SOME INVESTIGATIONS INTO THE LARVAL DEVELOPMENT ASSAY AND TRICHOSTRONGYLID NEMATODES OF SHEEP.

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SHAABAN BAKARI HOZA.

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Abstract

Two experiments were conducted to investigate the change over time in LD_{50} values in an *in vitro* larval development assay.

In each experiment, six field-reared Romney lambs were effectively treated with an anthelmintic and housed. In experiment I, six three month old lambs were given a single infection of 35000 infective larvae of *T. colubriformis*. From ten days post infection (DPI) three lambs (Group 1.1) were treated twice weekly with 0.5mg/kg of dexamethasone trimethylacetate whilst the other three (Group 1.2) served as controls and remained untreated. In experiment II, three lambs (Group 2.2), six month old were infected with a single dose of 22000 infective larvae of *T. colubriformis* whilst the other three (Group 2.1) of the same age were trickle-infected with 2000 infective larvae once weekly for 14 weeks. Larval development assays were conducted weekly for 14 weeks with ivermectin in Experiment I and ivermectin, avermectinB2 and levamisole in Experiment II.

In Experiment I and II for ivermectin, the LD_{50} values rose to a 4x increase between 50-70 DPI and fell again. The general pattern seen following a single infection with all anthelmintics was for the LD_{50} values to be relatively constant from 21-35 DPI, then rose 2.5-7x increase to peak 49-56 DPI and declined at the same rate again to original starting values by 84 DPI where they remained until the end of the experiment.

In Experiment I, the steroid treated group started with similar values but had a 5x fall by 42 DPI which was not seen in the single infection group. The trickle infection group in Experiment II generally resulted in a small increase of 1-1.5x from 42-77 DPI and then declined again to starting values until the remainder of the experiment.

The study demonstrates that there is a similar change in the LD_{50} values

with time for *T. colubriformis* with all three anthelmintics tested and that the change was prevented in steroid treated animals and was less apparent in trickle-infected animals.

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CHAPTER 1

1.1 INTRODUCTION.

Resistance of sheep, goats and cattle nematode parasites to commonly used anthelmintics is a subject of great concern in the world. Scientific studies have revealed that resistance is genetically controlled (Prichard,1990). The genetics of anthelmintic resistance have been well studied in the benzimidazole (BZ) and pro-benzimidazole group of anthelmintics (Roos,1990; Roos *et al.*,1990; Roos et *al.*,1993; Roos *et al.*,1995; Kwa *et al.*,1993;1994; Elard *et al.*1996; Warwick and Mascord,1996). It is important to recognise that understanding the mechanism of anthelmintic resistance requires an understanding of the modes of actions of anthelmintics, together with the biology and population dynamics of trichostrongylid nematodes.

A number of anthelmintic resistance detection methods have been described which can be divided into *in vivo* and *in vitro* techniques. The *in vivo* methods are suitable for all types of anthelmintics but are expensive in terms of labour and animals (Lacey *et al.*,1990).

The *in vitro* methods are essentially more rapid, sensitive and economical (Lacey *et al.*,1990; Taylor,1992) but with some limitations (Johansen,1989). For example the egg-hatch assay is technically demanding, presents problems with interpretation when mixed infections are involved and requires freshly collected eggs or eggs stored under special conditions (Le Jambre,1976). The larval motility assay for detection of levamisole and morantel resistance (Martin and Le Jambre,1979) is another *in vitro* technique which has limitations. It has been

shown that high concentrations of levamisole are less effective than lower concentrations in immobilising third stage larvae which can make estimation of LD_{50} values difficult. Some subjectivity also occurs in judging whether or not larvae are paralysed and the assay can not be preserved for later counting. Its usefulness is limited to levamisole/morantel and the ivermectin groups of anthelmintics. The tubulin binding assay for benzimidazoles (Lacey and Prichard,1986) requires the use of expensive laboratory equipment and involves radioactive isotypes which need approved laboratory facilities and trained personnel.

A reliable *in vitro* assay known as the larval development assay (LDA) which measures the success of larval development in varying concentrations of anthelmintics has been shown to offer considerable promise for all anthelmintic action families (Coles *et al.*,1988; Taylor,1990). A variety of different variations of this general concept have been described with each successive method offering advantages over earlier descriptions. Hubert and Kerboeuf (1992) described a microlarval development test in which, eggs were cultured to third stage larvae in the presence of Earles' Balanced Salt solution, yeast extracts and bacteria in a total volume of 150µl with or without varying dilutions of anthelmintics added. The technique gave a high level of larval development, a good linear dose-effect relationship so that the LD_{50s} could be estimated, and counting the larvae was easy due to the small culture volume.

One potential problem that has been identified with some *in vitro* assays is an apparent change in LD_{50} values with time after infection. This was originally observed with *Haemonchus contortus* in an egg-hatch assay (Borgsteede and Couwenberg,1987) and more recently with *Ostertagia circumcincta* with a Larval Development Assay (Amarante *et al.*,1997). This thesis presents results of a research investigation into this phenomenon with *Trichostrongylus colubriformis* using a Larval Development Assay.

1.2 LITERATURE REVIEW.

1.2.1 ANTHELMINTIC RESISTANCE.

Resistance can be described as "a heritable change in the ability of individual parasites to survive the recommended therapeutic dose of anthelmintics" (Taylor and Hunt,1989). Three sub-classifications have been made. Side-resistance, which occurs among chemically related compounds with the same mode of action, has been reported to occur among the closely related benzimidazoles, salicylanilides and more recently macrocyclic lactones (avermectins/milbemycins), cross resistance which is a result of selection with one chemical group rendering the nematode also resistant to a chemically unrelated compound such as between the imidazothiazole drug levamisole and the tetrahydropyrimidine compound morantel. Multiple resistance involves resistance in one nematode species to at least two compounds with different modes of action (Green *et al.*,1981; Coles,1992; Pomroy *et al.*,1992).

Resistance has been reported in the trichostrongylid nematodes of small ruminants (sheep and goats) particularly *H. contortus*, *O. circumcincta*, *T. colubriformis* and to a lesser extent *Cooperia* and *Nematodirus* species. To date trichostrongylids have shown resistance to various benzimidazoles, pro-benzimidazoles, levamisole, morantel, rafoxanide, closantel, naphthalophos, and most recently ivermectin which are representatives of each of the major classes of anthelmintic drugs (Prichard,1990). The severity of anthelmintic resistance tends to be associated with a variety of factors such as geographical location and animal

production management systems. Although this problem is worldwide, it is more severe in the southern hemisphere especially the low latitude areas. This may be due to a warmer climate leading to a short generation period hence a faster establishment and spread of resistant populations and the need for effective parasite control which heavily rely on anthelmintic use. Areas where resistance is more prevalent include Australia, New Zealand, South Africa and South America (Donald,1983; Kettle,1983; McKenna and Watson,1987; Hughes,1988; Van Wyk and Malan,1988; Van Wyk *et al.*,1989; Waller,1987; Echevarria and Trindade,1989; Waller *et al.*,1996).

1.2.2 **Modes of action of anthelmintics and the mechanism of anthelmintic resistance.**

Studies have shown that resistance is genetically controlled as an inherited trait (Prichard,1990). An understanding of the genetics of anthelmintic resistance requires an understanding of the drugs' mode of action. A summary of what is known about the most important anthelmintics used in New Zealand follows.

1.2.3 **Benzimidazoles and Pro-benzimidazoles.**

The mode of action of benzimidazoles and pro-benzimidazoles is to disrupt the formation of microtubules in nematodes and fungi at concentrations harmless to mammalian cells (Lacey,1988). Microtubules form an intracellular cytoskeleton and are associated with the performance of various physiological functions of the cells. These are summarised as follows; the formation of mitotic

spindles during cell division, maintenance of cell shape, cell motility, cellular secretion, nutrient absorption and intracellular transport (Lacey,1988).

Microtubules comprise a series of protofilaments made up of cytoskeletal proteins (tubulin), and are in active equilibrium where they are gaining and losing soluble tubulin units at opposite terminals of the developing microtubule (Lacey, 1988). Tubulin exists as a dimer of closely related proteins α and β-tubulin which are about 50,000 molecular weight and sequence length of 450 and 445 amino acids respectively. Growth of microtubules depends on the rate of addition (polymerization) or loss of tubulin units. Disruption of tubulin-microtubule equilibrium results in a loss of cellular homeostasis.

Studies of the binding of benzimidazoles to the β-tubulin protein fraction of *H. contortus* indicated that tubulin from resistant parasites had reduced binding affinity for benzimidazoles implying that there were structural changes in the β-tubulin molecule. It was further reported that the binding affinity of benzimidazoles for host tubulin is much lower than for that of the parasite, which is consistent with the high general safety index of benzimidazoles (Lacey,1988). At the molecular level, it has been shown that only one β-tubulin gene is present (isotype 1) in resistant *H.contortus* worms tested and resistance was associated with the amino acid phenylalanine (Phe) being in place of tyrosine (Tyr) at position 200 on the derived amino acid sequence of the gene (Roos *et al.*,1995). A similar change of Phe for Tyr at position 200 in the amino acid sequence of the corresponding gene has been shown to confer resistance to benzimidazole anthelmintics in the free living nematode *Caenorhabditis elegans* and in the

fungus *Aspergillus nidulans* (Kwa *et al.*,1993; 1994; Elard *et al.*,1996). Further studies have shown that the amino acid 200 on β-tubulin is close to the position 205 - 208 which is the binding domain for β-tubulin with α-tubulin. It has been postulated that any change at this site may affect the equilibrium, assembly and disassembly rates of microtubules (Roos *et al.*,1993). It is further suggested that rather than the mutation being acquired, selection of isotype 1 gene is through a deletion of susceptible alleles from the population as Isotype 1 gene is present in susceptible populations of *H.contortus*. At higher levels of resistance a further genetic change is observed which involves deletion of isotype 2 gene (Roos *et al.*,1995). However, the role of this gene is not understood. All these earlier studies were with *H. contortus*. A similar finding has also been made with *Trichostrongylus colubriformis* β-tubulin gene (tcb-1). After selection for BZ resistance, polymorphism at the tcb-1 locus disappeared leaving a single tcb-1 allele in the resistant population (Warwick and Mascord,1996) implying that these genes are determinants of benzimidazole susceptibility and BZ-resistance. Similarly for *O. circumcincta* it has been demonstrated that a change of the Phe for Tyr at position 200 in the corresponding gene is associated with a change from susceptibility to resistance (Elard *et al.*,1996).

1.2.4 **Levamisole**.

Levamisole is a cholinergic blocking agent at the ganglion level causing reversible paralysis through sustained muscle contraction resulting in the nematode to be removed from the gastrointestinal tract by normal peristalsis

(Prichard *et al.*,1980; Booth and McDonald,1988). Unfortunately, little has been reported so far to determine the genetic mechanism of resistance to levamisole and other cholinergic agonists. However, Martin and McKenzie (1990) have reported that the inheritance of resistance to these drugs was of a sex-linked recessive character controlled by a single gene. The mechanism of resistance of the trichostrongylid nematodes *H. contortus* and *T. colubriformis* is through a reduction in the number of nicotinic acetylcholine receptors (Sangster *et al.*,1988: Hoekstra *et al.*,1997).

1.2.5 **Ivermectin.**

The mode of action of ivermectin is associated with opening of glutamate-gated chloride ion channels in the cells which increases membrane permeability to chloride ions of nematodes leading to a hyperpolarization which cannot subsquently transmit an action potential (McKellar and Benchaoui,1996; Shoop,1993). What changes occur in resistant nematodes is not yet understood.

Studies on ivermectin resistant *H. contortus* (Le Jambre,1993) suggest that resistance is inherited as a single dominant allele. However this strain was essentially a laboratory isolate and to date no genetic studies have been reported on field isolates. The physiological and molecular basis for IVM-resistance is not yet known.

1.3 THE *IN VITRO* ASSAYS.

1.3.1 Introduction

The *in vitro* assays are a group of anthelmintic resistance detection techniques which includes those for egg-hatch, larval development, larval motility, micromotility, tubulin binding and calorimetric assays. Generally the *in vitro* assays are more precise, reliable and economical than the *in vivo* assays and different assays are suitable for different anthelmintics.

1.3.2 The Egg-Hatch Assay for Benzimidazoles.

This is used for the diagnosis of benzimidazole resistance and relies on the ovicidal activity of BZs. The basic principle is to incubate eggs in serial dilutions of the anthelmintic for 24 hours at 26^{o}C, add Lugols' iodine solution as a stain and then count the proportion which have failed to hatch (Johansen,1989; Le Jambre,1976; Coles and Simpkin,1977 ; Hall et al.,1978; Whitlock et al.,1980; Donald,1983). The percentage of eggs that hatch is then corrected for the natural mortality in the controls. The data are usually subjected to probit analysis from which LD_{50} estimates can be calculated. Eggs of BZ-resistant *H. contortus* and *T. colubriformis* have shown to be resistant to the ovicidal activity of benzimidazoles (Coles and Simpkin,1977). A key limitation of this assay, as reported by most authors, is to ensure that all the eggs are fresh (undeveloped) and no more than one hour after being collected per rectum or are at the same stage of development. However, Hunt and Taylor (1989) found that anaerobic storage of eggs up to 7 days has little effect on the usefulness of the assay,

implying that the technique can be used widely for diagnosis (except for *Nematodirus*) as samples can be couriered by post. Smith-Buijs and Borgesteede (1986) stored faecal samples on ice during transportation to the laboratory and found that there was no significant effect on the LD_{50} values compared with fresh eggs.

Variations also existed in the floatation media used during the egg recovery procedure. Different media have been reported to have no obvious effect on the results as long as eggs are cleaned thoroughly before incubation. Salt solutions will kill eggs and sugar residues act as nutrient for fungal growth in the control samples unless they are removed (Obendorf *et al.*,1986).

The technique can be used for determining the presence and level of BZ-resistance (Hall *et al.*,1978). It is more reliable than the Faecal Egg Count Reduction Technique and has a high repeatability. Its limitation is that it requires skilled personnel, (Donald,1985) and is only suitable for benzimidazole anthelmintics.

1.3.3 **The Egg-Hatch Assay for Levamisole.**

The basic principle of this assay is to compare the difference between resistant and susceptible strains of trichostrongylid nematodes in the rate of recovery from paralysis of unhatched larvae in serial dilutions of levamisole as measured by the number that subsquently successfully hatch (Dobson et al.,1986). Fresh undeveloped eggs are recovered, concentrated, then incubated at 26°C in microtitre plates until one hour before hatching when the anthelmintic is added.

Following further incubation for 24 hours, the plates are snap cooled to -15°C for 5 minutes following which chilled formaldehyde is added. The numbers of hatched larvae are then counted.

The technique is accurate if good timing between adding the drug to eggs and terminating the experiment is adhered to. However, it is difficult to compare results between different laboratories and therefore it is not recommended for field diagnosis of resistance (Dobson *et al.*,1986; Johansen,1989).

1.3.4 **Larval Paralysis / Motility Assays.**

A number of different procedures for measuring larval paralysis or motility have been described. The overall principle of these assays is to measure the ability of anthelmintics to paralyse infective third stage larvae. The original assay was described for levamisole and morantel (Martin and Le Jambre,1979). Third stage larvae are recovered from faecal cultures and incubated in serial dilutions of anthelmintic for 24 hours. The larvae are then viewed under a microscope and classified as normal (moving) or paralysed (not moving) over a 5 second observation period. The percentage of paralysed larvae is then calculated and the LD_{50} calculated, commonly using log dose-probit lines (Le Jambre *et al.*,1976). The procedure is generally fast and simple to carry out.

Following the original work different variations have been described. Sutherland and Lee (1990) described a modified larval paralysis assay for detecting thiabendazole (TBZ) resistance in which TBZ-resistant larvae in the presence of the acetylcholinesterase inhibitor eserine, became paralysed more

slowly than susceptible larvae. This assay usually relies on visual assessment of the motility of larvae. However, other methods for assessing motility have been used as well. An instrument for detecting the motility of larvae after incubation with anthelmintics (albendazole, levamisole, haloxon, morantel and ivermectin) was described by Bennett and Pax (1986). The instrument uses microprocessor technology to measure light refraction at the meniscal interface, whereby the angle of light refraction entering the photodiode is altered by larval movement. The change in light refraction are then measured by a computer to give a motility index. Folz *et al.*, (1987; 1988) described a different motility meter which they used to test the motility of *H. contortus* infective larvae to various anthelmintics (albendazole, cambendazole, fenbendazole, ivermectin and levamisole) and observed differences in motility between BZ-resistant and susceptible strains but not between levamisole resistant and susceptible ones (Coles *et al.*,1989). Use of instruments to detect motility has some limitations in that if only a few larvae are present they produce insufficient movement to register any significant effect whilst too many motile larvae move the dead ones thus leading to an exaggerated or misleading results. The technique is not suitable for testing more than one sample at a time.

The inhibition of larval motility by ivermectin was described by Gill *et al.*,(1991). The basic principle of this assay is to incubate L3 larvae in the dark on an agar matrix containing serial dilution of ivermectin for 24 hours at 25°C before exposing them to light sufficient to activate more than 90 % of larvae in the control wells (1-2) minutes. The numbers of non-motile larvae as a

proportion of the total larvae present at each concentration are calculated and a log concentration-logit model is fitted to estimate LD_{50} values. Normally third stage (L3) larvae of *H. contortus* isolates resistant to ivermectin show a decreased sensitivity to induced paralysis *in vitro*. The technique is sensitive, precise, quick and cheap to perform and the authors suggest that it could be used in detection of ivermectin resistance in the other trichostrongylid nematodes *T. colubriformis* and *O. circumcincta* (Gill *et al.*,1991).

1.3.5 **Biochemical Assays**.

These have only been used for the benzimidazole anthelmintics . The tubulin binding assay is based on the differential binding of tubulin extracts of eggs, infective larvae or adult nematodes to tritiated benzimidazole carbamates by resistant and susceptible parasites (Lacey and Prichard,1986; Lacey and Snowdon,1988). The tubulin extract is incubated with tritiated labelled benzimidazole until equilibrium is reached (Lacey and Snowdon,1988). The free drug is then removed with charcoal, leaving the amount of tritium-benzimidazole-tubulin complex to be estimated with a liquid scintillation spectrometer. The protein concentration (μg/assay) is plotted against the amount of bound tritiated benzimidazole. Usually tubulin from BZ-resistant parasites binds substantially less drug than that from susceptible parasites (Lacey and Prichard, 1986). The assay is rapid and can detect very low frequencies of resistance and is suitable for field conditions (Lacey and Snowdon,1988; Johansen,1989). Its only limitation is that it requires the use of expensive laboratory facilities and trained personnel.

Sutherland and Lee (1988; 1989) described a colorimetric assay which is a modified version of the aphid tile test used to diagnose insecticide resistance. This assay compares the levels of non-specific esterases and acetylcholinesterases of benzimidazole-resistant and susceptible *H. contortus, O circumcincta* and *T. colubriformis*. In this assay greater esterase or acetylcholinesterase activity occurs in the BZ-resistant strain which is assessed by visual examination or through the use of a densitometer. The assay is said to be accurate, rapid, inexpensive and can detect resistance even in mixed infections except for *Nematodirus* species (Johansen,1989).

1.3.6 **The Larval Development Assay**.

The larval development assay (LDA) is an *in vitro* assay which has been used for the detection of resistance to benzimidazoles, levamisole, benzimidazole/levamisole combinations and avermectin/milbemycin anthelmintics in three major gastrointestinal nematode parasites of small ruminants, *H. contortus*, *O. circumcincta*, *T. colubriformis* (Lacey *et al.*,1990; Gill *et al.*,1995). Various descriptions of larval development assays have been described (Coles *et al.*, 1988; Taylor,1990; Gill *et al.*, 1995). The techniques differ in the media used (whether liquid or agar culture), in the volume of culture and culture apparatus, and egg recovery procedures but the basic principle remains the same, i.e. to culture eggs through to infective larvae in serial dilutions of anthelmintic. The proportion of infective larvae is determined and the LD_{50} values calculated. The original description was by Ibarra and Jerkins (1984) in

which the media used for cultivation of parasites consisted of one part distilled water and one part faecal suspension of rat faeces rich in bacteria on which the developing larvae fed. In the same year Hubert and Kerboeuf (1984) described a nutritive medium consisting of Earle's Balanced Salt solution and yeast extracts. These were modified in the report by Coles *et al.*, (1988) in which lyophilised *E. coli* was used as a nutrient source for the larvae to feed on. Taylor (1990) further modified the assay by incubating nematode eggs on agar in a nutritive medium consisting of Earle's Balanced Salt solution and yeast extracts in 96 well microtitre plates. Hubert and Kerboeuf (1992) described a technique where eggs were cultured in a nutrient medium comprising of Earles' Balanced Salt solution, yeast extract and killed bacteria in tubes at 23°C for 7 days. Gill *et al.*,(1995) further modified the assay in which doubling dilutions of anthelmintic were mixed with agar and placed in 96 well microtitre plates. Eggs, nutritive medium similar to that described by Hubert and Kerboeuf (1992), and amphotericinB (to prevent fungal growth) were layered over the agar and the plates were incubated at 25°C for seven days. All stages (eggs, first, second and third stage larvae) were then counted after staining with Lugol's iodine solution to determine the proportion of ensheathed L3s and this was corrected against control wells and a log-dose response curve is fitted to calculate the LD_{50} values. With these modifications good dose response data were obtained with TBZ, LEV, Pyrantel and IVM allowing the determination of LD_{50} values. In this variation of the assay, water-soluble drugs are serially diluted in distilled water, whilst in-soluble

compounds can be serially diluted in 2 % dimethylsulphoxide (DMSO) (Gill *et al.*,1995).

The assay procedure is simple to carry out, several anthelmintics can be tested simultaneously and differentiation of species in mixed infection is easier than with other *in vitro* assays (Coles *et al.*,1988; Taylor,1990). The technique has proved to show good repeatability, therefore it is suitable for both field screening and research work because eggs are easily obtained in large numbers in faeces. It has been developed to the point that a commercial kit called "DrenchRite" is now available, essentially using the technique described by Gill *et al.*,(1995). Since the assay relies on the effect of anthelmintics on growth of the first stage larvae, the age of the faecal sample is not as critical as with the egg-hatch assay and that faecal samples may be preserved for up to 7 days and yet the eggs remain viable for the test (Coles *et al.*,1988; Taylor,1990). The assay is applicable for screening for BZ-resistance in the field even in mixed infections except for *Nematodirus* species (Taylor,1990; Johansen,1989). The most important aspect of this assay is its ability to detect resistance to the three main broad-spectrum anthelmintic classes; benzimidazoles, levamisole / morantel and avermectins. However, there are reports that concentration of avermectins (avermectin B1; B2) necessary to kill *H. contortus* are rarely the same as those required to kill *O. circumcincta* and *T. colubriformis* (Shoop *et al.*,1995) which may complicate its use. Also problems such as bacterial overgrowth of the agar in the wells and difficulty in interpretation of results when using levamisole been

reported as the dose response tends to vary between species and nature of media (Johansen,1989).

1.4 THE LIFE CYCLE OF *Trichostrongylus colubriformis*.

The life cycle of trichostrongylid nematodes has been well documented by Dunn (1978). *T. colubriformis* like the other trichostrongylids has a direct life cycle with six stages (Soulsby,1982). Essentially males mate with females, eventually the females lay eggs containing an embryo which are passed in the faeces of the host. Under favourable conditions of temperature, approximately 10-30°C (optimum 25-27°C), enough moisture (about 80 % relative humidity) and a plentiful supply of oxygen, the embryo develops hatches to produce larvae stage one (L1). Basically, growth occurs during each stage followed by a short resting period (lethargus) then moulting occurs (Dunn,1978). Normally there are 4 moults, 2 outside the host and 2 inside the host (Soulsby,1982). The larval stage one (L1) develops into larval stage two (L2) but the L2 then undergoes an incomplete moult in which the cuticle separates, but is not shed resulting in the ensheathed larval stage three (L3) which cannot feed. The L1, L2 and L3 stages are free living, but only the L1 and L2 can feed on bacteria. The ensheathed L3 relies on stored metabolites for its energy.

