

# REASSESSING ANTHELMINTIC RESISTANCE - ITS EMERGENCE, DETECTION AND METHODS TO COMBAT

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## INTRODUCTION

Gastro intestinal nematode infections pose a significant threat to the productivity of livestock worldwide. Anthelmintics offer an easy and effective means for controlling helminth infection and have been used as the first line of treatment in the past four to five decades. However, indiscriminate and irrational use of anthelmintics over the years has led to the emergence of resistance against many of the commonly used classes of anthelmintics.

Resistance is the heritable change in the ability of parasites to survive the therapeutic dose of an anthelmintic. As per Prichard *et al.* (1980), "Resistance is present when there is a greater frequency of individuals within a population able to tolerate doses of compound than in a normal population of the same species and is heritable". The condition is usually suspected when a farmer or veterinarian reports or detects poor clinical response after repeated anthelmintic treatment and gradual loss of weight. The emergence of resistance to anthelmintics is highly prevalent in small ruminants and has been recorded to a lesser extent in horses and pigs and recently observed in cattle also.

The history of anthelmintic resistance

dates back to 1957 with the first report of phenothiazine resistance from United States of America (USA). The currently available anthelmintics belong to three major classes *viz.*, benzimidazoles, imidazothiazoles/ tetrahydropyrimidines and macrocyclic lactones. Benzimidazoles were launched into the market in the 1960s and within a few years, resistance was reported against the drug (Drudge *et al.*, 1964). Thereafter resistance was reported to all major classes of anthelmintics within a few years of their introduction into the market. Resistance to imidazothiazoles was reported by Le-Jambre (1976) and the first report of ivermectin resistance was by Carmichel *et al.* (1987) from Africa. Now anthelmintic resistance has evolved as a significant problem confronting the successful control of parasitic nematodes in livestock in many parts of the world. The situation is found to be critical in South Africa, Australia, New Zealand and Latin American countries, where the resistance scenario is complicated by cross resistance and multidrug resistance resulting in total drug failure (Varady *et al.*, 2011). The gravity of the situation is signified by the fact that resistance has already been documented to the recently developed

drug like monepantel (Leathwick *et al.*, 2013; Scott *et al.*, 2013). Research on the development of new classes of anthelmintics is limited by economic constraints and therefore the prospects of development of novel anthelmintics is poor. These facts signify the need for conserving the efficacy of the currently available anthelmintics and the judicious use of existing drugs so as to reduce the incidence of anthelmintic resistance. This requires coordinated efforts for routine testing for anthelmintic sensitivity and implementation of resistance management protocols such as targeted selective treatment to prevent further selection for resistance.

#### Status of anthelmintic resistance in India

In India, anthelmintic resistance was first reported by Varshney and Singh (1976) against phenothiazine and thiabendazole in sheep. Subsequently, development of resistance to all major classes of anthelmintics was reported from different states including Haryana (Yadav, 1990; Yadav and Uppal, 1993), Uttar Pradesh (Srivastava *et al.*, 1995), Rajasthan (Singh *et al.*, 1995), Gujarat (Gill, 1996), Tamil Nadu (Gill, 1996; Jeyathilakan *et al.*, 2003; Arunachalam *et al.*, 2005), Karnataka (Dhanalakshmi *et al.*, 2003; Kumar *et al.*, 2012) etc.

In Kerala, Deepa and Devada (2007) first reported multiple anthelmintic resistance in an organized goat farm while Asha *et al.* (2013) documented benzimidazole resistance in small holder farmer flocks in Thrissur. Asha (2017) surveyed the resistance status to benzimidazoles in organized government/ private farms and small holder flocks in Kerala and reported resistance in hundred per cent of organized farms screened while 43.75 per cent of the

small holder farmers flocks were found resistant.

#### Detection of resistance

Several *in vivo* and *in vitro* techniques have been developed for the detection of anthelmintic resistance.

