MODULE 2. HELMINTHS: ANTHELMINTIC RESISTANCE: DIAGNOSIS, MANAGEMENT AND PREVENTION

1 INTRODUCTION

The era of modern anthelmintics started in the middle of the 20th century with the introduction of phenothiazine and piperazine, products that are considered to be the first generation of the broad spectrum drugs. The 2nd generation of truly broad spectrum anthelmintics were released in the 1960s and included the benzimidazoles, the phenothiazine, the imidazothiazoles and the tetra-hydro-pyrimidines. Following the early success of the introduction of the benzimidazoles, extensive research programmes were initiated during which successful structural modification resulted in the production of a series of benzimidazoles. Most recently, a 3rd generation of broad spectrum anthelmintics, the macrocyclic lactones, emerged in the early nineteen eighties.

In addition, other compounds with a narrower spectrum have also been available on the market. These include substituted salicylanilides, phenols and organophosphates.

Thus the pharmaceutical industry has, during the last 35 years, been able to produce a string of highly effective, broad and narrow spectrum anthelmintics, and veterinarians and livestock producers have used these extensively for parasite control either by drenching or injecting cattle, sheep and goats.

The access to efficient drugs and the ease with which they could be applied, combined with the immense progress made in establishing the epidemiology of the gastro-intestinal nematodes of ruminants, led to a period of relative success in the control of worms, particularly in the livestock production systems of the industrialized countries. However, the false assumption that worm control is easy and can be accomplished by using broad spectrum drugs without an epidemiological database was also being promoted, preventing or delaying the epidemiological studies that are a prerequisite for effective control. Further complicating the situation today and for future parasite control programmes is the fact that all the economically important parasite species of sheep and goats have developed resistance to all four groups of anthelmintics.

2 RESISTANCE DEVELOPMENT: HELMINTHS

Resistance is probably an inevitable consequence of the use of anthelmintics, and the history of parasite resistance to anthelmintics starts with the first report on phenothiazine resistance in 1957. It is apparent from the records of the reported resistance to the major anthelmintics presented in Table 1 that resistance tends to develop only a few years after the introduction of the new drugs. It should also be noted that in most cases, Haemonchus contortus was the first nematode to develop resistance against the different anthelmintics. There is substantial evidence that when a parasite has developed resistance to one anthelmintic from a certain group it will usually also be resistant to other products from the same group.

There are several phases in the process of resistance development. Firstly, there is an initial phase of susceptibility where the number of resistant individuals within the parasite population is low. With continued exposure to the same drug group, an intermediate phase then follows in which the frequency of heterozygous resistant individuals within the population increases. Finally, sustained selection pressure results in a resistant phase where homozygous resistant individuals predominate within the population. The speed of this process will depend on how severe the selection pressure is on the parasite population. It is known that this is linked to the frequency of treatment and the fact that widespread and excessive use (8 to 12 times per year) of these drugs in sheep, without considering the epidemiology and ecology of the parasites, has led to the development of resistance of the sheep parasites to drugs from all four chemical groups.
Table 1. The first reports of anthelmintic resistance in nematodes of sheep to drugs with different modes of action (Coles et al., 1994)

<table>
<thead>
<tr>
<th>Year</th>
<th>Country</th>
<th>Drug</th>
<th>Nematode</th>
</tr>
</thead>
<tbody>
<tr>
<td>1957</td>
<td>USA</td>
<td>Phenothiazine</td>
<td>H. contortus</td>
</tr>
<tr>
<td>1964</td>
<td>USA</td>
<td>Thiabendazole</td>
<td>H. contortus</td>
</tr>
<tr>
<td>1968</td>
<td>USA</td>
<td>OP-compounds</td>
<td>T. circumcinctus</td>
</tr>
<tr>
<td>1976</td>
<td>Australia</td>
<td>Levamisole/Morantel</td>
<td>H. contortus</td>
</tr>
<tr>
<td>1980</td>
<td>S. Africa</td>
<td>Rafoxanide</td>
<td>H. contortus</td>
</tr>
<tr>
<td>1987</td>
<td>S. Africa</td>
<td>Ivermectin</td>
<td>H. contortus</td>
</tr>
</tbody>
</table>

Under-dosing, which is a common problem, is likely to favour the survival of heterozygous individuals, possibly enhancing the selection pressure for resistance. Persistence and initial efficacy of the drugs were found to be far more important in determining the rate of selection for resistance as drug efficacy declined, than was the selection of resistant third larval stage (L3) parasites (Dobson et al., 1996). There is also evidence that strategic treatments have contributed to resistance development, particularly at times when the free-living component of the parasite population has been small.

3 RESISTANCE TO ANTHELMINTICS: CURRENT STATUS

The first reports of anthelmintic resistance were made on farms attached to parasitological research establishments where anthelmintics were often used intensively. Because of the seemingly formidable chemotherapeutic arsenal at the disposal of the stockowner, such reports were often considered to be merely parasitological oddities and their potential significance was overlooked. It was not until control failed on a substantial number of farms that relied heavily on intensive anthelmintic treatment to maintain productivity, that the potential implications were fully realized. Since the early 1980s resistance has been detected among the gastro-intestinal nematode parasites of sheep and goats throughout the world, and large scale surveys have shown that the situation is critical in many Latin American countries, South Africa, Australia and New Zealand. Many farms have resistance to at least two of the anthelmintic groups and a substantial number have resistance to all four groups. An extensive literature search of all the main life sciences databases for the period 1993 to 1998 found 142 publications on resistance. These have been analysed to determine the species of parasites in which resistance has been diagnosed and to what drugs. The results are presented in Table 2; the reported cases of resistance in sheep and goat helminths to the different anthelmintic groups, from 1993 until the present, are presented in Table 3. In sheep, narrow spectrum anthelmintics such as the salicylanilides, rafoxanide, organophosphates and thiophanate, could be alternative tools where resistance to the major anthelmintic groups is present.

Table 2. Reported cases (X) of resistance in helminth parasites of sheep and goat according to class of anthelmintic

<table>
<thead>
<tr>
<th>Parasite</th>
<th>BZ</th>
<th>IMZ</th>
<th>ML</th>
<th>SAL</th>
<th>OP</th>
<th>RA</th>
<th>TH</th>
</tr>
</thead>
<tbody>
<tr>
<td>H. contortus</td>
<td>X</td>
<td>rare</td>
<td>X</td>
<td>X</td>
<td>X</td>
<td>X</td>
<td>X</td>
</tr>
<tr>
<td>Ostertagia spp.</td>
<td>X</td>
<td>X</td>
<td>X</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Trichostrongylus spp.</td>
<td>X</td>
<td>X</td>
<td>X</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Nematodirus spp.</td>
<td>X</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Fasciola hepatica</td>
<td>X</td>
<td>-</td>
<td>X</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
</tbody>
</table>

BZ= Benzimidazole; IMZ= Imidazothiazole; ML= Macrocyclic lactones; SAL=Salicylanilide; OP= Organophosphate; RA= Rafoxanide; TH=Thiophanate
Based on data from Survey of OIE member countries, FAO questionnaires (1998) and literature search (1999)

* The countries have reported "No resistance". However this is not necessarily based on the results of randomized countrywide surveys.
** The countries did not reply to the questionnaires.
### Table 3. Reported cases of resistance in sheep and goat helminths listed for each anthelmintic group.