Under the influence of light and in the presence of water films the infective stages migrate randomly from the faecal mass and are stranded on the herbage (Rogers and Sommerville,1963). When ingested by a grazing ruminant the infective third stage larvae (L3) are initially deposited in the rumen. The

gastrointestinal tract provides a chemical stimulus (example carbonic acid) to stimulate exsheathment from the cuticle retained by the infective larvae. In the case of *T. colubriformis* exsheathment occurs in the abomasum in response to "exsheathment fluid" influenced by a host stimulus comprising of unionized bicarbonate-carbon dioxide buffer, undissociated CO_2 and dissolved gaseous CO_2 (Soulsby,1982). Aided by their own movement, the larvae then escape from the broken sheath. The L3 then pass and occupy the fisrt three meters of the small intestine, where they enter the superficial mucosal gland crypts, feed, grow and moult into the fourth stage larvae (L4). These larvae then emerge on to the surface of the mucosa, feed, grow and moult into immature adults who feed, grow and mate. Finally the females lay eggs to complete the life cycle. The minimum prepatent period (PPP) is 18-21 days (Soulsby,1982; Rahman and Collins,1990).

1.5 **The Population Dynamics of *Trichostrongylus colubriformis* in the host.**

1.5.1 **The Effect of Immune Response**.

Scientific evidence show that with *T. colubriformis* infections a primary build-up of an infection of adult worms is followed by expulsion and subsquent immunity and in later life only a small short lived adult infection is established while the infective larvae are expelled without any further development (Dobson *et al.*,1990c). Immunological studies suggest that following nematode infections, humoral and cellular changes occur resulting in the production of antibodies and

various immunological mediarors. Pomroy (1994) summarised a hypothetical chain of events and suggested that excretory-secretory products from the nematodes cross the mucus membrane and are then exposed to T cells which stimulate the T_H subset. Cytokines are then produced which subsequently drives the immune response with mastocytosis, increased IgE, IgG_1 and IgA and increased tissue eosinophil production. Mucus secreting goblet cells increase as well as other pathological changes in the mucosa *per se*. This is followed by the release of inflammatory mediators after being triggered by antigen cross-linking antibody on cell membrane receptors. Nematode incoming larvae are then exposed to the inflammatory mediators such as leukotrines, whilst the mucus layer acts as a further line of defence. The antibodies also contribute directly by binding to the stroma, excretory pore or cuticle with subsequent harmful effect.

1.5.2 **Population Dynamics.**

Recent studies on the population dynamycs of *T. colubriformis* by Dobson *et al.* (1990) have suggested that with a low infection rate establishment of worms remained at maximum levels for the first four weeks, then falls to a rate of about 5% per week after 7, 10 and 14 weeks of continuous L3 intake for higher, medium and low infection rates respectively, implying that a threshold of worm exposure was necessary before resistance to establishment developed. Once the threshold was achieved there was a similar rate of decline of establishment irrespective of the infection rat. The studies have also shown that fecundity expressed as eggs per female per day had an inverse relationship with the rate of infection. Fecundity remained high for 5 weeks at low infection rate

but only maintained at this level for about 3 weeks at medium and high infection rates.

Further studies show that resistance to T colubriformis is expressed as a suppression of establishment of larvae whilst adult worm numbers are unaffected (Dobson *et al.*,1990c). It is further suggested that rejection of adult parasites began at approximately the same time regardless of infection rate and takes about 9 weeks to complete commencing when establishment of incoming larvae declines to approximately 1 % (Dobson *et al.*,1990c). About 20 % of adults are rejected by week 10 in high infection rate and is suggested that this might have commenced at week 7.

Likewise, the rate of development of resistance to new infections was studied (Dobson *et al.*,1990b) using three month and nine month old lambs and was found to be faster in the older than the younger hosts. The logical conclusions to these observations are firstly, there is a threshold worm burden that must be exceeded before any substantial resistance to develop, secondly, once the threshold is exceeded the rate of development of resistance is independent of infection rate but determined by host age only; thirdly, arrested development is induced by host immunity; and fourthly, acquired immunity observed in indoor reared sheep can develop naturally under field conditions.

These above findings indicate that firstly, the mechanism of adult worm loss was rejection by the host and not by worm age-related mortality. Secondly, variability between host worm burden increased over the course of infection implying that the onset of host immunity varies between individuals.

In conclusion it is apparent that as adult establishment decline to low levels, worm burdens accumulated to levels that relate to, but are not directly proportional to the infection rate. When this happens other effects of the immune response are observed. Fecundity decline rapidly before the onset of worm expulsion began. Decline in establishment, reduced fecundity, adult rejection all represent an immune response by the host. However, the time at which the immune mechanisms are active varies according to infective rate, host age and individual host responsiveness.

CHAPTER 2

2.0 LARVAL DEVELOPMENT ASSAY OPTIMIZATION.

Some preliminary trials were carried out in order to determine the relationship between the success of larval development and incubation period and number of eggs per well. Gill *et al.*,(1995) described a modification of the larval development assay which involves culturing eggs in a nutritive medium layered over agar gels containing anthelmintic in 96-well microtitre plates. The following investigation was undertaken to optimise this technique for use with *T. colubriformis* in particular.

2.1 Methodology.

2.1.1 Egg Recovery.

Fresh faeces were collected from a sheep infected with *T. colubriformis*. The egg recovery procedure was largely as described by Hubert and Kerboeuf (1992). About 10g of faeces were made into a slurry with water, sieved through a 1mm aperture sieve then through a 100μm aperture sieve with eggs subsquently collected on a 20μm aperture sieve. The eggs were further concentrated by centrifugation in 20 % magnesium sulphate (density 1.10) at 1500g for 5 minutes. The eggs were recovered, washed and the number of eggs/ml estimated on the basis of 10 counts and their concentration adjusted in order to provide approximately 40, 80 or 200 eggs/60μl.

2.1.2 The Larval Development Assay

The larval development assay is essentially as described by Gill *et al.*, (1995) and is described in Appendix V. Briefly, 100μl of 2 % agar was layered in the wells in 96-well microtitre plates. For investigation of the effect of egg concentration on developmental success 100μl of egg suspension comprising

40µl of the nutritive medium and 60µl of egg suspension to give approximately 40, 80, or 200 eggs were added on top of the agar matrix (n=12 for each concentration). A nutritive medium (NM) was added to the egg suspension so that 100µl of the mixture contained 40µl of NM plus 60µl of eggs. To investigate the incubation time needed, three replicates with 80 eggs/well were set up. After incubating the plates for 3, 5, 7 and 9 days at 26°C, the numbers of eggs, first, second and third stage larvae in each well were counted. For both investigations plates were stored at 4°C until counting. All counts were made within three days of the end of incubation. Counting was done after staining each aliquot with Lugol's iodine solution.

2.1.3 **Statistical Analysis**.

The proportion of L3 in each well was analysed by means of One Way Analysis of Variance (Statistix 4.1). In order to analyse the proportion of L3s by one way analysis of variance, it was necessary to transform them (arcsin√proportion) to normalise the distribution Means were compared by Tukey Comparison of means.

2.1.4 **Results.**

The numbers of each stage are shown in Tables 2.1 and 2.2 below. The results clearly demonstrate that the highest percentage success of development to the third stage (L3) infective larvae (88 %) was after an incubation period of 7 days Table 2.1 and with few eggs per well (40-80) (87-75 %) than when the number is 200 eggs (63 %) see Table 2.2.

Table 2.1. Incubation time relationship.

Incubation time	Mean % of each stage			
Days	**Eggs**	**L1**	**L2**	**L3**
3	2.3	42.6	55.1	0
5	2.8	18.1	67.1	12
7	2.5	6.4	3.1	88
9	2.3	1.5	45.1	50.1

Table 2.2

Larval development on number of eggs per well relationship. ♠=number of each respective stage/well; ♣=proportion of L3 of the total number of eggs+larvae.

40 Eggs/well					80 Eggs/well					200 Eggs/well				
♠Egg	♠ L1	♠ L2	♠ L3	♣ Prop	♠Egg	♠ L1	♠L 2	♠L3	♣ Prop	♠Egg	♠L 1	♠ L2	♠L3	♣ Prop
0	0	7	38	0.84	7	0	14	55	0.72	4	0	0	126	0.96
1	0	26	13	0.83	13	0	17	47	0.61	5	0	1	183	0.85
0	0	3	41	0.93	2	0	6	72	0.90	1	0	36	140	0.79
0	0	0	56	1.00	2	0	1	76	0.96	2	0	37	157	0.80
2	0	2	34	0.89	3	0	22	55	0.68	0	0	34	172	0.81
0	0	1	41	0.98	1	0	5	73	0.92	2	0	13	151	0.90
0	0	4	37	0.90	2	0	6	73	0.90	4	0	46	146	0.82
0	0	2	39	0.95	6	0	5	69	0.86	3	0	73	132	0.78
0	0	2	38	0.95	0	0	8	70	0.90	1	0	88	94	0.70
0	0	1	43	0.98	3	0	13	70	0.81	2	0	65	79	0.68
0	0	2	40	0.95	2	0	6	6	0.89	2	0	55	107	0.65
0	0	4	34	0.89	2	0	15	64	0.79	0	0	45	85	0.65
% mean L3 prop				0.87					0.75					0.63

These results showed that there was a significantly higher ($p<0.05$) proportion of L3s in 40eggs/well than in the 80 eggs/well and 200egga/well but

there was no significant difference ($p>0.05$) between 80 eggs/well and 200 eggs/well.

2.1.5 **Discussion.**

Different authors have used different incubation temperature regimes in the larval development assay. In most studies incubation was at temperatures between 25-27°C (Taylor,1990; Le Jambre *et al.*,1995; Gill *et al.*,1995) which was not very different anyway. At temperatures above 27°C development is rapid, but larval survival is reduced by larvae being very active and utilising metabolic energy very quickly (Hubert and Kerboeuf,1992). At temperature below 23°C development is prolonged. The results reported by Hubert and Kerboeuf (1992) at temperatures of 23°C was 85 %, and those of Gill *et al.*,(1995) at 25°C was 90 %. In this LDA optimization experiment cultures were made at 26oC with 75-87 % development to L3 being achieved. This is a little less than some published results but was considered acceptable. The results in Table 2.1 show that an incubation time of at least 7 days is required.

Experience has shown that the smaller the number of eggs per well the better are the results. Hubert and Kerboeuf (1992) layered 100 eggs per well in a total volume of 150µl and the percentage development of L3 stage larvae was 85 %, whilst Gill *et al.*, (1995) layered 80 eggs per well in a total volume of 100µl obtaining 90 % development. The results of the egg concentration experiment (Table 2.2) demonstrated with 40 eggs/well there was 87 % success whilst with 80 eggs/well there was only 75 % which was significantly less. With the incubation time experiment (Table 2.1) which also involved incubating 80 eggs/well at 26°C for 7 days there was a higher development success of 88 % which is as high as was achieved with 40 eggs/well in the egg concentration

experiment and as high as was achieved in earlier published reports. As 80 eggs /well gives greater flexibility in setting up and reading the assay ,this was the chosen number to be used for subsequent experiments. When the larval development assay was first developed, most workers were tempted to use large volume apparatus which in one way or another forced them to incubate large numbers of eggs which made counting tedious but also resulted in unsatisfactory larval development. With the use of smaller number of eggs and reduced volumes it was possible to obtain high and consistent development rates as well as facilitating counting (Gill *et al.*, 1995). In addition, the use of agar gels in microtitre plates with this technique eliminates solubility problems with anthelmintic and leads to more reproducible development because a free equilibrium of the drug between the agar matrix and larvae is maintained (Lacey *et al.*,1990).

These trials have confirmed that using small volumes and relatively small numbers of *T. colubriformis* eggs per well yielded highly satisfactory results using the procedure described by Gill *et al.*,(1995). Therefore, the following conditions were adopted for subsequent experiments (1) optimum temperature of 26°C, (2) 7 days incubation period, (3) the use 100µl of 2 % agar matrix, (4) laying about 80 eggs per well in total volume of 100µl, (5) laying eggs in 96-well microtitre plates, (6) the use of nutritive media, (7) use of amphotericin B to prevent fungal growth.

CHAPTER 3

3.0 EXPERIMENT I.

3.1 Introduction.

Previous results of an egg hatch assay with TBZ following a single experimental infection with *H. contortus* showed that the LD_{50} values changed with time rising to a peak at about 50 DPI and then declining to base line values (Borgesteede and Couwenberg,1987). Similar changes were observed with *O. circumcincta* with IVM, TBZ and LEV with a larval development assay (Amarante *et al.*,1997). The aim of this experiment was to investigate if the same phenomenon occurs with *T. colubriformis* and to investigate what effect immunosuppression of the host sheep using corticosteroid treatment has on this change in LD_{50}values.

3.2 Materials and Methods.

3.2.1 Animals

Six field-reared Romney lambs about 3 months old were effectively drenched and housed. They were fed a ration of lucerne and barley based nuts and given access to hay and water *ad-libitum.*

3.2.2 **Experimental Design.**

Animals were randomly divided into two groups of three, and were each given a single infection of 35000 infective larvae of *T. colubriformis* on Day 0. From 10 day post infection (DPI) three lambs in group 1.1(S1,S2,S3) were treated with 0.5mg/kg body weight of dexamethasone trimethylacetate (Dexavet DP 5mg/ml; Bomac Laboratories Ltd.) twice weekly until 112 DPI, whilst the other remaining three lambs in group 1.2 (S4,S5,S6) were left as untreated controls. Faecal samples were collected once weekly from 21 DPI.

3.2.3 **Egg Recovery Procedure**.

This was as described previously and as detailed in Appendix V. Briefly, 50g faeces were made into a slurry with water, sieved through a 1mm sieve, into a 100μm sieve with eggs collected on a 20μm sieve. Further concentration was achieved by centrifugation in 20 % magnesium sulphate (1.10 density) at 1500g for 5 minutes initially and then collecting the eggs on a 20μm sieve and washing the eggs with water to remove the magnesium sulphate. The concentration of eggs was adjusted to 1333 eggs/ml so that there were 80 eggs in 60μl.

3.2.4 **The Larval Development Assay**.

The larval development assay was similar to that described by Gill *et al.* (1995) using 96-well microtitre plates. A nutritive medium comprising yeast extract, Earles' Balanced Salt solution, lyophilised *E. coli* and amphotericin B was added to the egg suspension (Amarante *et al.*,1997) to make a final concentration of 800 eggs/ml. A stock solution of 24μg/ml of ivermectin (IVM) in dimethylsulphoxide (DMSO) was prepared by diluting commercial product (Ivomec drench for sheep and goats MSDAgvet). The stock solution was the serially diluted 1:2 with DMSO to obtain 15 concentrations ranging from

0.0007325 to 24µg/ml (see Appendix V). The plates were prepared the day before each assay was commenced. Two µl of each dilution of anthelmintic was put in each successive wells and mixed with 100µl of 2 % agar. The following day 100µl of egg suspension containing nutritive medium and approximately 80 eggs were added on top of the agar matrix. After incubating the plates for 7 days at 26°C, the contents of each well including 3 control wells containing DMSO instead of anthelmintic was removed by pipette and the numbers of eggs, first, second and third stage larvae in each well were counted after staining with Lugol's iodine solution.

3.2.5 **Parasitological Procedure.**

Faecal egg counts (FEC) were carried out using a modified McMaster technique in which each egg counted represents 50 eggs per gram as per Appendix I.

3.2.6 **Statistical Analysis.**

The number of third stage (L3) larvae in the test wells was adjusted by the proportion that developed in the control wells. The adjusted proportion was fitted to a sigmoid curve as shown in Fig. 3.1 after log transformation (Slidewrite Version 6, Advanced Graphics Software Inc.) to calculate the LD_{50} values. This programme utilises the Levenberg-Marquardt algorithm in an unconstrained optimization approach to estimate the coefficient by minimising the sum of the squared deviations. No data were available for day 28. As the actual concentrations of anthelmintic in the aqueous phase is unknown, the concentration reported was that in the DMSO before it was added to the agar. The change in LD_{50} values with time were analyse by a two-way analysis of variance using the software programme Statistix 4.1 (Analytical Software, USA).

The correlation between LD_{50} values and the mean proportion of L3s in the control wells were investigated by sheep using the Statistix 4.1.

3.2.7 **Results.**

The FEC results are shown in Fig. 3.2 with numerical data in Appendix II. Group 1.1 FEC showed a rise and fall within a range of about 1000-3000 eggs per gram (epg) throughout the experiment, whereas group 1.2 also had showed a rise and fall within a range of 500-2000 eggs per gram throughout the experiment with the exception of sheep 6 (S6) whose egg count declined to low levels 56 DPI and remained so until the remainder of the experiment.

The LD_{50} results are shown in Fig. 3.3 and the raw data in Appendix IV. On 21 and 35 DPI both groups had similar LD_{50} values of about 0.05μg/ml. Then those of group 1.1 declined to about 0.01μg/ml on 42 DPI at which level they remained for the duration of the experiment. The LD_{50} values for group 1.2 continued to remain at about the starting value until about 49 DPI after which they climbed steadily to a peak of 0.2μg/ml on 70 DPI (which represents about a 4-fold increase) and then steadily declined to the starting values of about 0.05μg/ml by 84 DPI and remained at this level for the remainder of the experiment. It is important to note that the drug concentrations reported in this document are those in DMSO and not concentrations in agar and/ or liquid phase.

Two way analysis of variance (ANOVA) showed there was overall a significant difference between the 2 groups and a significant time interaction. When the results were analysed by time after infection there was a significant

difference between groups ($p<0$) and interaction from days 42 to 84 ($p=0.000$). Between days 84 to 112 there was a significant difference between groups ($p<0.01$) but no interaction ($p>0.05$). When examined by group there was no significant correlation ($p>0.05$) between egg counts and LD_{50}. There was no significant correlation ($p>0.05$) between LD_{50} and the mean proprtion of L3s in the control wells when examined by sheep except for sheep 3. As this was an animal that received corticosteroids, this finding does not help to explain the change in LD_{50} values with time.

The coefficient of determination for fitting the sigmoid curve (COD) (r^2) ranged from 0.88 to 0.99 as shown in Appendix VII. The mean r^2 was 0.97 indicating good dose response curve were achieved with good data repeatability.

3.2.8 **Discussion**.

These results from group1.2 show that there is a change with time in non-steroid treated sheep which is similar to that seen with the egg-hatch assay for benzimidazoles with *H. contortus,* and with benzimidazoles, levamisoles, and ivermectin for *O. circumcincta* in a larval development assay. However the peak occurred at 70 DPI which is 10-20 days later than the two earlier reports with *H. contortus* and with *O. circumcincta* using ivermectin (Borgsteede and Couwenberg,1987; Amarante *et al.*,1997). These findings suggest that this change in LD_{50} values is a general phenomenon in trichostrongylid nematodes with all anthelmintics. The LD_{50} values of group 1.1 which were given sufficient steroids treatment to suppress the immune response (Dobson *et al.*,1990a) suggest that it is the host immune system which is in some way responsible for this change in the LD_{50} values. An alternative hypothesis is that the steroids had a direct influence on the nematodes themselves (due to the high dose given). The fall from starting levels in LD_{50} values in group 1.1 was unexpected. The lambs

were field-reared and would have developed at least partial immunity to *T. colubriformis* and the fall may reflect inhibition of this degree of immunity using steroids. The fall in LD_{50} values in group 1.1 occurred from Day 35 although steroid treatment commenced on Day 10. This is somewhat later than might have been expected if suppression of the immune response is the primary reason.

The inconsistency observed in the rise and fall of the FEC was unexpected. The animals were faecal sampled consistantly during the evening after sufficient feeding. Therefore there is no clear explanation to this inconsistency.

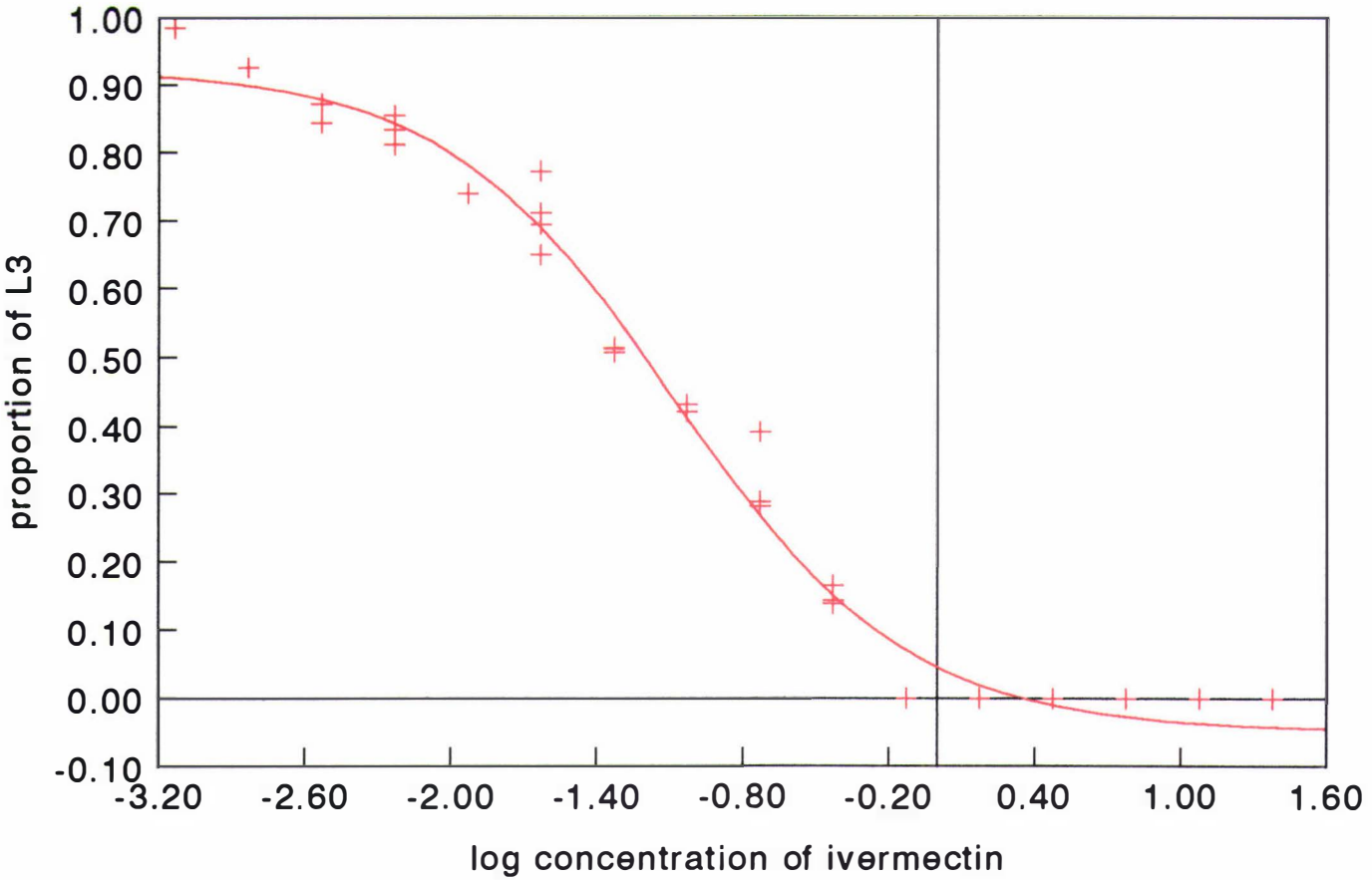

Fig. 3.1 A typical dose response curve relating the proportion of developed L3 to log concentration of ivermectin. This example if for Sheep 6 on Day 112.