A. <i>In vivo</i> Test	Spectrum	Application
FECRT	All anthelmintics	Wide spread
Critical anthelmintic test	All anthelmintics	Limited
Controlled test	All anthelmintics	Limited
B. <i>In vitro</i> Test	Spectrum	Application
Egg hatch test	BZ	Widespread
Egg embryonation	BZ	limited
Larval paralysis test	LEV/MT	limited
Larval developmental test	BZ, LEV, MT	Commercialized
Tubulin binding	BZ	Research
Esterase activity	BZ	Research
Esterine induced paralysis	BZ	Research
Tubulin probe	BZ	Research

Of these, the World Association for the Advancement of Veterinary Parasitology (WAAVP) recommends Faecal Egg Count Reduction Test (FECRT) and Egg Hatch Test (EHT) as field tests to detect resistance in livestock.

#### *In vivo* detection of benzimidazole resistance

##### Faecal Egg Count Reduction Test (FECRT)

This test assesses anthelmintic efficacy *in vivo* by comparing faecal egg counts

in the treated and untreated hosts before and after treatment, from which the per cent reduction in faecal egg count is calculated. The test is suitable for detection of resistance to all classes of anthelmintics. The WAAVP guidelines for FECRT have been described by Coles *et al.* (1992). In this test, young animals (< 3-6 months of age) that have not been dewormed for 8-12 weeks prior to the test are divided into treatment and control groups with 10 to 15 animals in each group. The animals in the treatment group are given the prescribed dose of the drug. Dung samples are collected and processed on day 0 and day 14 after treatment to determine eggs per gram (EPG). The values of mean pre-treatment EPG and mean post treatment EPG are applied to a software computer programme "RESO" downloadable from the website [www.sheepwormcontrol.com](http://www.sheepwormcontrol.com) to get the percentage of egg count reduction and the 95 per cent confidence limits. Resistance is concluded to be present if the per cent egg count reduction is less than 90 per cent and the lower 95 per cent confidence level is less than 90 per cent. Resistance is suspected if only one of the two criteria is met.

#### ***In vitro* tests for detection of anthelmintic resistance**

*In vitro* tests assess the efficacy of the anthelmintics by determining its effect on hatching, development or motility of the pre-parasitic stages of the nematodes *in vitro*. A number of tests are available, of which the egg hatch assay and larval development assay are the most widely used.

#### ***Egg Hatch Assay (EHA)***

Egg hatch assay is based on the ovicidal

properties of benzimidazoles and evaluates the ability of nematode eggs to embryonate and hatch in the presence of increasing concentrations of benzimidazoles. The test was originally described by Le-Jambre (1976) and was subsequently reported by several workers for *in vitro* benzimidazole resistance detection.

Egg hatch assay is simpler, easier and faster when compared to other detection tests. In EHA the undeveloped nematode eggs are incubated in different concentrations of thiabendazole which is the commonly used reference drug due to its high solubility. The percentage egg hatch at each concentration is determined and corrected for natural egg mortality in control wells. A dose-response line is then plotted using the probit values of the egg hatching and logarithm of the dose, from which the ED<sub>50</sub> is calculated (Taylor *et al.*, 2002). Samson-Himmelstjerna *et al.* (2009a) described standardized protocols for drug dilution, sample preparation and storage of eggs to reduce protocol variations in EHA between different laboratories.

The sensitivity of EHT could be increased significantly by using discriminating doses (DD) which is the dose that prevented 99 per cent of the susceptible eggs from hatching. A DD of 0.1µg/ml was established for the major nematode species of small ruminants and the proportion of eggs hatching at this DD gives a direct estimate of the resistant worms in the population (Coles *et al.*, 2006).