<table>
<thead>
<tr>
<th>Drug</th>
<th>Anthelmintic resistance reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>Benzimidazoles</td>
<td>Coles et al., 1998a; Romero et al., 1998; Chaudhri et al., 1997; Maingi et al., 1998; Chartier et al., 1998; Waruiru et al., 1998; Kerboeuf et al., 1997; Campos Ruelas et al., 1997; Mukaratiwira et al., 1997; Corba et al., 1998; Farias et al., 1997; Waruiru, 1997a; de Souza et al., 1996; Borgsteede et al., 1997; Balicka Ramisz et al., 1997; Barre et al., 1997; Waruiru et al., 1997; Waruiru, 1997b; Van Wyk et al., 1997; Boersema and Pandey, 1997; Sutherland et al., 1997; Singh and Yadav, 1997; Sani and Chandrawathani, 1996; Wanyangu et al., 1996; Maingi et al., 1997a; Maingi et al., 1997b; Borgsteede et al., 1996; Chartier et al., 1996; Dhirendra Singh et al., 1995; Bauer et al., 1996; Hong et al., 1996; Waruiru et al., 1996; Soccol et al., 1996; Yadav et al., 1996; Mage et al., 1994; Eddi et al., 1996; Echevarria et al., 1996; Maciel et al., 1996a; Maciel et al., 1996b; Miller and Craig, 1996; Mwamachi et al., 1995; Cabaret et al., 1995; Yadav et al., 1995; Srivastava et al., 1995; Watson et al., 1993; Waruiru, 1994; Varady et al., 1995; McKenna et al., 1995; Whelan et al., 1995; Charles and Medeiros, 1993; Uppal et al., 1995; Kochapakdee et al., 1995; Waruiru et al., 1994; Burger et al., 1994; Borgsteede et al., 1995; Dhirendra Singh et al., 1995; Coles and Simkins, 1996; Eddi et al., 1996; Maciel et al., 1996a; Maciel et al., 1996b; Miller and Craig, 1996; Mwamachi et al., 1995; Nilsson et al., 1995; Yadav and Uppal, 1993; Singh et al., 1992; Pandey and Sivaraj, 1994; Overend et al., 1994; Nilsson et al., 1992; Hunt et al., 1994; Chartier and Pors, 1994; Van Wyk et al., 1991; Campos Ruelas et al., 1992; Varady et al., 1994; Rahman, 1994b; Sivaraj et al., 1994; Vieira et al., 1992; Romero et al., 1992; Taylor and Hunt, 1993; Rudby Martin and Nilsson, 1991; Yadav et al., 1993; Oosthuizen and Erasmus, 1993; Echevarria et al., 1993; Louw and Reinecke, 1993; Gray et al., 1993; Corba et al., 1993; Uppal et al., 1993; Rothwell and Sangster, 1993; Maingi, 1993.</td>
</tr>
<tr>
<td>Imidathiazole/Morantel</td>
<td>Coles et al., 1998a; Romero et al., 1998; Maingi et al., 1998; Chartier et al., 1998; Waruiru et al., 1998; Mukaratiwira et al., 1997; Corba et al., 1998; Hoekstra et al., 1997; Farias et al., 1997; Waruiru, 1997a; Sharma, 1996; Waruiru et al., 1997; Waruiru, 1997b; Van Wyk et al., 1997; Boersema and Pandey, 1997; Singh and Yadav, 1997; Sani and Chandrawathani, 1996; Wanyangu et al., 1996; Maingi et al., 1997a; Maingi et al., 1997b; Hong et al., 1996; Soccol et al., 1996; Coles and Simkins, 1996; Eddi et al., 1996; Echevarria et al., 1996; Maciel et al., 1996a; Maciel et al., 1996b; Miller and Craig, 1996; Mwamachi et al., 1995; Yadav et al., 1995; Santos et al., 1993; Praslicka et al., 1995a; Varady et al., 1995; McKenna et al., 1995; Sangster and Bjorn, 1995; Dorny et al., 1994; Hong et al., 1994; Chartier and Pors, 1994; Varady et al., 1994; Sivaraj et al., 1994; Yadav et al., 1993; Corba et al., 1993; Uppal et al., 1993; Rothwell and Sangster, 1993; Maingi, 1993.</td>
</tr>
<tr>
<td>Macrocyclic lactones</td>
<td>Romero et al., 1998; Kotze, 1998; Gill et al., 1998a; Gill et al., 1998b; Farias et al., 1997; Rolfe and Fitzgibbon, 1996; Besier, 1996; Waruiru, 1997a; de Souza et al., 1997; Sutherland et al., 1997; Sani and Chandrawathani, 1996; Maingi et al., 1997a; Maingi et al., 1997b; Soccol et al., 1996; Eddi et al., 1996; Echevarria et al., 1996; Maciel et al., 1996a; Maciel et al., 1996b; Miller and Craig, 1996; Watson et al., 1996; Mwamachi et al., 1995; Le Jambre et al., 1995; Watson et al., 1993; Varady et al., 1995; Charles and Medeiros, 1993; Le Jambre, 1993; LeJambre et al., 1995; Miller and Barras, 1994; Shoop et al., 1993; Le Jambre et al., 1995; Van Wyck and Schallwyc, 1991; Varady et al., 1994; Sivaraj and Pandey, 1994; Sivaraj et al., 1994; Vieira et al., 1992; Oosthuizen and Erasmus, 1993; Echevarra et al., 1993; Pomroy and Wheelan, 1993; Corba et al., 1993; Rothwell and Sangster, 1993.</td>
</tr>
<tr>
<td>Salicylanilide</td>
<td>Anonymous, 1996; Soccol et al., 1996; Echevarria et al., 1996; Mwamachi et al., 1995; Oosthuizen and Erasmus, 1993; Echevarria et al., 1993; Louw and Reinecke, 1993; Rothwell and Sangster, 1993.</td>
</tr>
<tr>
<td>Organophosphate</td>
<td>Lorenzelli et al., 1996; Van Wyk et al., 1997; Soccol et al., 1996.</td>
</tr>
<tr>
<td>Rafoxanide</td>
<td>Waruiru et al., 1998; Boersema and Pandey, 1997; Dhirendra Singh et al., 1996; Van Wyk et al., 1991.</td>
</tr>
<tr>
<td>Thiophanate</td>
<td>Waruiru et al., 1996; Yadav et al., 1996; Chartier, 1993.</td>
</tr>
</tbody>
</table>
The problem of anthelmintic resistance in cattle parasites has not been investigated nearly as intensively as it has for small ruminants (McKenna, 1996a). Assessing the situation in cattle, it should be taken into account that in general, cattle parasites are nearly always a sub-clinical problem. It is possible that even efficacy levels of 50 to 70 percent could well hide the adverse effects of parasites on cattle, so the same frequency of resistant worms in a population might be less likely to be detected in cattle as they are in sheep. However, more and more reports are being published and it appears that anthelmintic resistance in cattle parasites is an emerging problem, with the potential of developing to similar proportions as experienced with sheep and goat parasites (Coles et al., 1998b). Analyses of the currently available publications are presented in Tables 4 and 5.

Table 4. Reported cases of resistance in helminth parasites of cattle according to class of anthelmintic

<table>
<thead>
<tr>
<th>Parasite</th>
<th>BZ</th>
<th>IMZ</th>
<th>ML</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>H. contortus</em></td>
<td>X</td>
<td>X</td>
<td></td>
</tr>
<tr>
<td><em>Ostertagia</em> spp.</td>
<td>X</td>
<td>X</td>
<td></td>
</tr>
<tr>
<td><em>Trichostrongylus</em> spp.</td>
<td>X</td>
<td>X</td>
<td></td>
</tr>
<tr>
<td><em>Cooperia</em> spp.</td>
<td>X</td>
<td>X</td>
<td>X</td>
</tr>
</tbody>
</table>

BZ= Benzimidazole; IMZ= Imidazothiazole; ML=Macrocyclic lactones;

Table 5. Reported cases of resistance in helminth parasites of cattle listed for each anthelmintic group

<table>
<thead>
<tr>
<th>Drug</th>
<th>Anthelmintic resistance reference</th>
</tr>
</thead>
</table>

In conclusion, the available data from the literature review showed that the vast majority of economically important gastro-intestinal nematodes of sheep and goats, and to a lesser extent cattle, have developed resistance to all of the available anthelmintics.

Resistance to anthelmintics could also become a problem for wildlife. The role of wild ruminants in spreading anthelmintic-resistant nematodes between flocks of sheep or goats has already been confirmed (Praslicka *et al.*, 1995b).

Considering the situation, it would be natural to look to the pharmaceutical industry for new drugs (Coles, 1998). We are however led to believe that there are likely to be few forthcoming for the following reasons. The expenses incurred by the companies from the identification of a new chemical until it is tested and marketed are in excess of US$100 million. Thus the companies are looking very carefully at the potential markets before investing, and the industry has most recently focused on the human market and on products for pets.

It is fair to say that parasite control is at a crossroads and it is essential that we:

Preserve and safeguard the drugs we have available and use them wisely.

Realize that the time of easy parasite control based on the use of anthelmintics only is over.

Understand, that in the future we will have to rely on a combination of strategies which will require more work and more monitoring.
Understand that when resistance has been detected, the drugs will have reduced or no effect and the use of the compound, or any from the same family of drugs, is likely to be a waste of resources.

4 DIAGNOSIS OF RESISTANCE: AN OVERVIEW OF METHODOLOGIES

With the development and spread of anthelmintic resistance in nematodes of livestock, the need for methods to detect resistance has evolved simultaneously. Different *in vivo* and *in vitro* tests are now available and there is an ongoing effort to refine, standardize and validate these tests. The development of molecular tests (Beech *et al.*, 1994) is also progressing and is trying to apply DNA-probe and polymerase chain reaction (PCR) technology.

**Diagnostic methods: In vivo**

**Faecal egg count reduction test. (FECRT)**

This is the most common test to study anthelmintic resistance. This test was originally designed for sheep, but can be used also for cattle, swine and horses.

Modern broad spectrum anthelmintics are highly efficacious, and treatment should normally result in a reduction of faecal egg counts by more than 95 percent. Thus this test provides an estimation of anthelmintic efficacy by comparing faecal egg counts of animals before and ten days after treatment (Gill *et al.*, 1998a). For monitoring of normal fluctuation, the treated group is generally compared with non-treated controls.

This test is particularly suitable for field surveys and it has the advantage that the number of groups can be increased if appropriate, to test the efficacy of a range of broad or narrow spectrum anthelmintics at one time.

**The controlled test**

In this test, the efficacy of an anthelmintic is determined by comparing parasite populations in groups of treated and non-treated animals. Basically, the procedure compares worm burdens of animals artificially infected with susceptible or suspected resistant isolates of nematodes. The parasitized animals are randomly separated into medicated and non-medicated groups and at a suitable interval after treatment (10 to 15 days), a necropsy is carried out and the parasites are recovered, identified and counted. This test is not extensively used, except in cases of special interest or when confirmation of resistance is required at species level, and for evaluation of the effect on larval stages (Reinecke and Louw, 1994).