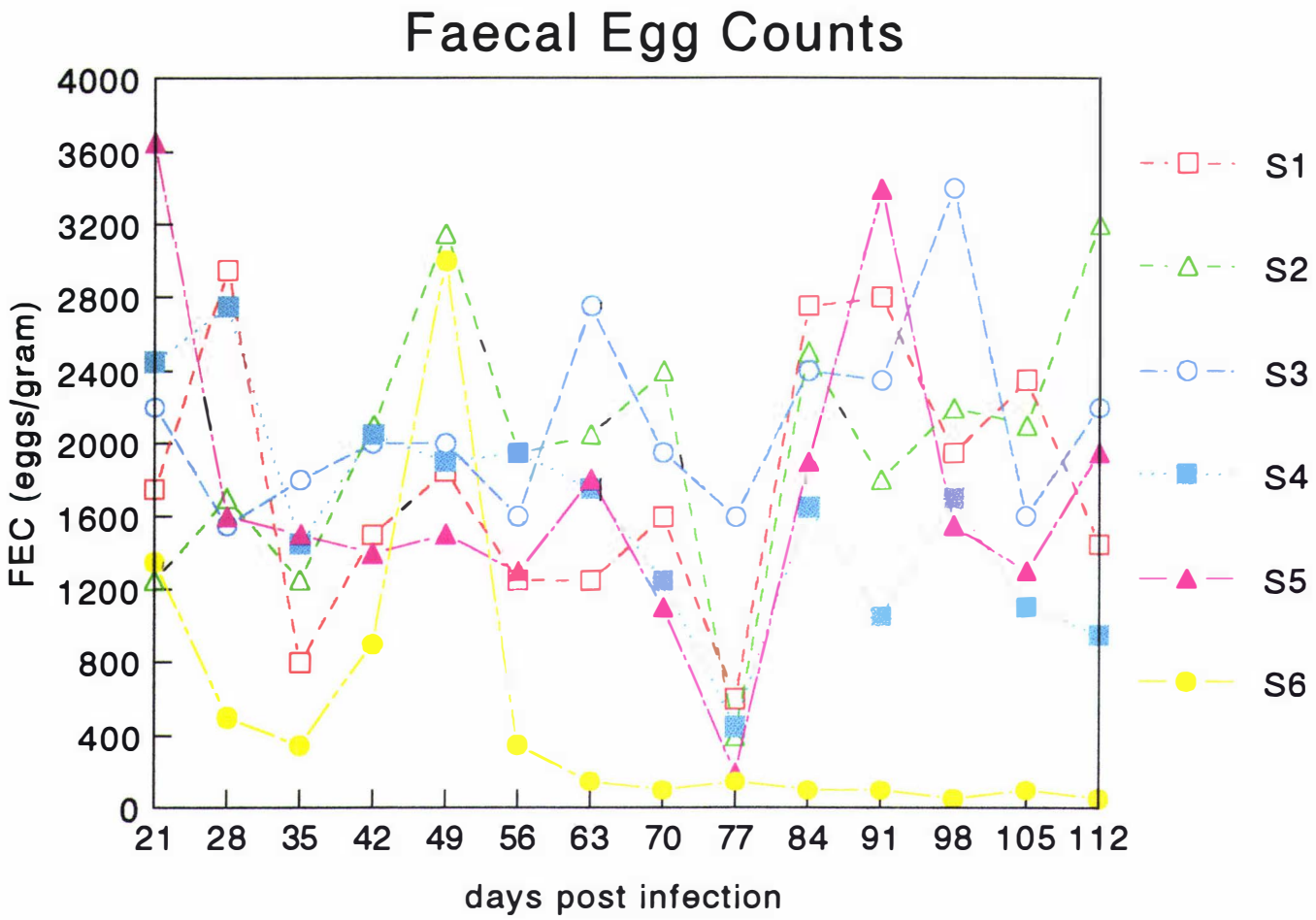

Fig. 3.2 FEC (epg) values for 3 lambs (S1, S2, S3) treated with 0.5mg/kg dexamethasone trimethylacetate from Day 10 twice weekly and 3 control lambs (S4, S5, S6) all infected with 35000 L3 on Day 0

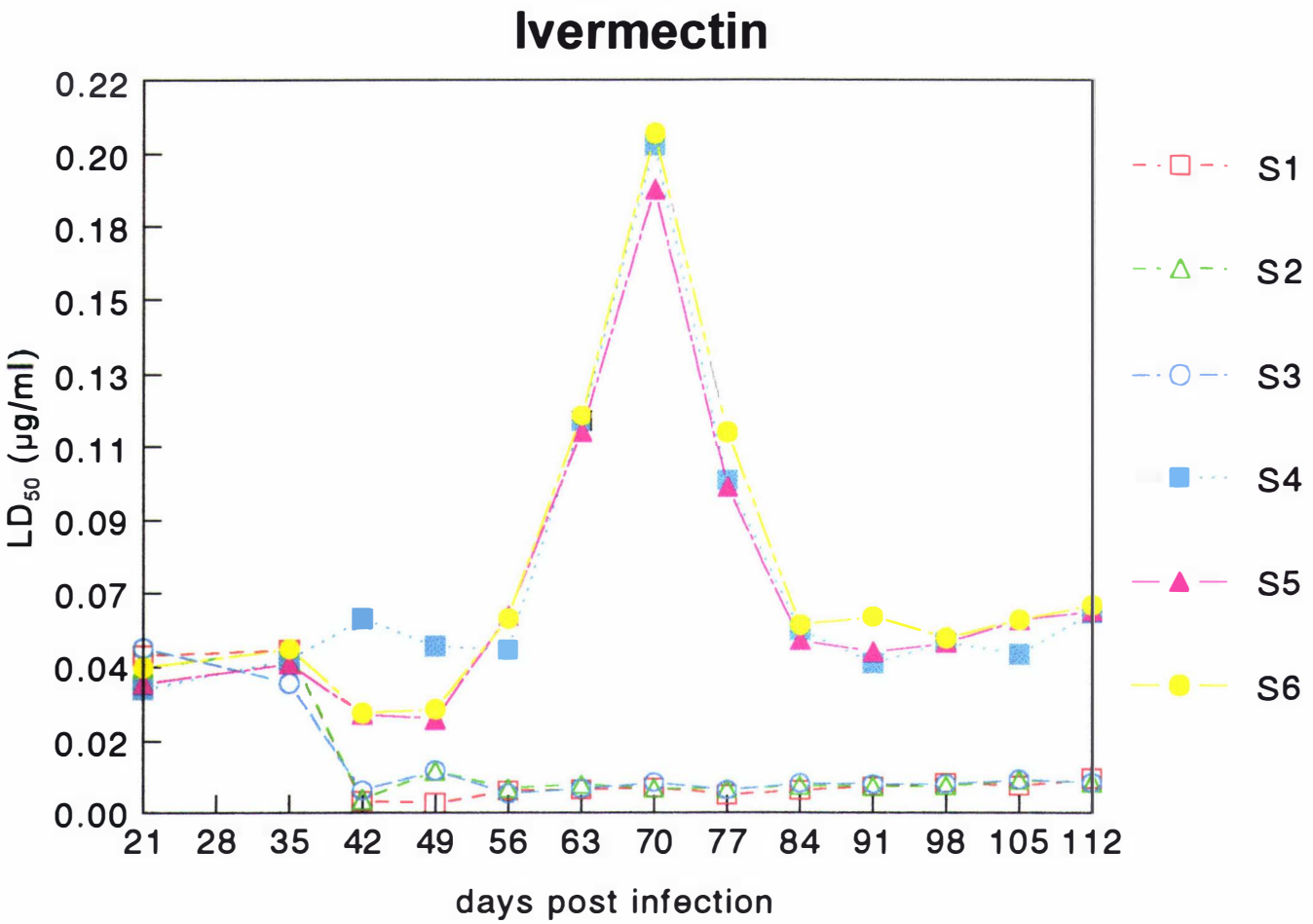

Fig. 3.3 LD_{50} values of ivermectin (µg/ml) for 3 lambs (S1, S2, S3) treated with 0.5mg/kg dexamethasone trimethylacetate from Day 10 twice weekly and 3 control lambs (S4, S5, S6) all infected with 35000 L3 on Day 0

CHAPTER 4

4.0 EXPERIMENT II

4.1 Introduction.

The results of Experiment I suggested the possibility of an involvement of the host's immune response with the rise and fall of the LD_{50} values with time. The aim of Experiment II was to investigate whether this phenomenon is apparent with *T. colubriformis* with (1) trickle infection (simulating a field situation) and (2) if it also occurs with other anthelmintics (avermectin B2 and levamisole).

4.2 Materials and Methods.

4.2.1 Animals

Six field-reared Romney lambs about 6 months old were weighed and then effectively drenched and housed. They were fed as in Experiment I on a ration of lucerne and barley based nuts and given access to hay and water *ad-libitum*.

4.2.2 Experimental Design.

From Day 10 post-drenching, animals were randomly divided into two groups of three. The three lambs in group 2.1 (S4,S5,S6) were infected with 2000 infective (L3) larvae of once weekly for 14 weeks until day 112, whilst three lambs group 2.2 (S1,S2,S3) were given a single infection of 22000 infective (L3) stage of *T. colubriformis*. Faeces were collected once weekly from 21 DPI. At the end of the experiment about 140 DPI, two sheep from group 2.2 were killed to confirm that only *T. colubriformis* was present in the animals.

4.2.3 **Egg Recovery Procedure**.

This was as described in section 3.2.3

4.2.4 **The Larval Development Assay**.

Essentially this was as described in section 3.2.4. Stock solutions of anthelmintics were serially diluted 1:2 with DMSO (for ivermectin and avermectin B2 (AVMB2)) (Gill *et al.*,1995) or distilled water for levamisole (Amarante *et al.*,1997) to obtain 15 concentrations as shown in Appendix V. The egg suspension was prepared the day before each assay commenced. In other respects the assay procedure was as described in section 3.2.3.

4.2.5 **Parasitological Procedure.**

This was as described in section 3.2.5 and as in Appendix I. The worm counting procedure was as described in Appendix VIII, in which $1/10^{th}$ of the sample was examined and one worm counted represents 10 worms.

4.2.6 **Statistical Analysis.**

Essentially this was as described in section 3.2.6. As in Experiment I the concentrations of IVM and AVMB2 reported are those in DMSO and for LEV those after dilution in water before being added to the agar.

4.2.7 **Results.**

The FEC results are shown in Fig.4.1 and as in Appendix II. The FEC of group 2.1 started at low levels of about 100 epg on 21 DPI and rose to a peak of about 1100 epg by 42 to 84 DPI then declined to low levels again and remained at this level until the end of the experiment. The FEC of group 2.2 started at about 500-1000 eggs per gram on 21 DPI the increased to maximum of about 2500 epg from 35 to 84 DPI then declined to low levels again as for those of group 2.1 with the exception of sheep 4 (S4) whose FEC continued to rise until the end of the experiment.

The LD_{50} results are shown on Figs. 4.2 , 4.3 and 4.4 and the raw data are given in Appendix IV. The LD_{50} values for IVM (Fig.4.2) for group 2.2 started at a starting values of 0.04μg/ml on 21 DPI, fell slightly to about 0.02μg/ml on 35 DPI, increased to starting values on 42 DPI and then steadily increased to a peak of about 0.14μg/ml 49 to 56 DPI (which represents an almost 3.5x increase) then steadily declined to starting values of about 0.03μg/ml from 84 DPI where they remained until the end of the experiment. The LD_{50} values of the trickle-infected group 2.1 were initially the same as those of group 2.2 including a small rise on 42 DPI. After this time they remained at a constant level of about 0.05μg/ml until 77 DPI when they fell in line with values for group 2.1 to about 0.02μg/ml for the remainder of the experiment.

The LD_{50} for AVMB2 (Fig. 4.3) behaved in a similar way to those for IVM. Those of group 2.2 which were given a single infection started at values of about 0.06μg/ml on 21 DPI and remained at about this value with some

fluctuations until 35 DPI, then rose to a peak of 0.15μg/ml by 49 to 56 DPI (representing about a 2.5x increase) then declined to about 0.03μg/ml on 77 DPI where they remained until the end of the experiment. The LD_{50} values of group 2.1 given a trickle infection were similar to those of group 2.2 up to 42 DPI. They remained at about the 42 DPI level until 56 DPI and then declined to about 0.04μg/ml on 63 DPI where they remained until the end of the experiment. From 77 DPI they were again similar to the LD_{50} values of group 2.2.

The LD_{50} values of LEV (Fig. 4.4) behaved in a similar manner to those of IVM and AVMB2. Those of the group 2.2, given a single infection, started at a value of 0.13μg/ml on 21 DPI and remained at about this value until 42 DPI. They then increased steadily to a peak of 0.6-0.9μg/ml (representing about a 4-7x increase) on 56 DPI and declined steadily to starting values on 84 DPI of about 0.15μg/ml where they remained until the end of the experiment. The LD_{50} values of the group 2.1 given a trickle infection was the same as those of group 2.2 from 21 to 35 DPI, but increased slightly to 0.2μg/ml from 42 to 77 DPI then declined to starting values of about 0.13μg/ml on 84 DPI and remained at this level until the end of the experiment.

The LD_{50s} values of all three anthelmintics (IVM, AVMB2 and LEV) for group 2.1 showed a peculiar sharp fall on 63 DPI then rose slightly on 70 DPI and then declined steadily to low values on 84 DPI. This peculiar rise and fall may have been associated with or caused by dietary changes which occurred at this time. There was a change in formulation of the concentrate feed which resulted in the death of young goats due to ruminal acidosis in an unrelated experiment but which were eating the same ration.

For each anthelmintic, Two way analysis of variance showed there was overall a significant difference between the 2 groups ($p<0.05$) and a significant

time interaction ($p<0.01$). When examined by group there was no significant correlation ($p>0.05$) between egg counts and LD_{50}. There was no significant correlation's ($p>0.05$) between LD_{50} values and the mean proportion L3 in controls except in sheep 4 with levamisole where the correlation was –0.6032 ($p=0.02$).This result may have been due to chance given the number of correlations examined.

The COD (r^2) values are as shown in Appendix VII. The mean was 0.98 (0.89-0.99); 0.98 (0.87-0.99); and 0.98 (0.89-0.99) for IVM, AVMB2 and LEV respectively. In general all these respective curves showed good fits with the data and little variation between anthelmintics.

During the experimental period only *T. colubriformis* larvae were seen in the samples. However, when 2 sheep from the single infection group were killed 140 DPI to confirm the presence of worms in the gut, some 30 *H. contortus* in the abomasum were seen in each sheep but this may be due to late contamination from the hay. Also larval cultures from faeces collected 142 DPI from the remaining 4 sheep showed that out of 212 third stage infective larvae identified, 67 (32 %) were *H. contortus* and the remaining 145 (67 %) were *T. colubriformis*.

4.2.8 **Discussion**

The LD_{50} values of group 2.2 given a single infection for the three anthelmintics all generally followed the same trend. There was a tendency with all three athelmintics for the LD_{50} values to fall slightly from 21 DPI to 35 DPI, then increase to starting values by about 42 DPI and continue to a peak value on 49 and 56 DPI, and then decline steadily to low values on 77-84 DPI similar or slightly lower than the starting value on 21 DPI. They then remained at or about

this low level for the remainder of the experiment There was an unexpected peculiar sharp fall on 63 DPI as described in section 4.2.7. Although this may have been due to some change in nutrition, the formula of the nut ration did not change over the course of Experiment I or II. How this could affect the sensitivity of nematode eggs is also difficult to explain.

With all three anthelmintics, the LD_{50} values of the trickle-infected sheep (group 2.1) generally followed those of group 2.2 except between 42-77 DPI. During this period they generally remained at a constant level although with AVMB2 they declined to final steady levels by 63 DPI, which was somewhat earlier than with the other two anthelmintics. It was hypothesised that the trickle infection would mean that there would be a relatively constant ratio of different ages of *T. colubriformis* throughout the experiment and that this would mean the LD_{50s} would initially parallel those of the single infection but would not show the dramatic rise and fall seen with these animals from 42-84 DPI. Rather they would rise slightly and then remain at a constant, slightly elevated value for the remainder of the experiment.

The absence of a dramatic rise is consistent with this hypothesis but the decline to low levels again from 84 DPI is not. The original hypothesis did not take account of the developing immune response and its impact on the dynamics of *T. colubriformis* in the sheep, particularly as those in this experiment were more than six months of age and thus able to develop an immune response (Dobson *et al.*,1990b).

The FEC of Sheep 5 and 6 (S5, S6) group 2.1 started at low levels, increased and then fell again 84 DPI and continued at low levels until the end of the experiment with the exception of sheep 4 (S4). Those of sheep1 and 2 (S1,

S2) group 2.2 also started at low levels (low infection rate), rose and then fell again except sheep 3 (S3) which fell later on 98 DPI. This rise and fall is consistent with the results reported by Dobson *et al.*,(1990a) and is thought to be due to the fact that at this time there was no further worm establishment in the host.

Generally the mean range of COD (r^2) values was 0.98 indicating the goodness of fit of the sigmoid curves was good.

Borgsteede and Couwenberg (1987) were unable to give a reason for the rise and fall of the LD_{50} values of an egg hatch assay TBZ with *H. contortus*. The results of these experiments are also unable to provide a solution to this potential problem. More research needs to be carried in future to further investigate this problem.

These results demonstrate that this change in LD_{50} values is a general phenomenon in trichostrongylid nematodes with all anthelmintics as it has been seen with egg-hatch assay for BZs with *H. contortus* and with BZs, LEV and IVM for *O. circumcincta* in a larval development assay.

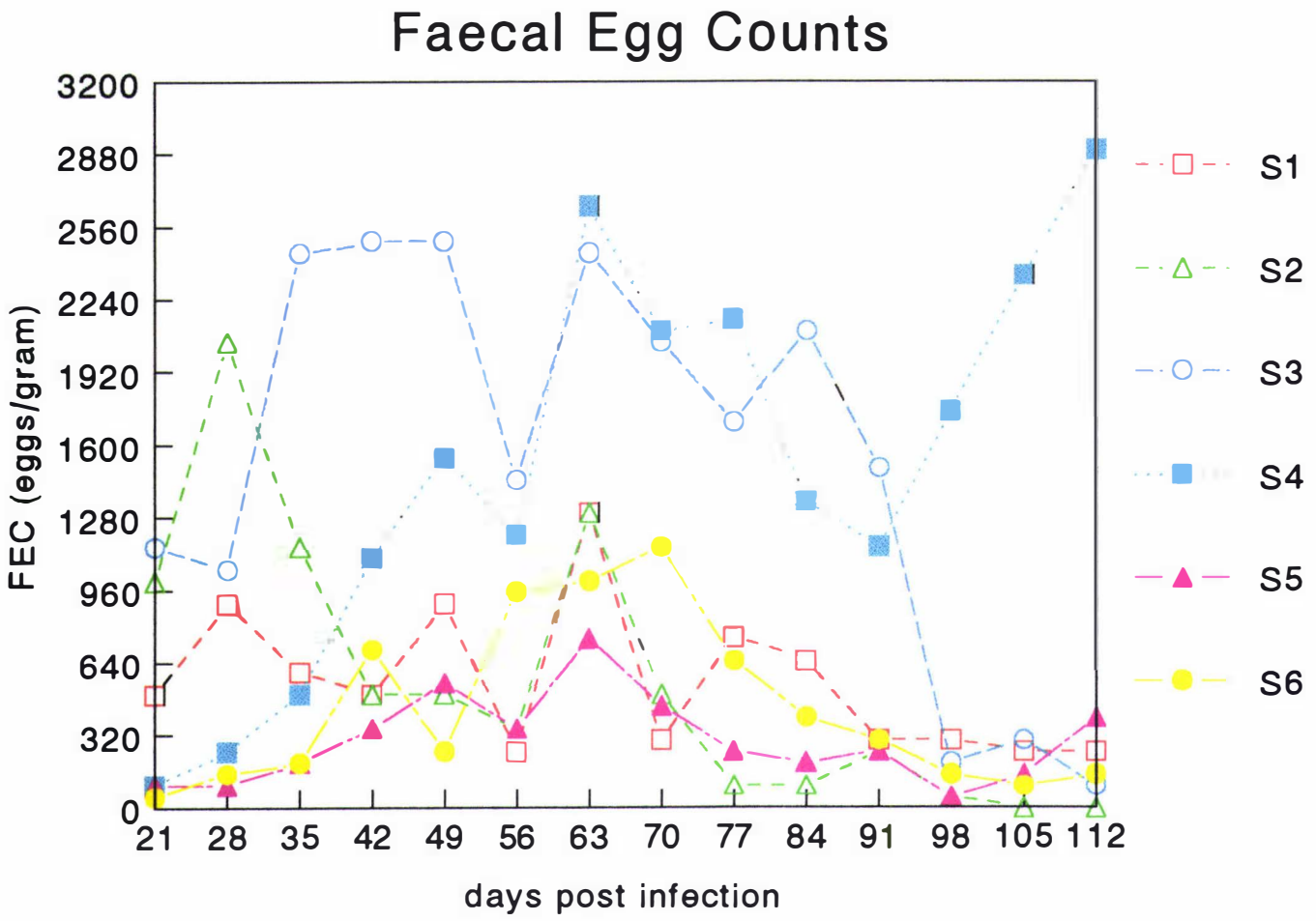

Fig. 4.1 FEC (epg) values for 3 lambs (S1, S2, S3) infected with 22000 L3 on Day 0 and 3 lambs (S4, S5, S6) infected with 2000 L3/week from Day 0.

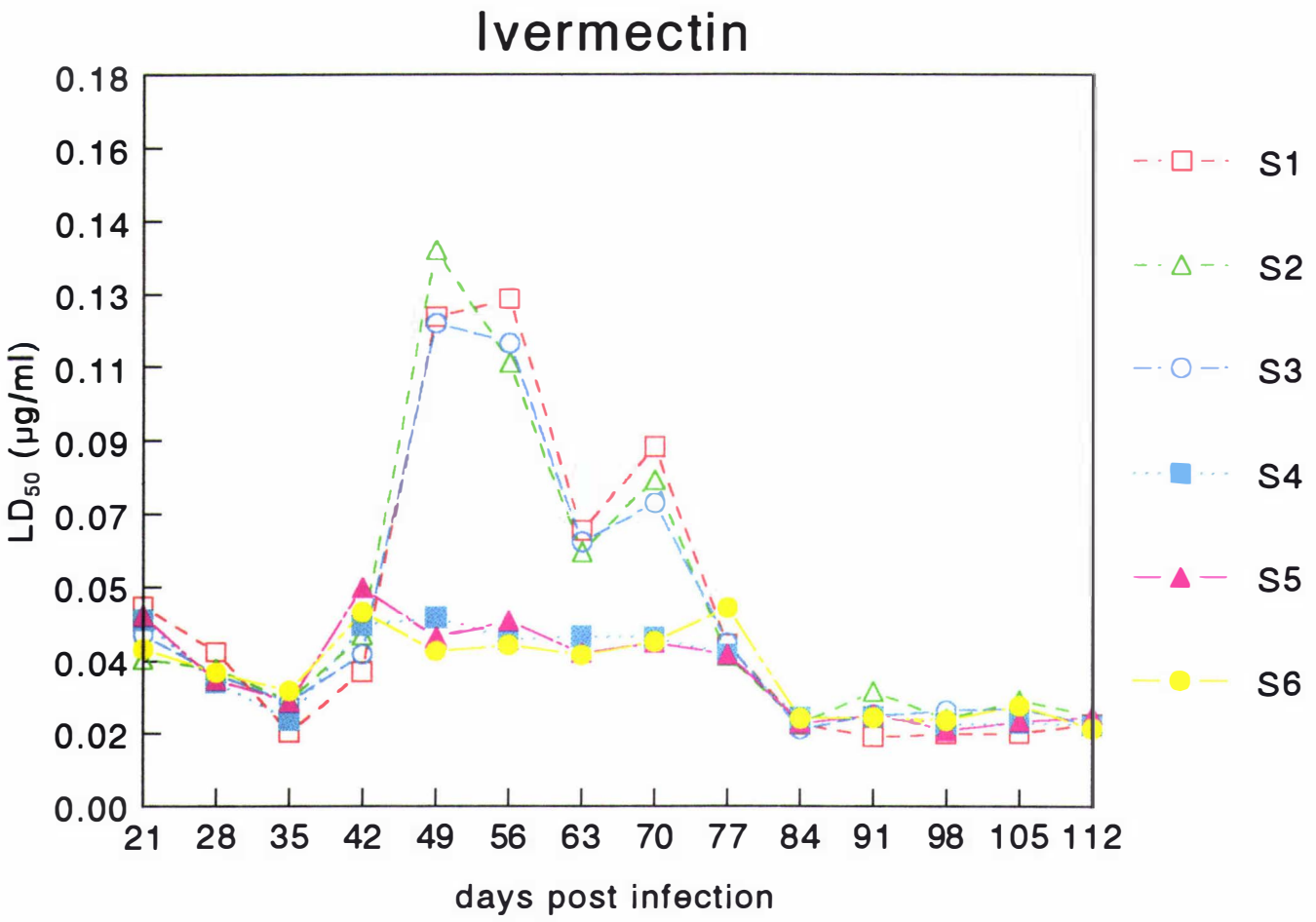

Fig. 4.2 LD_{50} values of ivermectin (µg/ml) for 3 lambs (S1, S2, S3) infected with 22000 L3 on Day 0 and 3 lambs (S4, S5, S6) infected with 2000 L3/week from Day 0.

