00027 μg/ml + 1 ml of DMSO = 0.000135 μg/ml = 0.0000018 μg/ml in the agar phase
22. 1 ml of 0.000135 μg/ml + 1 ml of DMSO = 0.0000675 μg/ml = 0.0000009 μg/ml in the agar phase
23. 1 ml of 0.0000675 μg/ml + 1 ml of DMSO = 0.0000338 μg/ml = 0.00000045 μg/ml in the agar phase
24. 1 ml of 0.0000338 μg/ml + 1 ml of DMSO = 0.0000169 μg/ml = 0.000000225 μg/ml in the agar phase
25. 1 ml of 0.0000169 μg/ml + 1 ml of DMSO = 0.0000084 μg/ml = 0.000000113 μg/ml in the agar phase

(d) Moxidectin

Moxidectin (1 mg/ml) (Moxidectin oral drench, Cyanamid, New Zealand Ltd.)
Therefore 1 ml of 1000 μg/ml of Moxidectin + 30 ml Dimethyl suphoxide (DMSO) gives 33.33 μg/ml of moxidectin in stock solution.

The following dilutions of moxidectin were used. The final concentration is shown when 150 μl of agar was added to 2 μl of each moxidectin dilution

1. 2 μl of stock solution (33.33 μg/ml) in 150 μl of agar = 0.44 μg/ml in the agar phase
2. 1 ml of 33.33 μg/ml + 1 ml of DMSO = 16.665 μg/ml = 0.22 μg/ml in the agar phase
3. 1 ml of 16.665 μg/ml + 1 ml of DMSO = 8.33 μg/ml = 0.11 μg/ml in the agar phase
4. 1 ml of 8.33 μg/ml + 1 ml of DMSO = 4.17 μg/ml = 0.0556 μg/ml in the agar phase

5. 1 ml of 4.17 μg/ml + 1 ml of DMSO = 2.08 μg/ml = 0.0278 μg/ml in the agar phase
6. 1 ml of 2.08 μg/ml + 1 ml of DMSO = 1.04 μg/ml = 0.0139 μg/ml in the agar phase
7. 1 ml of 1.04 μg/ml + 1 ml of DMSO = 0.52 μg/ml = 0.0069 μg/ml in the agar phase
8. 1 ml of 0.52 μg/ml + 1 ml of DMSO = 0.26 μg/ml = 0.00347 μg/ml in the agar phase
9. 1 ml of 0.26 μg/ml + 1 ml of DMSO = 0.13 μg/ml = 0.00174 μg/ml in the agar phase
10. 1 ml of 0.13 μg/ml + 1 ml of DMSO = 0.065 μg/ml = 0.000868 μg/ml in the agar phase
11. 1 ml of 0.065 μg/ml + 1 ml of DMSO = 0.0325 μg/ml = 0.000434 μg/ml in the agar phase
12. 1 ml of 0.0325 μg/ml + 1 ml of DMSO = 0.01627 μg/ml = 0.000217 μg/ml in the agar phase
13. 1 ml of 0.01627 μg/ml + 1 ml of DMSO = 0.00814 μg/ml = 0.000108 μg/ml in the agar phase
14. 1 ml of 0.00814 μg/ml + 1 ml of DMSO = 0.00407 μg/ml = 0.0000542 μg/ml in the agar phase
15. 1 ml of 0.00407 μg/ml + 1 ml of DMSO = 0.00203 μg/ml = 0.000027 μg/ml in the agar phase
16. 1 ml of 0.00203 μg/ml + 1 ml of DMSO = 0.00102 μg/ml = 0.0000136 μg/ml in the agar phase

(e) Levamisole hydrochloride (Rycozole^{R}, oral drench, Young's Animal Health, New Zealand)

Concentration of Levamisole in drench = 40 g/litre i.e. 40 μg/μl

Therefore 250 μl of drench contain 10000 μg of levamisole

So 250 μl of drench + 77.5 ml distilled water = 129 μg/ml of levamisole in stock solution

The following dilutions were used for levamisole. The final concentration is shown when 150 µl of agar was added to 2 µl of each levamisole dilution

1. 2 µl of stock solution (129 µg/ml) in 150 µl of agar = 1.72 µg/ml in the agar phase
2. 1 ml of 129 µg/ml + 1 ml of H_2O = 64.5 µg/ml = 0.86 µg/ml in the agar phase
3. 1 ml of 64.5 µg/ml + 1 ml of H_2O = 32.25 µg/ml = 0.43 µg/ml in the agar phase
4. 1 ml of 32.25 µg/ml + 1 ml of H_2O = 16.13 µg/ml = 0.215 µg/ml in the agar phase
5. 1 ml of 16.13 µg/ml + 1 ml of H_2O = 8.06 µg/ml = 0.108 µg/ml in the agar phase
6. 1 ml of 8.06 µg/ml + 1 ml of H_2O = 4.03 µg/ml = 0.0538 µg/ml in the agar phase
7. 1 ml of 4.03 µg/ml + 1 ml of H_2O = 2.02 µg/ml = 0.0269 µg/ml in the agar phase
8. 1 ml of 2.02 µg/ml + 1 ml of H_2O = 1.01 µg/ml = 0.0134 µg/ml in the agar phase
9. 1 ml of 1.01 µg/ml + 1 ml of H_2O = 0.504 µg/ml = 0.0067 µg/ml in the agar phase
10. 1 ml of 0.504 µg/ml + 1 ml of H_2O = 0.252 µg/ml = 0.0034 µg/ml in the agar phase
11. 1 ml of 0.252 µg/ml + 1 ml of H_2O = 0.126 µg/ml = 0.0017 µg/ml in the agar phase
12. 1 ml of 0.126 µg/ml + 1 ml of H_2O = 0.063 µg/ml = 0.00084 µg/ml in the agar phase
13. 1 ml of 0.063 µg/ml + 1 ml of H_2O = 0.0314 µg/ml = 0.00042 µg/ml in the agar phase
14. 1 ml of 0.0314 µg/ml + 1 ml of H_2O = 0.016 µg/ml = 0.00021 µg/ml in the agar phase
15. 1 ml of 0.016 µg/ml + 1 ml of H_2O = 0.0079 µg/ml = 0.00011 µg/ml in the agar phase
16. 1 ml of 0.0079 µg/ml + 1 ml of H_2O = 0.0039 µg/ml = 0.00005 µg/ml in the agar phase

(f) Thiabendazole (99.3% powder, kindly donated by MSD AgVet, New Zealand Ltd.)

12.9 mg powder was dissolved in 50 ml of DMSO. This gave a stock solution of 258 µg/ml of thiabendazole.

The following dilutions were used for thiabendazole. The final concentration is shown when 150 µl of agar was added to 2 µl of each thiabendazole dilution

1. 2 µl of stock solution (258 µg/ml) in 150 µl of agar = 3.44 µg/ml in the agar phase
2. 1 ml of 258 µg/ml + 1 ml of DMSO = 129 µg/ml = 1.72 µg/ml in the agar phase
3. 1 ml of 129 µg/ml + 1 ml of DMSO = 64.5 µg/ml = 0.86 µg/ml in the agar phase
4. 1 ml of 64.5 µg/ml + 1 ml of DMSO = 32.25 µg/ml = 0.43 µg/ml in the agar phase
5. 1 ml of 32.25 µg/ml + 1 ml of DMSO = 16.12 µg/ml = 0.22 µg/ml in the agar phase
6. 1 ml of 16.12 µg/ml + 1 ml of DMSO = 8.06 µg/ml = 0.11 µg/ml in the agar phase
7. 1 ml of 8.06 µg/ml + 1 ml of DMSO = 4.03 µg/ml = 0.0538 µg/ml in the agar phase
8. 1 ml of 4.03 µg/ml + 1 ml of DMSO = 2.02 µg/ml = 0.0268 µg/ml in the agar phase
9. 1 ml of 2.02 µg/ml + 1 ml of DMSO = 1.01 µg/ml = 0.013 µg/ml in the agar phase
10. 1 ml of 1.01 µg/ml + 1 ml of DMSO = 0.5039 µg/ml = 0.0067 µg/ml in the agar phase
11. 1 ml of 0.5039 µg/ml + 1 ml of DMSO = 0.252 µg/ml = 0.00336 µg/ml in the agar phase
12. 1 ml of 0.252 µg/ml + 1 ml of DMSO = 0.1260 µg/ml = 0.001680 µg/ml in the agar phase
13. 1 ml of 0.1260 µg/ml + 1 ml of DMSO = 0.0630 µg/ml = 0.00084 µg/ml in the agar phase
14. 1 ml of 0.0630 µg/ml + 1 ml of DMSO = 0.0315 µg/ml = 0.00042 µg/ml in the agar phase
15. 1 ml of 0.0315 µg/ml + 1 ml of DMSO = 0.0157 µg/ml = 0.00021 µg/ml in the agar phase
16. 1 ml of 0.0157 µg/ml + 1 ml of DMSO = 0.000787 µg/ml = 0.00011 µg/ml in the agar phase

Nutritive media

One gram of yeast extract (Y-1000, Sigma) was added in 90 ml of 0.85% saline solution. The yeast solution was then autoclaved and stored in 9 ml bottles at -20^{O}C until used. At the time of assay 1 ml of Earle's Balanced Salt solution (E7510, Sigma) was added to every 9 ml of yeast solution.

E. coli suspension

15 mg of lyophilised *E. coli* (Strain W (ATCC) 9637, Sigma) was added to 100 ml of distilled water. The suspension was dispensed into 3 ml bottles, sterilized by autoclaving and stored at -20^{O}C until used.

Amphotericin B solution

25 mg of Amphotericin B (A-9525, Sigma) was dissolved in 100 ml of distilled water. The suspension was dispensed into 0.5 ml aliquots and stored in freezer until used.

Agar Matrix (2%)

Two grams of agar (Bacto-agar, Y-1000, Sigma) was dissolved in 100 ml of distilled water by heating a microwave until dissolved. The agar was kept warm (approximately 85^{O}C) on a heating plate during the application of assay.

Magnesium sulphate solution (20%, specific gravity 1.1)

100g of magnesium was dissolved in 500 ml of tap water.

Lugol's iodine solution

5g of iodine and 10g of potassium iodide were dissolved in 100 ml of tap water and mixed thoroughly.

Dimethyl sulphoxide (DMSO) (Sigma, New Zealand Ltd.)

Procedure

Nematode eggs recovery

Nematode eggs were recovered as described by Hubert and Kerboeuf (1992). The steps are as follows:

1. Nematode egg counts (see Appendix I) were carried out before recovering the nematode eggs. A known amount of faeces, depending on the total number of eggs required for an assay, was put in water in a beaker for 30 minutes to soften.
2. The faeces were worked through a 1mm sieve into a 1 litre beaker and the faecal residuals in the sieve were discarded. Then the faeces were then washed through a 100 μm sieve into a 2 litre beaker. The faecal residues in 100 μm sieve were discarded. The washings were then washed through a fine 20 μm sieve and the eggs were retained by the sieve. The eggs were then collected in a small beaker with a jet of distilled water from a squeeze bottle.
3. The egg suspension was transferred into 50 ml plastic centrifuge tubes and filled to the 50 ml mark. These tubes were centrifuged at 1500 rpm for 10 minutes to concentrate the eggs at the bottom.
4. The supernatant was discarded, tubes were filled with 20% magnesium sulphate up to 50 ml mark, shaken properly and centrifuged at 1500 rpm for another 5 minutes.
5. The supernatant was filtered through a 60 μm sieve and then through a 20 mm sieve (60 μm sieve placed in side 20 μm sieve). The eggs were retained by the 20 μm sieve. The eggs were washed thoroughly to remove the traces of magnesium sulphate. The eggs were then collected in another clean plastic centrifuge tube with a jet of water form a squeeze bottle.
6. Since a long exposure to magnesium sulphate damages the eggs, this step was completed quickly (Hubert and Kerboeuf, 1984).
7. The egg suspension was then transferred into a one litre cylinder, filled with deionised water and allowed to settle for one and a half-hours. The supernatant was then discarded and the volume was reduced to 50 ml. This step was repeated if the egg suspension was still dirty.
8. The egg suspension was then transferred to a 50 ml plastic centrifuge tube and allowed to stand for one hour. The volume was then reduced to 15 ml.
9. The tube was shaken vigorously, five 20 μl samples were taken out and eggs were counted under 100x magnification of microscope.
10. The final volume of egg suspension was adjusted to approximately 833 eggs/ml (50 eggs/60μl).

Preparation of larval development assay plates

1. The test was carried out in 96 well microtitre plates largely described by Gill *et al.* (1995).
2. Two replicates per anthelmintic dilution were prepared. Two µl of anthelmintic dilution was added to each well and mixed with 150 µl (or 200 µl) of 2% hot agar. Sixteen different concentrations of the anthelmintic (25 concentrations for ivermectin aglycone) to be tested were prepared as described previously.
3. Two µl of DMSO was added in each of three control wells and mixed with 150 µl (or 200 µl) of 2% hot agar.
4. The plates were allowed to stand for half an hour at room temperature.

Preparation of cultures

1. Nine ml of egg suspension was mixed with 3 ml of sterilized *E. coli* suspension, 3 ml nutritive media (9 ml yeast extract + 1 ml Earle's balanced salt solution) and 180 µl of amphotericin B.
2. The solution was then mixed, and 100 µl was laid on top of agar matrix in each well with Eppendorp pipette.
3. The plates were placed in desiccator with water at the bottom to ensure high humidity and incubated at 25^{O}C for 7 days.
4. The liquid phase from each well was removed and transferred to a scored glass slide by means of a Pasteur pipette.
5. The number of eggs, first, second and third stage larvae were counted in each well after staining with Lugol's iodine solution.
6. The data were corrected for the mean number of larvae not developed to L_3 in three control wells.
7. The proportion of L_3's was fitted into a sigmoid curve after $\log_{10}$ transformation of values for the different anthelmintic concentrations. The curve was fitted using the software package SlideWrite (Advanced Graphic Software Inc., U.S.A.)
8. The LC_{50} values were calculated using the same software programme.

Appendix for the experiments described in Chapter 2

Appendix 2.1

Statistical analysis of LC_{50} values, pasture and soil larvae counts

(a) Analysis of LC_{50} values

(Group = 150 µl or 200 µl of agar)

General Linear Models Procedure
Dependent Variable: LOGLD50

Source	DF	Sum of Squares	Mean Square	F Value	Pr > F
Model	6	0.01542503	0.00257084	1.34	0.3815
Error	5	0.00956440	0.00191288		
Corrected Total	11	0.02498944			

R-Square	C.V.	Root MSE	LOGLD50 Mean
0.617262	-6.715223	0.043736	-0.651304

Source	DF	Type I SS	Mean Square	F Value	Pr > F
GROUP	1	0.00222221	0.00222221	1.16	0.3303
DAYS	5	0.01320282	0.00264056	1.38	0.3661

General Linear Models Procedure
Tukey's Studentized Range (HSD) Test for variable: LOGLD50

Means with the same letter are not significantly different.

Tukey Grouping	Mean	N	GROUP
A	-0.63770	6	150
A			
A	-0.66491	6	200

(b) Analysis of pasture larvae counts

(Group = 3030, 6060, 9090, 12120 or 15150 larvae; Replicate = I, II or III)

General Linear Models Procedure
Dependent Variable: LOGCOUNT

Source	DF	Sum of Squares	Mean Square	F Value	Pr > F
Model	6	6.81645635	1.13607606	386.32	0.0001
Error	8	0.02352598	0.00294075		
Corrected Total	14	6.83998232			

R-Square	C.V.	Root MSE	LOGCOUNT Mean
0.996561	0.641786	0.054229	8.449648

Source	DF	Type I SS	Mean Square	F Value	Pr > F
Group	4	6.74661508	1.68665377	573.55	0.0001
Replicate	2	0.06984127	0.03492063	11.87	0.0040

General Linear Models Procedure
Tukey's Studentized Range (HSD) Test for variable: LOGCOUNT

Means with the same letter are not significantly different.