In an attempt to reduce the cost and labour required for this test, laboratory animal models have been used and guidelines for evaluating anthelmintic efficacy using the controlled test have been published (Powers *et al.*, 1982; Presidente, 1985).

**Diagnostic methods: In vitro**

Several different *in vitro* tests are available but the majority is almost exclusively used for research purposes. These tests can be used to quantify the level of resistance but they require considerable technical expertise and in some cases, expensive laboratory equipment. Ideally, these tests require mono-specific infections because there can be difficulties in the interpretation of results with field infections, which usually consist of multiple parasite species. The maintenance of standard laboratory strains, both drug susceptible and resistant is necessary for comparative purposes (D’Assonville *et al.*, 1996). The main bioassays are listed in Table 6.
Table 6. Bioassays for the diagnosis of anthelmintic resistance

<table>
<thead>
<tr>
<th>Tests</th>
<th>Diagnosis of resistance to:</th>
</tr>
</thead>
<tbody>
<tr>
<td>Egg hatch</td>
<td>Benzimidazoles-Levamisole/Morantel</td>
</tr>
<tr>
<td>Larval paralysis</td>
<td>Levamisole/Morantel</td>
</tr>
<tr>
<td>Tubulin binding</td>
<td>Benzimidazoles</td>
</tr>
<tr>
<td>Larval development</td>
<td>All drugs</td>
</tr>
<tr>
<td>Adult development</td>
<td>Benzimidazoles</td>
</tr>
</tbody>
</table>

Several attempts have been made to establish the sensitivity of the different tests. For example, comparative studies to determine the resistance to thiabendazole and levamisole have used (a) an egg hatch assay, (b) an egg hatch paralysis assay, (c) a larval development assay, (d) a larval paralysis assay, (e) a larval paralysis assay with physostigmine and (f) a larval micro-motility assay.

Of all the available tests, the larval development test is the most sensitive for quantitatively measuring thiabendazole and levamisole resistance. The egg hatch assay is also sensitive and accurate in determining benzimidazole resistance. It was concluded that the other methods were unsuitable for use in field monitoring of resistance (Varady and Corba, 1999).

The egg hatch assay

The egg-hatch test has been developed to differentiate between resistant and susceptible strains of gastro-intestinal nematodes for the benzimidazoles and for the levamisoles. It provides an accurate method for assessing the susceptibility of mixed nematode populations, and it is comparatively more rapid and economic to conduct than the FECRT. It is based on the determination of the proportion of eggs that fail to hatch in solutions of increasing drug concentration in relation to the control wells, enabling the user of the test to develop a dose response line plotted against the drug concentration. To obtain meaningful data, eggs for the egg hatch test must be fresh and should be used within three hours of being shed from the host, as sensitivity to some benzimidazoles decreases as embryonation proceeds. The test has only been shown to work on nematode species in which eggs hatch rapidly. Due to difficulties in the interpretation of the results this assay is not widely used for field surveys.

Larval paralysis and motility assay

The test is used for levamisole and morantel resistance. This assay discriminates between resistant and susceptible strains of parasites, by estimating the proportion of third stage larvae in tonic paralysis after incubation with a range of levamisole and morantel drug concentrations. It is relatively easy to carry out, stocks of infective larvae are readily obtained and it is reported that there is a fairly good reproducibility of the test, any differences in repeatability being attributed to the age of larvae. However, the interpretation is complicated by the fact that if the anthelmintic is added to the egg suspension too early, the development has not proceeded far enough; if it is added too late the drug has no effect.

A modification of the technique was developed using the micro-motility meter, an instrument for measuring the motility of larval and adult nematodes after incubation with benzimidazole and levamisole. A further modification of the larval paralysis assay has been made in order to apply it for the detection of thiabendazole resistance. Some lack of repeatability in this method has been attributed to the reversibility of paralysis.
Module 2: Helminths

Tubulin binding assay

This test is based on the mode of action of the drugs. The mechanism of benzimidazole resistance appears to be associated with a reduced affinity of tubulin for the anthelmintics. The test is based on the differential binding of benzimidazoles to tubulin, an intracellular structural protein from susceptible and resistant nematodes. The test involves the incubation of a crude tubulin extract from adult parasites, infective larvae or eggs, with a tritiated benzimidazole until equilibrium is reached. The free, unbound drug in test suspension after incubation is removed using charcoal, and the tubulin-bound label is sampled and counted by liquid scintillation spectrophotometry. Tubulin extracts from resistant parasites bind substantially less strongly than do those from susceptible parasites. The test is claimed to be rapid, robust, highly reproducible and sensitive to minor changes in the resistance status of parasite populations, but it requires relatively large numbers of larvae, making it unsuitable for routine field assays. Moreover, it requires access to expensive laboratory apparatus for high performance liquid chromatography (HPLC) estimations and a source of radiolabelled drug.

Larval development assay (LDA)

The larval development tests are the only ones that allow the detection of resistance against all the drugs, irrespective of their mode of action. Several methods have been described, but reproducibility, linearity of the dose-response and susceptibility differ. The LDA is an in vitro assay for the detection of resistance to benzimidazole, levamisole, combinations of benzimidazole and levamisole, and avermectin and milbemycin drenches in the major gastrointestinal nematode parasites of sheep, *Haemonchus contortus*, *Trichostrongylus colubriformis* and *Ostertagia circumcincta*. In this test nematode eggs, isolated from faecal samples submitted by producers, are applied to the wells of a micro-titre plate and larvae hatch and develop to the L3 stage in the presence of anthelmintic. The concentration of anthelmintic required to block development is related to an anticipated in vivo efficacy.

Adult development assay

The adult development assay for detecting benzimidazole resistance in trichostrongylid nematodes has advanced significantly and *Haemonchus contortus* has been cultured through to the adult egg-laying stages, although this test is mainly for research purposes.

Research and development of new tests

Lately, gene probes, allele frequencies, trans-membrane functional analysis, PCR and flow cytometry have been investigated as tools for the determination of anthelmintic resistance. Currently, these procedures are exclusively for research purposes (Kwa *et al.*, 1998).

Gene probes have been used to analyse restriction fragment length polymorphism between susceptible isolates and isolates of *Haemonchus* resistant to benzimidazole; levamisole and benzimidazole; or benzimidazole, ivermectin and closantel. A P-glycoprotein gene probe was also isolated from *Onchocerca volvulus* and an *Onchocerca*-specific PCR was developed for detection of resistance strains (Kwa *et al.*, 1998).

Analyses of allele frequencies showed significant differences between the unselected and the drug-selected derived strains. In all three drug-selected strains, an apparent selection for the same allele was observed. It is suggested that P-glycoprotein (Pgp) may be involved in resistance to both ivermectin and moxidectin in *H. contortus* (Blackhall *et al.*, 1998).

A functional analysis of trans-membrane transport of drugs in drug-resistant helminths was undertaken using a flow cytometry method on two isolates of *Haemonchus contortus* that were susceptible or resistant to benzimidazoles and ivermectin. The results confirm those obtained with biological drug assays, using both anthelmintics and verapamil, which suggest the involvement of
Pgp in drug resistance, and provide a quantitative and effective methodology for the functional study of multi-drug resistance in nematodes (Kerboeuf et al., 1999).

A very sensitive PCR test was developed that can detect benzimidazole resistance in the sheep parasite *Haemonchus contortus*. With this assay, the population genetics of benzimidazole susceptible and resistant worms can be studied in more detail under different conditions of selection. This may lead to a better control and a delay in the development of anthelmintic resistance (Roos et al., 1994).

Flow cytometry could be applied to the analysis of nematode populations. Forward-scatter emission can be used as a discriminating parameter for egg size. The hatching rate and side-scatter emission have a significantly positive relationship. The rate of resistance to the anthelmintic can be observed as a significant regression on the native green-fluorescence pulses that might reflect the state of oxidation of associated flavin molecules (Kerboeuf et al., 1996).

5. DETECTION OF ANTHELMINTIC RESISTANCE: PROTOCOLS FOR RECOMMENDED METHODOLOGIES

Considering all the limitations of the various tests, such as difficulties in repeatability, requirement for complex and expensive laboratory procedures and lack of sensitivity, the guidelines will provide the protocol for two methods of detecting anthelmintic resistance, namely the faecal egg count reduction test (FECRT, *in vivo*) and the larval development assay (LDA, *in vitro*). If the FECRT is inconclusive it can be complemented by the efficacy test which will be briefly described.

**Faecal egg count reduction test (FECRT) (In vivo)**

The FECRT remains the most practical method of determining resistance by nematodes in sheep to anthelmintics.

**Principle:** The ability of the anthelmintic in question to reduce the concentration of eggs per gram of faeces (EPG) by more than 95 percent, measured ten days after treatment, in comparison with the EPG measured at the time of treatment. Failure to do so is indicative of resistance.

**Prerequisites:** The required group size is at least ten animals per group per anthelmintic to be tested. In addition, a non-treated control group is required. The pre-treatment EPGs must exceed 150 to 200.

**Advantages:** It is a simple and robust test that does not require highly trained personnel, expensive resources, sophisticated equipment or facilities, and it can be used for testing anywhere and for any of the anthelmintics routinely used. This procedure can be used for testing in sheep, goats, cattle, horses and pigs.