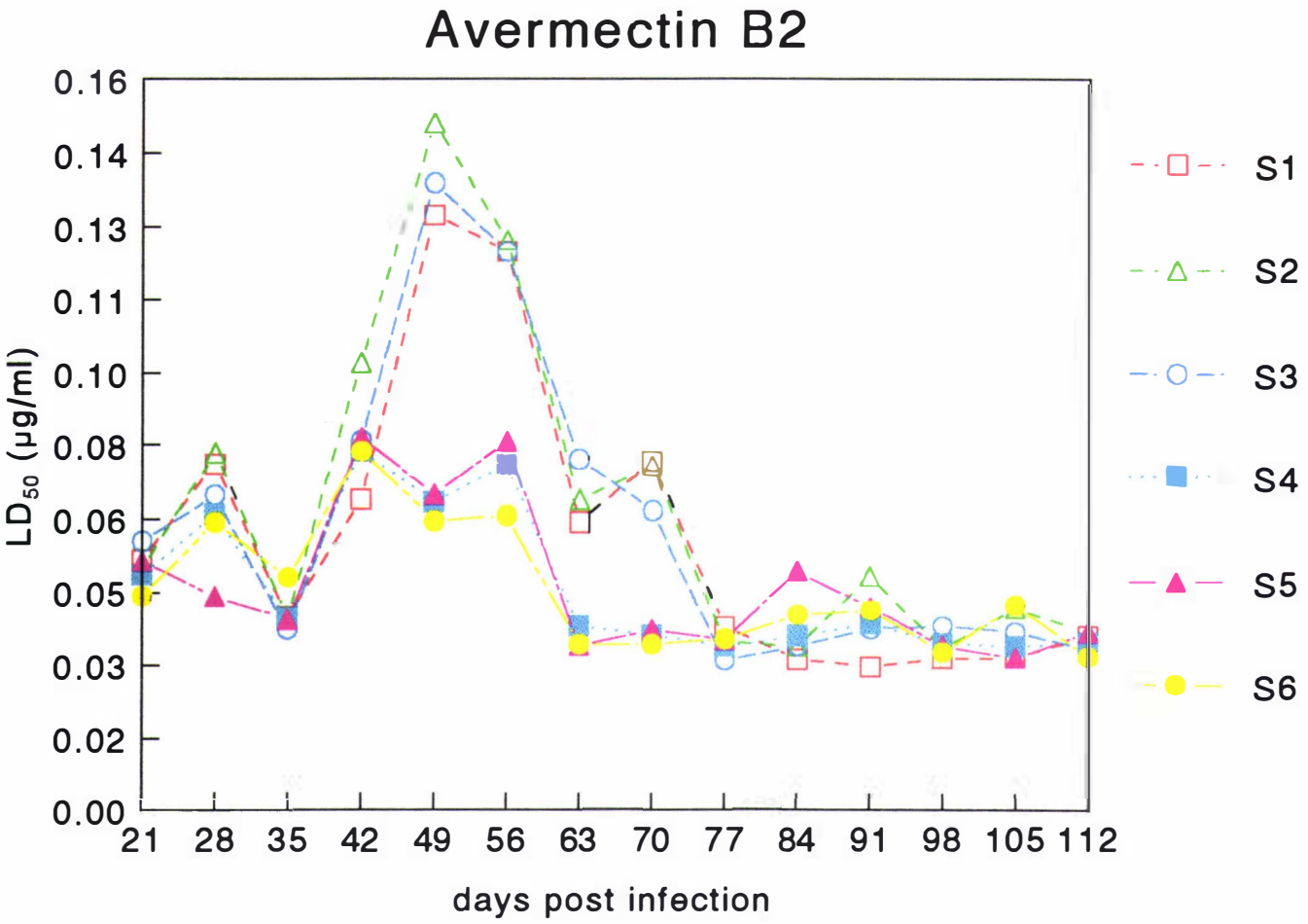

Fig. 4.3 LD_{50} values of avermectin B2 (µg/ml) for 3 lambs (S1, S2, S3) infected with 22000 L3 on Day 0 and 3 lambs (S4, S5, S6) infected with 2000 L3/week from Day 0.

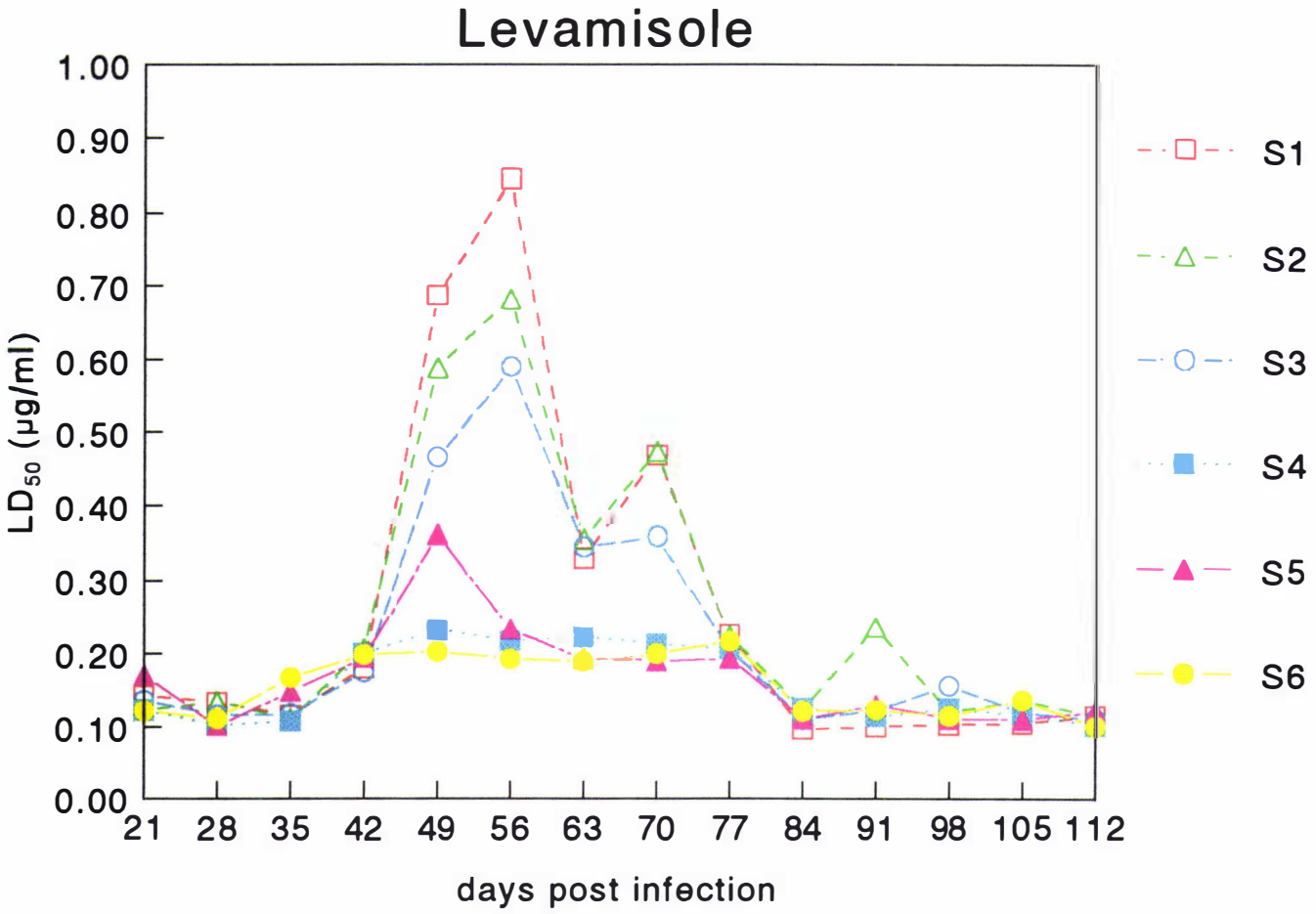

Fig. 4.4 LD_{50} values of levamisole hydrochloride (µg/ml) for 3 lambs (S1, S2, S3) infected with 22000 L3 on Day 0 and 3 lambs (S4, S5, S6) infected with 2000 L3/week from Day 0.

CHAPTER 5

5.0 EXPERIMENT III.

5.1 Introduction.

As a consequence of finding a small burden of *H. contortus* in the sheep slaughtered after Experiment II it was decided to investigate the LD_{50} values of *H. contortus*. This would allow comparison of LD_{50} values of *H. contortus* with those of *T. colubriformis* to determine if they may have influenced the results of Experiment II.

5.2 Materials and Methods.

5.2.1 Animals.

Fresh faecal samples were obtained from three month old field-reared Romney lambs given a single infection of 3000 infective third stage (L3) larvae of *H. contortus*. The samples were collected once weekly on 28, 35 and 42 DPI. The egg counts results were not recorded.

5.2.2 Egg Recovery Procedure.

This was as described in section 3.2.3.

5.2.3 The Larval Development Assay.

This was as described in section 3.2.4. Only two athelmintics were used i.e. ivermectin and levamisole.

5.2.4 **Parasitological Procedure.**

They were as described in section 3.2.5.

5.2.5 **Statistical Analysis.**

LD_{50} values were estimated as described in section 3.2.6.

5.2.6 **Results.**

When expressed as the concentration of anthelmintic in water the LD_{50} values obtained for levamisole ranged from 0.43 to 0.46μg/ml and those of ivermectin (in DMSO) ranged from 0.011 to 0.012μg/ml on 28 , 35 and 42 DPI. The mean COD (r^2) values for goodness of fit for the sigmoid curve for IVM and LEV were 0.99. The actual values are as shown on Table 5.1.

Table 5.1 LD_{50} and COD (r^2) values for IVM and LEV with *H. contortus* by day.

Anthelmintic	**Day**		
	28	35	42
Ivermectin(μg/ml)	0.011	0.012	0.012
COD (r^2)	0.993	0.991	0.996
Levamisole (μg/ml)	0.42	0.45	0.45
COD (r^2)	0.994	0.995	0.989

5.2.7 **Discussion.**

The LD_{50} values with both IVM and LEV were similar at the three sampling times. They were different from those for *T.colubriformis* on these days. The LD_{50} values for IVM obtained with *T. colubriformis* ranged from 0.04 to 0.05μg/ml whereas those with *H. contortus* were 0.01μg/ml which was lower than those of Experiment II. The LD_{50} values for levamisole with *T. colubriformis* were 0.13μg/ml and those with *H. contortus* were about 0.43μg/ml which were higher than those of Experiment II.

These results cannot give a clear explanation as to the relevance of the *H. contortus* found at the end of Experiment II. However, since during the time of Experiment II there were no *H. contortus* isolated in the control wells, it is suggested that those few *H. contortus* found in the abomasum of the two sheep killed 140 DPI were due to a late contamination from the hay.

CHAPTER 6.

6.0 GENERAL DISCUSSION.

Previous reports (Borgsteede and Couwenberg,1987; Amarante *et al.*,1997) have shown that the LD_{50} values in *in vitro* assays change with age of the infection. This is regardless of the anthelmintics used. They tend to increase to a peak and then decline again. This peak value has been shown to occur at about 50-60 DPI as seen in Table 6.1 below.

Table 6.1 Time of Peak in LD_{50} values.

Nematode species	Peak Day
T. colubriformis with IVM	70 DPI Experiment I
T. colubriformis with IVM; AVMB2; LEV	49-56 DPI Experiment II
H. contortus with TBZ	50 DPI Borgsteede & Couwenberg,1987.
O. circumcincta with TBZ; IVM; LEV	52 DPI Amarante *et al.*,1997

Although some differences in LD_{50} values were observed throughout the patent period of infection in Experiments I and II, a similar pattern was still apparent (Figs. 3.3, 4.2, 4.3, 4.4) in that there was an initial period when the LD_{50} values were relatively constant before they rose to a peak and then declined to starting values at the same rate as they increased. They then remained at those starting values for the remainder of both experiments. The factors which may be responsible for this change in LD_{50} values include the population dynamics of the parasites and the host's immune response.

In Experiment I, the host sheep were 3-6 months old which was too young for a solid immunity to become established (Dobson *et al.*,1990c). However,

given that they had been field-reared, it is reasonable to suggest that some immunity was developing during the experiment and possibly some before the experiment started. It is, however, not clear as to why the LD_{50} peaked at 70 DPI which was 20 days later than expected based on the previous reports and subsequently, in Experiment II. Although the sheep in Experiment II and in the report by Borgsteede and Couwenberg (1987) were nine months old, those used by Amarante *et al.*,(1997) were of similar age to those of Experiment I. So a mature immune response does not appear to be required for this change to occur. The non-steroid treated sheep had a 4-fold increase in the LD_{50} values. In contrast, the steroid treated sheep had a 5-fold decrease in the LD_{50} values without any further increase or decrease until the end of the experiment. However, the mechanism by which the steroids express their effects is not yet understood.

The size of the increase in LD_{50} values was generally similar for all drugs in Experiment I and II ranging from 2.5x for AVMB2 in Experiment II to 4-7x for LEV in the same experiment. In previous reports (Borgsteede and Couwenberg,1987; Amarante *et al.*,1997) with different nematodes and anthelmintics in an egg-hatch assay and larval development assay was generally less being about a 2-fold increase.

Experiment II was designed to see if the phenomenon of a change in LD_{50} value with time would occur with continuing infection as occurs in the field. The hypothesis was that the LD_{50} values of the trickle-infected animals would rise to a much smaller degree at about the same time as those of single infection group, but

they would remain at this slightly elevated level for the remainder of the experiment as the infection would comprise nematodes of varying ages.

The LD_{50} values for the trickle infection group were initially similar and rose in time with the values for the single infection group but only by a factor of 1.5 which is consistent with the hypothesis. However, they also declined with the single infection group to starting values. This decline was not expected and it was contrary to our starting hypothesis. One explanation for the fall in LD_{50} values may be that after the initial establishment of worms, the immune response developed and most of the newly infected larvae were rejected by the host immune response instead of becoming established, implying that the worm burden was almost of uniform age composition which is consistent with the findings reported by Dobson *et al*,(1990a). However, if this is true, it does not explain why the LD_{50} values only rose by a factor of 1-1.5 rather than the 2.5-7x increase seen in the single infection groups. The influence of 42-70 day old nematodes may have been expected to have a greater effect on the LD_{50} values. If age of the parasites is involved, this indicates that mature worms produce more resistant eggs, implying that maybe their receptors bind less drug. However, given their different modes of action why then should this phenomenon occur in all anthelmintics? Surely this is still difficult to explain.

The LD_{50} values of *T. colubriformis* compared with *H. contortus* and *O. circumcincta* obtained in Experiments I, II, III and those published by others are shown in Table 6.2. All values for Experiments I, II, and III are concentrations in the agar and thus been corrected by a factor of 50. For some other reports in

Table 6.2 particularly those with the drug in agar gel it is not always clear what the concentration refers to but it is assumed to be in the agar or the total liquid volume.

Table 6.2 The LD_{50} values of *T. colubriformis*, *H. contortus* and *O. circumcincta*.

Nematode species	**Drug concentration (μg/ml)**			**Author/Expt.**
	LEV	IVM	AVMB2	
T. colubriformis	0.127	0.00056-0.0015		Lacey *et al.*,1990
		0.0002-0.004		Experiment I
	0.0026-0.018	0.0008-0.0028	0.0012-0.003	Experiment II
O. circumcincta	0.08	0.0018		Lacey *et al.*,1990
	0.102	0.007		Taylor,1990
	0.006-0.016	0.00024-0.0006		Amarante *et al.*,1997
H. contortus	0.00084-0.0092	0.0002-0.00024		Experiment III
	0.14	0.0004		Lacey *et al.*,1990
	0.5	0.0001		Taylor,1990
		0.0005-0.001	0.0008-0.00114	Gill *et al.*,1995
		0.0004		Hubert & Kerboeuf,1992

The LD_{50} values for LEV with *T. colubriformis* were not consistent to those reported by Lacey *et al.*,(1990) whilst those for IVM were although there was no indication of the age of the infection involved. The LD_{50} values for IVM with *H. contortus* showed some consistency to those reported by Lacey *et al.*,(1990); Hubert and Kerboeuf,(1992); Gill *et al.*,(1995) even though there was no indication of the age of the infection involved as well. However, it seems to be

difficult to make a comparison of the LD_{50} values obtained from larval development assays from different laboratories.

6.1 **CONCLUSION**.

These results suggest three factors which may be involved in the rise and fall of the LD_{50} values. (1) probably the immune response of the host is involved. However, the mechanism and the way it causes the rise and fall in the LD_{50} values is unclear. (2) maybe the population dynamics of the worms in the host is involved. In particular, the age of the parasites, with mature nematodes in some way being more resilient to the effects of anthelmintics rather than very young or slightly older nematodes. (3) a combination of the first two in some way acting in sequence.

These results have demonstrated that the phenomenon of having a rise and fall at about 50-60 DPI is apparent in *T. colubriformis* as with the other trichostrongylid nematodes investigated. They also demonstrate that the phenomenon is a general one with all anthelmintics. It is also apparent there are some variations in the LD_{50} values between different laboratories.

Appendix I

The McMaster Egg Count Technique.

Equipment.

McMaster counting slide - Olympic Equine ; grid size 1cm^2 ,volume under each grid 0.15ml. Small stainless bowl; size 9.2 x 3.5cm, volume 100ml.

Microscope (Olympus).

Pasteur's pipette.

Domestic strainer 1mm aperture, 6cm diameter.

Electronic balance (Delta Range^R USA) 0.1g.

Domestic plastic tea spoon.

Universal bottle volume 28ml.

Saturated NaCl spesific gravity 1.2.

Procedure.

1. Weigh out 2g faeces into sieve within bowl.
2. Add 28 ml of saturated salt solution and work faeces through them until thoroughly broken down, press out solution through sieve then discard sieve and residue.
3. Whilst mixing remove 2 samples with a pasteur pipette and fill both chambers of a McMaster slide.
4. Leave slide 2 minutes before counting to allow eggs to float to the surface.
5. Count eggs within grid and both sides (chambers) , multiply by 50 to get egg per gram.

Appendix II (1.0)

Faecal egg count by day by sheep.

DAY	S1	S2	S3	S4	S5	S6
21	1750	1250	2200	2450	3650	1350
28	2950	1700	1550	2750	1600	500
35	800	1250	1800	1450	1500	350
42	1500	2100	2000	2050	1400	400
49	1850	3150	2000	1900	1500	3000
56	1250	1950	1600	1950	1300	350
63	1250	2050	2750	1750	1800	150
70	1600	2400	1950	1250	1100	100
77	600	400	1600	450	200	150
84	2750	2500	2400	1650	1900	100
91	2800	1800	2350	1050	3400	100
98	1950	2200	3400	1700	1550	50
105	1350	2100	1600	1100	1300	100
112	1450	3200	2200	950	1950	50

Key:(S1, S2, S3=Group 1.1); (S4, S5, S6=Group 1.2).

Appendix II (2.0)

Faecal egg count by day by sheep.

DAY	S1	S2	S3	S4	S5	S6
21	500	1000	1150	100	100	50
28	900	2050	1050	250	100	150
35	600	1150	2450	500	200	200
42	500	500	2500	1100	350	700
49	900	500	2500	1550	550	250
56	250	350	1450	1200	350	950
63	1300	1300	2450	2650	750	1000
70	300	500	2050	2100	450	1150
77	750	100	1700	2150	250	650
84	650	100	2150	1350	200	400
91	300	250	1500	1150	250	300
98	300	50	200	1750	50	150
105	250	0	300	2350	150	100
112	250	0	100	2900	400	150

Key:(S1, S2, S3=Group 2.2); (S4, S5, S6=Group 2.1).

Appendix III.

Larval Culture.

Equipment.

Pasteur pipette,

Glass jars, or Agee with lids,

Incubator set at 26°C,

Vermiculite to retain moisture and air in the culture jars.

Glass or plastic funnel with rubber tubing attached and closed by a clip,

Stand for funnel,

Fine gauze or wire mesh or fine paper tissue.

Glass slides (grooved) and coverslips,

Graduated cylinders volume 500 ml,

Conical flask volume 100 ml; Laboratory counter (Clay Adams).

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

Faeces 10 g or more and Water.

Procedure.

1.Break up the faeces and mix with vermiculite (not less than 20 % the volume of faeces) and water, aiming for a mixture that is just wet enough to stick together, but not 'muddy'.

2. pack the mixture loosely into glass jars, leaving a large air space at the top.

3. Screw on the lids leaving a slight air-leak.

4. Incubate at 26°C for 10 days.

5. Add water as needed to ensure that culture do not dry out.

6. Recover larvae by the Baermann technique as follows;

i) Close clip on rubber tubing.

ii) Fill funnel to within 2 cm of the top with water.

iii) Place culture on gauze/ wire mesh with a layer of tissue underneath the faecal material so that they are just submerged in the water.

iv) Leave to stand overnight (8-24 hour).

v) Carefully release clip and run off 100-150ml of water + larvae from the rubber tubing into a graduated cylinder. Fill up with water to 500ml level mark.

vi) Allow it to stand for 1-2 hours so that larvae concentrate on the bottom.

vii) Suck off supernatant and leave about 100-150ml.

viii) Pour the content into a conical flask and allow it to stand for 1 hour.

ix) Using a pasteur's pipette take a sample from the bottom of the conical flask and put on a grooved counting microscope slide, put a drop of lugol's iodine solution and cover with coverslip.

x) Put under microscope and count samples under 10x magnification.

Appendix IV

Experiment 1 Results:

LD_{50} Values of Ivermectin (ug/ml) by animal by day

Day	S1	S2	S3	S4	S5	S6
21	0.048	0.044	0.05	0.037	0.039	0.044
28	ND	ND	ND	ND	ND	ND
35	0.049	0.049	0.039	0.046	0.045	0.049
42	0.004	0.004	0.007	0.058	0.03	0.03
49	0.003	0.013	0.013	0.05	0.027	0.037
56	0.007	0.008	0.006	0.049	0.59	0.058
63	0.007	0.009	0.008	0.118	0.116	0.12
70	0.008	0.008	0.01	0.201	0.19	0.204
77	0.006	0.007	0.007	0.1	0.1	0.115
84	0.007	0.008	0.009	0.055	0.052	0.057
91	0.008	0.008	0.009	0.045	0.049	0.059
98	0.009	0.009	0.009	0.051	0.051	0.053
105	0.009	0.01	0.011	0.048	0.058	0.058
112	0.011	0.009	0.01	0.06	0.061	0.063

Key:(S1, S2, S3=GR.1.1; S4, S5, S6=GR.1.2).

Experiment II Results:

LD_{50} Values of Ivermectin (ug/ml)

Day	S1	S2	S3	S4	S5	S6
21	0.049	0.036	0.043	0.046	0.047	0.039
28	0.038	0.034	0.033	0.031	0.031	0.033
35	0.018	0.026	0.026	0.021	0.026	0.029
42	0.033	0.043	0.038	0.045	0.054	0.048
49	0.121	0.137	0.119	0.047	0.042	0.038
56	0.125	0.109	0.114	0.042	0.046	0.04
63	0.068	0.063	0.065	0.042	0.038	0.038
70	0.088	0.081	0.075	0.042	0.041	0.04
77	0.04	0.037	0.041	0.038	0.038	0.049
84	0.02	0.021	0.019	0.022	0.021	0.021
91	0.018	0.029	0.023	0.022	0.023	0.022
98	0.018	0.022	0.024	0.02	0.019	0.021
105	0.018	0.026	0.024	0.021	0.021	0.025
112	0.021	0.023	0.023	0.02	0.022	0.019

Key:(S1,S2, S3=GR.2.2; S4, S5, S6=GR.2.1).