#### ***Larval Development Assay (LDA)***

The test is simple, reliable, inexpensive and suitable for field surveys for all major classes of anthelmintics. It determines the ability of an anthelmintic to arrest

the larval development in nematodes. In LDA, nematode eggs are cultured in serial dilutions of the anthelmintics for seven days at 26°C in the presence of a nutrient medium after which proportion of mature third stage larvae (L<sub>3</sub>) at each concentration is determined from which LD<sub>50</sub> is calculated (Hubert and Kerboeuf, 1984). Several modifications have been made with regard to the nutrient medium used. Lacey *et al.* (1990) described an agar based LDA which used a 96 well microtitre plate with agar matrix in which serial concentrations of the anthelmintics were incorporated. Currently a commercial version of this micro larval development test based on Lacey *et al.* (1990) and Gill *et al.* (1995) was developed by Commonwealth Scientific and Industrial Research (CSIRO), Australia and marketed as Drench Rite<sup>®</sup> LDA.

The major problem with the conventional tests for resistance detection like FECRT, EHA and LDA is their lack of sensitivity. These tests can detect resistance only when the frequency of resistant alleles is greater than 25 per cent in the worm population (Martin *et al.*, 1989). The application of DD in EHA and LDA could increase the sensitivity of the tests and reduce the number of drug concentration tested (Coles *et al.*, 2006). In a study conducted in Kerala Veterinary and Animal Sciences University (KVASU), the proportion of L<sub>3</sub> developing at DD in LDA was found to be significantly correlated with other resistance parameters indicating that it was a better criterion for resistance detection than LD<sub>50</sub> in LDA (Asha, 2017).

#### **Molecular detection of anthelmintic resistance**

The molecular basis of resistance

development has been clearly understood in the case of benzimidazoles, where the resistance has been associated with mutations in the gene encoding the drug target,  $\beta$ -tubulin. This has led to the development of PCR based detection tests which provide high level of sensitivity and specificity and facilitate early detection of genetic change linked with resistance. Genotyping the predominant nematode species for these resistance associated polymorphisms will help in accurate determination of the prevalence of benzimidazole resistance in nematode populations.

Several PCR based tests have been reported for detection of the single nucleotide polymorphisms (SNPs) in the  $\beta$ -tubulin gene of gastro intestinal (GI) nematodes. Elard *et al.* (1999) and Silvestre and Humbert (2000) reported allele specific PCR (AS-PCR) for genotyping adult and third stage larvae of predominant GI nematodes at the codon 200 of isotype 1  $\beta$ -tubulin gene. The test was subsequently used extensively for molecular detection of benzimidazole resistance by several authors. Tiwari *et al.* (2006) developed PCR-RFLP for detection of SNP at codon 200 of  $\beta$ -tubulin isotype 1 gene in *H. contortus* and described it to be easier, accurate, economic and more convenient than AS-PCR. Asha (2017) reported molecular detection of benzimidazole resistance in *Haemonchus* spp. and *Trichostrongylus* spp. in goats by PCR-RFLP targeting the codons 167, 198 and 200 of isotype 1  $\beta$ -tubulin gene.

Other molecular tests currently available for detection of benzimidazole resistance associated SNPs are real time PCR (Alvarez-Sanchez *et al.*, 2005) and pyro-

sequencing assays (Samson-Himmelstjerna *et al.*, 2009b). These assays allow estimation of frequencies of resistance alleles in pools of nematodes and are therefore less laborious as they do not require genotyping of individual larvae. However, these tests require expensive and less widely available equipments making them more expensive than the other tests (Samson-Himmelstjerna *et al.*, 2009b).

The molecular basis of resistance development to levamisole and macrocyclic lactones is not fully understood and the development of molecular tools for resistance detection to the above drugs is in the phase of research.

#### **Factors leading to the development of resistance**

Prolonged use of a single group anthelmintic and under dosing are the two important factors leading to the development of resistance.

Frequent use of the same anthelmintic is the most important factor that leads to resistance development (Chagas *et al.*, 2016). High frequency of treatment cause considerable selection pressure on worm populations resulting in rapid emergence of resistance. Vijayasarithi *et al.* (2016) reported fenbendazole resistance in field flocks of Madras Red sheep in Tamil Nadu and attributed it to the regular and frequent use of benzimidazoles in these flocks. Similarly, Asha (2017) reported highly significant correlation between the frequency of deworming in goat farms/flocks and benzimidazole resistance development. The lower prevalence of resistance observed in small holder farmers' flocks might be attributed to the less intensive use of drugs in these flocks compared to that

in organized farms (Chaudhry *et al.*, 2016; Asha, 2017).