**Disadvantages:** Required group size and relatively high pre-treatment EPGs. False positive and negative indications of resistance to levamisole (Grimshaw et al., 1996). It is not reliable for the detection of low levels of anthelmintic resistance (McKenna, 1997).

**Protocol for FECRT: sheep and goats**

1. **Selection of animals**

   Use young animals, three to six months of age, which have been bred on the farm. Older animals can be used if individual egg counts are above 150 eggs per gram of faeces (EPG). Animals should not have been treated in the previous 8 to 12 weeks. If animals have been recently treated, the test may be conducted on 'pre-selected' worms but will not represent the normal distribution of the parasitic population.
Randomly allocate animals or allocate by ranking of faecal egg counts into control and treatment groups of at least 10 and preferably 15 animals each. Create one group for each of the anthelmintics to be tested. A control (untreated) group should be used to allow for monitoring of natural changes in egg counts during the test period.

2. **Treatment**

Animals are treated with the anthelmintic according to the manufacturer's recommended dose either as (a) the accurate dose in mg/kg (for research purposes) or (b) according to the weight of the heaviest animal in the group (for clinical diagnosis). Anthelmintics should be administered with a syringe or a drench gun that has been previously calibrated.

3. **Sampling procedures**

For pre-screening of animals for sufficient egg counts, a minimum of 5 g (10 to 15 pellets) of faeces should be collected from each animal directly from the rectum. The same procedure should be followed at the post-treatment sampling. Samples must be placed in individually sealed containers and returned rapidly to the laboratory for egg counts. If group mean egg counts are below 150 EPG (limit of sensitivity of McMaster counting technique is 50 EPG), assessment of resistance will not be reliable. Group mean egg counts below 150 EPG can be common in adult sheep.

The post-treatment collection of faecal samples should be 10 to 14 days after treatment. Sampling earlier may give misleading results with a number of anthelmintics.

4. **Processing of samples**

For faecal egg counts, a modified McMaster method should be used.

Weigh 3 g of faeces into a suitable container.

Add 42 ml of water and soak for a few minutes to 1 h, until the faeces are soft.

Homogenise using a laboratory stirrer or place in a shaker jar with about $45 \times 8$ mm diameter glass beads and shake until all the pellets have been broken up.

Pour through a 100 mesh (0.15 mm aperture), 20 cm diameter sieve into a bowl.

Swirl the liquid and pour 15 ml into a 17 ml centrifuge tube.

Centrifuge for 2 min at about $300 \times g$ (approximately 1500 rev/min on a bench-top centrifuge).

Gently pour or suck off the supernatant.

Agitate the tube to loosen the sediment.

Add suitable flotation fluid to give the same volume as before (15 ml).

Invert the tube five or six times.

Immediately withdraw a sample with a Pasteur pipette.

Fill the chamber of a McMaster slide.

Repeat the process of inversion and fill the second chamber.

At $10 \times$ magnification count all the eggs under the two ruled grids (total volume 0.3 ml).

Multiply the number of eggs by 50 to give the EPG in the faecal sample. For greater sensitivity count all the eggs in each chamber (total volume 1 ml and multiply by 15 to give the EPG).
5. Analysis and interpretation of data

Calculate the arithmetic mean, percentage reduction and 95 percent confidence interval. The arithmetic mean is preferable to the geometric mean as: (a) it is easier to calculate; (b) it provides a better estimate of the worm egg output; (c) it is a more conservative measure of anthelmintic activity.

The percentage reduction, \( \text{FECR} \% = 100(1 - \frac{X_t}{X_c}) \)

Where \( X_t \) is the mean egg count of the treated group at 10-14 days and \( X_c \) is that of the control group at 10–14 days.

Details of the calculation of the 95 percent confidence intervals are given in an example in Table 7. A computer program, RESO, is available for this calculation. Resistance is considered to be present if:

(i) the percentage reduction in egg counts is less than 95 percent and

(ii) the 95 percent confidence level is less than 90 percent.

If only one of the two criteria is met, resistance is suspected.

6. Factors affecting the result

There might be disagreement in the presentation of the results from calculated FECR depending on whether they are based on the use of the arithmetic mean or the geometric mean for EPG calculations. Similarly, inclusion of pre-treatment EPG or control group EPG in the calculation of FECR percentage could influence the determination of resistance (Maingi et al., 1997b). In general it is recommended to use the arithmetic mean, because it will give more conservative results. Where the control group is included in the calculations using geometric means, the percentage efficacy is corrected for changes that occur in this group by the equation:

\[ \text{FECR} \% = \left[ 1 - \frac{(C_1/C_2)}{(T_2/T_1)} \right] \times 100 \]

Where \( T \) and \( C \) are the geometric means for the treated and control groups, and subscripts 1 and 2 designate the counts before and after treatment, respectively (Presidente, 1985; Hotson et al., 1970).

FECRT results may not estimate anthelmintic efficacy accurately because nematode egg output does not always correlate well with worm numbers, and the test only measures the effects on egg production of mature worms. A good correlation was found between faecal egg counts and worm counts of \( H. contortus \), and \( O. circumcincta \) but not for \( T. colubriformis \). Egg counts for \( Nematodirus \) spp. are generally low and bear little relationship to actual worm burdens (Martin, 1985).

7. Diagnosis of genus and species present

The FECRT might not provide sufficient information on its own for correct interpretation. The failure of an anthelmintic to effectively reduce egg counts indicates resistance, but as most natural infections include a mixture of species and only one species may be resistant, there is a need to determine the resistant species. Third-stage larvae are therefore cultured from the eggs in faeces from controls and from treated groups separately. If remaining samples are to be cultured for the determination of nematode species, samples should not be stored at 4 °C as it may affect the hatching of \( Haemonchus contortus \). The procedure is as follows:
Module 2: Helminths

a. Collect about 50 g of faeces by combining similar size samples from each animal in one treatment group.

b. Break up faeces finely using a spatula. They should be moist and crumbly but not really wet. With wet faeces add vermiculite, crushed charcoal or sterile peat moss.

c. Fill glass culture dishes (e.g. crystallizing dish) with the mixture, covering but not sealing them, and culture for seven days at 22–27ºC.

d. Collect the larvae in a Baermann apparatus or by suspending the mixture in water in muslin, or by standing the mixture in a Petri dish containing water.

e. Treat the larvae with Lugol's iodine and identify 100 larvae. Identification guides are given in the chart below.

The nematode genera that are represented at the time of routine FECRT must be taken into account to reduce the likelihood of being misled when undertaking assessments of farm resistance status (McKenna, 1996b; Hotson et al., 1970; Martin, 1985; Taylor, 1992; Kerboeuf, 1994; Dash et al., 1985).

A modification of the FECRT has been described in which no pre-treatment samples are taken. The authors argued that statistically, both treatment and control groups are taken from the same population mean and as a result, the pre-treatment sample can discounted.

Protocol for FECRT: cattle, horses and pigs

The procedures are the same as those described above for sheep.

Table 7. Example of FECRT calculations

<table>
<thead>
<tr>
<th>Data</th>
<th>Post treatment egg counts (10–14 days)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Control</td>
</tr>
<tr>
<td>525</td>
<td>15</td>
</tr>
<tr>
<td>450</td>
<td>0</td>
</tr>
<tr>
<td>270</td>
<td>0</td>
</tr>
<tr>
<td>540</td>
<td>30</td>
</tr>
<tr>
<td>90</td>
<td>0</td>
</tr>
<tr>
<td>765</td>
<td>0</td>
</tr>
<tr>
<td>120</td>
<td>0</td>
</tr>
<tr>
<td>945</td>
<td>0</td>
</tr>
<tr>
<td>465</td>
<td>45</td>
</tr>
<tr>
<td>225</td>
<td>0</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Calculations</th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td>Number in group $n_i$: ($N = \sum n_i$)</td>
<td>10</td>
</tr>
<tr>
<td>Arithmetic mean count $\bar{X}<em>i = \frac{\sum X</em>{ij}}{n_i}$</td>
<td>9</td>
</tr>
<tr>
<td>Variance of counts $s^2_i = \frac{\sum (X_{ij} - \bar{X}_i)^2}{n_i-1}$</td>
<td>74062</td>
</tr>
<tr>
<td>Percent reduction $R = 100\left(1 - \frac{X_t}{X_c}\right)$</td>
<td>0</td>
</tr>
<tr>
<td>Variance of reduction (on log scale) $\nu = [s^2_c/(n_cX^2_c)] + [s^2_t/(n_tX^2_t)]$</td>
<td>0.36</td>
</tr>
<tr>
<td>Approximate 95% confidence interval for $R$</td>
<td>99</td>
</tr>
<tr>
<td>Upper confidence limit $100[1-(X_t/X_c)\exp(-2.1\sqrt{\nu})]$</td>
<td>74</td>
</tr>
<tr>
<td>Lower confidence limit $100[1-(X_t/X_c)\exp(+2.1\sqrt{\nu})]$</td>
<td>93</td>
</tr>
</tbody>
</table>

Where $i$ denotes either the treated ($t$) or control ($c$) groups

$s_i^2$ denotes each sheep in the group

$s^2_t$ denotes the variance on the arithmetic scale, calculated as above or:

$s^2_t = \frac{\sum (X_{ij} - \bar{X}_t)^2}{n_t-1}$
In order to make valid comparison between results of different investigators, the conduct and the interpretation of the procedure require standardization (Waller, 1986; Cawthorne and Cheong, 1984).