Tukey Grouping		Mean	N	Replicate
	A	8.52910	5	I
	A			
B	A	8.45736	5	III
B				
B		8.36249	5	II

(c) Analysis of soil larvae counts

(Group = 3000, 6000, 9000, 12000 or 15000 larvae; Replicate = I, II or III)

General Linear Models Procedure
Dependent Variable: LOGCOUNT

Source	DF	Sum of Squares	Mean Square	F Value	Pr > F
Model	6	6.21256700	1.03542783	20.60	0.0002
Error	8	0.40213027	0.05026628		
Corrected Total	14	6.61469727			

R-Square	C.V.	Root MSE	LOGCOUNT Mean
0.939207	3.325074	0.224201	6.742751

Source	DF	Type I SS	Mean Square	F Value	Pr > F
GROUP	4	5.89763739	1.47440935	29.33	0.0001
Replicate	2	0.31492961	0.15746480	3.13	0.0989

General Linear Models Procedure
Tukey's Studentized Range (HSD) Test for variable: LOGCOUNT

Means with the same letter are not significantly different.

Tukey Grouping	Mean	N	Replicate
A	6.8520	5	II
A			
A	6.8383	5	III
A			
A	6.5380	5	I

Appendices for the experiments described in Chapter 3

Appendix 3.1

Statistical analysis of worm counts and LC_{50} values

(a) Comparison of establishment rate of *T. colubriformis* between sheep and goats
(SPECIES = Sheep or Goat)

TTEST PROCEDURE
Variable: **LOGWORM**

SPECIES	N	Mean	Std Dev	Std Error	Minimum	Maximum
1	6	3.03773415	0.13293999	0.05427252	2.79239169	3.17897695
2	5	3.16998269	0.21404307	0.09572297	2.79934055	3.34242268

Variances	T	DF	Prob>|T|
Unequal	-1.2018	6.5	0.2719
Equal	-1.2572	9.0	0.2403

For HO: Variances are equal, F' = 2.59 DF = (4,5) Prob>F' = 0.3244

(b) Comparison of establishment rate of *Ostertagia* spp. between sheep and goats
(SPECIES = Sheep or Goat)

TTEST PROCEDURE
Variable: **LOGWORM**

SPECIES	N	Mean	Std Dev	Std Error	Minimum	Maximum
1	6	2.22453751	0.14958995	0.06106984	2.11394335	2.43136376
2	5	2.19403775	0.13362517	0.05975899	2.00000000	2.32221929

Variances	T	DF	Prob>|T|
Unequal	0.3570	8.9	0.7294
Equal	0.3529	9.0	0.7323

For HO: Variances are equal, F' = 1.25 DF = (5,4) Prob>F' = 0.8509

(c) Comparison of worm counts *Ostertagia* spp. in lambs between control and treatment group
(GROUP = Control or Treatment)

TTEST PROCEDURE Variable: **LOGCOUNT**

GROUP	N	Mean	Std Dev	Std Error
1	6	2.22453751	0.14958995	0.06106984
2	6	2.05857616	0.12585213	0.05137892

Variances	T	DF	Prob>|T|

Unequal	2.0795	9.7	0.0651
Equal	2.0795	10.0	0.0643

For HO: Variances are equal, F' = 1.41 DF = (5,5) Prob>F' = 0.7138

(d) Comparison of worm counts of *T. colubriformis* in lambs between control and treatment group

(GROUP = Control or Treatment)

TTEST PROCEDURE
Variable: **LOGCOUNT**

GROUP	N	Mean	Std Dev	Std Error
1	6	3.03773415	0.13293999	0.05427252
2	6	2.64024057	0.45559649	0.18599649

Variances	T	DF	Prob>|T|
Unequal	2.0515	5.8	0.0875
Equal	2.0515	10.0	0.0673

For HO: Variances are equal, F' = 11.74 DF = (5,5) Prob>F' = 0.0172

(e) Comparison of worm counts of *Ostertagia* spp. in goats between control and treatment group

(GROUP = Control or Treatment)

TTEST PROCEDURE
Variable: **LOGCOUNT**

GROUP	N	Mean	Std Dev	Std Error
1	5	2.19403775	0.13362517	0.05975899
2	5	2.24521682	0.07473096	0.03342070

Variances	T	DF	Prob>|T|
Unequal	-0.7475	6.3	0.4819
Equal	-0.7475	8.0	0.4762

For HO: Variances are equal, F' = 3.20 DF = (4,4) Prob>F' = 0.2865

(f) Comparison of worm counts of *T. colubriformis* in goats between control and treatment group

(GROUP = Control or Treatment)

TTEST PROCEDURE

Variable: **LOGCOUNT**

GROUP	N	Mean	Std Dev	Std Error
1	5	3.16998269	0.21404307	0.09572297
2	5	3.12669050	0.13522331	0.06047370

Variances	T	DF	Prob>|T|
Unequal	0.3824	6.8	0.7140
Equal	0.3824	8.0	0.7122

For H0: Variances are equal, F' = 2.51 DF = (4,4) Prob>F' = 0.3954

(g) Comparison of LC_{50} values of ivermectin between control and treatment groups of sheep before ivermectin treatment

(Group = Control/treatment)

General Linear Models Procedure
Dependent Variable: **LOGLC50**

Source	DF	Sum of Squares	Mean Square	F Value	Pr > F
Model	23	0.04691752	0.00203989	.	.
Error	0	.	.		
Corrected Total	23	0.04691752			

R-Square	C.V.	Root MSE	LOGLC50 Mean
1.000000	0	0	0.229617

Tests of Hypotheses using the Type III MS for ANIMAL as an error term

Source	DF	Type III SS	Mean Square	F Value	Pr > F
GROUP	1	0.00215344	0.00215344	1.86	0.3057

Tests of Hypotheses using the Type III MS for GROUP*ANIMAL*TIME as an error term

Source	DF	Type III SS	Mean Square	F Value	Pr > F
TIME	3	0.00108390	0.00036130	0.12	0.9440

Tests of Hypotheses using the Type III MS for GROUP*ANIMAL*TIME as an error term

Source	DF	Type III SS	Mean Square	F Value	Pr > F
GROUP*TIME	3	0.00078159	0.00026053	0.09	0.9644

(h) Comparison of LC_{50} values of ivermectin between control and treatment groups of goat before ivermectin treatment

(Group = Control/treatment)

General Linear Models Procedure
Dependent Variable: **LOGLC50**

Source	DF	Sum of Squares	Mean Square	F Value	Pr > F
Model	23	0.01728914	0.00075170	.	.
Error	0	.	.		
Corrected Total	23	0.01728914			

R-Square	C.V.	Root MSE	LOGLC50 Mean
1.000000	0	0	0.266852

```
Tests of Hypotheses using the Type III MS for ANIMAL as an error term
Source                  DF     Type III SS     Mean Square    F Value     Pr > F
GROUP                    1      0.00018682      0.00018682       0.28     0.6471

Tests of Hypotheses using the Type III MS for
GROUP*ANIMAL*TIME as an error term
Source                  DF     Type III SS     Mean Square    F Value     Pr > F
TIME                     3      0.00154279      0.00051426       0.58     0.6381

Tests of Hypotheses using the Type III MS for
GROUP*ANIMAL*TIME as an error term
Source                  DF     Type III SS     Mean Square    F Value     Pr > F
GROUP*TIME               3      0.00182134      0.00060711       0.68     0.5764
```

(i) Comparison of LC_{50} values of avermectin B_2 between control and treatment groups of sheep before ivermectin treatment
(Group = Control/treatment)

```
General Linear Models Procedure
Dependent Variable: LOGLC50
                                  Sum of          Mean
Source                  DF        Squares        Square   F Value     Pr > F
Model                   23     0.04691752     0.00203989         .          .
Error                    0              .              .

Corrected Total         23     0.04691752

                R-Square             C.V.        Root MSE          LOGLC50 Mean
                1.000000                0               0              0.229617

Tests of Hypotheses using the Type III MS for ANIMAL as an error term
Source                  DF     Type III SS     Mean Square    F Value     Pr > F
GROUP                    1      0.00215344      0.00215344       1.86     0.3057

Tests of Hypotheses using the Type III MS for
GROUP*ANIMAL*TIME as an error term
Source                  DF     Type III SS     Mean Square    F Value     Pr > F
TIME                     3      0.00108390      0.00036130       0.12     0.9440

Tests of Hypotheses using the Type III MS for
GROUP*ANIMAL*TIME as an error term
Source                  DF     Type III SS     Mean Square    F Value     Pr > F
GROUP*TIME               3      0.00078159      0.00026053       0.09     0.9644
```

(j) Comparison of LC_{50} values of avermectin B_2 between control and treatment groups of goat before ivermectin treatment
(Group = Control/treatment)

```
General Linear Models Procedure
Dependent Variable: LOGLC50
                                  Sum of          Mean
Source                  DF        Squares        Square   F Value     Pr > F
Model                   23     0.01661660     0.00072246         .          .
Error                    0              .              .
Corrected Total         23     0.01661660
```

R-Square	C.V.	Root MSE	LOGLC50 Mean
1.000000	0	0	0.183057

Tests of Hypotheses using the Type III MS for ANIMAL as an error term

Source	DF	Type III SS	Mean Square	F Value	Pr > F
GROUP	1	0.00240585	0.00240585	952.27	0.0810

Tests of Hypotheses using the Type III MS for GROUP*ANIMAL*TIME as an error term

Source	DF	Type III SS	Mean Square	F Value	Pr > F
TIME	3	0.00344689	0.00114896	1.52	0.2534

Tests of Hypotheses using the Type III MS for GROUP*ANIMAL*TIME as an error term

Source	DF	Type III SS	Mean Square	F Value	Pr > F
GROUP*TIME	3	0.00015996	0.00005332	0.07	0.9748

(k) Comparison of LC_{50} values of ivermectin between sheep and goats

(Treatment = control/treatment group; Species = Sheep/Goat; Stage = Before treatment/After treatment)

Split-Split-Split Plot Design: Iso Experiment
General Linear Models Procedure
Dependent Variable: LOGLC50

Source	DF	Sum of Squares	Mean Square	F Value	Pr > F
Model	33	0.22039617	0.00667867	3.70	0.0001
Error	62	0.11186369	0.00180425		
Corrected Total	95	0.33225986			

R-Square	C.V.	Root MSE	LOGLC50 Mean
0.663325	13.98137	0.042477	0.303808

Source	DF	Type I SS	Mean Square	F Value	Pr > F
TREAT	1	0.06210056	0.06210056	34.42	0.0001
SPECIES	1	0.01767972	0.01767972	9.80	0.0027
TREAT*SPECIES	1	0.00060518	0.00060518	0.34	0.5646
ANIMAL	2	0.00264102	0.00132051	0.73	0.4851
STAGE	1	0.07053251	0.07053251	39.09	0.0001
STAGE*TREAT	1	0.04814501	0.04814501	26.68	0.0001
STAGE*SPECIES	1	0.00132875	0.00132875	0.74	0.3941
STAGE*TREAT*SPECIES	1	0.00049090	0.00049090	0.27	0.6038
TIME	6	0.01028768	0.00171461	0.95	0.4662
TREAT*TIME	6	0.00395176	0.00065863	0.37	0.8983
SPECIES*TIME	6	0.00195392	0.00032565	0.18	0.9812
TREAT*SPECIES*TIME	6	0.00067915	0.00011319	0.06	0.9990

(l) Comparison of LC_{50} values of avermectin B_2 between sheep and goats

(Treatment = control/treatment group; Species = Sheep/Goat; Stage = Before treatment/After treatment)

Split-Split-Split Plot Design: Iso Experiment (avermectin B_2)
General Linear Models Procedure

Dependent Variable: **LOGLC50**

Source	DF	Sum of Squares	Mean Square	F Value	Pr > F
Model	33	0.40492124	0.01227034	10.85	0.0001
Error	62	0.07014328	0.00113134		
Corrected Total	95	0.47506452			

R-Square	C.V.	Root MSE	LOGLC50 Mean
0.852350	13.55201	0.033635	0.248195

Source	DF	Type I SS	Mean Square	F Value	Pr > F
TREAT	1	0.07151857	0.07151857	63.22	0.0001
SPECIES	1	0.06376436	0.06376436	56.36	0.0001
TREAT*SPECIES	1	0.00747012	0.00747012	6.60	0.0126
ANIMAL	2	0.00036025	0.00018013	0.16	0.8532
STAGE	1	0.18472211	0.18472211	163.28	0.0001
TREAT*STAGE	1	0.06008364	0.06008364	53.11	0.0001
STAGE*SPECIES	1	0.00002263	0.00002263	0.02	0.8880
TREAT*STAGE*SPECIES	1	0.00082314	0.00082314	0.73	0.3970
TIME	6	0.00995555	0.00165926	1.47	0.2045
TREAT*TIME	6	0.00159870	0.00026645	0.24	0.9633
SPECIES*TIME	6	0.00230074	0.00038346	0.34	0.9137
TREAT*SPECIES*TIME	6	0.00230143	0.00038357	0.34	0.9136

(m) Comparison of LC_{50} values of ivermectin between sheep and goats (treatment groups after ivermectin treatment)

(Group = Sheep/Goat)

General Linear Models Procedure
Dependent Variable: **LOGLC50**

Source	DF	Sum of Squares	Mean Square	F Value	Pr > F
Model	23	0.01728914	0.00075170	.	.
Error	0	.	.		
Corrected Total	23	0.01728914			

R-Square	C.V.	Root MSE	LOGLC50 Mean
1.000000	0	0	0.266852

Tests of Hypotheses using the Type III MS for ANIMAL as an error term

Source	DF	Type III SS	Mean Square	F Value	Pr > F
GROUP	1	0.00018682	0.00018682	0.28	0.6471

Tests of Hypotheses using the Type III MS for GROUP*ANIMAL*TIME as an error term

Source	DF	Type III SS	Mean Square	F Value	Pr > F
TIME	3	0.00154279	0.00051426	0.58	0.6381

Tests of Hypotheses using the Type III MS for GROUP*ANIMAL*TIME as an error term

Source	DF	Type III SS	Mean Square	F Value	Pr > F
GROUP*TIME	3	0.00182134	0.00060711	0.68	0.5764

(n) Comparison of LC_{50} values of avermectin B_2 between sheep and goats (treatment groups after ivermectin treatment)
(Species = Sheep/Goat)

General Linear Models Procedure
Dependent Variable: **LOGLC50**

Source	DF	Sum of Squares	Mean Square	F Value	Pr > F
Model	23	0.01871967	0.00081390	.	.
Error	0	.	.		
Corrected Total	23	0.01871967			

R-Square	C.V.	Root MSE	LOGLC50 Mean
1.000000	0	0	0.344373

Tests of Hypotheses using the Type III MS for ANIMAL as an error term

Source	DF	Type III SS	Mean Square	F Value	Pr > F
SPECIES	1	0.00995339	0.00995339	26.26	0.0360

Tests of Hypotheses using the Type III MS for SPECIES*ANIMAL*TIME as an error term

Source	DF	Type III SS	Mean Square	F Value	Pr > F
TIME	3	0.00288649	0.00096216	3.09	0.0618

Tests of Hypotheses using the Type III MS for SPECIES*ANIMAL*TIME as an error term

Source	DF	Type III SS	Mean Square	F Value	Pr > F
SPECIES*TIME	3	0.00075696	0.00025232	0.81	0.5095

Appendix 3.2

LC_{50} values of ivermectin and avermectin B_2 for the susceptible strain of *T. colubriformis* for sheep

LC_{50} values of ivermectin of susceptible strain (Chapter 5)*				
Trial I	**LC_{50} values (ng/ml)**			
	23 DPI	**27 DPI**	**31 DPI**	**35 DPI**
	0.479	0.510	0.515	0.538
Trial II	**23 DPI**	**30 DPI**	**37 DPI**	**44 DPI**
	0.515	0.474	0.513	0.589
Mean LC_{50} value of ivermectin = 0.517				
LC_{50} values (ng/ml) of avermectin B_2 of susceptible strain at 24 DPI (Chapter 7)*				
Sheep No. 7		**Sheep No. 9**		**Sheep No. 10**
0.611		0.638		0.660
Mean LC_{50} value of avermectin B_2 = 0.636				

* used for calculation of resistant ratios

Appendix 3.3

LC_{50} values of ivermectin for the control group goats

Days post infection	LC_{50} values (ng/ml of agar)			Mean LC_{50} value (ng/ml of agar)
	Goat No. 14	Goat No. 17	Goat No. 25	
22	2.0303	1.5267	1.7371	1.7647
24	1.7672	1.754	1.8727	1.798
26	1.7853	1.8974	1.9058	1.8628
28	2.0277	1.8382	1.961	1.9423
31	1.8307	1.9244	1.8137	1.8563
32	1.7917	1.9433	1.8665	1.8672
33	1.8319	1.9529	1.8021	1.8623
35	1.7753	1.9951	1.8616	1.8773