Experiment II Results:

LD_{50} Values of Avermectin (ug/ml)

Day	S1	S2	S3	S4	S5	S6
21	0.055	0.053	0.059	0.052	0.055	0.047
28	0.076	0.078	0.069	0.065	0.047	0.063
35	0.043	0.043	0.04	0.042	0.042	0.051
42	0.068	0.098	0.081	0.078	0.081	0.078
49	0.131	0.151	0.138	0.067	0.069	0.063
56	0.123	0.125	0.123	0.075	0.081	0.064
63	0.063	0.068	0.077	0.041	0.036	0.036
70	0.076	0.075	0.066	0.038	0.039	0.037
77	0.041	0.037	0.033	0.036	0.037	0.037
84	0.033	0.036	0.036	0.038	0.052	0.043
91	0.032	0.052	0.04	0.041	0.044	0.044
98	0.034	0.036	0.041	0.036	0.036	0.035
105	0.034	0.045	0.039	0.036	0.033	0.045
112	0.039	0.039	0.036	0.037	0.039	0.034

LD_{50} Values of Levamisole (ug/ml)

Day	S1	S2	S3	S4	S5	S6
21	0.143	0.123	0.136	0.123	0.171	0.122
28	0.134	0.135	0.116	0.103	0.126	0.112
35	0.116	0.111	0.118	0.108	0.148	0.167
42	0.18	0.206	0.116	0.2	0.195	0.199
49	0.69	0.59	0.465	0.233	0.361	0.203
56	0.85	0.68	0.589	0.217	0.233	0.193
63	0.33	0.355	0.345	0.223	0.192	0.189
70	0.47	0.47	0.358	0.213	0.189	0.199
77	0.23	0.22	0.206	0.205	0.193	0.217
84	0.1	0.125	0.11	0.126	0.112	0.122
91	0.101	0.235	0.123	0.114	0.129	0.123
98	0.104	0.121	0.157	0.125	0.112	0.115
105	0.106	0.136	0.119	0.115	0.111	0.137
112	0.116	0.115	0.111	0.103	0.122	0.102

Appendix V.

The Larval Development Assay.

Equipment.

Electronic balance (Delta Range^{R} USA) 0.1g, measuring cylinders, beakers-volume 250 to 500ml, plastic test tubes (Falcon) volume 50ml; Gilson 20µl, 200µl, 1000µl and 5000µl pipettes; multidose pipette (Multipette Plus); 2µl Nichiryo model 5000 pipette; grooved microscope slides, cover slips and 96-well (Nunclon^{MT}) round flat bottomed microtitre plates-volume of well 300µl. Desiccator (to incubate samples); electric shaker and heater; squeeze bottles-volume 250ml; sieves-1mm aperture diameter 12cm, 20µm aperture diameter 10.2cm, 60µm aperture diameter 7.5cm and 100µm aperture diameter10.2cm. Microscope (Olympus); Centrifuge (ILEC Centra-8 International Equipment Company) and Laboratory counter (Clay Adams).

Chemicals.

Anthelmintics.

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

0.08 % Ivermectin Drench = 0.8g/litre i.e. 0.8mg/ml i.e. 800µg/ml.

Therefore 1ml of 800ug/ml + 33.3 Dimethyl sulphoxide (DMSO) gives 24µg/ml stock solution. 2µl of 24µg/ml in 100µl of agar = 2x24/100 = 0.48µg/ml.

Then 1ml of 24µg/ml + 1ml DMSO = 12µg/ml = 0.24µg/ml in agar

1ml of 12µg/ml + 1ml DMSO = 6µg/ml = 0.12µg/ml in agar

1ml of 6μg/ml + 1ml DMSO = 3μg/ml = 0.6μg/ml in agar

1ml of 3μg/ml + 1ml DMSO = 1.5μg/ml = 0.3μg/ml in agar

1ml of 1.5μg/ml + 1ml DMSO = 0.75μg/ml = 0.015μg/ml in agar

1ml of 0.75μg/ml + 1ml DMSO = 0.375μg/ml = 0.0075μg/ml in agar

1ml of 0.375μg/ml + 1ml DMSO = 0.1875μg/ml = 0.0038μg/ml in agar

1ml of 0.1875μg/ml + 1ml DMSO =0.09375μg/ml = 0.0019μg/ml in agar

1ml of 0.09375μg/ml + 1ml DMSO = 0.046875μg/ml = 0.00094μg/ml in agar

1ml of 0.046875μg/ml + 1ml DMSO = 0.02343μg/ml = 0.00047μg/ml in agar

1ml of 0.02343μg/ml + 1ml DMSO = 0.01172μg/ml = 0.000234μg/ml in agar

1ml of 0.01175μg/ml + 1ml DMSO = 0.005859μg/ml = 0.000117μg/ml in agar

1ml of 0.005859μg/ml + 1ml DMSO = 0.002929μg/ml = 0.00006μg/ml in agar

1ml of 0.002929μg/ml + 1ml DMSO = 0.001465μg/ml = 0.00003μg/ml in agar

1ml of 0.001465μg/ml + 1ml DMSO = 0.0007325μg/ml = 0.000015μg/ml in agar

AvermectinB2 (Kindly donated by MSDAgVet, New Zealand Ltd.)

Molecular weight 875g

1 molar = 875g in 1000ml i.e. 875g x 10^6 in 1000ml or 875g x 10^3 in 1ml

1μM = 0.875μg/ml

50μM = 0.875 x 50 = 43.75μg/ml,

50μl 43.75 x 50/1000 = 2.19mg/ml (concentration)

Then 2.19mg + 50ml DMSO = 43.75μg/ml and 2μl in 100υl of agar =0.875μg/ml

Then 2.19mg + 50ml DMSO = 43.75µg/ml 2µl in 100µl of agar = 0.875µg/ml

1ml of 43.75µg/ml + 1ml DMSO = 21.9µg/ml 0.437µg/ml

1ml of 21.9µg/ml + 1ml DMSO = 10.95µg/ml 0.214µg/ml

1ml of 10.95µg/ml + 1ml DMSO = 5.475µg/ml 0.109µg/ml

1ml of 5.475µg/ml + 1 ml DMSO = 2.7375µg/ml 0.0546µg/ml

1ml of 2.7375 ıg/ml + 1ml DMSO = 1.3688µg/ml 0.027µg/ml

1ml of 1.3688µg/ml + 1ml DMSO = 0.6844µg/ml 0.0136µg/ml

1 ml of 0.6844µg/ml + 1ml DMSO = 0.3422µg/ml 0.0068µg/ml

1ml of 0.3422µg/ml +1ml DMSO = 0.1711µg/ml 0.0034 ıg/ml

1ml of 0.1711µg/ml + 1ml DMSO = 0.0855µg/ml 0.0017µg/ml

1ml of 0.0855µg/ml + 1 ml DMSO = 0.0428µg/ml 0.00085µg/ml

1ml of 0.0428µg/ml + 1 ml DMSO = 0.0214µg/ml 0.00042µg/ml

1ml of 0.0214µg/ml + 1 ml DMSO = 0.0107µg/ml 0.00021µg/ml

1ml of 0.0107µg/ml + 1ml DMSO = 0.00535µg/ml 0.00011µg/ml

1ml of 0.00535µg/ml + 1ml DMSO = 0.00267µg/ml 0.00005µg/ml

1ml of 0.00267µg/ml + 1ml DMSO = 0.001336µg/ml. 0.000026µg/ml

Levamisole hydrochloride (Rycozole^{R} , Young's Animal Health New Zealand Ltd.) Oral drench (2.58µg/ml concentration)

2µl drench = contain 129µl/ml

100 ıl of egg solution = 2 x 129/100 = 2.58µg/ml.

Therefore 250 ıl (Rycozole drench) + 77.5ml distilled H_2O = 129µg/ml stock solution. 2µl of 129µg/ml in 100 ıl of agar = 2.58µg/ml

1ml of 129µg/ml + 1ml H_2O = 64.5µg/ml	1.29µg/ml
1ml of 64.5µg/ml + 1 ml H_2O = 32.25µg/ml	0.645µg/ml
1ml of 32.25µg/ml + 1ml H_2O = 16.125µg/ml	0.32µg/ml
1ml of 16.125µg/ml + 1ml H_2O = 8.0625µg/ml	0.16µg/ml
1ml of 8.0625µg/ml + 1ml H_2O = 4.03µg/ml	0.081µg/ml
1ml of 4.03µg/ml + 1 ml H_2O = 2.015µg/ml	0.04µg/ml
1 ml of 2.015µg/ml + 1ml H_2O = 1.008µg/ml	0.02µg/ml
1ml of 1.008µg/ml + 1 ml H_2O = 0.5039µg/ml	0.01µg/ml
1ml of 0.5039µg/ml + 1ml H_2O = 0.252µg/ml	0.005µg/ml
1 ml of 0.252µg/ml + 1ml H_2O = 0.126µg/ml	0.0025µg/ml
1ml of 0.126µg/ml + 1ml H_2O = 0.063µg/ml	0.00125µg/ml
1ml of 0.063µg/ml + 1ml H_2O = 0.0314µg/ml	0.00063µg/ml
1ml of 0.0314µg/ml + 1ml H_2O = 0.01575µg/ml	0.00032µg/ml
1ml of 0.01575µg/ml + 1ml H_2O = 0.007879µg/ml	0.00016µg/ml
1ml of 0.007879µg/ml + 1ml H_2O = 0.003939µg/ml	0.00008µg/ml

Nutritive media.

1g yeast extract (Y-1000 Sigma) was added in 90 ml of 0.85 % saline solution (Oxoid). To the final solution was added 10ml of Earle's Balanced Salt solution (E7510,Sigma) which was put in the freezer until the prepared stock is used up.

E. coli suspension.

15mg of the lyophilised *E.coli* (Strain W (ATCC) 9637, Sigma) were added to 100ml of distilled water. The suspension was sterilized by autoclaving.

Amphotericin B solution.

25mg of amphotericin B solubilised (A-9525, Sigma) were dissolved in 100ml of distilled water.

2 % Agar matrix.

2g agar (Bacto-Agar; Y-1000 Sigma) were dissolved I 98 ml of distilled water by heating in a microwave for about 3 minutes.

20 % Magnesium sulfate solution.

100g of magnesium sulfate were dissolved in 500ml of water.

Lugol's iodine solution.

5 % iodine and 10 % potassium iodide in water.

2 % dimethylsulphoxide (DMSO) Sigma New Zealand Ltd.)

Procedure.

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

1. To recover nematode eggs from faces, about 50g of faeces (the amount depending on the egg count of the faeces under test) were added to approximately 200ml of water in a 500ml beaker.
2. The faeces were mixed until the faecal material was in suspension. The suspension was poured through a 1mm aperture sieve into a100μm sieve into a another beaker.
3. The suspension was then washed on a 20um aperture sieve which retained the eggs. The eggs were washed off the screen with a jet of water from a squeeze bottle and collected in a beaker.
4. The next step is to transfer the egg suspension to a plastic test tube which is filled to the 50ml mark level with water and centrifuged at 1500g for 10 minutes in order to concentrate the eggs.
5. The supernatant was discarded and the eggs were further cleaned from the organic debris by centrifugation in $MgSO_4$ (density 1.10) for 5 minutes at 1500g.
6. The supernatant was then filtered through a 60um aperture sieve and the eggs were collected and washed on a 20um aperture sieve. The eggs were then washed off the screen with a jet of water from a squeeze bottle and collected in a beaker. As lengthy exposure to $MgSO_4$ damages the eggs, it is important to complete this quickly (Hubert and Kerboeuf,1984).
7. The egg suspension was transferred to a graduated 50ml plastic test tube and allowed to settle. The supernatant was removed to reduce the volume to about 15ml and finally, the concentration of eggs was estimated in ten 20μl samples.

8. Each sample was counted under 10x magnification and the final volume of egg suspension was adjusted to approximately 1333 eggs/ml (80 eggs/60µl of suspension).

Preparation of plates.

A hundred (100)µl of hot 2 % agar matrix were mixed with 2µl of anthelmintic in the wells of microtitre plates. The control wells contained 2µl of DMSO (for ivermectin and avermectinB2) or distilled water (for levamisole) instead of anthelmintic.

Preparation of cultures.

The test was carried out in 96-well microtitre plates as described by Gill *et al*., 1995.

1. Mix 9ml of egg suspension + 3ml nutritive medium + 3ml *E. coli* suspension + 180µl amphotericin B.
2. A hundred (100)µl of this mixture is layered on top of agar matrix in the microtitre plate.
3. The plates were then incubated at 26°C for 7 days in a desiccator over water to ensure a high humidity.
4. The liquid media containing parasites were transferred to a glass slide by means of a pipette.
5. The number of eggs, first, second and third stage larvae in each well then counted after staining with Lugol's iodine solution
6. The number of third stage larvae in the test wells was adjusted by the proportion that developed in the control wells.

Appendix VI

Proportion of L3 by animal by day by mean controls.

1S1 Days post infection.

Conc.(ug/ ml) IVM	21	28	35	42	49	56	63	70	77	84	91	98	105	112
0.0007	0.97	ND	0.93	1.04	1.04	0.93	1.09	0.96	0.98	1.03	1.05	0.88	0.9	0.96
0.0015	0.94	ND	1.01	0.83	0.71	0.83	0.98	0.92	0.77	0.85	0.86	0.83	0.89	0.91
0.0029	0.91	ND	0.9	0.55	0.53	0.69	0.56	0.79	0.56	0.65	0.72	0.65	0.62	0.74
		ND	ND	0.54	0.43	0.56	0.73	0.69	0.62	0.64	0.69	0.63	0.63	0.75
		ND	ND	0.55	0.47	0.6	0.67	0.69	0.54	0.68	0.75	0.65	0.64	0.73
0.0059	0.84	ND	0.93	0.44	0.39	0.56	0.53	0.57	0.5	0.49	0.55	0.48	0.48	0.56
		ND	ND	0.36	0.4	0.49	0.57	0.51	0.5	0.48	0.56	0.48	0.49	0.57
		ND	ND	0.3	0.29	0.51	0.55	0.5	0.47	0.49	0.53	0.49	0.48	0.55
0.0117	0.8	ND	0.89	0.24	0.14	0.43	0.44	0.43	0.42	0.4	0.43	0.45	0.42	0.49
		ND	ND	0.21	0.07	0.41	0.42	0.4	0.35	0.46	0.44	0.42	0.39	0.5
		ND	ND	0.28	0.08	0.42	0.43	0.44	0.35	0.48	0.45	0.43	0.46	0.52
0.0234	0.66	ND	0.63	0.6	0	0.28	0.36	0.29	0.36	0.38	0.32	0.38	0.39	0.39
	0.57	ND	0.65	0.18	0	0.29	0.3	0.34	0.37	0.38	0.38	0.37	0.32	0.43
	0.63	ND	0.61	0.24	0	0.32	0.31	0.28	0.34	0.39	0.37	0.35	0.35	0.43
0.0469	0.52	ND	0.55	0.14	0	0.14	0.14	0.1	0.12	0.13	0.17	0.12	0.18	0.21
	0.49	ND	0.49	0	0	0.06	0.17	0.07	0.13	0.17	0.14	0.12	0.16	0.23
	0.54	ND	0.42	0.16	0	0	0.2	0.03	0	0.13	0.21	0.18	0.18	0.2
0.0938	0.38	ND	0.54	0	0	0	0	0	0	0.06	0.04	0	0.06	0.14
	0.42	ND	0.43	0.04	0	0	0	0	0	0.08	0	0	0.09	0.14
	0.42	ND	0.4	0.02	0	0	0	0	0	0.12	0	0	0.12	0.11
0.1875	0.05	ND	0.05	0	0	0	0	0	0	0	0	0	0	0
	0.01	ND	0.13	0	0	0	0	0	0	0	0	0	0	0
	0.06	ND	0	0	0	0	0	0	0	0	0	0	0	0
0.375	0.01	ND	0	0	0	0	0	0	0	0	0	0	0	0
0.75	0	ND	0	0	0	0	0	0	0	0	0	0	0	0
1.5	0	ND	0	0	0	0	0	0	0	0	0	0	0	0
3	0	ND	0	0	0	0	0	0	0	0	0	0	0	0
6	0	ND	0	0	0	0	0	0	0	0	0	0	0	0
12	0	ND	0	0	0	0	0	0	0	0	0	0	0	0
24	0	ND	0	0	0	0	0	0	0	0	0	0	0	0
Mean control	0.95	ND	0.91	0.9	0.88	0.93	0.89	0.91	0.97	0.97	0.89	0.9	0.91	0.87

Key:1S1 = Experiment I and Sheep 1 (S1) 2S1 = Experiment II and sheep 1 (S1).

1S2 Days post infection.

Conc.(ug/ ml) IVM	21	28	35	42	49	56	63	70	77	84	91	98	105	112
0.0007	0.98	ND	1.01	0.98	0.97	0.89	0.97	0.94	0.97	1.02	0.96	0.87	0.88	0.96
0.0015	0.95	ND	0.98	0.91	0.73	0.93	0.78	0.85	0.95	0.89	0.85	0.82	0.81	0.87
		ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	0.76	ND
		ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	0.76	ND
0.0029	1	ND	0.97	0.61	0.67	0.64	0.76	0.64	0.64	0.66	0.69	0.64	0.69	0.72
		ND	ND	0.54	0.62	0.67	0.6	0.66	0.74	0.71	0.75	0.62	0.62	0.7
		ND	ND	0.57	0.63	0.73	0.63	0.69	0.62	0.73	0.7	0.67	0.66	0.71
0.0059	0.84	ND	0.97	0.41	0.56	0.51	0.58	0.51	0.51	0.57	0.53	0.48	0.47	0.53
		ND	ND	0.34	0.53	0.52	0.58	0.47	0.52	0.54	0.52	0.48	0.49	0.53
		ND	ND	0.41	0.6	0.53	0.59	0.5	0.51	0.54	0.51	0.48	0.47	0.54
0.0117	0.8	ND	0.87	0.21	0.52	0.39	0.41	0.52	0.39	0.51	0.37	0.43	0.41	0.49
		ND	ND	0.28	0.56	0.42	0.44	0.4	0.36	0.42	0.46	0.45	0.43	0.51
		ND	ND	0.29	0.49	0.37	0.45	0.38	0.47	0.42	0.42	0.38	0.46	0.46
0.0234	0.62	ND	0.64	0.36	0.44	0.4	0.34	0.39	0.34	0.38	0.35	0.33	0.37	0.37
	0.58	ND	0.64	0.14	0.29	0.27	0.37	0.39	0.31	0.39	0.38	0.32	0.34	0.42
	0.55	ND	0.57	0.14	0.34	0.4	0.38	0.41	0.43	0.38	0.41	0	0.38	0.42
0.0469	0.52	ND	0.54	0.22	0	0.22	0.3	0.14	0.15	0.02	0.18	0.12	0.19	0.21
	0.51	ND	0.54	0.28	0	0.12	0.23	0.12	0.13	0.14	0.18	0.12	0.17	0.19
	0.41	ND	0.47	0.26	0	0.14	0.16	0.13	0.11	0.14	0.14	0.16	0.17	0.14
0.0938	0.39	ND	0.58	0.11	0	0.04	0	0.04	0	0.07	0	0	0.16	0.14
	0.38	ND	0.5	0.14	0	0.07	0	0.02	0	0.12	0	0	0.06	0.14
	0.58	ND	0.36	0.07	0	0.07	0	0.01	0	0.14	0	0	0.12	0.09
0.1875	0.04	ND	0.04	0	0	0	0	0	0	0	0	0	0	0
	0.03	ND	0.02	0	0	0	0	0	0	0	0	0	0	0
	0.06	ND	0	0	0	0	0	0	0	0	0	0	0	0
0.375	0.01	ND	0	0	0	0	0	0	0	0	0	0	0	0
0.75	0	ND	0	0	0	0	0	0	0	0	0	0	0	0
1.5	0	ND	0	0	0	0	0	0	0	0	0	0	0	0
3	0	ND	0	0	0	0	0	0	0	0	0	0	0	0
6	0	ND	0	0	0	0	0	0	0	0	0	0	0	0
12	0	ND	0	0	0	0	0	0	0	0	0	0	0	0
24	0	ND	0	0	0	0	0	0	0	0	0	0	0	0
Mean control	0.98	ND	0.92	0.91	0.87	0.93	0.87	0.94	0.93	0.9	0.92	0.9	0.87	0.89

1S3 Days post infection.

Conc.(ug/ ml) IVM	21	28	35	42	49	56	63	70	77	84	91	98	105	112
0.0007	0.95	ND	1	1.09	1.1	0.99	1.05	0.9	1.02	1.06	0.97	0.89	0.88	1.04
0.0015	0.99	ND	0.94	0.97	0.98	0.9	0.86	1.04	0.97	0.97	0.81	0.86	0.84	0.86
		ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	0.83	ND
		ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	0.84	ND
0.0029	0.89	ND	0.84	0.61	0.76	0.61	0.72	0.77	0.72	0.71	0.73	0.64	0.64	0.73
		ND	ND	0.54	0.77	0.53	0.66	0.81	0.72	0.71	0.74	0.64	0.72	0.7
		ND	ND	0.58	0.69	0.68	0.65	0.73	0.68	0.7	0.74	0.65	0.64	0.72
0.0059	0.79	ND	0.87	0.47	0.64	0.42	0.55	0.53	0.54	0.58	0.53	0.48	0.5	0.56
		ND	ND	0.3	0.56	0.52	0.57	0.51	0.52	0.53	0.52	0.48	0.49	0.53
		ND	ND	0.47	0.54	0.55	0.56	0.53	0.54	0.53	0.54	0.49	0.49	0.55
0.0117	0.78	ND	0.82	0.4	0.54	0.41	0.38	0.47	0.34	0.53	0.42	0.42	0.5	0.46
		ND	ND	0.58	0.55	0.42	0.41	0.4	0.36	0.49	0.47	0.44	0.45	0.51
		ND	ND	0.42	0.66	0.33	0.45	0.49	0.41	0.51	0.45	0.41	0.38	0.49
0.0234	0.77	ND	0.6	0.22	0.49	0.32	0.32	0.43	0.28	0.36	0.38	0.38	0.37	0.42
	0.58	ND	0.57	0.32	0.53	0.26	0.36	0.37	0.32	0.42	0.36	0.39	0.38	0.42
	0.67	ND	0.54	0.4	0.43	0.28	0.29	0.41	0.33	0.43	0.42	0.37	0.38	0.42
0.0469	0.52	ND	0.46	0.26	0.3	0.19	0.24	0.18	0.13	0.22	0.22	0.12	0.19	0.21
	0.51	ND	0.46	0.31	0.28	0.11	0.13	0.14	0.14	0.14	0.16	0.16	0.2	0.19
	0.48	ND	0.56	0.29	0.32	0.07	0.14	0	0.11	0.14	0.13	0.19	0.2	0.16
0.0938	0.4	ND	0.39	0.21	0	0.11	0	0.08	0.11	0.02	0	0	0.12	0.15
	0.45	ND	0.35	0	0	0.04	0	0.07	0.13	0	0	0	0.08	0.14
	0.37	ND	0.38	0	0	0.03	0	0	0	0	0	0	0.13	0.14
0.1875	0.01	ND	0.03	0	0	0	0	0	0	0	0	0	0	0
	0.03	ND	0	0	0	0	0	0	0	0	0	0	0	0
	0.01	ND	0.03	0	0	0	0	0.02	0	0	0	0	0	0
0.375	0.01	ND	0	0	0	0	0	0	0	0	0	0	0	0
0.75	0	ND	0.01	0	0	0	0	0	0	0	0	0	0	0
1.5	0	ND	0	0	0	0	0	0	0	0	0	0	0	0
3	0	ND	0	0	0	0	0	0	0	0	0	0	0	0
6	0	ND	0	0	0	0	0	0	0	0	0	0	0	0
12	0	ND	0	0	0	0	0	0	0	0	0	0	0	0
24	0	ND	0	0	0	0	0	0	0	0	0	0	0	0
Mean control	0.93	ND	0.96	0.85	0.87	0.93	0.89	0.89	0.91	0.89	0.91	0.91	0.9	0.89

1S4 Days post infection.