**Efficacy test**

When results from an FECRT undertaken 10 days post-treatment are not definitive, the efficacy test can be carried out to reach final conclusions. A complete parasitological necropsy should be performed in five animals selected at random from both groups used in the FECRT to determine the worm burden in each test group.

The gastrointestinal tract should be processed for worm recovery according to the standard procedures; the abomasum through to the large intestine (each segment tied off at both ends) and the lungs and complete trachea must be collected from each carcass. All viscera must be processed for worm recovery within one hour of slaughter.

**Analysis of the data**

The arithmetic mean must be calculated for each nematode species in each group. Efficacy, expressed as a percentage (PE) for each species, will be obtained, using the following formula:

$$ PE \% = \left( \frac{MC - MT}{MC} \right) \times 100 $$

*Where*  

$ MC = $ mean number of worms in the control group.  

$ MT = $ mean number of worms in the treated group.

Resistance is considered to exist when the PE is below 90 percent.

**In vitro tests for anthelmintic resistance**

Of all the available *in vitro* tests, the larval development test (LDA) is the most sensitive for the quantitative determination of thiabendazole and levamisole resistance. The egg hatch assay is also sensitive and accurate in determining resistance to benzimidazoles, levamisole and macrocyclic lactones. It was concluded that the other *in vitro* methods described in the literature were unsuitable for use in field monitoring of resistance (Varady and Corba, 1999).

1. **Larval development assay (LDA)**

The LDA is an *in vitro* assay for the detection of resistance to benzimidazoles, levamisoles and macrocyclic lactones in nematodes parasites of sheep (Taylor, 1990; Varady et al., 1996), horses (Kerboeuf, 1994), pigs (Varady et al., 1996) and cattle. There is a commercial version of the LDA available which measures the resistance status of these anthelmintics.

**Principle:** Nematode eggs are isolated from a faecal sample, placed into wells of a microtitre plate and allowed to develop through to infective L3 larvae in the presence of a range of concentrations of anthelmintic.

**Advantages:** The LDA allows simultaneous evaluation of all broad spectrum anthelmintics in a single farm visit with minimal on-farm work.

**Disadvantages:** The counting procedure to determine the LD₅₀ is time consuming. As in other *in vitro* techniques, susceptible and resistant strains of parasites are necessary for comparison. In the case of macrocyclic lactones the test provides only an indication of anthelmintic resistance.
Protocol

1. Field collection of the samples

At least 10 animals are selected at random from the flock to be tested and no less than 100 g of faeces are collected as a pool in a plastic bag. The test must be carried out on faeces with a mean EPG above 200.

2. Nematode egg recovery technique

a. Suspend 20 g of faeces in water for 10 minutes, break up with a pestle and remove organic debris by filtration through a 100-mesh sieve.

b. Collect the filtrate and centrifuge at 2000 rev/min for 10 minutes.

c. Discharge supernatant and agitate the tubes to loose the sediment, then refill the centrifuge tubes with saturated sucrose solution until a meniscus forms above the tube.

d. Add a cover slip and centrifuge again at 1000 rev/min for 5 minutes. Gently pluck the cover slip off the tube and wash off the eggs to another centrifuge tube.

e. Fill the tube with distilled water and centrifuge for another 5 minutes at 1000 rev/min. This last step is to further clean the eggs isolated from the sucrose solution.

f. Remove the supernatant and estimate the eggs per ml and dilute to the required concentration (80 eggs approx. in 20 µl of water) if necessary.

3. Test procedure

a. The isolated eggs will be incubated in a micro-titre plate for 7 days at 25 ºC. To prevent dehydration a wet sponge should be introduced under the plate and the system should be covered with a pouch. The water in the wells must be checked every day during incubation.

b. Add 90 µl of fungizone per ml of egg suspension and mix the solution.

c. Dispense 20 µl egg suspension (80 eggs approx.) to each well of the micro-titre plate.

d. Incubate the plate at 25 ºC for 24 hours. After hatching occurs supplement all the wells with 20 µl of growth nutritive medium. Abandon the assay if 60 percent or more of eggs fail to hatch after 48 hours. This may happen if the eggs were exposed to high temperature.

e. After the addition of growth medium, add 10 µl of distilled water to the control wells.

f. Prepare a range of dilutions of anthelmintics with dimethyl sulphoxide (DMSO) 1% and put 10 µl of each drug concentration in the other wells of the plate. Drug concentrations are tested in duplicate.

g. Return the plate to the incubator for another 6 days.

h. At day 7 of incubation, kill all the larvae by adding 10 µl of a dilute solution of Lugol’s iodine and examine the plate under a binocular microscope. For every anthelmintic row, count the L3 in each well till they are reduced to 50 percent of the average number of L3 in the control wells.

4. Results

The results are expressed as the concentration of each drug that inhibits to 50 percent, the development of larvae to infective third stage in relation to control wells (LC₅₀). Resistance factors (RF) can be determined for comparison with known reference isolates.
DETECTION OF RESISTANCE IN FLUKES

Resistance of *Fasciola hepatica* to common flukicides should be detected as follows:

- the faecal egg count reduction test (FECRT);
- efficacy trials (with or without previous isolation of the resistant strain).

1. The faecal egg count reduction test (FECRT)

Six to ten animals should be allocated into two groups. Group 1 should be treated with the drug to be tested, while group 2 will be considered as an untreated control. If enough infected animals are available, a third group should be treated with a drug of known efficacy. Since *F. hepatica* is frequently resistant to triclabendazole, the third group should be treated with closantel.

Individual faecal samples should be collected on the day of treatment (day 0) and at least seven days later (day 7). It should be noted that it is valid to do the trial with faecal samples taken between day 7 and day 21 post-treatment.

The percentage efficacy in terms of reduction of the egg counts is determined using the following formula:

\[
PE(\%) = \left(\frac{MC - MT}{MC}\right) \times 100
\]

Where, \( MC = \text{mean egg count on day 0} \)
\( MT = \text{mean egg count on day 7, 10 or 21} \)

Resistance is considered to exist when the PE is below 90%.

Due to extreme EPG values, calculation of the geometric mean is recommended over the arithmetic mean.

6. EPIDEMIOLOGY AND CONTROL OF GASTRO-INTESTINAL NEMATODES

The most important single requirement for the successful implementation of rational and sustainable helminth parasite control programmes in grazing animals, is a sound knowledge of the epidemiology of the parasite as it interacts with the host in a specific climatic, management and production environment (Barger *et al*., 1999). The epidemiological knowledge base has been established through extensive studies and field trials in many developed countries, and mostly in the context of industrialized livestock production. This is not the case for the majority of developing countries and countries in transition, and if information is available it rarely covers the diversity of their production systems. The reasons for this are often the obvious lack of human, economic and infra-structural resources. However, it is also often wrongly assumed that the epidemiological work conducted in one climatic region or production system can be extrapolated to another, or that the availability of modern broad spectrum anthelmintics eliminates the need for epidemiological knowledge.
In the absence of appropriate epidemiological knowledge the approach by which anthelmintics are administered is limited to two.

The livestock owner can choose to treat in a suppressive manner at intervals close to or at the end of the pre-patent period, or if drugs with residual effect are used, coincident with the length of persistence of the drug.

Treat whenever clinical signs of infection appear (curative approach).

The first option is the most effective at reducing the parasite populations and production losses in the short term, but this approach will select heavily for drug resistance in the parasites. The second option is associated with considerable risk of uncontrolled production losses and possible clinical disease, but will select less strongly for resistance.

Thus a prerequisite for the development and implementation of successful sustainable parasite control programmes is epidemiological knowledge.

7 CURRENTLY AVAILABLE CONTROL STRATEGIES

The currently available tools for gastro-intestinal nematode control consist of chemical and non-chemical technologies. The chemical technology relies entirely on treatment with different formulations of anthelmintics used in different control strategies according to whether epidemiological knowledge is absent or available. The non-chemical technology is based on, among other things, pasture and breeding management and nutritional interventions.

8 CHEMICAL TECHNOLOGY

During the last 35 years the pharmaceutical industry has produced a succession of highly effective, broad spectrum anthelmintics, and veterinarians and livestock producers have come to expect that worm control is easy, either by drenching or injecting cattle, sheep and goats with these products. This has made helminth control easy but has not fostered conservative use of the products. The following are strategies for the use of chemical anthelmintics.
Module 2: Helminths

Suppressive (systematic) treatments

This is a strategy that has been widely applied, particularly for parasites of small ruminants in the tropics and sub-tropics, where epidemiological knowledge is limited or absent. Without this knowledge owners of sheep and goats have been forced to treat regularly to keep their animals alive.

Principle: Regular treatments at intervals at or near the length of the pre-patent period of the parasite, or if drugs with residual effect are used, the length of the effective persistence of the drug, whichever is greater.

Prerequisites: Availability of the chosen drugs at affordable prices.