Under dosing can result from improper means of assessment of body weight of animals, larger feed intake increasing the risk of digesta flow thus leading to reduced absorption and reduced efficacy of drug, use of sheep dose in goats and cattle dose in buffaloes etc. As per Niciura *et al.* (2012) visual estimation of body weight of animals for treatment which could lead to under dosing was the most important factor leading to resistance development.

In goats, rapid hepatic metabolism of benzimidazoles leads to lower bio availability of drugs. Thus, use of sheep dose in goats could lead to suboptimal dosing for prolonged periods resulting in resistance development (Rialch *et al.*, 2013). These resistant worms subsequently may get transferred to sheep while grazing and thus, keeping sheep and goat herds together in farms could lead to resistance development (Iliev *et al.*, 2014).

Use of anthelmintics without knowing the type of worms present and unnecessary drenching of animals without assessing the worm burden are the other factors leading to the development of resistance.

#### **Management of anthelmintic resistance**

As there is no practical measure to replace the use of anthelmintics in controlling helminth infections, the available drugs should be used judiciously in order to preserve their efficacy. Proper anthelmintic treatment strategies, together with alternative measures for parasite control and proper grazing management, offer to be the only sustainable way in the coming years.

### **Correct anthelmintic dosage and administration**

It is critically important that all the animals in a flock receive the full therapeutic dose. In flocks or herds this can be ensured by weighing representative animals from each age or sex class and calculating the dose based on the heaviest animal in an age or sex group.

Sub-dosing is an important factor in the development of resistance in goats because of the lower bioavailability of drugs in their system. The general rule of the thumb is that goats need twice the anthelmintic dosage as sheep in case of benzimidazoles and one and a half times the sheep dose in case of imidazothiazoles and ivermectin (Harikrishnan, 2012).

Anthelmintics should be administered orally over the tongue of the animal. Improper drenching leads to closure of oesophageal groove and the drug directly reaches the abomasum and is rapidly eliminated. Fasting 12 to 24 hr prior to treatment or two treatments given 12 hr apart is also beneficial.

### **Targeted Selective treatment**

Generally in a flock, only a small number of animals are heavily infected whereas majority will have a moderate worm burden without much exhibition of clinical effects or production loss. In targeted selective treatment (TST), we limit the anthelmintic treatment to the most susceptible animals in a flock such as lambs or kids, high producers etc and around 2 to 5 per cent of the animals are left untreated. By selective treatment we can maximize the refugia in grasslands and pasture. The term "refugia" refer to the proportion of worm population which has not been exposed

to a particular drug thereby escaping the selection for resistance or the possibility to develop resistance. Maximising refugia helps to dilute the selection pressure on the population thereby delaying the development of resistance. In this regard, a technique called FAMACHA<sup>©</sup> system was developed in South Africa which helps in clinical evaluation of anaemia in haemonchosis and to limit anthelmintic treatment to the most needed ones (Van-Wyk and Bath, 2002). In this system, a color eye chart depicting varying degrees of anaemia is used to determine the need for anthelmintic treatment in sheep. Only those animals showing physical signs of infection (with pale mucous membrane and PCV 15%) are dewormed while the others are left untreated.

Marykutty and Syamala (2016) evaluated the efficacy of FAMACHA<sup>©</sup> eye colour chart in the assessment of parasitic load and anaemia in Attapady Black goats in Kerala and reported positive and significant correlation between FAMACHA<sup>©</sup> score and haematocrit indicating FAMACHA<sup>©</sup> score as a good indicator of parasitism and anaemia in goats of humid tropics. Marykutty and Syamala (2017) also reported that FAMACHA scores of 3, 4 and 5 indicated anaemia in goats, thereby imparting optimum sensitivity of the system in Attapady Black goats of Kerala.