Conc.(ug/ ml) IVM	21	28	35	42	49	56	63	70	77	84	91	98	105	112
0.0007	0.97	ND	0.87	1.1	0.98	1	1.01	0.94	1.03	0.95	0.96	0.96	1.04	1.06
0.0015	0.96	ND	0.82	0.95	0.94	0.95	0.95	0.96	0.92	0.92	0.95	0.97	0.94	1
0.0029	0.97	ND	0.8	0.93	0.9	0.9	0.95	0.93	0.91	0.88	0.84	0.9	0.9	0.87
		ND	ND	ND	ND	ND	ND	ND	ND	ND	0.83	ND	ND	0.89
		ND	ND	ND	ND	ND	ND	ND	ND	ND	0.87	ND	ND	0.89
0.0059	0.89	ND	0.74	0.79	0.8	0.85	0.92	0.87	0.8	0.8	0.8	0.84	0.87	0.82
		ND	ND	ND	ND	ND	ND	ND	ND	ND	0.79	ND	ND	0.82
		ND	ND	ND	ND	ND	ND	ND	ND	ND	0.81	ND	ND	0.79
0.0117	0.83	ND	0.69	0.75	0.72	0.78	0.84	0.85	0.78	0.7	0.68	0.73	0.73	0.78
		ND	ND	ND	ND	ND	ND	ND	ND	0.74	0.68	0.71	0.71	0.78
		ND	ND	ND	ND	ND	ND	ND	ND	0.73	0.72	0.76	0.7	0.75
0.0234	0.6	ND	0.6	0.67	0.62	0.66	0.82	0.78	0.75	0.7	0.65	0.6	0.64	0.7
	0.59	ND	0.53	0.61	0.64	0.69	0.78	0.77	0.77	0.62	0.61	0.65	0.56	0.72
	0.56	ND	0.61	0.61	0.63	0.63	0.81	0.75	0.76	0.67	0.61	0.63	0.64	0.72
0.0469	0.39	ND	0.48	0.58	0.51	0.52	0.7	0.88	0.67	0.48	0.48	0.52	0.5	0.51
	0.52	ND	0.51	0.57	0.53	0.52	0.7	0.7	0.61	0.5	0.47	0.48	0.49	0.51
	0.49	ND	0.45	0.53	0.54	0.52	0.73	0.71	0.71	0.5	0.45	0.5	0.49	0.51
0.0938	0.4	ND	0.45	0.42	0.45	0.37	0.51	0.72	0.49	0.45	0.41	0.44	0.42	0.42
	0.28	ND	0.44	0.37	0.4	0.28	0.54	0.66	0.5	0.35	0.39	0.39	0.4	0.42
	0.14	ND	0.42	0.54	0.4	0.38	0.49	0.57	0.51	0.47	0.4	0.41	0.42	0.44
0.1875	0.06	ND	0.6	0.31	0.21	0.18	0.48	0.48	0.45	0.32	0.32	0.31	0.29	0.18
	0.02	ND	0	0.29	0.13	0.17	0.42	0.52	0.3	0.27	0.26	0.28	0.27	0.29
	0	ND	0.03	0.29	0.14	0.22	0.44	0.57	0.36	0.32	0.28	0.3	0.3	0.31
0.375	0	ND	0.03	0.14	0.02	0.07	0.28	0.41	0.31	0.19	0.13	0.14	0.11	0.17
		ND	0.03	0.15	0	0.17	0.16	0.16	0.27	0.13	0.16	0.13	0.14	0.14
		ND	0	0.19	0	0.14	0.24	0.42	0.21	0.19	0.11	0.12	0.14	0.14
0.75	0	ND	0	0.07	0	0	0.18	0.24	0.12	0.06	0.1	0.06	0.07	0.07
		ND	0	0.04	0	0	0.07	0.07	0.14	0.11	0.06	0.11	0.04	0.07
		ND	0	0.01	0	0	0.08	0.28	0.18	0.2	0.06	0.06	0.1	0.13
1.5	0	ND	0	0	0	0	0	0	0	0	0	0	0	0
3	0	ND	0	0	0	0	0	0	0	0	0	0	0	0
6	0	ND	0	0	0	0	0	0	0	0	0	0	0	0
12	0	ND	0	0	0	0	0	0	0	0	0	0	0	0
24	0	ND	0	0	0	0	0	0	0	0	0	0	0	0
Mean control	0.89	ND	0.9	0.89	0.89	0.91	0.87	0.88	0.88	0.93	0.95	0.92	0.9	0.9

1S5 Days post infection.

Conc.(ug/ ml) IVM	21	28	35	42	49	56	63	70	77	84	91	98	105	112
0.0007	0.99	ND	1.06	1.08	1	0.98	1.06	0.98	0.97	1	0.94	0.96	0.99	0.99
0.0015	1.02	ND	0.91	0.96	0.87	0.93	0.99	0.95	0.91	0.96	0.91	0.9	0.95	0.95
0.0029	0.99	ND	0.85	0.84	0.85	0.84	0.97	0.94	1	0.89	0.82	0.81	0.95	0.84
		ND	ND	ND	ND	ND	ND	ND	ND	ND	0.8	ND	ND	0.87
		ND	ND	ND	ND	ND	ND	ND	ND	ND	0.85	ND	ND	0.84
0.0059	1.02	ND	0.82	0.82	0.68	0.82	0.89	0.88	0.86	0.84	0.81	0.73	0.85	0.79
		ND	ND	ND	ND	ND	ND	ND	ND	ND	0.79	0.7	ND	0.78
		ND	ND	ND	ND	ND	ND	ND	ND	ND	0.78	0.73	ND	0.81
0.0117	0.92	ND	0.69	0.62	0.59	0.73	0.88	0.89	0.81	0.71	0.68	0.66	0.78	0.76
		ND	ND	ND	ND	ND	ND	ND	ND	0.72	0.7	0.61	0.79	0.73
		ND	ND	ND	ND	ND	ND	ND	ND	0.75	0.72	0.66	0.79	0.77
0.0234	0.48	ND	0.65	0.46	0.57	0.71	0.73	0.85	0.74	0.69	0.66	0.48	0.69	0.71
	0.57	ND	0.66	0.46	0.51	0.66	0.81	0.8	0.73	0.67	0.63	0.5	0.62	0.69
	0.51	ND	0.58	0.59	0.5	0.74	0.84	0.75	0.75	0.68	0.65	0.49	0.69	0.72
0.0469	0.56	ND	0.51	0.42	0.47	0.55	0.65	0.68	0.63	0.43	0.48	0.45	0.52	0.52
	0.5	ND	0.48	0.49	0.43	0.56	0.77	0.76	0.62	0.46	0.49	0.35	0.52	0.5
	0.56	ND	0.49	0.43	0.43	0.55	0.71	0.66	0.63	0.5	0.49	0.41	0.52	0.51
0.0938	0.29	ND	0.43	0.28	0.29	0.41	0.51	0.63	0.49	0.41	0.38	0.33	0.45	0.43
	0.29	ND	0.43	0.31	0.3	0.4	0.55	0.61	0.5	0.38	0.4	0.28	0.39	0.42
	0.32	ND	0.49	0.48	0.36	0.44	0.52	0.62	0.49	0.44	0.39	0.34	0.46	0.45
0.1875	0	ND	0.04	0.19	0.15	0.2	0.5	0.56	0.33	0.3	0.26	0.13	0.23	0.35
	0	ND	0	0.22	0.14	0.13	0.34	0.56	0.38	0.28	0.26	0.14	0.27	0.28
	0	ND	0.07	0.3	0.14	0.17	0.37	0.54	0.43	0.31	0.28	0.09	0.3	0.3
0.375	0	ND	0	0.07	0.08	0	0.33	0.45	0.32	0.14	0.12	0.07	0.12	0.16
	0	ND	0	0.12	0.05	0	0.15	0.36	0.22	0.14	0.13	0.04	0.15	0.14
	0	ND	0	0.1	0	0	0.21	0.39	0.23	0.15	0.13	0.11	0.16	0.13
0.75	0	ND	0	0	0	0	0.15	0.15	0.16	0.06	0.06	0	0.12	0.07
	0	ND	0	0	0	0	0.17	0.16	0.14	0.09	0.08	0	0.07	0.07
	0	ND	0	0	0	0	0.19	0.14	0.27	0.12	0.1	0	0.06	0.11
1.5	0	ND	0	0	0	0	0	0	0	0	0	0	0	0
3	0	ND	0	0	0	0	0	0	0	0	0	0	0	0
6	0	ND	0	0	0	0	0	0	0	0	0	0	0	0
12	0	ND	0	0	0	0	0	0	0	0	0	0	0	0
24	0	ND	0	0	0	0	0	0	0	0	0	0	0	0
Mean control	0.89	ND	0.9	0.89	0.89	0.91	0.87	0.88	0.88	0.93	0.95	0.92	0.9	0.9

1S6 Days post infection.

Conc.(ug/ ml) IVM	21	28	35	42	49	56	63	70	77	84	91	98	105	112
0.0007	0.85	ND	0.94	1.04	1.03	1.05	1.01	0.98	1.06	1.04	1	0.98	0.98	0.98
0.0015	0.82	ND	0.94	0.95	0.89	0.99	0.98	0.95	1	1.01	0.92	0.95	0.95	0.92
0.0029	0.81	ND	0.83	0.84	0.93	0.97	0.91	0.91	0.96	0.94	0.85	0.91	0.92	0.87
		ND	ND	ND	ND	ND	ND	ND	ND	ND	0.92	ND	ND	0.84
		ND	ND	ND	ND	ND	ND	ND	ND	ND	0.86	ND	ND	0.84
0.0059	0.79	ND	0.8	0.73	0.63	0.84	0.74	0.88	0.9	0.9	0.89	0.84	0.85	0.81
		ND	ND	ND	ND	ND	ND	ND	ND	ND	0.81	ND	ND	0.85
		ND	ND	ND	ND	ND	ND	ND	ND	ND	0.86	ND	ND	0.83
0.0117	0.78	ND	0.72	0.61	0.57	0.78	0.93	0.87	0.85	0.73	0.75	0.76	0.79	0.74
	0.78	ND	ND	ND	ND	0.71	ND	ND	ND	0.78	0.73	0.72	0.78	ND
	0.7	ND	ND	ND	ND	0.72	ND	ND	ND	0.79	0.75	0.75	0.78	ND
0.0234	0.58	ND	0.6	0.52	0.57	0.53	0.79	0.8	0.79	0.73	0.69	0.61	0.69	0.71
	0.57	ND	ND	0.57	0.54	0.58	0.85	0.85	0.78	0.59	0.68	0.63	0.68	0.77
	0.56	ND	ND	0.53	0.62	0.56	0.85	0.84	0.82	0.68	0.7	0.66	0.69	0.69
0.0469	0.48	ND	0.52	0.42	0.49	0.42	0.76	0.77	0.71	0.51	0.53	0.5	0.52	0.51
	0.54	ND	0.43	0.51	0.47	0.44	0.76	0.71	0.65	0.49	0.52	0.49	0.5	0.5
	0.41	ND	0.49	0.45	0.47	0.43	0.74	0.76	0.64	0.51	0.51	0.52	0.51	0.51
0.0938	0.43	ND	0.5	0.37	0.31	0.13	0.53	0.65	0.45	0.44	0.48	0.41	0.43	0.43
	0.36	ND	0.36	0.31	0.3	0.09	0.55	0.71	0.54	0.4	0.42	0.43	0.42	0.43
	0.37	ND	0.5	0.37	0.22	0.14	0.54	0.64	0.55	0.45	0.36	0.28	0.29	0.27
0.1875	0.15	ND	0.04	0.15	0.14	0	0.38	0.5	0.42	0.36	0.28	0.29	0.27	0.39
	0.15	ND	0	0.23	0.15	0	0.41	0.52	0.48	0.29	0.25	0.28	0.28	0.28
	0.15	ND	0	0.15	0.15	0	0.36	0.5	0.42	0.29	0.3	0.31	0.29	0.28
0.375	0.17	ND	0	0.28	0.09	0	0.31	0.41	0.29	0.14	0.13	0.12	0.09	0.14
	0.15	ND	0	0.14	0.09	0	0.25	0.42	0.29	0.14	0.14	0.14	0.14	0.16
	0.15	ND	0	0.11	0	0	0.04	0.42	0.26	0.14	0.14	0.13	0.14	0.13
0.75	0	ND	0	0.13	0	0	0	0.15	0.14	0.07	0.04	0.08	0.07	0
		ND	0	0.07	0	0	0	0.28	0.16	0.11	0	0.07	0.04	0
		ND	0	0.04	0	0	0	0.14	0.12	0.07	0.02	0.12	0.08	0
1.5	0	ND	0	0	0	0	0	0	0	0	0	0	0	0
3	0	ND	0	0	0	0	0	0	0	0	0	0	0	0
6	0	ND	0	0	0	0	0	0	0	0	0	0	0	0
12	0	ND	0	0	0	0	0	0	0	0	0	0	0	0
24	0	ND	0	0	0	0	0	0	0	0	0	0	0	0
Mean control	0.89	ND	0.91	0.86	0.89	0.89	0.92	0.92	0.88	0.88	0.9	0.91	0.9	0.9

KEY: ND (No Data)

2 S1 Days post infection.

Conc.(ug/ ml) IVM	21	28	35	42	49	56	63	70	77	84	91	98	105	112
0.0007	1.04	1.01	0.99	1.04	0.94	0.95	0.96	1.01	0.99	0.96	0.99	0.98	0.96	1
0.0015	ND	ND	ND	ND	0.86	0.82	0.87	0.96	0.93	0.92	0.9	0.94	0.83	0.95
0.0029	ND	ND	ND	ND	0.87	0.76	0.82	0.88	0.87	0.84	0.79	0.78	0.76	0.81
0.0059	ND	ND	ND	ND	0.81	0.71	0.76	0.84	0.8	0.81	0.71	0.74	0.72	0.75
0.0117	0.96	0.93	0.52	0.76	0.75	0.72	0.65	0.79	0.72	0.66	0.6	0.62	0.63	0.62
	1	0.89	0.55	0.68	0.75	0.72	0.68	0.8	0.72	0.63	0.62	0.6	0.59	0.67
	1.02	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND
0.0234	0.62	0.55	0.46	0.61	0.68	0.65	0.68	0.77	0.66	0.44	0.42	0.45	0.46	0.45
	0.55	0.56	0.48	0.57	0.68	0.65	0.72	0.79	0.64	0.43	0.44	0.45	0.46	0.48
	0.58	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND
0.0469	0.39	0.48	0.35	0.45	0.66	0.6	0.68	0.66	0.46	0.29	0.28	0.26	0.28	0.28
	0.47	0.44	0.4	0.45	0.66	0.62	0.63	0.64	0.46	0.3	0.26	0.26	0.27	0.31
	0.53	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND
0.0938	0.43	0.28	0.14	0.22	0.55	0.44	0.6	0.49	0.31	0.17	0.16	0.21	0.25	0.23
	0.46	0.3	0.17	0.2	0.57	0.43	0.61	0.49	0.33	0.17	0.19	0.17	0.2	0.19
	0.47	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND
0.1875	0.3	0	0.06	0.19	0.45	0.26	0.42	0.33	0.12	0.13	0.09	0.1	0.12	0.16
	0.16	0.12	0.1	0.18	0.42	0.21	0.43	0.29	0.07	0.09	0.1	0.09	0.11	0.16
	0.04	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND
0.375	0	0	0	0	0.33	0.06	0.24	0.21	0	0	0	0	0	0.07
	0	0	0	0.08	0.26	0.07	0.27	0.22	0	0	0	0	0	0.08
0.75	0	0	0.06	0	0.1	0	0.07	0	0	0	0	0	0	0
	0	0	0	0	0.06	0	0.13	0	0	0	0	0	0	0
1.5	0	0	0	0	0	0	0	0	0	0	0	0	0	0
3	0	0	0	0	0	0	0	0	0	0	0	0	0	0
6	0	0	0	0	0	0	0	0	0	0	0	0	0	0
12	0	0	0	0	0	0	0	0	0	0	0	0	0	0
24	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Mean control	0.82	0.89	0.97	0.92	0.94	0.93	0.95	0.87	0.89	0.96	0.99	0.95	0.93	0.91

KEY: ND (Not Done)

2 S2 Days post infection.

Conc.(ug/ ml) IVM	21	28	35	42	49	56	63	70	77	84	91	98	105	112
0.0007	1.08	1.01	1.02	1	0.97	0.98	0.96	1.04	0.98	1.02	1.09	0.99	1.04	0.97
0.0015	ND	ND	ND	ND	0.74	0.9	0.96	1	0.95	0.96	1.03	0.96	1	0.92
0.0029	ND	ND	ND	ND	0.85	0.83	0.88	0.9	0.81	0.88	0.93	0.84	0.94	0.82
0.0059	ND	ND	ND	ND	0.8	0.75	0.79	0.84	0.71	0.76	0.8	0.77	0.86	0.76
0.0117	0.97	0.87	0.76	0.8	0.82	0.76	0.73	0.82	0.69	0.69	0.77	0.73	0.72	0.68
	0.88	0.9	0.76	0.82	0.82	0.76	0.75	0.8	0.72	0.61	0.74	0.69	0.69	0.64
	1.02	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND
0.0234	0.58	0.56	0.46	0.6	0.69	0.64	0.68	0.79	0.63	0.48	0.54	0.46	0.52	0.46
	0.55	0.51	0.49	0.62	0.66	0.65	0.71	0.76	0.61	0.45	0.53	0.44	0.5	0.48
	0.92	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND
0.0469	0.32	0.44	0.31	0.5	0.68	0.59	0.64	0.67	0.45	0.27	0.38	0.27	0.38	0.33
	0.45	0.43	0.32	0.49	0.65	0.59	0.68	0.66	0.47	0.3	0.35	0.27	0.32	0.32
	0.45	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND
0.0938	0.43	0.13	0.23	0.31	0.61	0.46	0.58	0.49	0.3	0.23	0.25	0.18	0.22	0.22
	0.44	0.26	0.23	0.31	0.57	0.45	0.54	0.47	0.34	0.16	0.23	0.16	0.24	0.21
	0.41	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND
0.1875	0.15	0.08	0.13	0.17	0.46	0.26	0.44	0.29	0.19	0.06	0.15	0.09	0.16	0.11
	0.07	0.12	0.12	0.16	0.45	0.23	0.45	0.25	0.16	0.08	0.18	0.11	0.14	0.1
	0.04	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND
0.375	0	0	0.07	0	0.36	0.15	0.26	0.14	0.13	0	0.03	0	0	0
	0	0.04	0.06	0	0.2	0.13	0.2	0.13	0.12	0	0.03	0	0	0
0.75	0	0	0	0	0.14	0	0.12	0	0	0	0	0	0	0
	0	0	0	0	0.13	0	0.13	0	0	0	0	0	0	0
1.5	0	0	0	0	0	0	0	0	0	0	0	0	0	0
3	0	0	0	0	0	0	0	0	0	0	0	0	0	0
6	0	0	0	0	0	0	0	0	0	0	0	0	0	0
12	0	0	0	0	0	0	0	0	0	0	0	0	0	0
24	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Mean control	0.85	0.88	0.96	0.82	0.92	0.93	0.92	0.87	0.91	0.92	0.82	0.92	0.85	0.9

2 S3 Days post infection.

Conc.(ug/ ml) IVM	21	28	35	42	49	56	63	70	77	84	91	98	105	112
0.0007	1.05	1.06	0.99	1.07	0.95	0.99	0.96	0.93	1.01	1.03	1.03	1.01	1.01	1
0.0015	ND	ND	ND	ND	0.86	0.95	0.94	0.94	0.97	0.96	0.97	1	0.94	0.92
0.0029	ND	ND	ND	ND	0.82	0.85	0.85	0.86	0.88	0.85	0.93	0.91	0.89	0.83
0.0059	ND	ND	ND	ND	0.76	0.78	0.8	0.76	0.83	0.77	0.83	0.79	0.77	0.74
0.0117	0.95	0.79	0.75	0.82	0.75	0.74	0.76	0.74	0.69	0.62	0.68	0.7	0.68	0.64
	0.87	0.8	0.71	0.8	0.78	0.76	0.8	0.74	0.72	0.64	0.65	0.69	0.66	0.67
	0.89	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND
0.0234	0.58	0.54	0.48	0.57	0.68	0.65	0.68	0.72	0.67	0.46	0.48	0.48	0.67	0.47
	0.54	0.5	0.49	0.63	0.69	0.66	0.69	0.73	0.64	0.47	0.5	0.49	0.46	0.45
	0.62	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND
0.0469	0.44	0.42	0.38	0.49	0.67	0.61	0.63	0.6	0.49	0.28	0.28	0.34	0.29	0.27
	0.37	0.47	0.27	0.5	0.64	0.62	0.63	0.63	0.49	0.27	0.31	0.34	0.28	0.27
	0.45	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND
0.0938	0.27	0.19	0.18	0.23	0.58	0.47	0.59	0.47	0.31	0.17	0.21	0.25	0.2	0.2
	0.38	0.28	0.26	0.26	0.57	0.45	0.61	0.47	0.28	0.12	0.18	0.21	0.18	0.2
	0.16	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND
0.1875	0.07	0.14	0.17	0.14	0.43	0.28	0.45	0.26	0.19	0.07	0.1	0.11	0.12	0.09
	0.07	0.17	0.13	0.07	0.42	0.25	0.43	0.25	0.21	0.1	0.08	0.1	0.11	0.11
	0	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND
0.375	0.02	0.07	0	0.04	0.26	0.06	0.22	0.2	0.08	0	0	0	0	0
	0	0.07	0.11	0.02	0.28	0	0.21	0.14	0.07	0	0	0	0	0
0.75	0	0	0	0	0.12	0	0.15	0	0	0	0	0	0	0
	0	0	0	0	0.09	0	0.07	0	0	0	0	0	0	0
1.5	0	0	0	0	0.06	0	0	0	0	0	0	0	0	0
3	0	0	0	0	0	0	0	0	0	0	0	0	0	0
6	0	0	0	0	0	0	0	0	0	0	0	0	0	0
12	0	0	0	0	0	0	0	0	0	0	0	0	0	0
24	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Mean control	0.85	0.96	0.96	0.88	0.89	0.92	0.87	0.9	0.9	0.88	0.92	0.91	0.91	0.92

2 S4 Days post infection.

Conc.(ug/ ml) IVM	21	28	35	42	49	56	63	70	77	84	91	98	105	112
0.0007	1.02	1.04	1.04	0.98	0.98	1.03	0.97	0.99	0.98	0.98	0.97	1.02	0.98	0.97
0.0015	ND	ND	ND	ND	0.84	1	0.93	0.94	0.95	0.96	0.9	0.94	0.94	0.89
0.0029	ND	ND	ND	ND	0.87	0.88	0.83	0.88	0.87	0.87	0.87	0.87	0.87	0.82
0.0059	ND	ND	ND	ND	0.79	0.81	0.79	0.81	0.74	0.76	0.76	0.74	0.77	0.72
0.0117	0.87	0.75	0.54	0.83	0.78	0.76	0.73	0.75	0.71	0.69	0.69	0.69	0.64	0.65
	0.91	0.86	0.76	0.8	0.85	0.74	0.75	0.76	0.69	0.65	0.64	0.63	0.65	0.65
	0.95	NA	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND
0.0234	0.54	0.5	0.44	0.58	0.71	0.64	0.65	0.64	0.46	0.49	0.45	0.47	0.49	0.45
	0.52	0.5	0.49	0.56	0.72	0.66	0.61	0.63	0.49	0.49	0.48	0.46	0.45	0.45
	0.5	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND
0.0469	0.44	0.4	0.35	0.5	0.49	0.49	0.47	0.49	0.25	0.29	0.32	0.28	0.3	0.27
	0.57	0.31	0.24	0.49	0.44	0.5	0.48	0.47	0.28	0.29	0.31	0.28	0.27	0.3
	0.45	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND
0.0938	0.39	0.21	0.2	0.37	0.32	0.29	0.32	0.32	0.18	0.2	0.17	0.18	0.21	0.2
	0.42	0.25	0.29	0.36	0.31	0.32	0.33	0.29	0.14	0.17	0.2	0.16	0.19	0.22
	0.39	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND
0.1875	0.07	0.13	0.13	0.23	0.12	0.17	0.14	0.16	0	0.07	0.13	0.09	0.14	0.09
	0.15	0.12	0.17	0.21	0.17	0.19	0.2	0.21	0	0.05	0.13	0.07	0.09	0.11
	0.09	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND
0.375	0	0.07	0	0.04	0	0.1	0.08	0.07	0	0	0	0	0	0
	0	0.09	0.09	0.07	0	0.07	0.06	0.12	0	0	0	0	0	0
0.75	0	0.06	0	0	0.12	0	0.15	0	0	0	0	0	0	0
	0	0.13	0	0	0.09	0	0.07	0	0	0	0	0	0	0
1.5	0	0	0	0	0.06	0	0	0	0	0	0	0	0	0
3	0	0	0	0	0	0	0	0	0	0	0	0	0	0
6	0	0	0	0	0	0	0	0	0	0	0	0	0	0
12	0	0	0	0	0	0	0	0	0	0	0	0	0	0
24	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Mean control	0.91	0.9	0.97	0.87	0.94	0.92	0.9	0.92	0.89	0.89	0.89	0.87	0.91	0.92