Advantages: This approach is very effective in the short term in minimising parasite populations and production losses.

Disadvantages: Numerous examples from the field and modelling have clearly demonstrated that this strategy selects inexorably for drug resistance in the parasites. It is also not necessarily cost effective.

Epidemiological consequences: This strategy will initially lead to reduced contamination of pastures with parasite eggs and a subsequent lower challenge with infective larvae. However, resistance develops quickly because of the small refugia (parasites not exposed to the chemical agent) and consequent high selection rate. As resistance develops, the parasite epidemiology will change and control is lost.

Possible combination with other strategies: Suppressive strategies should not be promoted.

Curative treatments

Treatment based on clinical diagnosis was a common practice in the past. With the introduction and promotion of strategic treatments, this method went out of use in most of the areas with industrialized livestock production. It was however still used by many small-scale farmers. Due to the problems of anthelmintic resistance, tissue residues and possible negative impact of chemicals in the environment, this strategy is being re-evaluated.

Principle: Animals are treated therapeutically, whenever production losses and/or uncontrolled disease is considered to be significant. The trigger for treatment has been based on different criteria according to knowledge or interest and availability of support services. The triggers include:

Any clinical signs or evidence of sub-clinical disease.

Rise in faecal egg count. Attainment of threshold levels of EPG in groups of monitored animals indicates a need for treatment. The threshold will vary according to the composition of the parasite population, host type and geo-climatic conditions.

Anaemia in sheep and goats identified using systems such as the FAMACHA method (Van Wyk and Van Schalkwyk, 1990) (see below for more details).

In order to best utilize 2. and 3. it is necessary to know the composition of the parasite population on the farm. In cases where treatment has been initiated using 1. or 2. as indicators of infection level, two different approaches have been applied:

Treat all animals in the herd or flock.

Treat only those animals that are perceived to need treatment.

94
The use of FAMACHA specifically aims to differentiate between the animals in a group that need treatment and those that do not.

**Prerequisites:** A regularly applied monitoring system such as clinical examination, faecal egg counts or FAMACHA.

**Advantages:** Reduced expenses for anthelmintics as number of annual treatments will be lower and, if selective treatment is practised, the number of animals treated will be lower. The possibility of selecting for resistance is significantly reduced, and the risk of selection is delayed if only some animals are treated, as this will ensure the presence of a susceptible parasite population. Regular monitoring in the context of improved animal production and health management.

**Disadvantages:** Regular monitoring needs labour input.

**Epidemiological consequences:** These will depend on the variables. This strategy may not, however, reduce the overall contamination level and subsequent numbers of infective larvae on pasture. With EPG or FAMACHA monitoring, the sub-clinical effect should be controlled (Van Wyk and Schalkwyk, 1990).

**Possible combination with other strategies:** No validated, integrated strategies have yet been developed.

**Monitoring of Haemonchus infections using the FAMACHA system**

Based on the strong correlation that exists between the coloration of the mucous membranes of the conjunctival sac of sheep, and the degree of anaemia (packet cell volume (PCV)) caused by the blood-sucking parasite *Haemonchus contortus*, a standardized test known as the FAMACHA system has been developed by South African scientists (Van Wyk and Van Schalkwyk, 1990).
**Principle:** Based on the above-mentioned correlation this assay uses a standardized colour chart showing illustrations of sheep eyes with colour variations from bright pinkish red to almost white. Treatment is recommended when the colour of the mucous membranes of sheep matches a tint that is correlated with anaemia.

**Advantages:** The method is easy and cheap to apply for continuous monitoring and it is easily taught to farmers. The use of pictures and signs make it suitable for illiterate sheep owners. There is a substantial reduction in the costs of drenching. A lower rate of selection for anthelmintic resistance is expected.
Disadvantages: Currently the method only applies to infection with *Haemonchus contortus* and the assay is only validated for sheep.

Possible combination with other strategies: Based on records that identify which animals require repeated treatment, it is possible for the sheep owner to cull these and breed from the most resistant animals, increasing the overall resistance of the flock.
Current strategies based on modified use of anthelmintics

The “Worm Kill” principle

As a consequence of widespread anthelmintic resistance in sheep parasites in Australia, the “Worm Kill” programme was developed. The main aim of this programme was to reduce the number of treatments, while maintaining effective control of parasites. This was done by the use of a narrow spectrum drug, closantel, in combination with a minimum number of treatments with broad spectrum anthelmintics. Closantel is particularly effective against *Haemonchus* and has a persistent effect for 2 to 3 months.

Strategic treatments based on epidemiology

Clinical parasitic disease in ruminants usually occurs at or shortly after times of peak larval availability. The timing of peak larval availability on pasture is of crucial importance in understanding the population dynamics of the parasite population, because this is when the largest worm burdens are acquired. It is in order to prevent these seasonal peaks from developing that strategically timed control measures are implemented. Thus treatments are often administered at times when the larval challenge on pasture is low and the majority of the parasite population is in the host. This reduces the pool of susceptible parasites and may increase selection pressure for resistance. Due to the effect of the climate and weather on development, survival and transmission of free-living stages, weather conditions play a dominant role in determining the timing of strategic treatments. Geographical differences in the seasonal availability of infective larvae from the pastures have similarly been a key factor for determining the timing of strategic treatments. In temperate climatic zones, sequential treatments at the beginning of the grazing season, using intervals similar to the pre-patent period or pre-patent period plus the length of the residual effect of the drugs, have been used. Similar sequential treatments have been applied at the beginning of the rainy season in tropical zones, with one treatment added during the dry season when pastures would be almost sterile.
**Principle:** Strategic treatments are administered, not only for therapeutic purposes to rid the animals of worms, but also as a prophylactic measure to prevent future contamination of pasture and reduce the risk of future re-infection (Barger et al., 1999; Barger et al., 1994a).

**Prerequisites:** Knowledge of local epidemiology of relevant parasite species.

**Advantages:** Proven record of reducing contamination of pastures with parasite eggs, and subsequent challenge of grazing animals with infective larvae. This has resulted in significant increases in productivity.

**Disadvantages:** Some of the strategies are associated with strong selection pressure for anthelmintic resistance.

**Epidemiological consequences:** Significant reductions in egg excretion and pasture larval contamination (Barger et al., 1994b).

9 **RECOMMENDATIONS FOR BETTER USE (“SMART USE”) OF EXISTING DRUGS**

Successful worm treatment relies on effective on-farm management practices and recent studies have provided improved opportunities for maximizing drench action – giving the drench the best chance to work (Ali and Hennessy, 1995; Prichard and Van den Bossche, 1980).

In order to maintain the efficiency of benzimidazoles and avermectin-like drenches (macrocyclic lactones) it is worth remembering a few points. Increasing the efficiency of drenching means:

- More worms are removed, leaving healthier animals.
- Less pasture contamination.

1. **Place the drench gun over the tongue**

The value of weighing sheep and using the correct dose is reduced if the drench does not go to the right place.

The dose should go wholly into the rumen or first stomach, where it can be slowly released with prolonged exposure in the case of benzimidazoles and macrocyclic lactones, NOT levamisole. Depositing the drench in the front of the mouth can activate the oesophageal groove, allowing the drench to by-pass the rumen and dramatically shorten the time during which there is a sufficiently high concentration of the drug to kill the worms.

Care should be exercised to ensure that no drench is directed into the airways.

2. **Reduce feed before drenching**

Restricting access to feed for 24 hours before drenching slows the flow of gut contents containing the drench from the rumen. Reduced feed intake prolongs drench uptake, extending the effective duration of the killing effect.

Muster animals in the morning and provide little or no feed – especially not fresh green feed – for the rest of the day and overnight. Provide access to water. Drench the following morning. For maximum effect, keep animals off feed for a further six or so hours before returning them to pasture. For better farmer compliance, it may be useful to demonstrate the effect of this advice by comparing a group treated after reduced access to feed, with a group treated traditionally, and to use the reduced faecal egg counts as an indicator of improved drenching efficiency.
Some local conditions (e.g. poor feed availability, drought) will reduce the effectiveness of this application. You should not restrict feed if the sheep are heavily pregnant, severely stressed or in poor condition.

3. **Use only the recommended dose rate**

Recommended doses are designed to persist in the animal for a specific period. In some cases – where resistant worms are present – increased dose rates have been used. Because of the way drenches are removed from the animal, doubling the dose only marginally extends the “killing time”. This does not matter with levamisole because its action is related to peak concentration, but it is important with other drenches.

If resistance has reduced the efficiency of drenches other than levamisole, and a higher dose is considered, remember that increased dose rates may breach regulations and require written veterinary permission. Rather than double the dose rate, it is better to administer two single doses at the recommended dose rate, separated by 12 hours. The two separate doses are much more efficient than one double dose. If possible, the use of two separate doses can be combined with reduced feed intake before drenching.

Attention should be paid to the fact that goats metabolize drenches faster than sheep. Ensure that goats always receive a full dose. In some cases it is recommended, particularly if resistance is suspected, to give goats a second or third drench, each 12 hours after the previous dose. Because sheep and goats have the same species of worms, resistant strains will be passed from goats to sheep.