Appendix 3.4

LC_{50} values of ivermectin for the treatment group goats

Days post infection	LC_{50} values (ng/ml of agar)			Mean LC_{50} value (ng/ml of agar)
	Goat No. 6	Goat No. 12	Goat No. 23	
22	1.8362	1.9364	1.835	1.8692
24	1.8769	1.9402	1.7455	1.8542
26	1.9734	1.8011	1.831	1.8685
28	1.8719	1.9753	1.7242	1.8571
Treatment with 0.2 mg/kg b.wt. ivermectin orally on 28 DPI				
31	2.0881	2.2126	2.1452	2.1486
32	2.3394	2.345	2.3034	2.3293
33	2.1498	2.4313	2.2362	2.2724
35	2.358	2.3728	2.3273	2.3527

Appendix 3.5

LC_{50} values of ivermectin for the control group sheep

Days post infection	LC_{50} values (ng/ml of agar)			Mean LC_{50} value (ng/ml of agar)
	Sheep No. 6	Sheep No. B	Sheep No. E	
22	1.8938	1.9623	1.6553	1.8371
24	2.0252	2.0085	1.6559	1.8966
26	2.2407	2.024	1.7276	1.9975
28	2.309	1.8597	1.7985	1.9849
			Mean	1.9289
			Resistance Ratio	3.73
31	2.1801	1.8836	1.7054	1.9231
32	2.2981	1.8027	1.7809	1.9606
33	2.3547	1.8045	1.9549	2.0381
35	2.2034	1.8561	2.0087	2.0228
			Mean	1.9862
			Resistance Ratio	3.84

Appendix 3.6

LC_{50} values of ivermectin for the treatment group sheep

Days post infection	LC_{50} values (ng/ml of agar)			Mean LC_{50} value (ng/ml of agar)
	Sheep No. 5	Sheep No. F	Sheep No. A	
22	1.9584	1.5069	2.4137	1.9597
24	1.8552	1.7937	2.151	1.9334
26	1.8224	1.7615	2.3708	1.9849
28	1.8741	1.7723	2.3329	1.9931
			Mean	1.96776
			Resistance Ratio	3.80
Treatment with 0.2 mg/kg b.wt. ivermectin orally on 28 DPI				
31	2.2554	2.2613	2.5353	2.3507
32	2.2405	2.4877	2.6057	2.4453
33	2.4126	2.6546	2.7496	2.6056
35	2.4315	2.7848	2.8811	2.6992
			Mean	2.5252
			Resistance Ratio	4.88

Appendix 3.7

LC_{50} values of avermectin B_2 for the control group goats

Days post infection	LC_{50} values (ng/ml of agar)			Mean LC_{50} value (ng/ml of agar)
	Goat No. 14	Goat No. 17	Goat No. 25	
22	1.4196	1.388	1.5534	1.4536
24	1.2079	1.5089	1.3174	1.3447
26	1.441	1.6173	1.5159	1.5247
28	1.5047	1.6391	1.492	1.5452
31	1.5632	1.5283	1.6409	1.5774
32	1.6076	1.5044	1.7447	1.6189
33	1.6432	1.7515	1.6456	1.6801
35	1.5197	1.6357	1.5912	1.5822

Appendix 3.8

LC_{50} values of avermectin B_2 for the treatment group goats

Days post infection	LC_{50} values (ng/ml of agar)			Mean LC_{50} value (ng/ml of agar)
	Goat No. 6	Goat No. 12	Goat No. 23	
22	1.403	1.5562	1.5661	1.5084
24	1.559	1.5148	1.543	1.5389
26	1.6837	1.4657	1.591	1.5801
28	1.7175	1.5255	1.6182	1.6204
Treatment with 0.2 mg/kg b.wt. ivermectin orally on 28 DPI				
31	2.0108	2.0658	2.0649	2.0471
32	2.1298	2.1647	2.2725	2.189
33	2.233	2.0049	2.1757	2.1378
35	2.0036	2.1905	2.0117	2.0686

Appendix 3.9

LC_{50} values of avermectin B_2 for the control group sheep

Days post infection	LC_{50} values (ng/ml of agar)			Mean LC_{50} value (ng/ml of agar)
	Sheep No. 6	Sheep No. B	Sheep No. E	
22	1.8254	1.94	1.4566	1.7406
24	1.8092	1.8978	1.5074	1.7381
26	1.8273	1.7114	1.6084	1.7157
28	1.8413	1.824	1.6402	1.7685
			Mean	1.7407
			Resistance Ratio	2.74
31	1.8543	1.9259	1.7333	1.8378
32	1.9325	1.8668	1.7467	1.8486
33	2.0432	1.9542	1.734	1.9104
35	1.9811	1.9165	1.7862	1.8946
			Mean	1.8728
			Resistance Ratio	2.94

Appendix 3.10

LC_{50} values of avermectin B_2 for the treatment group sheep

Days post infection	LC_{50} values (ng/ml of agar)			Mean LC_{50} value (ng/ml of agar)
	Sheep No. 5	Sheep No. F	Sheep No. A	
22	1.5465	1.4068	1.9103	1.6212
24	1.6067	1.4451	1.8553	1.6357
26	1.8268	1.5082	1.8069	1.7139
28	1.6452	1.5277	1.9564	1.7097
			Mean	1.6701
			Resistance Ratio	2.63
Treatment with 0.2 mg/kg b.wt. ivermectin orally on 28 DPI				
31	2.1333	2.1721	2.3092	2.2048
32	2.2574	2.2922	2.4501	2.3332
33	2.4044	2.3375	2.4258	2.3892
35	2.4737	2.2406	2.3232	2.3458
			Mean	2.3183
			Resistance Ratio	3.64

Appendices for the experiments described in Chapter 4

Appendix 4.1

Faecal egg counts for goats grazed on paddocks previously grazed by goats - Replicate I

Date (Month/Year)	Pre Treatment Mean (S.E.) egg count	Pre-treatment larval composition (Percentage)					Post-treatment Mean (S.E.) egg count	Percent reduction in total egg count	Pre-treatment Mean egg count for *Ostertagia*	Post-treatment larval composition (Percentage)		Post-treatment mean egg count for *Ostertagia*	Percent reduction in egg count of *Ostertagia*
		Ost	*Trich*	*Hae*	*Coo*	*LT*				*Ost*	*Trich*		
7/97	983 (187)	89	4	2	4	1	433 (33)	56	875	97	3	423	52
8/97	1260 (352)	75	20	0	1	4	1080 (427)	14	945	100	0	1080	0
9/97	720 (102)	90	10	0	0	0	500 (71)	31	648	100	0	500	23
10/97	500 (71)	100	0	0	0	0	460 (242)	8	500	100	0	460	8
1/98	240 (100)	80	10	5	0	5	220 (49)	8	192	98	2	216	0
New Animals													
4/98	1420 (213)	70	25	2	1	1	1080 (66)	24	994	100	0	1080	0
5/98	725 (225)	78	21	0	1	0	860 (211)	0	566	96	4	828	0
5/98	450 (104)	60	40	0	0	0	575 (243)	0	270	78	22	449	0
6/98	575 (243)	78	22	0	0	0	575 (149)	0	449	90	10	518	0

Ost- Ostertagia, Trich - Trichostrongylus, Hae - Haemonchus, Coo - Cooperia, LT - Long tail larvae

Appendix 4.2

Faecal egg counts for goats grazed on paddocks previously grazed by goats - Replicate II

Date (Month/Year)	Pre Treatment Mean (S.E.) egg count	Pre-treatment larval composition (Percentage)					Post-treatment Mean (S.E.) egg count	Percent reduction in total egg count	Pre-treatment Mean egg count for *Ostertagia*	Post-treatment larval composition (Percentage)		Post-treatment mean egg count for *Ostertagia*	Percent reduction in egg count of *Ostertagia*
		Ost	*Trich*	*Hae*	*Coo*	*LT*				*Ost*	*Trich*		
7/97	717 (133)	90	3	1	6	0	650 (150)	9	645	100	0	650	0
8/97	717 (237)	82	10	1	6	1	1250 (411)	0	588	100	0	1250	0
9/97	700 (241)	91	8	1	0	0	1000 (154)	0	637	100	0	1000	0
10/97	1000 (154)	100	0	0	0	0	433 (92)	57	1000	100	0	433	57
1/98	250 (77)	68	19	4	0	9	250 (85)	0	170	99	1	248	0
New Animals													
4/98	1000 (328)	69	20	9	1	1	824 (388)	18	690	100	0	824	0
5/98	433 (123)	89	11	0	0	0	949 (304)	0	650	95	5	902	0
5/98	949 (304)	50	50	0	0	0	766 (225)	20	474	79	21	605	0
6/98	766 (225)	79	21	0	0	0	1073 (604)	0	605	89	11	955	0

Ost- Ostertagia, Trich - Trichostrongylus, Hae - Haemonchus, Coo - Cooperia, LT - Long tail larvae

Appendix 4.3

Faecal egg counts for goats grazed on paddocks previously grazed by sheep - Replicate I

Date (Month/Year)	Pre Treatment Mean (S.E.) egg count	Pre-treatment larval composition (Percentage)					Post-treatment Mean (S.E.) egg count	Percent reduction in total egg count	Pre-treatment Mean egg count for *Ostertagia*	Post-treatment larval composition (Percentage)		Post-treatment mean egg count for *Ostertagia*	Percent reduction in egg count of *Ostertagia*
		Ost	*Trich*	*Hae*	*Coo*	*LT*				*Ost*	*Trich*		
7/97	1750 (366)	70	14	0	6	10	483 (156)	72	1225	99	1	478	61
8/97	491 (59)	78	15	0	6	1	583 (170)	0	383	100	0	583	0
9/97	417 (60)	66	34	0	0	0	1017 (277)	0	275	100	0	1017	0
10/97	1017 (277)	100	0	0	0	0	950 (208)	7	1017	100	0	950	7
1/98	517 (40)	70	25	5	0	0	383 (70)	26	362	99	1	358	1
New Animals													
4/98	3660 (756)	68	20	4	3	5	760 (166)	79	2489	100	0	760	70
5/98	2840 (531)	37	63	0	0	0	1200 (141)	58	1051	93	7	1116	0
5/98	7550 (2776)	16	84	0	0	0	1350 (166)	82	1208	82	18	1107	8
6/98	1350 (166)	82	18	0	0	0	1050 (299)	22	1107	95	5	1052	0

Ost- Ostertagia, Trich - Trichostrongylus. Hae - Haemonchus, Coo - Cooperia. LT - Long tail larvae

Appendix 4.4

Faecal egg counts for goats grazed on paddocks previously grazed by sheep - Replicate II

Date (Month/Year)	Pre Treatment Mean (S.E.) egg count	Pre-treatment larval composition (Percentage)					Post-treatment Mean (S.E.) egg count	Percent reduction in total egg count	Pre-treatment Mean egg count for *Ostertagia*	Post-treatment larval composition (Percentage)		Post-treatment mean egg count for *Ostertagia*	Percent reduction in egg count of *Ostertagia*
		Ost	*Trich*	*Hae*	*Coo*	*LT*				*Ost*	*Trich*		
7/97	1367 (402)	80	18	0	1	1	483 (230)	65	1093	97	3	469	57
8/97	873 (159)	75	16	1	4	4	480 (275)	45	655	100	0	480	27
9/97	450 (98)	90	10	0	0	0	533 (250)	0	405	100	0	533	0
10/97	533 (250)	100	0	0	0	0	683 (320)	0	533	100	0	683	0
1/98	233 (42)	60	20	2	4	14	167 (33)	29	140	100	0	163	0
New Animals													
4/98	1283 (316)	52	35	5	2	6	667 (231)	48	667	100	0	667	0
5/98	1216 (192)	50	50	0	0	0	683 (241)	44	608	92	8	628	0
5/98	5350 (734)	10	90	0	0	0	1216 (117)	77	535	93	7	1131	0
6/98	1216 (117)	93	7	0	0	0	916 (400)	25	1131	98	2	898	21

Ost- Ostertagia, Trich - Trichostrongylus, Hae - Haemonchus, Coo - Cooperia, LT - Long tail larvae

Appendix 4.5

Faecal egg counts for sheep grazed on paddocks previously grazed by sheep

Date (Month/Year)	Pre Treatment Mean (S.E.) egg count	Pre-treatment larval composition (Percentage)					Post-treatment Mean (S.E.) egg count	Percent reduction in total egg count	Pre-treatment Mean egg count for *Ostertagia*	Post-treatment larval composition (Percentage)		Post-treatment mean egg count for *Ostertagia*	Percent reduction in egg count of *Ostertagia*
		Ost	*Trich*	*Hae*	*Coo*	*LT*				*Ost*	*Trich*		
Sheep on Sheep Plots - Replicate I- New replacement animals													
1/98	260 (87)	45	23	8	7	17	0	100	117	-	-	0	100
2/98	260 (75)	35	39	17	4	5	0	100	91	-	-	0	100
4/98	425 (48)	53	30	10	2	5	20 (20)	95	225	100	0	225	91
4/98	1075 (309)	25	75	0	0	0	25 (25)	98	269	100	0	25	91
5/98	125 (33)	45	55	0	0	0	0	100	56	-	-	0	100
Sheep on Sheep Plots - Replicate II													
1/98	940 (211)	61	22	10	0	7	20 (20)	98	573	100	0	20	96
2/98	1700 (349)	31	29	15	12	13	0	100	527	-	-	0	100
4/98	3733 1934)	62	20	12	2	4	100 (58)	97	2314	100	0	100	96
4/98	667 (328)	47	53	0	0	0	33 (33)	95	313	100	0	33	90
5/98	825 (214)	41	59	0	0	0	0	100	338	-	-	0	100

Ost- Ostertagia, Trich - Trichostrongylus, Hae - Haemonchus, Coo - Cooperia, LT - Long tail larvae

Appendix 4.6

Faecal egg counts for sheep grazed on paddocks previously grazed by goats

Date (Month/Year)	Pre Treatment Mean (S.E.) egg count	Pre-treatment larval composition (Percentage)					Post-treatment Mean (S.E.) egg count	Percent reduction in total egg count	Pre-treatment Mean egg count for *Ostertagia*	Post-treatment larval composition (Percentage)		Post-treatment mean egg count for *Ostertagia*	Percent reduction in egg count of *Ostertagia*
		Ost	*Trich*	*Hae*	*Coo*	*LT*				*Ost*	*Trich*		
Sheep on Goats Plots - Replicate I- New replacement animals													
1/98	2420 (307)	55	37	4	0	4	40 (24)	98	1331	100	0	40	97
2/98	1125 (390)	42	40	8	0	10	0	100	473	-	-	0	100
4/98	1050 (403)	55	25	10	5	5	300 (108)	71.5	578	100	0	300	48
4/98	1500 (349)	39	61	0	0	0	275 (125)	82	585	100	0	275	53
5/98	1950 (947)	41	59	0	0	0	325 (85)	83	800	100	0	325	59
Sheep on Goats Plots - Replicate II													
1/98	960 (183)	47	19	17	0	17	0	100	451	-	-	0	100
2/98	2340 (391)	38	29	11	4	22	20 (20)	99	889	100	0	20	98
4/98	1550 (435)	60	30	5	4	3	325 (75)	79	930	100	0	325	65
4/98	1275 (320)	45	55	0	0	0	250 (65)	80	574	99	1	248	57
5/98	550 (296)	38	62	0	0	0	100 (58)	82	209	100	0	100	52

Ost- Ostertagia, Trich - Trichostrongylus, Hae - Haemonchus, Coo - Cooperia, LT - Long tail larvae

Appendix 4.7

LC_{50} values (ng/ml of agar) of ivermectin and avermectin B_2 for *Ostertagia* spp. for all groups of sheep