2 S5 Days post infection.

Conc.(ug/ ml) IVM	21	28	35	42	49	56	63	70	77	84	91	98	105	112
0.0007	1.07	1.03	0.97	0.95	0.96	0.97	1.02	0.96	1.02	0.96	1.01	0.94	0.95	1.03
0.0015	ND	ND	ND	ND	0.9	0.92	0.93	0.9	0.94	0.95	0.99	0.92	0.92	1
0.0029	ND	ND	ND	ND	0.81	0.88	0.81	0.86	0.88	0.89	0.87	0.87	0.75	0.81
0.0059	ND	ND	ND	ND	0.73	0.79	0.8	0.79	0.83	0.75	0.82	0.68	0.7	0.74
0.0117	0.99	0.76	0.8	0.93	0.78	0.72	0.78	0.73	0.7	0.68	0.71	0.63	0.6	0.71
	0.99	0.85	0.71	0.87	0.74	0.73	0.74	0.73	0.73	0.63	0.68	0.66	0.62	0.69
	1.11	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND
0.0234	0.57	0.48	0.49	0.73	0.64	0.64	0.69	0.66	0.66	0.48	0.5	0.44	0.46	0.5
	0.53	0.48	0.46	0.76	0.66	0.59	0.65	0.62	0.62	0.46	0.49	0.45	0.45	0.47
	0.51	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND
0.0469	0.57	0.44	0.29	0.5	0.47	0.47	0.49	0.48	0.46	0.29	0.27	0.27	0.29	0.32
	0.36	0.39	0.34	0.5	0.46	0.47	0.51	0.46	0.47	0.28	0.29	0.22	0.3	0.29
	0.41	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND
0.0938	0.22	0.29	0.21	0.3	0.32	0.33	0.34	0.31	0.28	0.14	0.14	0.21	0.2	0.21
	0.44	0.27	0.23	0.32	0.27	0.29	0.29	0.29	0.27	0.16	0.16	0.17	0.2	0.21
	0.39	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND
0.1875	0.26	0.13	0.16	0.22	0.13	0.09	0.19	0.17	0.14	0.09	0.07	0.16	0.1	0.14
	0.16	0.1	0.17	0.24	0.13	0.1	0.21	0.14	0.12	0.07	0.08	0.13	0.09	0.11
	0.45	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND
0.375	0	0.06	0	0.07	0	0	0.11	0.09	0.12	0	0	0	0	0
	0	0	0	0.11	0	0	0.1	0.12	0.04	0	0	0	0	0
0.75	0	0	0	0	0	0	0	0	0	0	0	0	0	0
	0	0	0	0	0	0	0	0	0	0	0	0	0	0
1.5	0	0	0	0	0	0	0	0	0	0	0	0	0	0
3	0	0	0	0	0	0	0	0	0	0	0	0	0	0
6	0	0	0	0	0	0	0	0	0	0	0	0	0	0
12	0	0	0	0	0	0	0	0	0	0	0	0	0	0
24	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Mean control	0.86	0.96	0.91	0.84	0.92	0.88	0.92	0.92	0.91	0.91	0.88	0.93	0.94	0.87

2 S6 Days post infection.

Conc.(ug/ ml) IVM	21	28	35	42	49	56	63	70	77	84	91	98	105	112
0.0007	1.01	0.99	1.04	0.92	1	0.97	1	0.9	1.01	1.01	1.02	1.03	1.08	0.94
0.0015	ND	ND	ND	ND	0.86	0.92	0.92	0.93	0.92	1	0.94	0.97	1.02	0.9
0.0029	ND	ND	ND	ND	0.8	0.82	0.84	0.88	0.85	0.95	0.89	0.89	0.97	0.78
0.0059	ND	ND	ND	ND	0.77	0.76	0.78	0.76	0.76	0.8	0.76	0.79	0.83	0.69
0.0117	0.88	0.88	0.76	0.93	0.7	0.71	0.75	0.72	0.73	0.65	0.64	0.65	0.68	0.64
	0.99	0.86	0.76	0.94	0.76	0.69	0.74	0.71	0.71	0.68	0.68	0.66	0.73	0.64
	0.9	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND
0.0234	0.52	0.51	0.54	0.71	0.63	0.64	0.62	0.66	0.61	0.48	0.47	0.47	0.5	0.46
	0.53	0.51	0.5	0.67	0.64	0.64	0.65	0.64	0.63	0.48	0.49	0.49	0.49	0.46
	0.55	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND
0.0469	0.43	0.4	0.3	0.48	0.46	0.45	0.46	0.46	0.46	0.28	0.32	0.28	0.34	0.27
	0.43	0.44	0.4	0.48	0.48	0.45	0.46	0.48	0.47	0.3	0.31	0.21	0.33	0.28
	0.42	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND
0.0938	0.31	0.19	0.27	0.29	0.28	0.26	0.34	0.29	0.28	0.19	0.21	0.18	0.24	0.19
	0.34	0.22	0.25	0.31	0.2	0.28	0.3	0.27	0.84	0.23	0.19	0.18	0.23	0.18
	0.41	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND
0.1875	0.12	0.1	0.14	0.14	0.12	0.2	0.14	0.18	0.14	0.11	0.14	0.08	0.13	0.13
	0.07	0.12	0.13	0.14	0.06	0.17	0.2	0.2	0.17	0.13	0.07	0.09	0.12	0.13
	0.14	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND
0.375	0	0.3	0	0	0	0	0.04	0	0.07	0	0	0	0	0
	0	0.09	0.07	0.04	0	0	0	0	0.11	0	0	0	0	0
0.75	0	0	0.02	0	0	0	0	0	0	0	0	0	0	0
	0	0	0	0	0	0	0	0	0	0	0	0	0	0
1.5	0	0	0	0	0	0	0	0	0	0	0	0	0	0
3	0	0	0	0	0	0	0	0	0	0	0	0	0	0
6	0	0	0	0	0	0	0	0	0	0	0	0	0	0
12	0	0	0	0	0	0	0	0	0	0	0	0	0	0
24	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Mean control	0.9	0.95	0.85	0.88	0.92	0.93	0.91	0.92	0.9	0.85	0.89	0.89	0.84	0.95

2 S1 Days post infection.

Con.(ug /ml)AV MB2	21	28	35	42	49	56	63	70	77	84	91	98	105	112
0.0013	1.08	1.01	1	1.04	0.99	0.99	0.9	1.01	0.99	0.97	0.91	0.95	0.88	0.97
0.0027	1.1	ND	ND	ND	0.88	0.92	0.85	1	0.96	0.9	0.85	0.92	0.81	0.95
0.005	0.93	ND	ND	ND	0.88	0.92	0.77	0.93	0.93	0.81	0.79	0.77	0.76	0.81
0.0107	0.78	ND	ND	ND	0.81	0.87	0.73	0.87	0.64	0.73	0.7	0.74	0.68	0.77
0.0214	0.77	0.93	0.68	0.76	0.78	0.74	0.71	0.8	0.66	0.62	0.59	0.62	0.59	0.65
	0.7	0.93	0.78	0.78	0.79	0.72	0.7	0.76	0.65	0.57	0.58	0.62	0.61	0.64
0.0428	0.57	0.55	0.46	0.7	0.77	0.74	0.57	0.64	0.48	0.44	0.44	0.45	0.46	0.47
	0.55	0.53	0.46	0.61	0.81	0.72	0.62	0.67	0.5	0.48	0.43	0.45	0.44	0.47
0.0856	0.48	0.35	0.26	0.5	0.55	0.55	0.44	0.49	0.31	0.27	0.26	0.26	0.29	0.28
	0.38	0.48	0.27	0.5	0.57	0.62	0.44	0.49	0.27	0.3	0.29	0.25	0.27	0.31
0.1711	0.27	0.44	0.17	0.13	0.46	0.47	0.23	0.28	0.21	0.19	0.19	0.17	0.18	0.21
	0.32	0.45	0.23	0.12	0.44	0.46	0.24	0.27	0.18	0.13	0.19	0.17	0.17	0.21
0.3421	0	0.08	0.13	0.06	0.3	0.27	0.08	0.17	0.07	0.11	0.11	0	0.13	0.14
	0.14	0.08	0.19	0.04	0.25	0.28	0	0.13	0.11	0.1	0.09	0	0.08	0.14
0.684	0.15	0.02	0.11	0	0.09	0.06	0	0.04	0	0	0	0	0	0
	0	0	0	0	0.13	0	0	0	0	0	0	0	0	0
1.3688	0	0	0	0	0	0	0	0	0	0	0	0	0	0
	0	0	0	0	0	0	0	0	0	0	0	0	0	0
2.7375	0	0	0	0	0	0	0	0	0	0	0	0	0	0
5.475	0	0	0	0	0	0	0	0	0	0	0	0	0	0
10.95	0	0	0	0	0	0	0	0	0	0	0	0	0	0
21.9	0	0	0	0	0	0	0	0	0	0	0	0	0	0
43.8	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Mean control	0.82	0.89	0.97	0.92	0.95	0.93	0.95	0.87	0.89	0.96	0.99	0.95	0.93	0.91

2 S2 Days post infection.

Con.(ug/ml)AV MB2	21	28	35	42	49	56	63	70	77	84	91	98	105	112
0.0013	1.08	1.05	1.02	0.99	0.97	0.92	0.97	1.04	0.97	1	1.09	0.97	1.02	0.98
0.0027	1	ND	ND	ND	0.88	0.94	0.89	0.97	0.93	0.97	1.01	0.86	1.01	0.91
0.005	0.87	ND	ND	ND	0.85	0.93	0.78	0.93	0.85	0.88	0.9.	0.82	0.98	0.87
0.0107	0.72	ND	ND	ND	0.84	0.82	0.75	0.79	0.77	0.72	0.83	0.72	0.82	0.78
0.0214	0.77	0.97	0.69	0.85	0.8	0.74	0.73	0.73	0.64	0.67	0.81	0.66	0.71	0.68
	0.67	0.98	0.67	0.8	0.86	0.78	0.69	0.78	0.63	0.59	0.73	0.62	0.68	0.65
0.0428	0.56	0.56	0.46	0.74	0.79	0.7	0.61	0.64	0.48	0.46	0.52	0.45	0.51	0.47
	0.58	0.53	0.5	0.73	0.82	0.79	0.6	0.69	0.47	0.48	0.54	0.46	0.51	0.47
0.0856	0.38	0.31	0.3	0.54	0.67	0.54	0.46	0.5	0.28	0.29	0.35	0.27	0.3	0.28
	0.42	0.47	0.32	0.55	0.65	0.61	0.48	0.47	0.29	0.3	0.36	0.25	0.3	0.28
0.1711	0.2	0.43	0.23	0.32	0.47	0.45	0.27	0.3	0.14	0.2	0.24	0.18	0.22	0.18
	0.35	0.42	0.23	0.12	0.44	0.44	0.28	0.29	0.16	0.14	0.27	0.16	0.22	0.21
0.3421	0.07	0.21	0.12	0.2	0.21	0.27	0.15	0.18	0	0.08	0.12	0.11	0.12	0.1
	0.14	0.19	0.13	0.03	0.27	0.27	0.12	0.17	0.07	0.1	0.04	0.08	0.13	0.11
0.684	0.09	0	0	0.1	0.09	0.06	0	0	0	0	0	0	0	0
	0.07	0	0	0	0.06	0.08	0.04	0	0	0	0	0	0	0
1.3688	0	0	0	0	0	0	0	0	0	0	0	0	0	0
	0	0	0	0	0	0	0	0	0	0	0	0	0	0
2.7375	0	0	0	0	0	0	0	0	0	0	0	0	0	0
5.475	0	0	0	0	0	0	0	0	0	0	0	0	0	0
10.95	0	0	0	0	0	0	0	0	0	0	0	0	0	0
21.9	0	0	0	0	0	0	0	0	0	0	0	0	0	0
43.8	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Mean control	0.85	0.88	0.96	0.82	0.92	0.93	0.92	0.87	0.91	0.92	0.82	0.92	0.85	0.9

2 S3 Days post infection.

Con.(ug /ml)AV MB2	21	28	35	42	49	56	63	70	77	84	91	98	105	112
0.0013	0.99	1.06	0.99	1.07	0.94	0.93	0.97	0.95	1.02	1.02	1.05	0.93	0.99	0.97
0.0027	1.06	ND	ND	ND	0.9	0.94	0.95	0.9	0.96	0.93	0.97	0.89	0.92	0.94
0.005	0.91	ND	ND	ND	0.81	0.89	0.91	0.9	0.91	0.83	0.93	0.77	0.83	0.8
0.0107	0.87	ND	ND	ND	0.78	0.82	0.8	0.77	0.78	0.71	0.78	0.71	0.81	0.75
0.0214	0.75	0.94	0.73	0.73	0.78	0.73	0.78	0.73	0.66	0.65	0.69	0.66	0.68	0.61
	0.73	0.87	0.74	1	0.79	0.76	0.77	0.76	0.64	0.63	0.68	0.65	0.66	0.64
0.0428	0.55	0.54	0.44	0.61	0.75	0.7	0.67	0.6	0.47	0.48	0.51	0.5	0.47	0.47
	0.55	0.53	0.47	0.67	0.77	0.75	0.65	0.58	0.43	0.46	0.46	0.5	0.45	0.45
0.0856	0.5	0.37	0.15	0.5	0.57	0.56	0.47	0.44	0.16	0.28	0.28	0.29	0.28	0.27
	0.49	0.36	0.23	0.51	0.62	0.59	0.47	0.46	0.14	0.32	0.27	0.32	0.3	0.27
0.1711	0.22	0.28	0.23	0.3	0.47	0.46	0.28	0.26	0.04	0.17	0.17	0.22	0.2	0.17
	0.17	0.36	0.19	0.27	0.45	0.46	0.28	0.23	0.04	0.21	0.19	0.22	0.18	0.19
0.3421	0	0.28	0.1	0.14	0.21	0.28	0.18	0.17	0	0.08	0.08	0.11	0.14	0.12
	0	0.22	0.09	0.13	0.31	0.27	0.14	0.16	0	0.12	0.07	0.1	0.11	0.09
0.684	0	0.07	0.05	0	0	0	0.07	0.08	0	0	0	0	0	0
	0	0.11	0	0	0.12	0	0.09	0.13	0	0	0	0	0	0
1.3688	0	0	0	0	0	0	0	0	0	0	0	0	0	0
	0	0	0	0	0	0	0	0	0	0	0	0	0	0
2.7375	0	0	0	0	0	0	0	0	0	0	0	0	0	0
5.475	0	0	0	0	0	0	0	0	0	0	0	0	0	0
10.95	0	0	0	0	0	0	0	0	0	0	0	0	0	0
21.9	0	0	0	0	0	0	0	0	0	0	0	0	0	0
43.8	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Mean control	0.85	0.9	0.97	0.87	0.94	0.92	0.9	0.92	0.89	0.89	0.89	0.87	0.91	0.92

2 S4 2S4 Days post infection.

Con.(ug /ml)AV MB2	21	28	35	42	49	56	63	70	77	84	91	98	105	112
0.0013	0.97	0.94	1.04	0.99	0.91	0.97	1.07	1.02	0.97	0.99	0.96	1.02	0.96	0.97
0.0027	0.88	ND	ND	ND	0.89	0.91	0.97	0.96	0.94	0.94	0.94	0.94	0.87	0.93
0.005	0.83	ND	ND	ND	0.86	0.9	0.88	0.91	0.89	0.92	0.86	0.84	0.77	0.81
0.0107	0.78	ND	ND	ND	0.79	0.83	0.82	0.82	0.77	0.73	0.8	0.77	0.75	0.72
0.0214	0.73	0.88	0.76	0.78	0.65	0.82	0.67	0.65	0.61	0.69	0.68	0.63	0.63	0.66
	0.73	0.78	0.67	0.81	0.62	0.79	0.68	0.64	0.63	0.64	0.66	0.64	0.63	0.63
0.0428	0.53	0.48	0.45	0.66	0.64	0.62	0.5	0.49	0.47	0.47	0.5	0.47	0.48	0.47
	0.54	0.5	0.48	0.61	0.68	0.59	0.51	0.47	0.47	0.47	0.46	0.47	0.45	0.47
0.0856	0.42	0.39	0.33	0.48	0.47	0.48	0.29	0.29	0.28	0.27	0.27	0.28	0.27	0.27
	0.35	0.4	0.24	0.49	0.5	0.48	0.29	0.28	0.27	0.31	0.29	0.31	0.28	0.31
0.1711	0.18	0.34	0.19	0.29	0.31	0.31	0.16	0.14	0.18	0.21	0.22	0.18	0.21	0.2
	0.14	0.3	0.2	0.36	0.28	0.33	0.19	0.18	0.17	0.14	0.19	0.16	0.18	0.18
0.3421	0.08	0.26	0.11	0.14	0.12	0.13	0.04	0	0.07	0.07	0.09	0.09	0.14	0.09
	0.14	0.24	0.12	0.07	0.14	0.09	0	0	0.09	0.07	0.12	0.08	0.14	0.08
0.684	0	0.13	0	0	0	0	0	0	0	0	0	0	0	0
	0	0.1	0	0	0	0	0	0	0	0	0	0	0	0
1.3688	0	0	0	0	0	0	0	0	0	0	0	0	0	0
	0	0	0	0	0	0	0	0	0	0	0	0	0	0
2.7375	0	0	0	0	0	0	0	0	0	0	0	0	0	0
5.475	0	0	0	0	0	0	0	0	0	0	0	0	0	0
10.95	0	0	0	0	0	0	0	0	0	0	0	0	0	0
21.9	0	0	0	0	0	0	0	0	0	0	0	0	0	0
43.8	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Mean control	0.91	0.96	0.96	0.88	0.89	0.92	0.87	0.9	0.9	0.88	0.92	0.91	0.91	0.92

2 S5 Days post infection.

Con.(ug/ml)AV MB2	21	28	35	42	49	56	63	70	77	84	91	98	105	112
0.0013	1.01	1.03	0.97	1.14	0.92	1	1.01	0.99	0.98	0.98	1.02	0.94	0.94	1
0.0027	0.93	ND	ND	ND	0.81	0.94	0.93	0.93	0.91	0.91	0.99	0.9	0.91	0.96
0.005	0.91	ND	ND	ND	0.8	0.89	0.89	0.9	0.88	0.83	0.86	0.81	0.78	0.85
0.0107	0.8	ND	ND	ND	0.73	0.85	0.77	0.79	0.77	0.71	0.83	0.73	0.72	0.76
0.0214	0.71	0.89	0.62	0.84	0.67	0.8	0.63	0.67	0.69	0.66	0.71	0.63	0.6	0.65
	0.69	0.94	0.63	0.74	0.69	0.82	0.62	0.65	0.62	0.6	0.71	0.63	0.64	0.67
0.0428	0.55	0.48	0.48	0.72	0.62	0.65	0.48	0.48	0.47	0.46	0.49	0.46	0.43	0.47
	0.56	0.45	0.5	0.67	0.59	0.71	0.49	0.48	0.45	0.46	0.51	0.47	0.44	0.47
0.0856	0.46	0.28	0.37	0.52	0.46	0.48	0.27	0.32	0.27	0.27	0.31	0.26	0.28	0.29
	0.44	0.28	0.34	0.53	0.46	0.5	0.24	0.27	0.3	0.27	0.32	0.29	0.27	0.33
0.1711	0.23	0.31	0.22	0.29	0.31	0.28	0.21	0.13	0.14	0.2	0.2	0.17	0.17	0.21
	0.15	0.38	0.24	0.3	0.29	0.26	0.17	0.18	0.15	0.18	0.18	0.13	0.19	0.18
0.3421	0	0.2	0.07	0.22	0.13	0.12	0.08	0.04	0.08	0.09	0.11	0.09	0.09	0.14
	0	0.24	0.13	0.15	0.13	0.14	0.06	0	0.07	0.08	0.12	0	0.12	0.12
0.684	0	0.06	0.08	0	0	0	0	0	0	0	0	0	0	0
	0	0.09	0.04	0	0	0	0	0	0	0	0	0	0	0
1.3688	0	0	0	0	0	0	0	0	0	0	0	0	0	0
	0	0	0	0	0	0	0	0	0	0	0	0	0	0
2.7375	0	0	0	0	0	0	0	0	0	0	0	0	0	0
5.475	0	0	0	0	0	0	0	0	0	0	0	0	0	0
10.95	0	0	0	0	0	0	0	0	0	0	0	0	0	0
21.9	0	0	0	0	0	0	0	0	0	0	0	0	0	0
43.8	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Mean control	0.86	0.97	0.91	0.84	0.92	0.88	0.92	0.92	0.91	0.91	0.88	0.93	0.94	0.87

2 S6 Days post infection.

Con.(ug /ml)AV MB2	21	28	35	42	49	56	63	70	77	84	91	98	105	112
0.0013	1.02	0.96	1.04	1.13	0.96	0.93	0.97	0.97	0.98	1.03	0.99	1.03	1.1	0.93
0.0027	1	ND	ND	ND	0.89	0.94	0.95	0.91	0.98	0.98	0.95	1.02	1.03	0.86
0.005	0.85	ND	ND	ND	0.78	0.88	0.85	0.87	0.95	0.89	0.86	0.88	0.88	0.75
0.0107	0.79	ND	ND	ND	0.76	0.82	0.76	0.74	0.79	0.79	0.81	0.76	0.79	0.73
0.0214	0.75	0.77	0.67	0.74	0.65	0.76	0.64	0.64	0.64	0.69	0.74	0.65	0.72	0.63
	0.72	0.82	0.81	0.81	0.69	0.72	0.61	0.62	0.64	0.67	0.68	0.64	0.69	0.61
0.0428	0.55	0.52	0.51	0.71	0.63	0.61	0.49	0.48	0.47	0.5	0.51	0.48	0.52	0.44
	0.52	0.48	0.55	0.66	0.62	0.57	0.47	0.48	0.45	0.52	0.49	0.46	0.49	0.43
0.0856	0.3	0.41	0.38	0.52	0.47	0.44	0.28	0.27	0.28	0.3	0.29	0.24	0.37	0.28
	0.36	0.37	0.3	0.49	0.46	0.46	0.27	0.28	0.31	0.31	0.28	0.2	0.33	0.26
0.1711	0.05	0.34	0.29	0.28	0.21	0.27	0.15	0.15	0.18	0.14	0.21	0.21	0.24	0.21
	0	0.4	0.17	0.35	0.2	0.26	0.14	0.17	0.14	0.2	0.18	0.14	0.24	0.19
0.3421	0	0.19	0.15	0.14	0.07	0.13	0.02	0.02	0.07	0.2	0.14	0.11	0.12	0.13
	0	0.18	0.14	0.12	0.14	0.04	0.05	0.04	0.04	0.12	0.14	0.07	0.09	0.11
0.684	0	0.07	0.07	0.04	0	0	0	0	0	0.13	0	0	0	0
	0	0.11	0	0.02	0	0	0	0	0	0	0	0	0	0
1.3688	0	0	0	0	0	0	0	0	0	0	0	0	0	0
	0	0	0	0	0	0	0	0	0	0	0	0	0	0
2.7375	0	0	0	0	0	0	0	0	0	0	0	0	0	0
5.475	0	0	0	0	0	0	0	0	0	0	0	0	0	0
10.95	0	0	0	0	0	0	0	0	0	0	0	0	0	0
21.9	0	0	0	0	0	0	0	0	0	0	0	0	0	0
43.8	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Mean control	0.9	0.95	0.85	0.88	0.92	0.93	0.91	0.92	0.9	0.85	0.89	0.89	0.84	0.95