10. **NON-CHEMICAL TECHNOLOGY**

With the increasingly widespread problem of resistance to anti-parasiticides and the increase in consumer pressure for quality animal products without residues, the demand for alternative, non-chemical parasite control interventions will increase. Few of these methods have, however, been sufficiently validated to the point where they can be recommended for general use. It is also likely that they will have to be used in combination with other interventions in order to obtain the desired effect. Among the non-chemical tools available immediately for implementation are various forms of pasture management, breeding management and improved nutrition. Others, namely utilization of herbal remedies, use of copper particles and biological control, still require research, development and validation in different geo-climatic regions under a variety of production systems. They may, however, already be incorporated in integrated control strategies in some circumstances.

**Pasture management**

The thorough knowledge of epidemiology, including the seasonal variations in the pattern of larval development and availability on pasture, can form the basis for control of gastro-intestinal nematodes through pasture management. A number of different grazing systems have proved helpful in the control of these parasites.

**Rapid rotational grazing:**

Recently there has been increased interest in using rotational grazing of pastures for the optimization of pasture growth and productivity. This is an excellent tool, from the productivity point of view, as animals will consume a higher proportion of the available forage, which stimulates pasture re-growth (Barger *et al.*, 1999).

*Principle:* This is a grazing management technique involving subdivision of pastures in which each paddock is grazed for a short time and then rested for a relatively much longer time. The major requirement for parasite control would be sufficient resting time for most of the
Module 2: Helminths

infective larvae originating from the previous grazing to have died off. This is probably not achievable in temperate climates, given that substantial declines in pasture infectivity may take from 3 to 9 months, depending on the climate and time of the year. It may, however, be useful on wet tropical pastures where larval survival times are short. This makes it possible to design a practicable rotation that is short enough to prevent auto-re-infection within a single grazing period, because development from egg to infective larva can take as little as 4 or 5 days. A slightly modified version (strip-grazing) is based on making fresh pasture available for the animals between two movable electric fences, which prevent the animals from going back to previously grazed parts of the pastures.

**Prerequisites:** Suitable pastures and fencing.

**Advantages:** Continuous reductions in pasture larvae availability. Reduction in use of chemicals and reduced risk of resistance development. Better pasture utilization.

**Disadvantages:** Capital investment in fencing and watering facilities. Increased labour requirements.

**Epidemiological consequences:** Reduced pasture contamination levels creating safer pastures.

**Possible combination with other strategies:** An anthelmintic treatment at the time of introducing the rotational system. Following validation of biological control strategies these could be used in combination with rotational grazing, creating a parasite control strategy without chemicals.

**Safe pastures**

**Principle:** The safe pasture concept is based on the fact that the number of larvae in the pasture are reduced over time by resting the pasture during the period when they are normally being re-contaminated (spring, rainy season) or through the growing and harvesting of a crop of hay or silage, followed by a period of re-growth.

**Prerequisites:** Available and suitable pastures for hay or silage production and/or pastures in crop rotation.

**Advantages:** Reduction in number of larvae on pasture. Reduction in use of chemicals. Better pasture utilization.

**Disadvantages:** In order to have a substantial impact on transmission, the period of resting may be too long to be practical for the producers. The approach is only suitable in combined crop and livestock production systems. If combined with the use of anthelmintics (dose and move systems) it may increase the selection pressure for anthelmintic resistance development.

**Epidemiological consequences:** Reduction of the number of infective larvae on the pasture reduces the worm burden and subsequent contamination levels.

**Possible combination with other strategies:** The movement of animals to safe pasture has been combined with anthelmintic treatment. The possible increase in selection pressure for anthelmintic resistance should be considered before recommending this strategy.

**Alternate grazing**

**Principle:** Using alternate grazing for parasite control is based on different age groups of the same species, or different species grazing the pastures in sequence. In cases where different age groups are used it is common practice to graze calves followed by older cattle, taking advantage of higher resistance in the older animals. If the system is based on alternating between species (sheep – cattle) it utilizes the fact that many parasites show little cross-infectivity between adult
cattle and sheep and/or the reduced susceptibility of different host species. It should be kept in mind that cool moist weather prolongs larval survival, and it is likely that alternate grazing systems will be less efficient in controlling parasites in temperate climates compared to tropical and subtropical regions.

**Prerequisites:** This approach requires that two or more different species are available on the farm, or the operation of a management system where sufficient numbers of different age groups are grazed separately. Monitoring of faecal egg counts is also desirable with this approach.

**Advantages:** Older animals or a different species of livestock will act as a ‘vacuum cleaner’ reducing the number of infective larvae on pasture.

**Disadvantages:** In order for this to significantly reduce the number of infective larvae, the time intervals between returning the same age group or species to the pasture may have to be prolonged, depending on temperature and precipitation, making this system less attractive to the farmer. Some parasite species can survive and reproduce in different hosts. The system requires increased monitoring. It can only be used for different age groups of cattle, NOT for sheep.

Note: The annual variation in pasture production may negatively affect or prevent the use of this system. It is recommended that hay or silage production with subsequent re-growth is considered as a regulatory mechanism for an even feed distribution.

**Epidemiological consequences:** Reduced level of pasture larval contamination.

**Possible combination with other strategies:** This can be combined with anthelmintic treatment at the time of introducing another species or age group.

Note: A modification of the alternate grazing management strategy is the use of mixed grazing, where two or more different animal species graze together, resulting in removal of infective larvae by non-susceptible hosts. The effect of this in the context of parasite control is variable, but it may have an additive effect to other measures and it does contribute to better pasture utilization. It cannot be recommended as a stand-alone strategy (Barger et al., 1994).

**Supplementary feeding**

Gastro-intestinal parasitism of ruminants is a production-related phenomenon, enhanced by chronic malnutrition and under-nutrition, which is particularly common in the developing world. Research has shown that improved nutrition reduces production losses and mortality rates due to worm parasites of livestock. Strategic feed supplementation, particularly to susceptible classes of stock such as young and peri-parturient animals, can have long-term benefits.

**Principle:** Low-cost mineral and non-protein nitrogen supplements dramatically change the physiology of the rumen. These lead to greater feed intake and increased microbial protein production, resulting in increased protein for digestion and absorption in the small intestine.

**Prerequisites:** Availability of low-cost mineral and non-protein nitrogen supplements and the technology for the preparation of blocks, pellets and other feed supplement formulations.

**Advantages:** Productivity is increased and supplemented animals have an increased ability to withstand the effects of parasitism. The feed supplements can include locally produced surplus plant by-products, which will enhance the supply of nutrients without greatly increasing the cost to the farmer.

**Disadvantages:** Cost.

**Epidemiological consequences:** The ability of animals to better cope with parasites may result in lower egg output by the worms and a subsequent reduction in pasture infectivity levels.
**Possible combination with other strategies:** Supplementary feeding can and should be included in any parasite control programme. Several common livestock management procedures in the tropics/subtropics, particularly for small ruminants, lend themselves to the administration of low-cost supplementation of nitrogen and essential minerals by way of feed blocks. There are very simple methods for manufacturing such blocks; and some may prove to be suitable substrates for the growth of locally isolated strains of nematode-destroying fungi.

**Genetically resistant or resilient animals**

A substantial amount of evidence is now available to demonstrate that there are between breed and within breed differences in the ability of animals to respond to the challenge of gastrointestinal nematode infection (Bisset *et al.*, 1996; Albers *et al.*, 1987). Thus, breeding sheep, cattle and goats that require minimal anthelminthic treatment is an option for managing anthelminthic resistance, as well as providing control of helminth parasites.

In cattle, the relative economic importance and the dependence on anthelmintics are smaller, but the potential for rapid genetic progress is greater due to the opportunities afforded by more widespread use of artificial breeding strategies.

**Definitions:** Resistant animals have the ability to suppress the establishment and/or subsequent development of a parasitic infection. Resilient animals are animals that can maintain relatively unaffected production despite being subjected to parasite challenge. The heritability of resistance has been documented. The heritability of resilience is substantially lower than that of resistance, and selection progress for this trait could be slow.

**Principle:** Develop breeding programmes for the selection of resistant or resilient animals, thereby increasing the overall resistance of the flock or herd and reducing the requirement for the use of anthelmintics.
Module 2: Helminths

**Prerequisites:** The introduction of breeding programmes for resistance will require increased monitoring of the animals with regular worm egg counts or use of genetic markers when these become available.

**Breeding strategies:** The understanding of the reaction of sheep to nematode parasitism has increased significantly during the last 5 to 10 years, but it is not completely clear what is the most appropriate breeding strategy to achieve a flock which requires minimal anthelmintic treatment.

**Advantages:** Reduced use of anthelmintics. Reduced risk of residues in animal products.

**Disadvantages:** Requires increased monitoring and record keeping.

**Epidemiological consequences:** Increased genetic resistance in a herd or flock will reduce the contamination of pastures with parasite eggs, with a subsequent reduction in the number of infective larvae. This may not be the case with resilient animals where the worm burden is not necessarily lower, but resilient animals will be able to cope with the challenge. However, in cases where the flock or herd consists of a mixture of animals of carrying susceptibility, or if susceptible animals follow resilient animals on to pastures, the effect of having resilient animals may not be strong enough to prevent the negative effect of nematodes. This will also be the case at the beginning of breeding programmes for resistant animals.