The use of FAMACHA<sup>©</sup> is limited to haematophagous parasites that cause anaemia, especially *Haemonchus contortus*. Bath and Van-Wyk (2009) extended the principle of TST for use against non haematophagous worms and other important internal parasites of small ruminants and devised a five point check system which included nasal discharge (for nasal bots),

paling of ocular mucous membranes for anaemia (for haematophagous worms), submandibular oedema or bottle jaw (for haematophagous worms and conical fluke), body condition score (for worms causing loss of condition) and faecal fouling or dag score (for worms causing diarrhoea).

#### ***Strategic use of anthelmintics***

Strategic drenching aims to reduce the number of treatments by timing the treatment to complement environmental control of parasites. This includes treatment at strategic times of the year or treatment prior to lambing or kidding to prevent periparturient rise in worm egg production and pasture contamination. However, it has been observed that deworming at the time when the parasite population in the refugia was lesser than that in the host could increase the selection pressure leading to emergence of resistance (Sargison *et al.*, 2007). This is because, majority of the worms escaping anthelmintic treatment will be resistant worms and they will seed the pasture with resistant eggs when the refugia is low. Maharshi *et al.* (2011) reported that the practice of drenching flocks during extreme summer when there was limited refugia was a major reason for higher prevalence of resistant genotypes in the eastern and northern regions of Rajasthan. In regions with well defined wet and dry seasons, anthelmintic treatment in late monsoon is helpful in increasing the efficacy of anthelmintics by increasing the size of refugia (Swarnkar and Singh, 2017).

#### ***Non-chemotherapeutic measures for control of helminths***

The scenario of rapid emergence of resistance to the currently available

anthelmintics has led to a renewed interest in the use of alternative methods to reduce the dependence on drugs. Overuse of any new anthelmintics could quickly lead to anthelmintic resistance.

It is recognized that total parasite eradication is not practicable and generally not necessary to achieve an acceptable level of production in farm animals. Parasite control based on alternative management strategies has the objective not only to reduce the usage of drugs, the selection for resistant parasites and the release of chemicals on the environment but also exposure to different levels of the disease and the opportunity to the animals to manifest its natural immunity. This is also supported by an enhanced public concern for clean and residue free animal products.

Improvement of the host diet has been associated with two distinct benefits. It can enhance the host resistance i.e. its aptitude to regulate the worm populations as well as the host resilience, which means its ability to withstand the negative effects of nematode infections.

#### ***Protein supplementation***

Manipulation of protein nutrition to improve the host responses to nematode infection appears as an attractive option to reduce the reliance on chemotherapy to control parasitism. Protein supplementation around parturition could significantly reduce the periparturient rise in fecal egg counts and thus the pasture contamination with parasitic ova. Various studies demonstrated that supplementary feeding to browsing kids can improve resilience. Short periods of enhanced post weaning nutrition can have long-term benefits maintaining higher rates of live-weight gain

and lower fecal egg counts (FEC). Clear improvement in resilience is a point that farmers can possibly appreciate without means of laboratory techniques. In general, strategic supplementation should target those times when nutrient requirements are greatest and provide those nutrients which are deficient (protein, energy, minerals or trace elements). The economical viability of diet manipulation in developing countries have been illustrated by the positive effects obtained by use of low cost resources like non-protein nitrogen, energy sources like maize or molasses.

#### **Copper oxide wire particles (COWP)**

Copper oxide wire particles (COWP) were first developed to treat copper deficiency in ruminants. They are small pieces of copper oxide wire contained within a gelatin capsule. Following dissolution in the rumen, the copper particles pass to the abomasum where, they lodge in the mucosal folds and release ionic copper over an extended period of time. Several studies in sheep and goats have confirmed the efficacy of long lasting dissolving copper needles in the control of haematophagous nematodes. A dose of COWP as low as 0.5 g was found optimal to reduce the risk of copper toxicity and effective in reducing the FEC in young goats and 5.0 g was effective in older goats.