Date	Sheep on Sheep Plots (Rep I)	Resistant Ratio	Sheep on Sheep Plots (Rep II)	Resistant Ratio	Sheep on Goat Plots (Rep I)	Resistant Ratio	Sheep On Goat Plots (Rep II)	Resistant Ratio
Ivermectin LC_{50} values								
21/1/98	0.97	**1.59**	1.13	**1.85**	1.33	**2.18**	1.33	**2.18**
14/2/98	0.92	**1.51**	0.95	**1.56**	1.32	**2.16**	1.44	**2.36**
2/3/98	0.85	**1.39**	1.01	**1.66**	1.20	**1.97**	1.25	**2.05**
30/3/98	1.02	**1.67**	1.11	**1.82**	1.48	**2.43**	1.30	**2.13**
11/4/98	1.07	**1.75**	1.15	**1.89**	1.33	**2.18**	1.25	**2.05**
25/4/98*	ND	**ND**	ND	**ND**	2.29	**3.75**	2.20	**3.61**
11/5/98*	ND	**ND**	ND	**ND**	1.93	**3.16**	2.31	**3.79**
25/5/98	0.98	**1.61**	1.04	**1.70**	0.99	**1.62**	0.94	**1.54**
Avermectin B_2 LC_{50} values								
21/1/98	0.93	**1.75**	0.85	**1.60**	1.16	**2.19**	1.18	**2.23**
14/2/98	0.85	**1.60**	1.00	**1.89**	1.15	**2.17**	1.02	**1.92**
2/3/98	0.91	**1.72**	0.92	**1.74**	1.09	**2.06**	1.03	**1.94**
30/3/98	1.02	**1.92**	1.03	**1.94**	1.05	**1.98**	1.05	**1.98**
11/4/98	1.02	**1.92**	1.03	**1.94**	1.04	**1.96**	1.25	**2.36**
25/4/98*	ND	**ND**	ND	**ND**	1.80	**3.40**	ND	**ND**
11/5/98*	ND	**ND**	ND	**ND**	1.69	**3.19**	1.61	**3.04**
25/5/98	1.05	**1.98**	1.03	**1.94**	0.97	**1.83**	0.91	**1.72**

ND - Not done; * After treatment

Appendix 4.8

LC_{50} values of ivermectin and avermectin B_2 for the susceptible strain of *Ostertagia* spp. for sheep

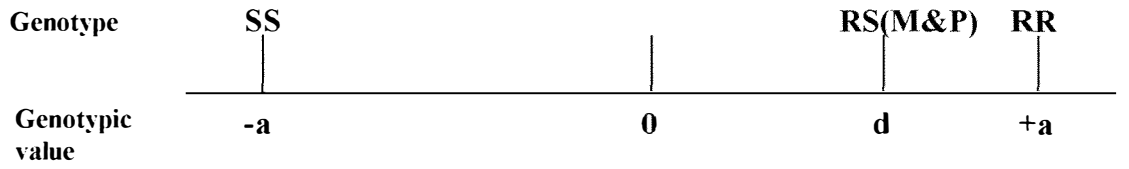
Animal No.	Days Post Infection		
	28 DPI	35 DPI	42 DPI
LC_{50} values for Ivermectin			
Black	0.63	0.63	0.69
18	0.59	0.59	0.56
24	0.63	0.55	0.61
Overall mean LC_{50} value for ivermectin = 0.61			
LC_{50} values for avermectin B_2			
Black	0.51	0.56	0.54
18	0.51	0.52	0.51
24	0.61	0.50	0.55
Overall mean LC_{50} value for avermectin B_2 = 0.53			

Appendices for the experiments described in Chapter 5

Appendix 5.1

Estimation of a and d values for trial I of the inheritance study

DAYS POST INFECTION	LC_{50} VALUES IN NG/ML OF AGAR			
	SS (Ivermectin-susceptible parent)-possibly homozygous	RR (Ivermectin-resistant parent)-possibly homozygous	RS(P) cross (Possibly heterozygous)	RS(M) cross (Possibly heterozygous)
23	0.479	2.63	2.14	2.29
27	0.501	3.09	2.05	2.27
31	0.515	3.13	2.12	2.78
35	0.538	3.22	2.19	2.79
Mean	**0.508**	**3.02**	**2.13**	**2.53**
			Mean value for two F_1 reciprocal crosses = **2.33**	



$$\text{Where } a = \frac{\text{Mean } LC_{50} \text{ value * for RR cross - Mean } LC_{50} \text{ value * for SS cross}}{2}$$

0 (Point of Zero) = Mid way mean LC_{50} value* for the ivermectin-resistant and ivermectin-susceptible parent crosses

d = Mean LC_{50} values* for the F_1 reciprocal crosses - Mid way mean LC_{50} value* between ivermectin-resistant and ivermectin-susceptible parent crosses

* Mean LC_{50} values on 23 to 35 days post infection

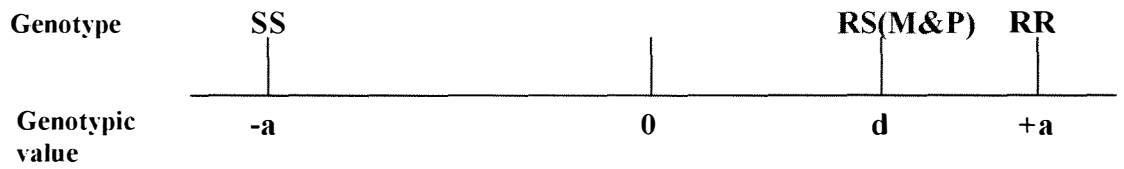
$$a = \frac{3.02 - 0.508}{2} = 1.26 \qquad 0 = \frac{3.02 + 0.508}{2} = 1.77$$

$$d = 2.33 - 1.77 = +0.57$$

Appendix 5.2

Estimation of a and d values for trial II of the inheritance study

DAYS POST INFECTION	LC_{50} VALUES IN NG/ML OF AGAR			
	SS (Ivermectin-susceptible parent)-possibly homozygous	RR (Ivermectin-resistant parent)-possibly homozygous	RS(P) Cross (Possibly heterozygous)	RS(M) Cross (Possibly heterozygous)
23	0.515	3.14	2.17	2.20
30	0.474	3.23	2.21	2.39
37	0.513	3.38	2.17	2.22
Mean	**0.50**	**3.25**	**2.18**	**2.27**
			Mean value for F_1 reciprocal crosses = **2.23**	



$$\text{Where } a = \frac{\text{Mean } LC_{50} \text{ value * for RR cross - Mean } LC_{50} \text{ value * for SS cross}}{2}$$

0 (Point of Zero) = Mid way mean LC_{50} value* for ivermectin-resistant and ivermectin-susceptible parent crosses

d = Mean LC_{50} values* for the F_1 reciprocal crosses - Mid way mean LC_{50} value* between ivermectin-resistant and ivermectin-susceptible parent crosses

* Mean LC_{50} values on 23 to 37 days post infection

$$a = \frac{3.25 - 0.50}{2} = 1.375 \qquad 0 = \frac{3.25 + 0.50}{2} = 1.875$$

$$d = 2.23 - 1.875 = +0.355$$

Appendix 5.3

The percentage of eggs developing and surviving to infective third stage larvae in the larval development assay at 3.16 ng/ml concentration of ivermectin in the agar phase

DAYS POST INFECTION	PERCENTAGE OF EGGS DEVELOPING AND SURVIVING TO L_3*			
	SS (Ivermectin-susceptible parent)-possibly homozygous	RR (Ivermectin-resistant parent) -possibly homozygous	RS(P) Cross (Possibly heterozygous)	RS(M) Cross (Possibly heterozygous)
Trial I				
23	20	47	46	46
27	14	50	45	46
31	18	50	45	48
35	14	50	46	48
Trial II				
23	13	50	46	46
30	14	50	46	47
37	14	51	46	46
Overall mean percentage	**15**	**50**	**46.7**	**45.7**
			Mean value for F_1 reciprocal crosses = **46**	

* estimated from dose response curves for ivermectin-resistant parents (possibly homozygous), F_1 reciprocal crosses (possibly heterozygotes) and ivermectin-susceptible parents (possibly homozygous).

Appendices for the experiments described in Chapter 6

APPENDIX 6.1

Statistical analysis of egg counts, worms counts, development of eggs to infective larvae under natural and laboratory conditions, eggs per female, female and male worm length

(a) Faecal egg counts - Unbalanced ANOVA

(Strain = Ivermectin-resistant, Ivermectin-susceptible I or Ivermectin-susceptible II; Time = 21, 28, 35, 42, 49, 54, 63, 70, 77 or 84 days post infection)

General Linear Models Procedure
Dependent Variable: LOGEPG

Source	DF	Sum of Squares	Mean Square	F Value	Pr > F
Model	43	41.63725862	0.96830834	1.51	0.0325
Error	196	125.87388366	0.64221369		
Corrected Total	239	167.51114228			

R-Square	C.V.	Root MSE	LOGEPG Mean
0.248564	12.13808	0.801382	6.602219

Source	DF	Type III SS	Mean Square	F Value	Pr > F
TIME	9	15.25163435	1.69462604	2.64	0.0066
ANIMAL	14	13.28173817	0.94869558	1.48	0.1223
STRAIN*TIME	18	10.56666686	0.58703705	0.91	0.5622

Tests of Hypotheses using the Type III MS for ANIMAL as an error term

Source	DF	Type III SS	Mean Square	F Value	Pr > F
STRAIN	2	2.21326449	1.10663224	1.17	0.3400

General Linear Models Procedure
Tukey's Studentized Range (HSD) Test for variable: LOGEPG
Means with the same letter are not significantly different.

Tukey Grouping	Mean	N	STRAIN
A	6.7494	80	Ivermectin-resistant
A			
A	6.5831	80	Ivermectin-susceptible II
A			
A	6.4742	80	Ivermectin-susceptible I

(b) Worm counts - One way ANOVA - Animals killed on 28DPI

(Strain = Ivermectin-resistant, Ivermectin-susceptible I or Ivermectin-susceptible II)

Analysis of worm count data (28 DPI) - one way
General Linear Models Procedure
Dependent Variable: LOGCOUNT

Source	DF	Sum of Squares	Mean Square	F Value	Pr > F
Model	2	0.03007241	0.01503621	1.17	0.3438
Error	12	0.15440659	0.01286722		

Corrected Total	14	0.18447901			

R-Square	C.V.	Root MSE	LOGCOUNT Mean
0.163013	1.270770	0.113434	8.926382

Source	DF	Type I SS	Mean Square	F Value	Pr > F
STRAIN	2	0.03007241	0.01503621	1.17	0.3438
Source	DF	Type III SS	Mean Square	F Value	Pr > F
STRAIN	2	0.03007241	0.01503621	1.17	0.3438

(c) Worm counts - One way ANOVA - Animals killed on 42DPI

(Strain = Ivermectin-resistant, Ivermectin-susceptible I or Ivermectin-susceptible II)

Analysis of worm count data (42 DPI) - one way
General Linear Models Procedure
Dependent Variable: LOGCOUNT

Source	DF	Sum of Squares	Mean Square	F Value	Pr > F
Model	2	0.07913057	0.03956528	0.70	0.5145
Error	12	0.67563165	0.05630264		
Corrected Total	14	0.75476221			

R-Square	C.V.	Root MSE	LOGCOUNT Mean
0.104842	2.651285	0.237282	8.949690

Source	DF	Type I SS	Mean Square	F Value	Pr > F
STRAIN	2	0.07913057	0.03956528	0.70	0.5145
Source	DF	Type III SS	Mean Square	F Value	Pr > F
STRAIN	2	0.07913057	0.03956528	0.70	0.5145

(d) Worm counts - One way ANOVA - Animals killed on 84DPI

(Strain = Ivermectin-resistant, Ivermectin-susceptible I or Ivermectin-susceptible II)

Analysis of wormcount data (84 DPI) - one way
General Linear Models Procedure
Dependent Variable: LOGCOUNT

Source	DF	Sum of Squares	Mean Square	F Value	Pr > F
Model	2	0.39903949	0.19951975	0.55	0.5899
Error	16	5.85101201	0.36568825		
Corrected Total	18	6.25005150			

R-Square	C.V.	Root MSE	LOGCOUNT Mean
0.063846	6.851810	0.604722	8.825721

Source	DF	Type I SS	Mean Square	F Value	Pr > F
STRAIN	2	0.39903949	0.19951975	0.55	0.5899
Source	DF	Type III SS	Mean Square	F Value	Pr > F
STRAIN	2	0.39903949	0.19951975	0.55	0.5899

(e) Development of larvae under natural conditions - Multivariate analysis
(Strain = Ivermectin-resistant, Ivermectin-susceptible I or Ivermectin-susceptible II; Time = 24th April or 26th April; Devlp1 = Faeces; Devlp2 = Pasture; Devlp3 = Soil)

Analysis of Plot data-Fitness study
General Linear Models Procedure
Dependent Variable: DEVLP1 Faeces

Source	DF	Sum of Squares	Mean Square	F Value	Pr > F
Model	5	346.2234267	69.2446853	1.78	0.1541
Error	24	931.1593600	38.7983067		
Corrected Total	29	1277.3827867			

R-Square	C.V.	Root MSE	DEVLP1 Mean
0.271041	38.24731	6.228829	16.28567

Source	DF	Type I SS	Mean Square	F Value	Pr > F
STRAIN	2	217.7301117	108.8650558	2.81	0.0803
TIME	1	116.1513633	116.1513633	2.99	0.0964
STRAIN*TIME	2	12.3419517	6.1709758	0.16	0.8538

Dependent Variable: DEVLP2 Pasture

Source	DF	Sum of Squares	Mean Square	F Value	Pr > F
Model	5	9.58802137	1.91760427	3.01	0.0302
Error	24	15.30895880	0.63787328		
Corrected Total	29	24.89698017			

R-Square	C.V.	Root MSE	DEVLP2 Mean
0.385108	50.54338	0.798670	1.580167

Source	DF	Type I SS	Mean Square	F Value	Pr > F
STRAIN	2	7.54067387	3.77033693	5.91	0.0082
TIME	1	1.91066803	1.91066803	3.00	0.0963
STRAIN*TIME	2	0.13667947	0.06833973	0.11	0.8988

Dependent Variable: DEVLP3 Soil

Source	DF	Sum of Squares	Mean Square	F Value	Pr > F
Model	5	29.13934150	5.82786830	4.87	0.0033
Error	24	28.74103200	1.19754300		
Corrected Total	29	57.88037350			

R-Square	C.V.	Root MSE	DEVLP3 Mean
0.503441	73.66699	1.094323	1.485500

Source	DF	Type I **SS**	Mean Square	F Value	Pr > F
STRAIN	2	3.70120220	1.85060110	1.55	0.2337
TIME	1	21.59856750	21.59856750	18.04	0.0003
STRAIN*TIME	2	3.83957180	1.91978590	1.60	0.2221

Manova Test Criteria and F Approximations for
he Hypothesis of no **Overall STRAIN Effect**
H = Type I SS&CP Matrix for **STRAIN** **E** = Error SS&CP Matrix

Statistic	Value	F	Num DF	Den DF	Pr > F
Wilks' Lambda	0.54918581	2.5623	6	44	0.0624
Pillai's Trace	0.48674055	2.4660	6	46	0.0676
Hotelling-Lawley Trace	0.75545983	2.6441	6	42	0.0588
Roy's Greatest Root	0.65569105	5.0270	3	23	0.0680

Manova Test Criteria and Exact F Statistics for
the Hypothesis of no **Overall TIME Effect**
H = Type I SS&CP Matrix for **TIME**

Statistic	Value	F	Num DF	Den DF	Pr > F
Wilks' Lambda	0.31719364	15.7861	3	22	0.0001
Pillai's Trace	0.68280636	15.7861	3	22	0.0001
Hotelling-Lawley Trace	2.15264833	15.7861	3	22	0.0001
Roy's Greatest Root	2.15264833	15.7861	3	22	0.0001

TIME	**DEVLP1** LSMEAN	**DEVLP2** LSMEAN	**DEVLP3** LSMEAN
April24	18.2533333	1.32780000	0.63700000
April26	14.3180000	1.83253333	2.33400000

STRAIN	**DEVLP1** LSMEAN	**DEVLP2** LSMEAN	**DEVLP3** LSMEAN
Res	18.2550000	1.55690000	1.83650000
SusI	18.1255000	2.20550000	1.00560000
SusII	12.4765000	0.97810000	1.61440000

(f) Development of larvae under laboratory conditions - Unbalanced ANOVA

(Strain = Ivermectin-resistant, Ivermectin-susceptible I or Ivermectin-susceptible II; Time = 28, 42 or 84 days post infection)

General Linear Models Procedure
Dependent Variable: LOGEPG

Source	DF	Sum of Squares	Mean Square	F Value	Pr > F
Model	22	10.34396824	0.47018037	0.79	0.7310
Error	67	40.08453829	0.59827669		
Corrected Total	89	50.42850653			

R-Square	C.V.	Root MSE	LOGEPG Mean
0.205121	21.17537	0.773483	3.652751

Source	DF	Type III SS	Mean Square	F Value	Pr > F
TIME	2	0.17462220	0.08731110	0.15	0.8645
ANIMAL	14	6.37338903	0.45524207	0.76	0.7060
STRAIN*TIME	4	3.69158444	0.92289611	1.54	0.1999

Tests of Hypotheses using the Type III MS for ANIMAL as an error term

Source	DF	Type III SS	Mean Square	F Value	Pr > F
STRAIN	2	0.48454224	0.24227112	0.53	0.5987

Tukey's Studentized Range (HSD) Test for variable: LOGEPG
Means with the same letter are not significantly different.