KEY: ND (Not Done)

2 S1 Days post infection.

Conc.(ug/ml) LEV	21	28	35	42	49	56	63	70	77	84	91	98	105	112
0.0039	1.08	1	1	1.04	0.95	0.96	0.94	1.03	1	0.89	1.01	1.02	0.99	0.94
0.0079	1.07	ND	ND	ND	0.87	0.93	0.86	1	0.88	0.9	0.92	0.85	0.95	0.9
0.0158	0.93	ND	ND	ND	0.91	0.83	0.76	0.9	0.94	0.87	0.82	0.78	0.82	0.81
0.0315	0.91	0.88	0.75	0.77	0.72	0.87	0.71	0.91	0.9	0.7	0.77	0.71	0.71	0.74
0.063	0.74	0.64	0.7	0.66	0.66	0.77	0.68	0.85	0.78	0.59	0.63	0.65	0.65	0.63
	ND	ND	0.66	0.64	0.69	0.71	0.69	0.84	0.76	0.58	0.6	0.61	0.62	0.65
0.126	0.55	0.56	0.5	0.61	0.72	0.7	0.66	0.79	0.64	0.42	0.45	0.47	0.44	0.48
	0.53	0.53	0.46	0.63	0.68	0.69	0.67	0.78	0.64	0.44	0.42	0.47	0.46	0.47
0.252	0.38	0.28	0.25	0.48	0.63	0.61	0.6	0.69	0.47	0.29	0.28	0.27	0.27	0.31
	0.32	0.35	0.33	0.44	0.65	0.61	0.54	0.69	0.48	0.3	0.29	0.3	0.32	0.3
0.5039	0.14	0.11	0.06	0.28	0.57	0.55	0.44	0.5	0.3	0.17	0.16	0.2	0.25	0.25
	0.16	0.14	0.15	0.26	0.61	0.54	0.43	0.51	0.31	0.13	0.18	0.17	0.19	0.2
1.0078	0	0	0.1	0.24	0.46	0.48	0.26	0.29	0.14	0.06	0.1	0.09	0.12	0.16
	0	0	0.09	0.18	0.45	0.9	0.23	0.32	0.14	0.06	0.11	0.08	0.13	0.14
2.016	0	0	0	0	0.2	0.21	0.1	0.08	0.09	0	0	0	0	0.08
	0	0	0	0	0.21	0.27	0.11	0.07	0	0	0	0	0	0.07
4.03	0	0	0	0	0	0.13	0	0	0	0	0	0	0	0
	0	0	0	0	0	0.09	0	0	0	0	0	0	0	0
8.06	0	0	0	0	0	0	0	0	0	0	0	0	0	0
16.125	0	0	0	0	0	0	0	0	0	0	0	0	0	0
32.25	0	0	0	0	0	0	0	0	0	0	0	0	0	0
64.5	0	0	0	0	0	0	0	0	0	0	0	0	0	0
129	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Mean control	0.82	0.89	0.97	0.92	0.94	0.93	0.95	0.87	0.89	0.96	0.99	0.95	0.93	0.91

2 S2 Days post infection.

Conc.(ug/ml) LEV	21	28	35	42	49	56	63	70	77	84	91	98	105	112
0.0039	1.05	1.01	0.98	1	1.04	0.97	1	1	0.98	0.98	0.95	1.04	0.95	0.99
0.0079	1	ND	ND	ND	0.94	0.91	0.91	0.99	0.95	0.93	0.93	1	0.93	0.97
0.0158	0.9	ND	ND	ND	0.88	0.86	0.82	0.91	0.92	0.94	0.89	0.95	0.91	0.88
0.0315	0.87	0.92	0.8	0.87	0.85	0.84	0.75	0.45	0.83	0.84	0.88	0.84	0.81	0.81
0.063	0.68	0.67	0.61	0.7	0.75	0.75	0.7	0.8	0.76	0.71	0.78	0.79	0.68	0.66
	ND	ND	ND	0.72	0.7	0.77	0.68	0.79	0.77	0.66	0.81	0.71	0.71	0.64
0.126	0.51	0.5	0.45	0.63	0.66	0.75	0.66	0.74	0.67	0.47	0.59	0.54	0.51	0.47
	0.47	0.51	0.48	0.63	0.69	0.74	0.67	0.76	0.64	0.47	0.58	0.53	0.5	0.46
0.252	0.27	0.34	0.27	0.51	0.65	0.69	0.62	0.67	0.48	0.3	0.48	0.38	0.31	0.28
	0.25	0.29	0.29	0.51	0.62	0.68	0.59	0.67	0.5	0.32	0.46	0.36	0.32	0.28
0.5039	0.13	0.22	0.22	0.28	0.61	0.55	0.48	0.5	0.28	0.22	0.39	0.26	0.22	0.21
	0.12	0.19	0.23	0.19	0.63	0.58	0.45	0.51	0.28	0.2	0.31	0.22	0.22	0.19
1.0078	0.07	0.08	0.04	0.13	0.46	0.48	0.27	0.27	0.14	0.08	0.3	0.17	0.15	0.11
	0	0.1	0.13	0.16	0.45	0.46	0.27	0.22	0.14	0.06	0.23	0.14	0.14	0.09
2.016	0	0	0	0	0.16	0.2	0	0.1	0.06	0	0	0	0	0
	0	0	0	0.04	0.2	0.2	0.13	0.14	0	0	0	0	0	0
4.03	0	0	0	0	0	0	0	0	0	0	0	0	0	0
	0	0	0	0	0	0	0	0	0	0	0	0	0	0
8.06	0	0	0	0	0	0	0	0	0	0	0	0	0	0
16.125	0	0	0	0	0	0	0	0	0	0	0	0	0	0
32.25	0	0	0	0	0	0	0	0	0	0	0	0	0	0
64.5	0	0	0	0	0	0	0	0	0	0	0	0	0	0
129	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Mean control	0.85	0.88	0.92	0.82	0.92	0.93	0.92	0.87	0.91	0.92	0.82	0.92	0.85	0.9

2 S3 Days post infection.

Conc.(ug/ml) LEV	21	28	35	42	49	56	63	70	77	84	91	98	105	112
0.0039	1.01	1.06	0.99	1.07	1	0.97	0.98	0.96	1.02	1	1.02	1.04	1.04	0.92
0.0079	1	ND	ND	ND	0.95	0.94	0.93	0.89	0.93	0.97	0.96	1	0.95	0.94
0.0158	0.93	ND	ND	ND	0.82	0.9	0.84	0.86	0.93	0.93	0.93	0.95	0.89	0.8
0.0315	0.83	0.88	0.81	0.88	0.81	0.82	0.78	0.77	0.79	0.74	0.86	0.84	0.8	0.76
0.063	0.76	0.64	0.63	0.7	0.76	0.73	0.74	0.73	0.72	0.64	0.69	0.79	0.7	0.64
	ND	ND	ND	0.71	0.75	0.72	0.73	0.76	0.72	0.64	0.64	0.71	0.65	0.63
0.126	0.48	0.44	0.48	0.62	0.61	0.69	0.67	0.68	0.62	0.47	0.49	0.54	0.5	0.46
	0.47	0.49	0.46	0.5	0.64	0.69	0.67	0.71	0.65	0.47	0.51	0.53	0.47	0.46
0.252	0.33	0.25	0.32	0.49	0.59	0.61	0.57	0.48	0.47	0.27	0.32	0.38	0.3	0.27
	0.36	0.31	0.3	0.5	0.57	0.63	0.59	0.65	0.47	0.31	0.28	0.36	0.28	0.3
0.5039	0.22	0.2	0.21	0.25	0.55	0.57	0.48	0.46	0.27	0.19	0.14	0.26	0.21	0.21
	0.22	0.18	0.24	0.19	0.6	0.56	0.45	0.45	0.28	0.18	0.14	0.22	0.18	0.17
1.0078	0.07	0.14	0.13	0.14	0.44	0.5	0.28	0.27	0.17	0.11	0.07	0.17	0.12	0.12
	0	0.11	0.13	0.18	0.45	0.47	0.29	0.3	0.21	0.12	0.11	0.14	0	0.11
2.016	0	0	0	0.02	0.13	0.31	0.08	0.13	0.07	0	0	0	0	0
	0	0	0	0	0.16	0.25	0.07	0.12	0.1	0	0	0	0	0
4.03	0	0	0	0	0	0	0	0	0	0	0	0	0	0
	0	0	0	0	0	0	0	0	0	0	0	0	0	0
8.06	0	0	0	0	0	0	0	0	0	0	0	0	0	0
16.125	0	0	0	0	0	0	0	0	0	0	0	0	0	0
32.25	0	0	0	0	0	0	0	0	0	0	0	0	0	0
64.5	0	0	0	0	0	0	0	0	0	0	0	0	0	0
129	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Mean control	0.85	0.9	0.97	0.87	0.94	0.92	0.9	0.92	0.89	0.89	0.89	0.87	0.91	0.92

2 S4 Days post infection.

Conc.(ug/ml) LEV	21	28	35	42	49	56	63	70	77	84	91	98	105	112
0.0039	0.98	0.94	1.04	0.98	1	0.98	1.03	0.97	0.98	0.99	0.94	1.01	0.98	1
0.0079	0.91	ND	ND	ND	0.88	0.89	0.98	0.95	0.94	0.96	0.9	0.95	0.95	0.96
0.0158	0.88	ND	ND	ND	0.86	0.8	0.92	0.89	0.88	0.94	0.87	0.94	0.85	0.81
0.0315	0.77	83	0.78	0.87	0.83	0.69	0.89	0.81	0.81	0.79	0.76	0.84	0.76	0.72
0.063	0.7	0.78	0.6	0.72	0.75	0.76	0.78	0.75	0.72	0.69	0.67	0.65	0.63	0.64
	0.69	ND	ND	0.69	0.77	ND	0.81	0.72	0.72	0.64	0.63	0.68	0.68	0.62
0.126	0.45	0.59	0.47	0.69	0.66	0.62	0.66	0.67	0.64	0.51	0.48	0.49	0.49	0.46
	0.49	0.61	0.46	0.56	0.64	0.62	0.65	0.59	0.65	0.51	0.45	0.49	0.46	0.45
0.252	0.29	0.46	0.33	0.47	0.48	0.49	0.49	0.47	0.46	0.29	0.27	0.3	0.27	0.27
	0.28	0.44	0.27	0.48	0.49	0.48	0.48	0.48	0.46	0.35	0.31	0.31	0.31	0.3
0.5039	0.22	0.26	0.23	0.28	0.33	0.34	0.26	0.3	0.27	0.17	0.2	0.21	0.21	0.2
	0.25	0.25	0.2	0.25	0.29	0.31	0.27	0.28	0.25	0.18	0.18	0.21	0.18	0.17
1.0078	0.12	0.17	0	0.12	0.17	0.14	0.15	0.12	0.14	0.07	0.08	0.12	0.13	0.12
	0.09	0.15	0.1	0.04	0.18	0.13	0.13	0.14	0.14	0.08	0.07	0.14	0.09	0.08
2.016	0	0.07	0	0	0.07	0.07	0	0	0.07	0	0	0	0	0
	0	0	0	0	0.04	0.08	0	0	0.04	0	0	0	0	0
4.03	0	0	0	0	0	0	0	0	0	0	0	0	0	0
	0	0	0	0	0	0	0	0	0	0	0	0	0	0
8.06	0	0	0	0	0	0	0	0	0	0	0	0	0	0
16.125	0	0	0	0	0	0	0	0	0	0	0	0	0	0
32.25	0	0	0	0	0	0	0	0	0	0	0	0	0	0
64.5	0	0	0	0	0	0	0	0	0	0	0	0	0	0
129	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Mean control	0.91	0.96	0.96	0.88	0.89	0.92	0.87	0.9	0.9	0.88	0.92	0.91	0.91	0.92

2 S5 Days post infection.

Conc.(ug/ml) LEV	21	28	35	42	49	56	63	70	77	84	91	98	105	112
0.0039	1.04	1.03	0.97	1.14	0.95	0.99	0.89	0.99	0.98	0.96	0.99	0.99	0.86	1.03
0.0079	0.96	ND	ND	ND	0.67	0.93	0.83	0.93	0.94	0.91	0.95	0.96	0.85	0.98
0.0158	0.95	ND	ND	ND	0.75	0.88	0.68	0.89	0.89	0.81	0.9	0.88	0.8	0.85
0.0315	0.86	0.81	0.78	0.91	0.68	0.81	0.62	0.75	0.77	0.73	0.87	0.86	0.73	0.73
0.063	0.84	0.78	0.7	0.71	0.68	0.75	0.62	0.72	0.72	0.63	0.7	0.62	0.64	0.69
	ND	ND	ND	0.75	0.7	0.73	0.64	0.73	0.72	0.64	0.67	0.63	0.62	0.67
0.126	0.82	0.62	0.5	0.63	0.64	0.59	0.54	0.6	0.62	0.46	0.49	0.45	0.45	0.51
	0.7	0.6	0.52	0.61	0.59	0.58	0.5	0.59	0.6	0.48	0.49	0.47	0.45	0.5
0.252	0.55	0.43	0.4	0.52	0.96	0.51	0.35	0.45	0.46	0.31	0.32	0.27	0.32	0.32
	0.55	0.45	0.38	0.5	0.48	0.46	0.35	0.45	0.46	0.31	0.29	0.29	0.26	0.29
0.5039	0.39	0.26	0.28	0.28	0.28	0.3	0.2	0.27	0.23	0.16	0.19	0.2	0.19	0.23
	0.45	0.3	0.26	0.22	0.28	0.35	0.18	0.28	0.28	0.2	0.21	0.17	0.21	0.21
1.0078	0.24	0.22	0.12	0.12	0.13	0.2	0.1	0.13	0.18	0.08	0.1	0.13	0.09	0.11
	0.27	0.19	0.14	0.1	0.14	0.14	0.13	0.12	0.16	0.07	0.11	0.08	0.13	0.1
2.016	0.13	0.1	0.06	0	0.13	0.1	0.06	0	0.04	0	0	0	0	0
	0.07	0.11	0	0	0.08	0	0.05	0	0.05	0	0	0	0	0
4.03	0	0	0	0	0	0.09	0	0	0	0	0	0	0	0
	0.04	0	0	0	0	0	0	0	0	0	0	0	0	0
8.06	0	0	0	0	0	0	0	0	0	0	0	0	0	0
16.125	0	0	0	0	0	0	0	0	0	0	0	0	0	0
32.25	0	0	0	0	0	0	0	0	0	0	0	0	0	0
64.5	0	0	0	0	0	0	0	0	0	0	0	0	0	0
129	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Mean control	0.86	0.97	0.91	0.84	0.92	0.88	0.92	0.92	0.91	0.91	0.88	0.93	0.94	0.87

2 S6 Days post infection.

Conc.(ug/ml) LEV	21	28	35	42	49	56	63	70	77	84	91	98	105	112
0.0039	0.92	0.96	1.04	0.95	0.98	0.94	1.09	0.94	1.06	1.05	0.99	1	1.01	1.03
0.0079	0.94	ND	ND	ND	0.87	0.9	0.92	0.94	0.91	0.95	0.93	0.97	1.07	0.95
0.0158	0.88	ND	ND	ND	0.76	0.83	0.87	0.85	0.89	0.86	0.86	0.95	0.97	0.85
0.0315	0.83	0.82	0.89	0.93	0.72	0.79	0.78	0.74	0.8	0.82	0.77	0.77	0.84	0.76
0.063	0.63	0.78	0.7	0.64	0.62	0.73	0.73	0.73	0.74	0.67	0.67	0.65	0.7	0.61
	0.64	ND	0.84	0.71	0.61	0.76	0.74	0.73	0.71	0.69	0.69	0.65	0.73	0.61
0.126	0.5	0.6	0.48	0.62	0.48	0.62	0.61	0.62	0.66	0.49	0.48	0.48	0.49	0.44
	0.46	0.64	0.45	0.56	0.46	0.55	0.6	0.59	0.66	0.49	0.48	0.48	0.49	0.44
0.252	0.34	0.45	0.39	0.49	0.24	0.45	0.47	0.47	0.49	0.29	0.31	0.28	0.34	0.27
	0.28	0.5	0.46	0.49	0.31	0.45	0.45	0.45	0.47	0.3	0.32	0.28	0.33	0.3
0.5039	0.25	0.27	0.21	0.28	0.2	0.27	0.27	0.3	0.31	0.18	0.21	0.21	0.22	0.22
	0.2	0.26	0.28	0.27	0.13	0.23	0.27	0.27	0.28	0.15	0.22	0.21	0.21	0.23
1.0078	0.11	0.21	0.15	0.14	0.09	0.14	0.14	0.12	0.15	0.07	0.06	0.14	0.13	0.11
	0.13	0.19	0.1	0.12	0	0.12	0.12	0.14	0.19	0.1	0.07	0.12	0.1	0.13
2.016	0	0.13	0	0	0	0	0	0.04	0.07	0	0	0	0	0
	0	0.16	0	0	0	0	0	0.02	0.08	0	0	0	0	0
4.03	0	0	0	0	0	0	0	0	0	0	0	0	0	0
	0	0	0	0	0	0	0	0	0	0	0	0	0	0
8.06	0	0	0	0	0	0	0	0	0	0	0	0	0	0
16.125	0	0	0	0	0	0	0	0	0	0	0	0	0	0
32.25	0	0	0	0	0	0	0	0	0	0	0	0	0	0
64.5	0	0	0	0	0	0	0	0	0	0	0	0	0	0
129	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Mean control	0.89	0.95	0.85	0.88	0.92	0.93	0.91	0.92	0.9	0.85	0.89	0.89	0.84	0.95

KEY: ND (Not Done)

Annex to Appendix VI. Correlation between LD_{50} values and the mean proportion of L3 larvae in the control wells.

Sheep	Expt. IVM	Expt. II IVM	Expt.II AVMB2	Expt.II LEV
S1	0.19	-0.20	-0.11	-0.05
S2	0.45	0.22	0.02	0.28
S3	0.60*	-0.19	0.22	0.28
S4	-0.52	0.01	0.03	-0.60*
S5	-0.44	-0.46	-0.52	-0.17
S6	0.52	0.23	0.05	0.05

*** significant $p<0.05$**

Appendix VII

The coefficient of determination for fitting the sigmoid curve (COD)values by day by animal for each anthelmintic.

Experiment I Results with Ivermectin.

Day	S1	S2	S3	S4	S5	S6
21	0.97	0.94	0.96	0.97	0.95	0.97
28	ND	ND	ND	ND	ND	ND
35	0.95	0.94	0.93	0.95	0.95	0.94
42	0.97	0.94	0.88	0.97	0.96	0.97
49	0.97	0.93	0.93	0.97	0.97	0.96
56	0.96	0.97	0.96	0.99	0.98	0.98
63	0.96	0.95	0.97	0.98	0.97	0.97
70	0.97	0.95	0.95	0.96	0.97	0.98
77	0.94	0.96	0.98	0.98	0.98	0.99
84	0.96	0.97	0.95	0.98	0.99	0.98
91	0.97	0.96	0.96	0.99	0.99	0.98
98	0.96	0.97	0.95	0.99	0.98	0.99
105	0.97	0.98	0.97	0.98	0.99	0.99
112	0.98	0.97	0.97	0.98	0.98	0.98

KEY: ND means No Data. (S1, S2 S3=GR.1.1; S4, S5, S6=GR.1.2)

Experiment II Results with Ivermectin.

Day	S1	S2	S3	S4	S5	S6
21	0.93	0.94	0.97	0.94	0.89	0.95
28	0.97	0.98	0.98	0.95	0.98	0.98
35	0.97	0.99	0.98	0.97	0.98	0.96
42	0.98	0.99	0.99	0.98	0.99	0.99
49	0.98	0.97	0.98	0.96	0.99	0.98
56	0.97	0.98	0.98	0.99	0.99	0.99
63	0.98	0.98	0.98	0.99	0.99	0.99
70	0.98	0.99	0.98	0.99	0.99	0.99
77	0.99	0.98	0.99	0.98	0.99	0.92
84	0.99	0.99	0.99	0.99	0.99	0.99
91	0.99	0.99	0.99	0.99	0.99	0.99
98	0.99	0.99	0.99	0.99	0.99	0.99
105	0.98	0.99	0.98	0.99	0.98	0.99
112	0.99	0.99	0.99	0.99	0.99	0.99

Key: (S1, S2, S3=GR. 2.2; S4, S5 S6=GR. 2.1)

Results with Avermectin B_2

Day	S1	S2	S3	S4	S5	S6
21	0.98	0.98	0.98	0.99	0.98	0.98
28	0.95	0.95	0.97	0.97	0.87	0.98
35	0.98	0.99	0.99	0.98	0.99	0.98
42	0.98	0.99	0.97	0.99	0.98	0.98
49	0.99	0.99	0.98	0.98	0.99	0.98
56	0.98	0.99	0.98	0.99	0.99	0.99
63	0.99	0.99	0.99	0.99	0.99	0.99
70	0.99	0.99	0.99	0.99	0.99	0.99
77	0.99	0.99	0.99	0.99	0.99	0.99
84	0.99	0.99	0.99	0.99	0.93	0.99
91	0.99	0.99	0.99	0.99	0.99	0.99
98	0.99	0.99	0.99	0.99	0.99	0.99
105	0.99	0.99	0.99	0.99	0.99	0.99
112	0.99	0.99	0.99	0.99	0.99	0.99

Results with Levamisole.

Day	S1	S2	S3	S4	S5	S6
21	0.99	0.9	0.99	0.99	0.99	0.99
28	0.98	0.99	0.99	0.99	0.89	0.99
35	0.99	0.99	0.99	0.98	0.99	0.98
42	0.98	0.98	0.98	0.98	0.98	0.98
49	0.97	0.96	0.96	0.99	0.92	0.99
56	0.92	0.98	0.96	0.98	0.97	0.99
63	0.98	0.97	0.98	0.99	0.98	0.98
70	0.99	0.94	0.98	0.99	0.99	0.99
77	0.99	0.99	0.99	0.99	0.99	0.99
84	0.99	0.99	0.99	0.99	0.99	0.99
91	0.99	0.98	0.99	0.99	0.99	0.99
98	0.99	0.99	0.99	0.99	0.99	0.94
105	0.99	0.99	0.99	0.99	0.99	0.99
112	0.99	0.99	0.99	0.99	0.99	0.99

Appendix VIII.

Counting of Parasitic Worms at Necropsy.

Equipment.

Gut scissors, strings, plastic bucket 8 litres capacity marked at 2L and 4L, Agee preserving jars, 500ml size. Kitchen ladles about 25ml, one litre plastic beaker marked at 100ml intervals and 250ml plastic beaker marked at 50ml intervals. Wooden stirrer-flat in section 2cm x 0.5cm x 30-40cm long. 60-mesh/in (250μm aperture) sieve, Lugol's iodine (5% iodine and 10% potassium iodide in water), 1% sodium thiosulphate in "squeezy" bottles. Petri dish with scored lines and a dissecting microscope.

Procedure.

1. The carcase is opened with the right side upper most. The abomasum, small intestine, caecum and colon are located and tied with string ligatures around the omasum, about 2cm distal to the pylorus, distal ilium, caecum and distal rectum.
2. After removing the entire gastrointestinal tract from the carcase, the abomasum is opened by cutting through the distal part of the omasum and a second ligature is tied 2cm distal to the pylorus.
3. The abomasum is placed in a bucket and opened along its length. The mucosa is washed thoroughly. As for the small intestine, the first half (8-10 meters from the pylorus) is opened by means of scissors along its length. The opened intestine is pulled between the fingers under a trickle of water.

4. The contents of the bucket are made to 2 litres with water and mixed vigorously cross-stirring and at the same time ladling out samples into a beaker until one tenth (200ml) of the total volume is removed.

5. The sample is poured from the beaker into 250μm aperture sieve and washed gently with a steady flow of water until clear. The sieved material is washed back into the Agee jar.

6. The contents are stained with several drops of Lugol's iodine for about 3 minutes, then discolourised background with sodium thiosulphate.

7. The sample is then examined under a dissection microscope by means of a petri dish with lines scored on it.

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