**Possible combination with other strategies:** The selection for resistance can be combined with any of the other strategies as required during the process of creating resistant herds and flocks.

**11 CONTROL STRATEGIES UNDER DEVELOPMENT**

**Biological control**

Almost all strategies for the control of gastro-intestinal nematodes target the parasitic stages within the animal. In contrast, the biological control of nematode parasites is targeted at the free-living stages on pasture. Current biological control aims to exploit the nematode-destroying properties of certain micro-fungi, particularly *Duddingtonia flagrans.*
**Principle:** Biological control using nematophagus fungi is a prophylactic measure reducing the infectivity level of pastures. It has no effect on parasites once the grazing animals have acquired them. *Duddingtonia flagrans* produces many resistant, thick-walled resting spores, chlamydospores, which have the ability to successfully pass through the gastrointestinal tract and survive in the faecal material. Upon stimulation they rapidly germinate and spread on and in fresh dung, and capture the infective larvae of most gastrointestinal worm species including *Cooperia, Ostertagia, Haemonchus, Nematodirus* and *Trichostrongylus* before they can migrate to the pasture.

**Prerequisites:** Availability of large amounts of chlamydospores, a vehicle to administer the spores and a management system to supply these to the animals.

**Advantages:** It is anticipated that this will be an inexpensive, sustainable non-chemical control method particularly suited to the practice of housing animals at night. It would be augmented where animals are provided with feed supplementation when housed, so that fungal material could either be co-administered or grown directly on the supplement if it consists of plant by-product material.

**Disadvantages:** It is not anticipated that this will be a stand-alone control strategy. Currently there is not a standardized product available. Each country will have to produce the fungus and identify suitable means of fungal delivery.

**Possible combination with other strategies:** The whole philosophy of using a biological control agent against parasitic nematodes, is to reduce the number of infective stages available to be picked up by susceptible, grazing livestock. The reduction in infective stages on herbage will subsequently reduce the build-up of worm burdens in hosts, which otherwise would cause sub-clinical or clinical responses, in particular in young animals. A steadily increasing number of review articles has been published within the last 4 to 5 years on the subject of biological control of helminths, as well as on possibilities for the various elements in integrated control strategies. The introduction of micro-fungi as a biological control in an integrated control programme of gastrointestinal nematodes, could be as part of a feed supplement, or incorporated into various
kinds of feed blocks presented to animals. This technology is currently under development and research (Larsen, 1999; Waller, 1999).

**Vaccines**

Considerable resources have been and still are being allocated to research into the effector mechanisms of naturally acquired immunity to gastrointestinal helminth infections of sheep (Sutherland et al., 1999) and cattle, with the aim of facilitating the development of vaccines. However, the situation is complex, involving a combination of local hypersensitivity, in addition to cell mediated, antibody and inflammatory responses, and is complicated further by the natural unresponsiveness which exists in the young lamb or calf, and in the dam around parturition.

Using the successful development of the irradiated larval vaccine against the bovine lungworm, *Dictyocaulus viviparus* as a model, attempts have been made to produce vaccines against gut parasites in ruminants, but they have all been disappointing.

Likewise, this seemed to be the case with the vaccines based on antigenic fractions of parasitic material. Early attempts to immunize ruminants against gastrointestinal helminths, either with crude worm homogenate antigen or by ectopic infection, met with little or no success. Currently, attempts are being made to direct high titre antibody responses towards potentially susceptible targets on or secreted by the parasite. In the case of blood-feeding species, several target molecules have been identified on the surface of the intestine of the parasites. Because molecules on the luminal surface of the parasite’s intestinal cells are not normally recognized by the host during infection, these antigens are classified as "hidden". Several vaccines using "hidden" antigens were developed for *H. contortus* in sheep, and these provided 94 percent protection in relation to EPG and their efficacy reached 90 percent when worm burdens were studied (Newton et al., 1995).

Another way to induce protection has been to use "homologous" antigen; that is an antigen first shown to be protective against another helminth species. An example is the glutathione-S-transferases (GST) of *Fasciola hepatica*, which were chosen as candidate vaccine antigens because homologous protein from *Schistosoma mansoni* and *S. japonicum* had been shown to be protective in laboratory animal models of infection. Sheep and cattle immunized with native GSTs isolated from *F. hepatica*, have been protected on average by 49 and 29 percent respectively, although the results from individual trials have been quite variable (Morrison et al., 1996).

The expectation was that for any vaccine to be acceptable, it had to compete favourably with modern anthelmintics, not only in terms of cost but also with regards to spectrum and levels of efficacy. Attitudes are now changing, largely due to the revelations of mathematical modelling of the effects of vaccination. Simulation studies have shown that substantial benefits might be obtained even with a vaccine that produces only 60 percent efficiency in 80 percent of the flock. It is argued that a vaccine of moderate efficacy measured in terms of economic benefits, rather than one with the ability to induce sterile immunity, may well be effective in the field. This is achieved by priming the host for progressive development of immune regulation of parasites, and thus reducing the overall rate of parasitic population increase in the flock.

Accepting the potentially lower efficiency associated with the use of the irradiated larval technique and reviewing existing data, it appears that these vaccines can provoke levels of protection similar to molecular worm vaccines. Not only would irradiated larval vaccines undoubtedly be much easier and cheaper to produce, but also their manufacture would not be constrained by commercial protection, as the technology involved in larval irradiation is very much in the public arena.
Copper particles

Various preparations of herbs, copper, arsenic and other more or less toxic minerals were the only products available for the control of helminths prior to the marketing of the first generation of broad spectrum anthelmintics in the late 1930s. The interest in copper formulations was renewed when the importance of trace elements and mineral deficiency were identified. It was shown that there were great benefits from low-dose depot delivery of copper to the rumen of sheep and cattle grazing on deficient pastures, and also from the equally lengthy protection such boluses gave to sheep against *H. contortus*. This however coincided with the promotion of thiabendazole, the first of the safe, broad-spectrum anthelmintics. Hence the possibilities for control of *H. contortus* using low-dose administration of copper were overlooked.

Stimulated by the widespread resistance to anthelmintics, the interest in using alternative measures including copper is being re-evaluated. Currently the use of copper-oxide wire particles (COWP), delivered by way of a capsule, not only to treat copper deficiency, but also to ameliorate the effects of abomasal parasites is being tested. The capsules dissolve in the rumen, resulting in the passage of particles to the abomasum, where they lodge in mucosal folds and release ionic copper over an extended period of time. Administering 5 g capsules to young sheep resulted in 96 percent and 56 percent reductions respectively of *H. contortus* and *Ostertagia circumcincta* infections. However, there was no effect on the intestinal species, *Trichostrongylus colubriformis*. Further work has shown that the protective effect can last for the entire dissolution period of the particles, which is approximately 3 months.

The apparently prolonged action of COWP against *H. contortus* could prove to be of enormous benefit in restoring some measure of control in those regions of the world where this parasite predominates, and anthelmintic resistance is rampant.

**12 ANTHELMITIC RESISTANCE MANAGEMENT AND INTEGRATED WORM CONTROL:**

The prevention or control of parasite resistance has become a major problem for the livestock industry and in particular for the small ruminant producers who, in many countries, are facing an emergency situation of no anthelmintics with sufficient effect to prevent productivity losses. Thus, there is a growing need to combat the problem of anthelmintic resistance in the parasites of ruminants through the development of integrated parasite control methods.

**Prudent use of anthelmintics**

There is obviously no point in continuing to use a drug to which the target parasites are resistant. It is therefore recommended that a test for resistance be made before new control strategies are implemented. If any drugs from one or more groups of anthelmintics are still effective, they should be carefully used in the future to delay the development of resistance to them.

**Development of resistance**

There is a general conflict between the requirements for a high level of control of helminths, and the requirements for delaying the development of anthelmintic resistance. This is clear from the list of factors below that are likely to accelerate the development of resistance but it should be noted that elements in this list require further scientific validation before being considered as fact.

Factors likely to accelerate resistance include:

- Treatment of the flock at times of the year when the majority of the parasite population is in the host.
- Treatment of the whole flock instead of only the animals with the highest parasite burden.
Use of poor quality anthelmintics of uncertain concentration.

Use of anthelmintics with a prolonged, sub-lethal decay curve.

Under treatment. Although it is widely believed that under-dosing contributes to the development of resistance, the relationship between dose and selection varies depending on the mode of inheritance of resistance and the dose used in relation to the lethal dose for each genotype.

Use of slow-release devices at times of the year where the level of pasture contamination is low.

Prevention of resistance

Actions likely to prevent resistance:

Reduce the frequency of treatments

Apply the right dosage. Remember to dose according to the heaviest animal. Determine the weight by using a scale.

Buy products from reputable companies. They are normally a little more expensive but this is compensated for by their improved efficiency and subsequent maintenance of or increase in animal productivity.

Treat only animals that need to be treated. This will maintain a population of susceptible parasites on the pastures.

Avoid the use of slow release devices at times when pasture contamination is low.

Introduce quarantine measures for newly purchased or returning animals. Treat with a macrocyclic lactone and/or closantel, and to reduce the risk of introducing resistant nematodes, keep new stock off pasture until all nematode eggs have been passed.

MODULE 2 REFERENCES


Module 2: Helminths