Tukey Grouping	Mean	N	STRAIN

```
A           3.7067     30  Ivermectin-susceptible I
A
A           3.6560     30  ivermectin-resistant
A
A           3.5955     30  Ivermectin-susceptible II
```

(g) Analysis for eggs in female worms - Nested Design

(Strain = Ivermectin-resistant, Ivermectin-susceptible I or Ivermectin-susceptible II)

```
Nested ANOVA using PROC NESTED - Animals killed on 28 DPI
General Linear Models Procedure
Dependent Variable: LOGEGGS

Tests of Hypotheses using the Type III MS for ANIMAL(STRAIN) as an error term
Source                  DF     Type III SS    Mean Square   F Value     Pr > F
STRAIN                   2      0.30893510     0.15446755      0.79     0.4776

Tests of Hypotheses using the Type III MS for
REPL(STRAIN*ANIMAL) as an error term
Source                  DF     Type III SS    Mean Square   F Value     Pr > F
ANIMAL(STRAIN)          12      2.35720311     0.19643359      3.09     0.0004

Nested Random Effects Analysis of Variance for Variable LOGEGGS

            Degrees
Variance         of           Sum of                                    Error
Source      Freedom          Squares      F Value        Pr > F        Term
TOTAL           299        20.783478
STRAIN            2         0.308935        0.786        0.4776      ANIMAL
ANIMAL           12         2.357203        3.090        0.0004      ERROR
ERROR           285        18.117339


General Linear Models Procedure

Level of              -----------LOGEGGS-----------
STRAIN           N       Mean              SD
Resistant I    100     2.67487642       0.23130134
Susceptible I  100     2.63956741       0.23773930
Susceptible II 100     2.59640266       0.31111618

Nested ANOVA using PROC NESTED - Animals killed on 42 DPI

General Linear Models Procedure
Dependent Variable: LOGEGGS

Tests of Hypotheses using the Type III MS for ANIMAL(STRAIN) as an error term
Source                  DF     Type III SS    Mean Square   F Value     Pr > F
STRAIN                   2      1.90049120     0.95024560      0.82     0.4646

Tests of Hypotheses using the Type III MS for
REPL(STRAIN*ANIMAL) as an error term
Source                  DF     Type III SS    Mean Square   F Value     Pr > F
ANIMAL(STRAIN)          12     13.94645305     1.16220442     14.25     0.0001


Nested Random Effects Analysis of Variance for Variable LOGEGGS
```

Variance Source	Degrees of Freedom	Sum of Squares	F Value	Pr > F	Error Term
TOTAL	299	39.095530			
STRAIN	2	1.900491	0.818	0.4646	ANIMAL
ANIMAL	12	13.946453	14.247	0.0000	ERROR
ERROR	285	23.248586			

General Linear Models Procedure

Level of STRAIN	N	LOGEGGS Mean	LOGEGGS SD
Resistant	100	2.63994892	0.41836548
Susceptible I	100	2.62942991	0.30499802
Susceptible II	100	2.46609410	0.32810669

Nested ANOVA using PROC NESTED - **Animals killed on 84 DPI**

General Linear Models Procedure
Dependent Variable: LOGEGGS

Tests of Hypotheses using the Type III MS for ANIMAL(STRAIN) as an error term

Source	DF	Type III SS	Mean Square	F Value	Pr > F
STRAIN	2	6.32552604	3.16276302	0.16	0.8507

Tests of Hypotheses using the Type III MS for
REPL(STRAIN*ANIMAL) as an error term

Source	DF	Type III SS	Mean Square	F Value	Pr > F
ANIMAL(STRAIN)	12	231.6356189	19.3029682	79.52	0.0001

Nested Random Effects Analysis of Variance for Variable LOGEGGS

Variance Source	Degrees of Freedom	Sum of Squares	F Value	Pr > F	Error Term
TOTAL	299	307.146351			
STRAIN	2	6.325526	0.164	0.8507	ANIMAL
ANIMAL	12	231.635619	79.516	0.0000	ERROR
ERROR	285	69.185206			

General Linear Models Procedure

Level of STRAIN	N	LOGEGGS Mean	LOGEGGS SD
Resistant	100	1.87917218	1.06018886
Susceptible I	100	1.76078569	1.05465961
Susceptible II	100	2.11044644	0.89570468

(h) Analysis for female worm length - Nested Design

(Strain = Ivermectin-resistant, Ivermectin-susceptible I or Ivermectin-susceptible II)

Nested ANOVA using PROC NESTED - **Animals killed 28 DPI**

General Linear Models Procedure
Dependent Variable: LOGLENGT

Tests of Hypotheses using the Type III MS for ANIMAL(STRAIN) as an error term

Source	DF	Type III SS	Mean Square	F Value	Pr > F
STRAIN	2	0.09262091	0.04631046	0.57	0.5800

Tests of Hypotheses using the Type III MS for
REPL(STRAIN*ANIMAL) as an error term

Source	DF	Type III SS	Mean Square	F Value	Pr > F
ANIMAL(STRAIN)	12	0.97454641	0.08121220	17.29	0.0001

Nested Random Effects Analysis of Variance for Variable LOGLENGT

Variance Source	Degrees of Freedom	Sum of Squares	F Value	Pr > F	Error Term
TOTAL	299	2.406050			
STRAIN	2	0.092621	0.570	0.5800	ANIMAL
ANIMAL	12	0.974546	17.287	0.0000	ERROR
ERROR	285	1.338883			

General Linear Models Procedure

Level of	LOGLENGT		
STRAIN	N	Mean	SD
Resistant	100	1.83360608	0.07718350
Susceptible I	100	1.80269382	0.08423302
Susceptible II	100	1.79221469	0.10156516

Nested ANOVA using PROC NESTED - **Animals killed on 42 DPI**

General Linear Models Procedure
Dependent Variable: LOGLENGT

Tests of Hypotheses using the Type III MS for ANIMAL(STRAIN) as an error term

Source	DF	Type III SS	Mean Square	F Value	Pr > F
STRAIN	2	1.19027297	0.59513648	2.82	0.0019

Tests of Hypotheses using the Type III MS for
REPL(STRAIN*ANIMAL) as an error term

Source	DF	Type III SS	Mean Square	F Value	Pr > F
ANIMAL(STRAIN)	12	2.53216597	0.21101383	48.46	0.0001

Nested Random Effects Analysis of Variance for Variable LOGLENGT

Variance Source	Degrees of Freedom	Sum of Squares	F Value	Pr > F	Error Term
TOTAL	299	4.963549			
STRAIN	2	1.190273	2.820	0.0019	ANIMAL
ANIMAL	12	2.532166	48.456	0.0000	ERROR
ERROR	285	1.241110			

General Linear Models Procedure

Level of	LOGLENGT		
STRAIN	N	Mean	SD
Resistant	100	1.91651601	0.08118981
Susceptible I	100	1.90616208	0.13759686
Susceptible II	100	1.77802103	0.11220168

Nested ANOVA using PROC NESTED - **Animals killed on 84 DPI**

General Linear Models Procedure
Dependent Variable: LOGLENGT

Tests of Hypotheses using the Type III MS for ANIMAL(STRAIN) as an error term

Source	DF	Type III SS	Mean Square	F Value	Pr > F
STRAIN	2	0.05015669	0.02507834	0.13	0.8810

Tests of Hypotheses using the Type III MS for REPL(STRAIN*ANIMAL) as an error term

Source	DF	Type III SS	Mean Square	F Value	Pr > F
ANIMAL(STRAIN)	12	2.35099191	0.19591599	26.70	0.0001

Nested Random Effects Analysis of Variance for Variable LOGLENGT

Variance Source	Degrees of Freedom	Sum of Squares	F Value	Pr > F	Error Term
TOTAL	299	4.492695			
STRAIN	2	0.050157	0.128	0.8810	ANIMAL
ANIMAL	12	2.350992	26.696	0.0000	ERROR
ERROR	285	2.091546			

General Linear Models Procedure

Level of STRAIN	N	LOGLENGT Mean	LOGLENGT SD
Resistant	100	1.80703286	0.13400228
Susceptible I	100	1.77802699	0.10941328
Susceptible II	100	1.80354558	0.12225484

(i) Analysis for Male worm length - Nested Design

(Strain = Ivermectin-resistant, Ivermectin-susceptible I or Ivermectin-susceptible II)

Nested ANOVA using PROC NESTED - **Animals killed on 28 DPI**

General Linear Models Procedure
Dependent Variable: LOGLENGT

Tests of Hypotheses using the Type III MS for ANIMAL(STRAIN) as an error term

Source	DF	Type III SS	Mean Square	F Value	Pr > F
STRAIN	2	0.54829850	0.27414925	1.98	0.1807

Tests of Hypotheses using the Type III MS for REPL(STRAIN*ANIMAL) as an error term

Source	DF	Type III SS	Mean Square	F Value	Pr > F
ANIMAL(STRAIN)	12	1.66171503	0.13847625	13.19	0.0001

Nested Random Effects Analysis of Variance for Variable LOGLENGT

Variance Source	Degrees of Freedom	Sum of Squares	F Value	Pr > F	Error Term
TOTAL	299	5.201283			
STRAIN	2	0.548299	1.980	0.1807	ANIMAL
ANIMAL	12	1.661715	13.194	0.0000	ERROR
ERROR	285	2.991269			

General Linear Models Procedure

Level of STRAIN	N	LOGLENGT Mean	LOGLENGT SD
Resistant	100	1.64245005	0.13198411
Susceptible I	100	1.57904836	0.11195163
Susceptible II	100	1.53857132	0.13056365

Nested ANOVA using PROC NESTED - **Animals killed on 42 DPI**

General Linear Models Procedure
Dependent Variable: LOGLENGT

Tests of Hypotheses using the Type III MS for ANIMAL(STRAIN) as an error term

Source	DF	Type III SS	Mean Square	F Value	Pr > F
STRAIN	2	2.88610473	1.44305237	11.09	0.0019

Tests of Hypotheses using the Type III MS for REPL(STRAIN*ANIMAL) as an error term

Source	DF	Type III SS	Mean Square	F Value	Pr > F
ANIMAL(STRAIN)	12	1.56124333	0.13010361	12.68	0.0001

Nested Random Effects Analysis of Variance for Variable LOGLENGT

Variance Source	Degrees of Freedom	Sum of Squares	F Value	Pr > F	Error Term
TOTAL	299	7.370955			
STRAIN	2	2.886105	11.092	0.0019	ANIMAL
ANIMAL	12	1.561243	12.683	0.0000	ERROR
ERROR	285	2.923607			

General Linear Models Procedure

Level of STRAIN	N	LOGLENGT Mean	LOGLENGT SD
Resistant	100	1.61885246	0.10778118
Susceptible I	100	1.42070737	0.14527419
Susceptible II	100	1.40211317	0.11216125

Nested ANOVA using PROC NESTED - **Animals Killed on 84 DPI**

General Linear Models Procedure
Dependent Variable: LOGLENGT

Tests of Hypotheses using the Type III MS for ANIMAL(STRAIN) as an error term

Source	DF	Type III SS	Mean Square	F Value	Pr > F
STRAIN	2	0.01920489	0.00960245	0.03	0.9675

Tests of Hypotheses using the Type III MS for REPL(STRAIN*ANIMAL) as an error term

Source	DF	Type III SS	Mean Square	F Value	Pr > F
ANIMAL(STRAIN)	12	3.47809603	0.28984134	32.95	0.0001

Nested Random Effects Analysis of Variance for Variable LOGLENGT

Variance Source	Degrees of Freedom	Sum of Squares	F Value	Pr > F	Error Term
TOTAL	299	6.004029			
STRAIN	2	0.019205	0.0331	0.9675	ANIMAL
ANIMAL	12	3.478096	32.953	0.0000	ERROR
ERROR	285	2.506729			

General Linear Models Procedure

Level of	LOGLENGT		
STRAIN	N	Mean	SD
Resistant	100	1.52862010	0.10879863
Susceptible I	100	1.50908242	0.17624088
Susceptible II	100	1.52018645	0.13249447

Appendix 6.2

Faecal egg counts for animals infected with 15000 infective larvae of ivermectin-resistant strain of *T. colubriformis*

SHEEP NO.	DAYS POST INFECTION (DPI)									
	21	28^	35	42^	49	56	63	70	77	84^
2*	1050	133	-	-	-	-	-	-	-	-
11*	750	567	-	-	-	-	-	-	-	-
13*	1500	1083	-	-	-	-	-	-	-	-
18*	500	1283	-	-	-	-	-	-	-	-
48*	1250	1167	-	-	-	-	-	-	-	-
4**	1450	583	1550	3317	-	-	-	-	-	-
9**	1350	1733	1300	2550	-	-	-	-	-	-
40**	50	850	300	850	-	-	-	-	-	-
42**	850	850	850	1167	-	-	-	-	-	-
44**	2050	1200	350	683	-	-	-	-	-	-
19#	1650	1550	1250	2033	1700	1100	2150	1300	1050	1133
20#	600	617	350	1050	250	100	250	100	100	33
27#	2200	2900	1500	1650	1250	1050	1950	1550	2000	2017
33#	450	1017	400	850	650	1100	400	300	450	100
37#	1750	1250	2450	1950	1800	1500	1950	1250	1700	1000

^ Mean of three egg counts; * Killed at 28 DPI, ** Killed at 42DPI, # Killed at 84DPI

Appendix 6.3

Faecal egg counts for animals infected with 15000 infective larvae of ivermectin-susceptible strain I of *T. colubriformis*

SHEEP NO.	DAYS POST INFECTION (DPI)									
	21	28^	35	42^	49	56	63	70	77	84^
8*	700	1150	-	-	-	-	-	-	-	-
12*	900	1167	-	-	-	-	-	-	-	-
25*	1300	1950	-	-	-	-	-	-	-	-
30*	700	1200	-	-	-	-	-	-	-	-
31*	850	800	-	-	-	-	-	-	-	-
1**	1450	833	1350	1033	-	-	-	-	-	-
15**	800	583	700	833	-	-	-	-	-	-
17**	500	400	850	867	-	-	-	-	-	-
38**	650	1300	1700	1683	-	-	-	-	-	-
50**	50	133	250	333	-	-	-	-	-	-
29#	500	767	400	617	850	1300	550	750	850	1267
32#	150	617	500	550	250	850	550	550	600	633
35#	1000	900	850	833	800	750	900	1550	850	1717
39#	1000	483	800	667	500	750	350	200	100	33
41#	800	800	950	1450	1250	1950	250	600	600	83

^ Mean of three egg counts; * Killed at 28 DPI, ** Killed at 42DPI, # Killed at 84DPI

Appendix 6.4

Faecal egg counts for animals infected with 15000 infective larvae of ivermectin-susceptible strain II of *T. colubriformis*