#### **Herbal anthelmintics**

Traditional veterinary pharmacopeia, mainly based on phytotherapy or plant remedies, remains the principal resource to treat animals against helminths in a large part of the world. Since the onset of anthelmintic resistance, the interest for such anthelmintics is rising. Besides these

ethnoveterinary compounds, scientific evidence has also been growing for nutraceuticals. The beneficial properties of these forages or plants have generally been associated with the presence of plant secondary metabolites (PSM).

The anthelmintic activity of several herbal formulations has been established by *in vitro* studies in KVASU. Sujith *et al.* (2015a) demonstrated the *in vitro* ovicidal and larvicidal activity of *Vitex negundo* (Karinochi) leaf extracts on *Haemonchus contortus*. Similarly, the leaf extracts of *Allophylus cobbe* (Mukkannanpezhu) (Priya *et al.*, 2015) and *Ocimum sanctum* (Krishna tulsi), *Murraya koenigii* (curry leaves) and *Mallotus philippensis* (Kamala) (Sujith *et al.*, 2015b) showed potent ovicidal and larvicidal activity against *H. contortus*. The *in vitro* anthelmintic activity of the extracts of the flowers of *Mallotus philippensis* was established by the study of Deepa *et al.* (2015) indicating its value as a potent anthelmintic. Krishnaprasad *et al.* (2018) demonstrated that the fruit extract of *Piper longum* (Thippali) possessed potent broad spectrum anthelmintic activity. The isolation of active compounds of the above plants could provide a lead for the development of a novel and safe anthelmintic.

#### **Grazing management**

Although pasture lands are invariably less in our state due to urbanisation, grazing management is one mechanism which can effectively reduce the need for anthelmintic therapy. A grass land or paddock which has not been grazed for 60 days is considered safe for grazing as the infective larval stages will be destroyed by this time due to starvation. In organized farms, the grazing area may be divided into 10 paddocks



and the animals allowed grazing on each paddock only for 7 days. Thereafter they should be shifted to the next paddock. By this type of management, the animals will be returned to the first paddock only after 70 days and by this time the pasture becomes safe for grazing (Harikrishnan, 2012).

Long tufts of grasses provide protection to infective larvae and therefore grasses should be clipped regularly. Browsing on bushes and shrubs limits ingestion of larvae by animals due to limited climbing activity of infective third stage larvae. Therefore allowing goats to browse on trees and shrubs to minimize grazing is also found to be beneficial.

#### **Breeding for resistance**

Breed differences in resistance to nematode infections have been well documented in small ruminants (Bishop and Morris, 2007). The Garole breed of India, Red Massai sheep breed in Africa and the West African Dwarf goat breeds are classical examples of breeds showing resistance to nematode infection. Selection for genetic resistance of host to parasitism is often regarded as the best alternative approach for parasitic control in small ruminants. Selection for resistance is based on phenotypic indicators such as faecal egg count or by using different genetic markers. Preliminary research work done in this regard at KVASU explored the possibility of using FEC and PCV as indicators for selecting goats for host resistance. The study also indicated the possibility of studying the polymorphisms in IFN- $\gamma$  intron 1 for marked assisted selection of resistance in goats (Aparna, 2010).

#### **SUMMARY**

Despite the current developments in the non-chemotherapeutic methods of control, anthelmintics will remain as the main stay in control of helminth parasites in livestock. Thus it is vital that the efficacy of the current anthelmintics is preserved by adopting appropriate treatment strategies. This requires coordinated efforts of regular monitoring for resistance in farms and implementation of appropriate management strategies when needed. Increasing farmer perception is important in this regard. Farmers should be made aware of the dangers of improper medication and the need for selective and correct anthelmintic therapy which can strengthen the struggle in combating anthelmintic resistance. Future developments in the field of geographic mapping of helminth fauna in livestock, disease forecast based on geographic information system (GIS) and use of effective immunization are likely to reduce the dependence on chemotherapy for helminth control.

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