SHEEP NO.	DAYS POST INFECTION (DPI)									
	21	28^	35	42^	49	56	63	70	77	84^
6*	300	717	-	-	-	-	-	-	-	-
10*	1450	917	-	-	-	-	-	-	-	-
21*	800	1250	-	-	-	-	-	-	-	-
26*	200	1067	-	-	-	-	-	-	-	-
28*	550	1083	-	-	-	-	-	-	-	-
7**	50	333	500	433	-	-	-	-	-	-
22**	250	550	1200	317	-	-	-	-	-	-
23**	700	783	1000	883	-	-	-	-	-	-
36**	1550	933	1600	1150	-	-	-	-	-	-
43**	350	967	1650	683	-	-	-	-	-	-
5#	50	900	800	1233	1800	1800	1050	850	900	1333
14#	700	1350	800	1217	1600	1373	250	500	450	483
16#	800	983	2000	783	650	600	650	300	250	117
46#	850	1433	1650	1600	850	750	1300	950	900	1117
49#	50	1067	1350	917	900	1150	897	750	800	717

^ Mean of three egg counts; * Killed at 28 DPI, ** Killed at 42DPI, # Killed at 84DPI

Appendix 6.5

Worm counts for sheep infected with 15000 infective larvae of ivermectin-resistant strain of *T. colubriformis*

SHEEP NO.	28 DAYS POST INFECTION			SHEEP NO.	42 DAYS POST INFECTION			SHEEP NO.	84 DAYS POST INFECTION		
	Contents	Digest	Total		Contents	Digest	Total		Contents	Digest	Total
2	7790	190	7980	4	10520	100	10620	19	10540	60	10600
11	6240	60	6300	9	9620	50	9670	20	740	10	750
13	9020	150	9170	40	5700	40	5740	27	10360	140	10500
18	7560	160	7720	42	7580	50	7630	33	5620	100	5720
48	8560	130	8690	44	9340	50	9390	37	9420	90	9510

Appendix 6.6

Worm counts for sheep infected with 15000 infective larvae of ivermectin-susceptible strain I of *T. colubriformis*

SHEEP NO.	28 DAYS POST INFECTION			SHEEP NO.	42 DAYS POST INFECTION			SHEEP NO.	84 DAYS POST INFECTION		
	Contents	Digest	Total		Contents	Digest	Total		Contents	Digest	Total
8	6360	160	6520	1	9320	120	9440	29	7640	40	7680
12	6840	180	7020	15	7860	100	7960	32	6160	40	6200
25	7360	120	7480	17	6540	70	6610	35	7820	90	7910
30	7020	130	7150	38	7820	120	7940	39	7640	40	7680
31	7250	100	7350	50	4340	60	4400	41	9140	70	9210

Appendix 6.7

Worm counts for sheep infected with 15000 infective larvae of ivermectin-susceptible strain II of *T. colubriformis*

SHEEP NO.	28 DAYS POST INFECTION			SHEEP NO.	42 DAYS POST INFECTION			SHEEP NO.	84 DAYS POST INFECTION		
	Contents	Digest	Total		Contents	Digest	Total		Contents	Digest	Total
6	6420	90	6510	7	6960	120	7080	5	7880	10	7890
10	8630	110	8740	22	6540	50	6590	14	9580	10	9590
21	7120	90	7210	23	7100	50	7150	16	7940	90	8030
26	8460	20	8480	36	9440	50	9490	46	9700	70	9770
28	7200	100	7300	43	8600	40	8640	49	7840	40	7880

Appendix 6.8

Percentage of development of eggs of *T. colubriformis* to infective larvae under natural conditions from the faecal samples placed on pasture on 24th April

STRAIN	SHEEP NO.	FAECES PERCENT DEVELOPMENT			PASTURE PERCENT DEVELOPMENT (CORRECTED)			SOIL PERCENT DEVELOPMENT (CORRECTED)		
		Rep I	Rep II	Mean	Rep I	Rep II	Mean	Rep I	Rep II	Mean
IVERMECTIN-RESISTANT STRAIN	20	21.51	27.23	24.37	1.54	0.88	1.21	1.56	0.81	1.18
	24	17.52	22.24	19.88	1.30	1.79	1.55	0.62	0.75	0.69
	33	31.09	22.09	26.59	1.88	1.64	1.76	0.16	0.16	0.16
	37	17.26	15.00	16.13	0.91	1.11	1.01	0.36	0.17	0.27
	47	13.36	10.29	11.83	0.61	0.53	0.57	1.22	1.15	1.19
IVERMECTIN-SUSCEPTIBLE STRAIN I	29	14.62	8.62	11.62	1.00	1.52	1.26	0.76	0.29	0.53
	32	28.57	23.31	25.94	1.38	1.50	1.44	3.54	0.51	2.02
	35	12.61	14.00	13.31	1.04	1.71	1.38	0.16	0.27	0.21
	41	18.86	23.57	21.22	2.24	2.52	2.38	0.38	0.32	0.35
	45	30.27	33.58	31.92	3.31	3.35	3.33	0.11	0.28	0.19
IVERMECTIN-SUSCEPTIBLE STRAIN II	5	7.83	19.19	13.51	1.00	1.17	1.09	1.35	1.23	1.29
	14	14.65	21.67	18.16	0.48	0.75	0.61	0.32	0.14	0.23
	16	12.80	14.4	13.60	0.73	0.60	0.67	0.63	0.45	0.54
	4	9.36	6.57	7.97	0.38	0.83	0.61	0.22	0.45	0.34
	49	17.52	12.00	14.76	1.76	0.37	1.06	0.50	0.28	0.39

Appendix 6.9

Percentage of development of eggs of *T. colubriformis* to infective larvae under natural conditions from the faecal samples placed on pasture 26th April

STRAIN	SHEEP NO.	FAECES PERCENT DEVELOPMENT			PASTURE PERCENT DEVELOPMENT (CORRECTED)			SOIL PERCENT DEVELOPMENT (CORRECTED)		
		Rep I	Rep II	Mean	Rep I	Rep II	Mean	Rep I	Rep II	Mean
IVERMECTIN-RESISTANT STRAIN	20	24.13	24.00	24.07	2.84	5.44	4.14	4.70	4.63	4.66
	24	20.59	20.24	20.41	0.69	1.78	1.24	2.69	2.61	2.65
	33	14.49	13.48	13.99	2.95	0.95	1.95	4.22	0.91	2.57
	37	17.90	5.02	11.46	1.66	1.03	1.35	3.79	1.57	2.68
	47	11.41	10.23	10.82	0.82	0.76	0.79	2.20	2.46	2.33
IVERMECTIN-SUSCEPTIBLE STRAIN I	29	12.82	16.23	14.53	2.76	2.71	2.73	1.97	1.24	1.60
	32	17.00	16.25	16.63	2.58	3.25	2.92	3.48	1.57	2.52
	35	7.62	7.22	7.42	1.76	1.11	1.44	0.66	0.51	0.58
	41	15.70	18.47	17.09	4.39	1.81	3.10	0.98	0.36	0.67
	45	24.51	18.67	21.59	1.35	2.81	2.08	1.38	1.38	1.38
IVERMECTIN-SUSCEPTIBLE STRAIN II	5	5.64	7.11	6.37	1.27	1.27	1.27	2.64	0.11	1.37
	14	5.02	5.79	5.40	2.29	2.47	2.38	1.17	0.25	0.71
	16	16.87	15.75	16.31	0.71	0.46	0.58	3.25	3.36	3.31
	46	10.14	8.69	9.41	0.90	0.43	0.67	0.99	2.72	1.85
	49	20.43	18.14	19.28	1.23	0.47	0.85	9.22	3.04	6.13

Appendix 6.10

Percentage of development of eggs of *T. colubriformis* to infective larvae under laboratory conditions from the faecal samples collected at 28 days post infection

SHEEP NO.	IVERMECTIN-RESISTANT STRAIN IN TRIPLICATE				SHEEP NO.	IVERMECTIN-SUSCEPTIBLE STRAIN I IN TRIPLICATE				SHEEP NO.	IVERMECTIN-SUSCEPTIBLE STRAIN II IN TRIPLICATE			
	I	II	III	Mean		I	II	III	Mean		I	II	III	Mean
2*	41.79	49.78	83.44	58.34	8*	131.8	96.87	109.6	112.8	6*	3.12	7.35	ND	5.24
11*	32.62	32.33	ND	32.47	12*	58.15	71.98	ND	65.07	10*	51.81	25.22	18.18	37.74
13*	55.52	35.45	41.13	44.03	25*	94.58	65.69	69.03	76.43	21*	18.64	58.49	ND	53.50
18*	42.33	48.21	62.75	51.11	30*	52.64	60.06	ND	56.35	26*	59.26	89.11	84.38	77.58
48*	10.76	9.89	8.19	9.61	31*	45.19	42.79	38.99	42.32	28*	40.18	39.09	ND	39.64
4**	72.95	48.79	82.12	67.95	1**	39.25	63.31	65.55	56.07	7**	51.04	83.37	ND	67.21
9**	48.44	52.07	37.27	45.93	15**	48.00	54.00	51.00	51.00	22**	72.50	111.7	129.5	104.6
40**	34.93	39.14	22.68	32.25	17**	46.02	85.90	60.00	64.00	23**	77.74	79.95	63.05	73.58
42**	61.00	62.00	59.00	60.00	38**	3.98	2.66	2.73	3.12	36**	9.21	23.07	10.85	14.38
44**	77.90	93.82	ND	85.86	50**	74.02	52.83	ND	63.43	43**	37.53	31.80	25.15	31.49
19#	6.26	4.11	ND	5.19	29#	109.0	155.2	100.4	121.5	5#	4.55	7.76	11.20	7.84
20#	144.2	94.25	ND	104.2	32#	50.80	53.99	ND	53.39	14#	16.58	14.60	ND	15.59
27#	60.23	64.44	71.56	65.41	35#	64.94	36.34	97.83	66.37	16#	24.40	26.27	24.77	25.15
33#	16.72	22.19	35.55	24.82	39#	48.75	52.16	9.20	36.70	46#	39.49	49.57	53.66	47.57
37#	62.98	9.82	33.63	35.48	41#	77.85	39.87	60.70	59.47	49#	30.18	34.96	39.50	34.88

ND - Not done, * Killed at 28 days post infection, ** Killed at 42 days post infection, # Killed at 84 days post infection.

Appendix 6.11

Percentage of development of eggs of *T. colubriformis* to infective larvae under laboratory conditions from the faecal samples collected at 42 days post infection

SHEEP NO.	IVERMECTIN-RESISTANT STRAIN IN TRIPLICATE				SHEEP NO.	IVERMECTIN-SUSCEPTIBLE STRAIN I IN TRIPLICATE				SHEEP NO.	IVERMECTIN-SUSCEPTIBLE STRAIN II IN TRIPLICATE			
	I	II	III	Mean		I	II	III	Mean		I	II	III	Mean
4**	43.57	61.50	42.53	49.50	1**	11.70	21.31	32.57	21.86	7**	72.15	65.99	76.45	71.53
9**	27.62	42.65	50.12	40.13	15**	16.37	49.83	27.55	31.25	22**	32.58	42.03	20.03	31.55
40**	37.95	16.59	11.12	21.89	17**	26.72	15.78	21.97	21.49	23**	12.46	66.71	26.74	35.30
42**	76.00	68.33	28.59	57.64	38**	6.17	6.35	6.54	6.35	36**	25.84	35.08	33.78	31.57
44**	57.42	35.33	48.38	47.04	50**	81.36	64.11	77.36	74.28	43**	15.38	29.52	20.35	21.75
19#	73.85	60.20	53.59	62.61	29#	31.12	21.90	17.49	23.50	5#	28.95	40.28	24.63	31.29
20#	60.97	11.79	4.76	25.84	32#	19.27	25.18	23.48	23.64	14#	14.69	6.11	ND	10.40
27#	46.47	45.82	54.68	48.99	35#	69.93	11.91	39.89	40.57	16#	109.8	26.23	38.07	58.02
33#	5.71	24.33	23.64	17.89	39#	17.74	16.96	ND	17.35	46#	63.06	83.51	63.88	70.15
37#	41.78	31.53	ND	36.33	41#	52.55	51.60	50.41	51.52	49#	34.90	65.34	ND	50.12

ND - Not done, ** Killed at 42 days post infection, # Killed at 84 days post infection

Appendix 6.12

Percentage development of eggs of *T. colubriformis* to infective larvae under laboratory conditions from the faecal samples collected at 84 days post infection

SHEEP NO.	IVERMECTIN-RESISTANT STRAIN IN TRIPLICATE				SHEEP NO.	IVERMECTIN-SUSCEPTIBLE STRAIN I IN TRIPLICATE				SHEEP NO.	IVERMECTIN-SUSCEPTIBLE STRAIN II IN TRIPLICATE			
	I	II	III	Mean		I	II	III	Mean		I	II	III	Mean
19#	68.05	79.50	67.29	71.61	29#	82.09	66.89	87.35	78.77	5#	70.00	81.38	ND	75.69
20#	4.46	8.90	7.33	6.90	32#	99.19	60.98	69.58	76.58	14#	88.44	59.81	72.09	73.45
27#	50.55	45.94	49.36	48.62	35#	65.13	65.75	ND	65.44	16#	32.53	46.20	25.50	34.74
33#	79.55	ND	ND	79.55	39#	7.48	9.45	13.00	9.98	46#	76.38	71.54	96.16	81.36
37#	62.71	57.44	ND	60.08	41#	77.92	72.77	88.38	79.69	49#	31.10	64.38	39.40	31.55

ND - Not done, # Killed at 84 days post infection

Appendix 6.13

Female worm length and eggs per female for *T. colubriformis* collected from animals slaughtered at 28 days post infection

STRAIN	SHEEP NO.	WORM LENGTHS OF 20 FEMALES PER ANIMAL IN MM (NUMBER OF EGGS PER FEMALE)																			
		1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20
IVERMECTIN-RESISTANT STRAIN	2	6.35 (12)	5.80 (21)	6.37 (12)	6.24 (19)	6.82 (17)	6.96 (12)	6.13 (17)	6.12 (17)	6.26 (20)	5.82 (14)	5.90 (13)	5.68 (17)	6.61 (15)	6.71 (14)	6.79 (12)	6.36 (13)	5.63 (18)	6.30 (18)	6.08 (10)	7.43 (22)
	11	6.21 (14)	6.12 (12)	6.43 (21)	6.28 (12)	6.83 (17)	5.61 (12)	5.74 (13)	6.30 (18)	5.85 (11)	6.41 (17)	6.01 (13)	5.67 (19)	6.23 (15)	5.98 (12)	6.62 (13)	6.56 (16)	6.14 (16)	6.11 (16)	6.24 (18)	5.65 (13)
	13	6.12 (13)	6.38 (15)	7.10 (15)	6.41 (17)	6.38 (16)	6.54 (15)	6.61 (7)	6.69 (17)	6.64 (19)	6.91 (27)	7.43 (23)	7.26 (19)	6.89 (16)	6.57 (17)	6.33 (22)	7.15 (20)	6.97 (14)	6.31 (13)	6.74 (17)	6.58 (13)
	18	4.83 (10)	4.94 (12)	6.04 (11)	6.65 (18)	6.01 (21)	5.93 (15)	5.74 (15)	5.69 (22)	6.57 (13)	5.78 (13)	6.02 (10)	6.06 (13)	6.39 (12)	6.01 (12)	6.30 (15)	5.69 (14)	5.50 (12)	5.79 (13)	6.73 (16)	6.13 (11)
	48	6.15 (18)	6.84 (17)	5.53 (12)	6.57 (13)	6.06 (15)	6.49 (18)	6.32 (15)	6.00 (10)	6.96 (10)	5.50 (10)	6.33 (13)	6.01 (14)	6.94 (13)	6.39 (14)	6.13 (11)	6.36 (16)	5.81 (11)	6.62 (12)	6.41 (14)	5.89 (10)
IVERMECTIN-SUSCEPTIBLE STRAIN I	8	6.09 (17)	5.72 (11)	5.38 (17)	5.49 (14)	5.86 (12)	7.03 (15)	6.27 (9)	5.09 (14)	5.79 (16)	6.63 (17)	5.42 (11)	6.04 (13)	5.50 (10)	5.64 (14)	5.84 (13)	5.72 (19)	5.54 (16)	5.47 (11)	5.17 (8)	5.29 (10)
	12	5.84 (12)	5.66 (14)	5.50 (15)	5.59 (17)	6.09 (17)	6.00 (14)	6.07 (15)	5.38 (13)	5.42 (16)	5.70 (12)	5.51 (14)	6.28 (15)	5.64 (13)	6.41 (17)	5.40 (10)	6.04 (12)	6.20 (13)	5.82 (12)	6.14 (12)	5.76 (12)
	25	5.93 (16)	6.66 (17)	6.16 (19)	6.60 (14)	5.91 (10)	6.03 (17)	6.76 (15)	6.64 (21)	5.85 (13)	6.32 (23)	6.47 (17)	6.94 (17)	6.81 (22)	5.66 (9)	6.51 (9)	6.48 (16)	7.18 (22)	6.11 (12)	6.03 (11)	6.64 (20)
	30	6.58 (12)	6.27 (13)	6.48 (14)	5.74 (10)	6.25 (14)	6.51 (17)	6.16 (16)	6.80 (19)	5.15 (14)	6.03 (12)	5.52 (14)	6.21 (17)	5.80 (14)	6.15 (11)	5.53 (11)	5.66 (14)	6.30 (13)	5.01 (14)	6.11 (16)	5.91 (15)
	31	6.23 (10)	7.21 (16)	5.71 (8)	6.73 (20)	6.18 (9)	6.63 (15)	6.82 (14)	5.96 (10)	6.85 (23)	6.70 (18)	5.87 (14)	6.62 (15)	6.40 (11)	7.06 (16)	5.89 (12)	6.50 (20)	6.03 (17)	6.80 (14)	7.13 (22)	6.12 (14)
IVERMECTIN-SUSCEPTIBLE STRAIN II	6	5.92 (15)	4.88 (9)	5.46 (11)	5.05 (11)	4.75 (9)	5.18 (7)	5.55 (12)	5.12 (13)	5.20 (13)	5.38 (12)	5.45 (13)	5.58 (11)	5.87 (21)	5.33 (13)	6.51 (11)	5.26 (11)	5.78 (14)	5.13 (11)	5.80 (12)	5.10 (10)
	10	6.87 (15)	6.76 (17)	6.82 (18)	6.45 (16)	6.93 (17)	7.63 (25)	6.75 (14)	6.43 (14)	6.52 (17)	6.50 (25)	7.01 (18)	6.87 (21)	6.41 (17)	6.98 (11)	6.80 (15)	6.73 (21)	6.89 (17)	6.66 (15)	6.16 (3)	6.40 (14)
	21	6.11 (11)	5.83 (8)	5.49 (8)	4.87 (9)	4.79 (12)	5.24 (13)	6.25 (12)	5.62 (11)	5.54 (14)	6.08 (14)	5.47 (13)	5.80 (13)	5.62 (17)	6.08 (7)	5.32 (9)	5.73 (12)	5.70 (8)	6.11 (17)	5.28 (12)	6.27 (21)
	26	5.73 (18)	6.76 (28)	6.53 (10)	6.65 (16)	6.27 (14)	5.95 (10)	5.87 (12)	5.59 (11)	6.37 (16)	6.23 (16)	5.53 (15)	6.04 (17)	6.14 (14)	5.60 (11)	6.31 (13)	6.15 (15)	6.43 (11)	6.67 (15)	6.19 (17)	6.28 (17)
	28	6.86 (21)	6.33 (15)	5.59 (12)	6.62 (16)	7.19 (17)	5.95 (12)	5.37 (12)	5.64 (15)	6.02 (18)	6.63 (15)	6.13 (15)	5.56 (10)	6.65 (17)	6.61 (22)	6.39 (15)	5.93 (14)	6.28 (15)	6.20 (12)	6.10 (14)	5.91 (9)

Appendix 6.14

Female worm length and eggs per female for *T. colubriformis* collected from animals slaughtered at 42 days post infection

STRAIN	SHEEP NO.	WORM LENGTHS OF 20 FEMALES PER ANIMAL IN MM (NUMBER OF EGGS PER FEMALE)																			
		1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20
IVERMECTIN-RESISTANT STRAIN	4	6.62 (23)	6.94 (25)	7.44 (26)	7.34 (20)	6.37 (14)	7.52 (19)	6.68 (16)	6.80 (21)	6.66 (17)	7.27 (12)	7.25 (21)	7.69 (20)	7.00 (14)	6.81 (17)	6.65 (17)	7.29 (19)	7.17 (18)	7.25 (22)	7.86 (19)	6.92 (15)
	9	8.09 (25)	7.01 (18)	5.27 (10)	6.37 (14)	7.94 (16)	5.94 (23)	7.59 (20)	8.19 (25)	7.38 (13)	7.75 (20)	6.84 (15)	6.04 (9)	7.11 (7)	7.07 (13)	6.70 (21)	6.89 (17)	7.36 (26)	7.36 (15)	6.61 (18)	7.03 (14)
	40	7.00 (10)	6.96 (17)	6.75 (14)	6.43 (16)	5.62 (20)	7.43 (14)	7.19 (13)	5.75 (10)	6.23 (25)	6.41 (16)	5.89 (12)	6.39 (14)	7.55 (14)	6.12 (18)	7.54 (12)	6.73 (18)	7.20 (16)	6.50 (12)	6.63 (16)	6.11 (15)
	42	6.66 (15)	6.88 (16)	6.77 (17)	6.97 (16)	6.77 (15)	6.84 (14)	6.33 (20)	6.85 (8)	6.77 (15)	6.34 (15)	6.76 (17)	6.56 (12)	6.38 (18)	5.89 (10)	6.86 (12)	6.21 (17)	6.42 (12)	7.12 (17)	6.70 (12)	6.94 (11)
	44	6.78 (4)	6.83 (9)	7.57 (16)	7.08 (11)	6.44 (12)	5.53 (8)	6.57 (3)	6.89 (4)	6.66 (8)	7.02 (14)	6.87 (14)	6.12 (4)	6.11 (17)	6.04 (16)	7.06 (7)	6.48 (14)	6.81 (17)	7.01 (6)	7.11 (5)	6.92 (12)
IVERMECTIN-SUSCEPTIBLE STRAIN I	1	8.10 (27)	8.00 (19)	8.14 (21)	7.88 (18)	7.52 (21)	6.86 (16)	8.08 (16)	7.09 (16)	6.56 (14)	7.28 (18)	7.50 (8)	7.77 (20)	7.20 (12)	8.02 (21)	7.28 (20)	6.78 (21)	7.25 (15)	7.28 (17)	6.78 (12)	7.28 (14)
	15	6.80 (18)	7.97 (15)	7.54 (20)	7.21 (20)	7.44 (23)	7.51 (17)	6.89 (22)	6.72 (10)	6.96 (15)	7.53 (20)	7.53 (15)	7.21 (10)	7.21 (19)	7.94 (16)	7.81 (9)	6.80 (9)	7.20 (10)	7.52 (19)	7.20 (20)	6.80 (15)
	17	7.02 (12)	6.59 (15)	7.23 (12)	6.59 (12)	6.69 (15)	6.24 (13)	6.76 (11)	6.01 (12)	5.92 (9)	6.85 (14)	6.87 (15)	6.88 (17)	6.56 (17)	6.46 (10)	6.66 (10)	6.90 (10)	6.80 (16)	6.65 (10)	6.90 (10)	6.80 (16)
	38	7.52 (17)	7.83 (22)	7.49 (20)	7.67 (14)	7.11 (16)	6.14 (16)	7.30 (13)	7.15 (14)	6.87 (12)	6.73 (16)	7.60 (15)	7.42 (17)	7.07 (16)	7.59 (18)	6.62 (14)	7.78 (13)	7.39 (12)	7.74 (23)	6.47 (14)	7.17 (20)
	50	5.05 (10)	5.56 (12)	5.29 (12)	5.18 (10)	5.51 (10)	5.63 (12)	5.63 (8)	5.69 (10)	5.73 (14)	5.23 (10)	5.53 (12)	4.90 (7)	5.02 (10)	5.30 (9)	5.61 (13)	5.17 (7)	4.53 (8)	5.14 (13)	5.20 (7)	5.41 (10)
IVERMECTIN-SUSCEPTIBLE STRAIN II	7	5.46 (8)	4.98 (12)	5.19 (11)	5.17 (10)	5.00 (10)	5.65 (13)	4.87 (9)	4.83 (10)	5.22 (10)	4.85 (8)	4.63 (11)	4.74 (8)	5.21 (8)	4.75 (6)	4.63 (7)	5.34 (13)	5.56 (10)	5.08 (12)	5.38 (8)	5.35 (9)
	22	5.73 (11)	5.67 (11)	6.04 (10)	5.86 (12)	5.41 (12)	5.12 (8)	5.45 (11)	5.80 (12)	5.59 (13)	6.09 (11)	5.52 (10)	5.90 (13)	5.74 (10)	5.45 (9)	5.78 (9)	5.42 (10)	5.28 (8)	5.50 (9)	5.51 (10)	5.83 (12)
	23	6.01 (12)	6.42 (12)	6.14 (15)	5.80 (15)	6.67 (19)	6.13 (7)	5.67 (17)	6.45 (5)	6.50 (15)	6.36 (17)	5.73 (20)	6.13 (13)	5.90 (11)	6.52 (13)	6.15 (12)	6.30 (17)	6.01 (17)	6.84 (14)	5.59 (13)	6.31 (8)
	36	7.10 (15)	6.72 (20)	6.35 (22)	6.55 (20)	7.17 (19)	6.92 (16)	6.91 (17)	6.92 (17)	6.77 (19)	6.77 (18)	6.74 (18)	7.06 (13)	7.15 (17)	7.20 (19)	7.01 (12)	6.96 (15)	5.07 (4)	6.88 (13)	6.33 (18)	6.71 (11)
	43	6.60 (15)	6.26 (14)	5.72 (13)	5.81 (14)	5.89 (15)	6.11 (12)	6.14 (18)	6.65 (7)	6.38 (9)	6.11 (10)	5.78 (10)	6.70 (8)	6.14 (7)	5.31 (8)	6.29 (18)	5.95 (13)	5.61 (8)	6.08 (14)	6.39 (15)	6.06 (12)

Appendix 6.15

Female worm length and eggs per female for *T. colubriformis* collected from animals slaughtered at 84 days post infection

STRAIN	SHEEP NO	WORM LENGTHS OF 20 FEMALES PER ANIMAL IN MM (NUMBER OF EGGS PER FEMALE)																			
		1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20
IVERMECTIN-RESISTANT STRAIN	19	5.14 (17)	5.62 (19)	6.57 (13)	6.02 (16)	6.09 (10)	6.33 (17)	6.60 (0)	6.24 (18)	6.08 (14)	6.92 (18)	5.33 (10)	6.72 (18)	6.80 (18)	6.64 (5)	6.44 (17)	6.60 (11)	6.56 (17)	5.99 (17)	6.35 (14)	5.35 (10)
	20	4.44 (3)	5.21 (6)	4.89 (0)	5.60 (3)	5.78 (0)	5.28 (3)	5.32 (2)	5.47 (3)	5.47 (3)	5.32 (6)	5.28 (0)	5.78 (3)	5.60 (2)	4.89 (3)	5.21 (3)	4.44 (6)	5.47 (0)	5.32 (3)	5.28 (2)	5.78 (3)
	27	6.64 (18)	6.89 (12)	6.87 (15)	6.59 (13)	7.30 (20)	5.74 (16)	6.67 (17)	6.55 (11)	6.42 (18)	6.37 (20)	7.11 (22)	6.96 (17)	5.98 (10)	7.05 (19)	7.21 (14)	6.13 (16)	6.45 (16)	6.61 (18)	6.18 (18)	6.88 (18)
	33	5.81 (5)	5.06 (0)	5.13 (0)	5.05 (1)	8.24 (1)	6.88 (0)	5.35 (0)	5.84 (1)	5.46 (1)	5.64 (0)	5.13 (0)	5.09 (1)	5.75 (0)	5.14 (3)	6.78 (0)	6.79 (0)	8.79 (1)	8.95 (2)	5.67 (0)	4.56 (0)
	37	6.38 (12)	6.89 (10)	6.44 (14)	6.68 (8)	6.30 (12)	6.32 (10)	6.24 (9)	6.29 (4)	6.19 (11)	6.60 (10)	6.22 (18)	7.28 (11)	6.04 (7)	6.30 (13)	6.74 (9)	6.49 (9)	6.40 (8)	6.37 (15)	7.80 (12)	6.79 (6)
IVERMECTIN-SUSCEPTIBLE STRAIN I	29	6.49 (15)	5.91 (18)	5.67 (14)	6.73 (15)	6.17 (12)	7.30 (16)	6.09 (11)	4.95 (7)	5.81 (10)	5.83 (9)	6.04 (15)	6.00 (15)	6.00 (18)	5.84 (14)	5.61 (12)	6.23 (18)	6.00 (15)	6.00 (18)	5.81 (14)	5.83 (15)
	32	5.82 (10)	4.97 (6)	4.71 (5)	5.02 (5)	5.52 (11)	5.38 (10)	4.88 (10)	4.99 (10)	5.32 (7)	4.76 (8)	5.05 (9)	5.16 (8)	5.54 (10)	5.28 (10)	4.91 (2)	4.91 (8)	5.62 (8)	4.88 (7)	3.98 (1)	4.88 (5)
	35	6.04 (18)	6.63 (19)	6.58 (13)	6.30 (13)	5.98 (12)	6.31 (18)	6.17 (7)	6.56 (12)	6.77 (12)	6.71 (14)	6.01 (13)	5.68 (7)	6.09 (10)	6.06 (12)	5.77 (12)	5.28 (12)	6.35 (15)	5.78 (17)	6.63 (16)	6.45 (15)
	39	6.66 (3)	5.98 (4)	7.35 (4)	7.03 (6)	5.76 (0)	6.72 (0)	6.39 (0)	7.22 (0)	5.53 (0)	6.24 (0)	6.35 (2)	6.71 (0)	5.63 (0)	7.21 (1)	5.93 (0)	6.55 (3)	5.85 (0)	6.23 (0)	6.80 (0)	6.30 (0)
	41	5.73 (9)	6.31 (4)	5.27 (0)	6.17 (2)	5.90 (1)	6.41 (1)	5.89 (0)	5.22 (4)	6.06 (0)	5.88 (1)	5.83 (8)	6.61 (8)	5.89 (0)	6.20 (0)	6.13 (2)	6.25 (0)	6.18 (3)	6.49 (6)	6.30 (2)	6.05 (0)
IVERMECTIN-SUSCEPTIBLE STRAIN II	5	7.37 (17)	7.03 (14)	7.02 (13)	6.54 (14)	7.40 (13)	8.08 (27)	7.19 (19)	7.12 (12)	7.38 (20)	6.66 (20)	7.03 (13)	7.37 (10)	7.03 (17)	7.02 (14)	6.54 (l 3)	7.40 (14)	7.03 (13)	7.02 (19)	6.54 (12)	7.40 (l 3)
	14	5.89 (13)	5.85 (8)	6.46 (12)	5.51 (6)	6.31 (10)	5.81 (9)	5.93 (12)	5.88 (4)	5.87 (7)	6.14 (9)	6.39 (10)	5.66 (7)	5.65 (7)	5.97 (11)	5.55 (4)	5.64 (11)	6.71 (10)	5.63 (8)	5.72 (7)	5.82 (8)
	16	6.48 (2)	4.90 (0)	4.94 (0)	5.22 (0)	5.26 (2)	5.53 (2)	5.04 (2)	5.07 (0)	4.26 (0)	5.44 (10)	4.66 (1)	6.32 (1)	5.21 (0)	5.35 (0)	5.32 (0)	5.65 (3)	4.46 (0)	4.98 (0)	4.60 (0)	5.30 (3)
	46	6.14 (13)	6.27 (11)	5.83 (10)	6.45 (10)	6.22 (11)	6.55 (14)	5.95 (13)	6.45 (8)	6.18 (9)	6.15 (8)	6.08 (12)	6.24 (12)	5.78 (11)	6.45 (13)	6.41 (7)	5.89 (13)	6.42 (11)	6.60 (10)	6.68 (6)	5.50 (11)
	49	6.39 (19)	5.92 (14)	6.47 (16)	6.53 (18)	5.95 (11)	6.25 (11)	6.47 (11)	6.23 (19)	6.38 (13)	6.68 (l 5)	5.79 (8)	6.03 (12)	5.60 (16)	5.49 (9)	6.32 (5)	6.52 (16)	5.39 (4)	6.87 (16)	6.09 (12)	5.39 (11)

Appendix 6.16

Male worm length for *T. colubriformis* collected from animals slaughtered at 28 days post infection

STRAIN	SHEEP NO.	WORM LENGTHS OF 20 MALES PER ANIMAL IN MM																		
		1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20
Resistant strain	2	5.28	5.31	4.49	4.81	4.68	4.60	4.72	4.87	4.79	4.69	4.60	4.90	4.96	4.14	5.65	5.31	5.65	5.18	4.87	4.72
	11	4.24	4.70	6.13	4.73	4.21	4.74	4.59	5.21	4.73	3.83	5.39	4.20	4.37	5.09	4.29	4.60	4.58	5.34	6.14	4.99
	13	6.20	6.32	5.30	4.84	5.91	5.89	5.45	6.25	5.01	5.68	4.93	5.85	7.50	5.57	5.74	6.19	6.23	5.60	5.29	5.80
	18	3.94	4.98	4.17	4.53	4.60	4.53	5.28	5.24	4.49	5.07	5.44	4.15	4.23	4.64	5.57	5.22	4.88	5.20	4.35	4.63
	48	5.52	6.34	5.82	5.76	5.12	6.03	5.67	5.17	5.52	6.29	5.46	5.98	6.29	5.39	5.48	6.25	6.49	6.56	5.49	5.63
Susceptible strain I	8	4.09	5.16	5.24	3.82	5.18	5.03	5.05	3.67	5.43	4.79	4.63	5.12	4.09	5.16	3.81	5.18	5.03	5.05	3.69	5.43
	12	4.80	4.62	4.70	3.92	4.71	4.40	4.35	4.62	4.89	4.79	4.27	4.46	4.23	5.79	4.04	5.59	5.35	4.53	4.44	5.23
	25	4.81	4.69	4.72	5.99	4.17	5.03	4.36	5.22	4.42	5.27	5.60	4.24	4.70	4.34	4.54	4.33	4.75	4.54	4.84	4.59
	30	5.30	5.23	4.35	5.15	5.03	4.22	5.17	4.72	5.34	4.51	5.17	5.18	4.38	4.12	4.67	5.09	4.94	4.55	4.77	4.89
	31	4.59	5.47	5.00	5.22	5.51	5.84	5.60	5.23	6.05	5.42	5.38	4.95	6.31	5.55	5.52	4.92	4.72	5.27	5.56	5.60
Susceptible strain II	6	4.37	4.98	4.87	5.29	4.58	4.38	4.67	4.67	4.41	4.88	4.91	4.07	5.00	4.98	4.55	4.92	3.78	4.15	3.55	5.17
	10	5.28	5.38	5.81	4.20	5.52	5.50	4.59	5.52	5.58	3.88	4.68	6.00	5.44	5.28	5.43	5.53	5.09	5.32	5.76	5.41
	21	3.94	4.18	3.83	3.99	3.99	4.39	4.12	4.48	4.20	4.31	4.55	3.74	4.17	3.87	4.46	3.49	4.27	4.71	3.99	4.28
	26	3.71	4.92	5.75	4.54	4.74	4.12	5.34	6.33	4.44	4.74	4.38	4.87	4.50	4.78	5.46	4.77	4.20	4.76	4.35	4.24
	28	3.86	4.71	4.57	4.03	4.84	6.08	5.13	3.67	4.73	3.92	4.77	4.56	4.94	4.72	4.71	4.84	5.19	5.42	5.56	4.21

Appendix 6.17

Male worm lengths for *T. colubriformis* collected from animals slaughtered at 42 days post infection

STRAIN	SHEEP NO.	WORM LENGTHS OF 20 MALES PER ANIMAL IN MM																		
		1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20
Resistant strain	4	6.01	4.91	4.85	5.17	4.82	5.30	4.92	4.84	4.54	5.10	4.60	4.90	4.67	4.48	4.60	4.40	4.27	4.28	4.71	5.06
	9	5.56	5.63	5.17	4.94	5.44	5.36	3.85	5.15	5.71	6.29	6.02	5.75	5.26	4.53	5.61	5.02	5.20	5.58	5.42	6.58
	40	4.67	4.45	4.76	4.78	4.74	4.16	4.31	4.83	4.42	5.27	4.39	4.76	4.72	4.36	4.68	4.91	4.95	4.59	4.57	4.91
	42	5.25	4.82	5.31	5.42	6.36	5.64	4.51	5.80	5.52	5.36	5.66	5.19	5.68	4.36	5.12	5.13	4.35	5.43	5.58	5.82
	44	5.50	5.70	4.96	6.19	4.66	5.88	5.71	5.81	4.88	5.70	5.60	5.33	4.86	4.79	4.64	5.00	5.15	4.64	4.09	4.52
Susceptible strain I	1	4.34	3.38	4.17	4.21	4.77	4.57	4.92	4.43	4.27	3.57	3.63	4.51	4.10	4.85	4.06	4.21	4.16	3.69	4.58	4.24
	15	5.53	5.00	4.30	4.64	5.36	4.21	4.90	4.80	4.72	4.31	5.04	4.48	4.39	3.10	5.07	4.60	3.91	3.90	3.88	4.98
	17	4.35	3.51	3.53	3.74	4.14	4.16	3.14	4.23	4.46	3.79	3.26	3.86	3.86	4.25	4.05	4.29	4.23	4.13	3.59	3.60
	38	4.71	4.41	5.24	4.13	4.61	4.58	4.86	4.44	4.58	5.45	5.47	5.00	3.89	4.62	5.26	3.69	4.63	4.33	5.10	4.29
	50	3.81	3.52	3.49	3.14	3.28	3.71	3.23	3.52	3.78	3.46	3.69	4.40	3.55	3.44	4.02	3.67	3.88	3.22	3.17	3.10
Susceptible strain II	7	4.50	3.87	4.12	3.84	3.90	4.27	3.79	4.19	4.53	3.97	4.07	4.50	4.20	3.99	4.31	3.65	4.24	3.35	3.39	4.44
	22	4.12	4.09	4.12	4.54	3.78	4.13	3.66	4.39	3.72	3.93	4.14	4.48	4.32	3.57	4.58	4.46	4.33	3.86	3.89	3.76
	23	4.62	3.54	4.41	4.22	4.19	4.44	4.86	5.11	4.91	4.78	5.38	3.73	4.54	4.19	4.28	3.74	4.25	4.01	3.68	5.60
	36	4.54	4.41	3.64	3.69	4.45	3.75	4.46	4.42	4.72	4.55	4.08	3.62	3.95	3.38	3.98	4.09	3.85	4.44	4.13	4.14
	43	4.39	3.96	3.69	4.15	3.64	4.14	4.33	3.13	3.38	2.80	3.62	4.22	3.14	3.93	3.58	3.70	3.79	3.99	3.64	3.92

Appendix 6.18

Male worm lengths for *T. colubriformis* collected from animals slaughtered at 84 days post infection

STRAIN	SHEEP NO.	WORM LENGTHS OF 20 MALES PER ANIMAL IN MM																		
		1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20
Resistant strain	19	4.84	5.65	4.95	4.37	5.12	4.43	4.95	4.24	4.42	4.78	5.15	4.05	5.11	5.58	5.29	5.30	4.72	4.72	4.23	4.96
	20	4.93	5.24	5.09	4.45	4.97	4.52	4.87	4.93	5.23	5.09	4.45	4.97	4.53	4.87	5.23	5.09	4.93	4.97	5.09	5.23
	27	4.49	3.89	3.96	3.78	4.32	3.76	3.98	3.76	3.71	4.21	4.56	4.23	4.49	4.17	3.74	3.91	4.01	4.24	4.20	4.49
	33	4.05	4.46	4.87	4.99	4.19	5.12	4.69	4.29	4.76	4.50	4.66	4.67	4.44	4.44	4.57	4.08	4.75	4.65	4.78	4.05
	37	3.49	4.79	5.06	4.82	5.66	4.38	4.97	5.72	3.46	4.72	4.79	4.76	4.36	4.75	4.51	4.33	5.60	5.60	4.98	4.97
Susceptible strain I	29	3.86	3.99	4.43	4.16	3.57	4.20	4.43	4.13	4.34	3.77	3.75	4.03	4.10	4.47	4.75	4.28	4.22	4.89	3.89	4.59
	32	3.60	4.28	3.47	3.75	3.64	3.40	3.65	4.08	3.80	3.21	3.14	3.46	4.16	3.22	3.69	3.56	4.32	3.21	3.37	4.21
	35	4.12	4.18	4.50	4.43	5.00	4.08	4.36	4.53	4.17	4.44	4.18	4.54	5.31	3.80	4.06	4.09	4.04	3.96	4.77	4.31
	39	5.93	5.93	5.67	6.06	4.71	5.43	5.88	6.53	5.41	5.82	4.91	5.12	5.88	5.45	5.74	4.86	6.13	6.08	5.36	6.51
	41	4.99	4.68	5.27	5.17	4.82	4.88	5.61	4.74	5.30	4.77	4.11	5.16	4.91	5.39	5.38	5.33	5.64	5.29	4.91	5.56
Susceptible strain II	5	4.29	4.58	4.24	4.62	3.63	4.09	4.53	4.32	4.77	4.13	3.12	4.40	5.04	4.28	4.75	4.97	4.66	5.22	3.98	4.75
	14	5.08	5.24	6.10	5.43	6.12	5.75	5.44	5.44	5.96	4.86	5.75	5.53	5.97	4.11	5.88	5.14	4.70	6.12	4.23	5.37
	16	4.68	4.86	3.57	4.07	3.70	4.82	4.54	4.78	4.71	3.22	4.74	4.25	4.89	4.68	5.03	4.70	4.23	3.85	4.25	4.60
	46	4.18	4.24	3.93	4.19	4.77	4.89	4.81	4.43	4.43	3.98	4.26	4.28	3.93	4.26	4.18	4.44	4.46	4.18	4.24	3.93
	49	4.77	4.54	4.06	4.65	4.61	4.46	5.13	4.39	4.13	4.56	5.13	4.35	3.71	4.99	4.55	4.59	5.02	3.61	4.29	4.99

Appendix 6.19

LC_{50} values (ng/ml of agar) of thiabendazole, levamisole and ivermectin

STRAIN	ANIMAL NO.	IVERMECTIN 73 DPI	IVERMECTIN 76 DPI	THIABENDAZOLE 73 DPI	THIABENDAZOLE 76 DPI	LEVAMISOLE 73 DPI	LEVAMISOLE 76 DPI
Resistant strain	19	2.67	3.06	197.36	205.35	290.24	373.61
	24	2.70	3.15	195.28	206.32	311.44	380.16
	37	3.39	3.31	202.98	211.80	312.37	411.81
Mean		2.92	3.17	198.54	207.82	304.68	388.52
Susceptible strain I	29	0.69	0.62	14.30	15.06	1413 .57	985.74
	35	0.66	0.69	14.53	14.71	1689.85	1241.96
	41	0.69	0.63	17.87	17.42	1132.91	1274.10
Mean		0.68	0.65	15.57	15.73	1412.11	1167.27
Susceptible strain II	5	0.69	0.63	13.97	15.30	277.75	266.18
	46	0.70	0.64	14.01	14.53	259.44	264.21
	49	0.64	0.64	14.98	15.07	259.41	259.91
Mean		0.676	0.637	14.32	14.97	265.57	263.43

Appendix for the experiments described in Chapter 7

Appendix 7.1

Statistical analysis of faecal egg counts and worm counts

(a) Faecal egg counts at 0 day after treatment

(Groups = Control, Ivermectin, Moxidectin (Oral) or Moxidectin (injection))

General Linear Models Procedure
Dependent Variable: LOGCOUNT

Source	DF	Sum of Squares	Mean Square	F Value	Pr > F
Model	3	0.09390058	0.03130019	0.83	0.4940
Error	20	0.75608900	0.03780445		
Corrected Total	23	0.84998958			

R-Square	C.V.	Root MSE	LOGCOUNT Mean
0.110473	6.607406	0.194434	2.942662

Source	DF	Type I SS	Mean Square	F Value	Pr > F
GROUPS	3	0.09390058	0.03130019	0.83	0.4940

Tukey's Studentized Range (HSD) Test for variable: LOGCOUNT

Means with the same letter are not significantly different.

Tukey Grouping	Mean	N	GROUPS
A	2.9987	6	contol
A			
A	2.9714	6	Ivermectin
A			
A	2.9638	6	Moxiinj
A			
A	2.8367	6	Moxioral

(b) Faecal egg counts at 3 day after treatment

(Groups = Control, Ivermectin, Moxidectin (Oral) or Moxidectin (injection))

General Linear Models Procedure
Dependent Variable: LOGCOUNT

Source	DF	Sum of Squares	Mean Square	F Value	Pr > F
Model	3	31.31947454	10.43982485	27.57	0.0001
Error	20	7.57335676	0.37866784		
Corrected Total	23	38.89283130			

R-Square	C.V.	Root MSE	LOGCOUNT Mean
0.805276	29.64466	0.615360	2.075787

Source	DF	Type I SS	Mean Square	F Value	Pr > F
GROUPS	3	31.31947454	10.43982485	27.57	0.0001

Tukey's Studentized Range (HSD) Test for variable: LOGCOUNT

Means with the same letter are not significantly different.

Tukey Grouping	Mean	N	GROUPS
A	3.1079	6	contol
A			
A	3.0289	6	Moxiinj
B	1.8817	6	Ivermectin
C	0.2846	6	Moxioral

(c) Faecal egg counts at 7 day after treatment
(Groups = Control, Ivermectin, Moxidectin (Oral) or Moxidectin (injection))

General Linear Models Procedure
Dependent Variable: LOGCOUNT

Source	DF	Sum of Squares	Mean Square	F Value	Pr > F
Model	3	25.01887682	8.33962561	29.50	0.0001
Error	20	5.65493156	0.28274658		
Corrected Total	23	30.67380838			

R-Square	C.V.	Root MSE	LOGCOUNT Mean
0.815643	23.40793	0.531739	2.271620

Source	DF	Type I SS	Mean Square	F Value	Pr > F
GROUPS	3	25.01887682	8.33962561	29.50	0.0001

Tukey's Studentized Range (HSD) Test for variable: LOGCOUNT

Means with the same letter are not significantly different.

Tukey Grouping	Mean	N	GROUPS
A	3.1665	6	contol
A			
B A	3.0686	6	Moxiinj
B			
B	2.2327	6	Ivermectin
C	0.6186	6	Moxioral

(d) Faecal egg counts at 10 day after treatment
(Groups = Control, Ivermectin, Moxidectin (Oral) or Moxidectin (injection))

General Linear Models Procedure
Dependent Variable: LOGCOUNT

Source	DF	Sum of Squares	Mean Square	F Value	Pr > F
Model	3	36.40221545	12.13407182	256.32	0.0001
Error	20	0.94678300	0.04733915		
Corrected Total	23	37.34899845			

R-Square	C.V.	Root MSE	LOGCOUNT Mean
0.974650	10.36645	0.217576	2.098845

Source	DF	Type I SS	Mean Square	F Value	Pr > F
GROUPS	3	36.40221545	12.13407182	256.32	0.0001

Tukey's Studentized Range (HSD) Test for variable: LOGCOUNT

Means with the same letter are not significantly different.

Tukey Grouping	Mean	N	GROUPS
A	2.9803	6	contol
A			
A	2.9758	6	Moxiinj
B	2.4393	6	Ivermectin
C	0.0000	6	Moxioral

(e) Worm counts

(Groups = Control, Ivermectin, Moxidectin (Oral) or Moxidectin (injection))

General Linear Models Procedure
Dependent Variable: LOGCOUNT

Source	DF	Sum of Squares	Mean Square	F Value	Pr > F
Model	3	15.23863076	5.07954359	53.00	0.0001
Error	20	1.91666296	0.09583315		
Corrected Total	23	17.15529372			

R-Square	C.V.	Root MSE	LOGCOUNT Mean
0.888276	9.543674	0.309569	3.243712

Source	DF	Type I SS	Mean Square	F Value	Pr > F
GROUPS	3	15.23863076	5.07954359	53.00	0.0001

Tukey's Studentized Range (HSD) Test for variable: LOGCOUNT

Means with the same letter are not significantly different.

Tukey Grouping	Mean	N	GROUPS
A	3.8755	6	contol
A			
A	3.8475	6	Moxiinj
B	3.3375	6	Ivermectin
C	1.9143	6	Moxioral

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