



ANTHELMINTIC DRUG-RESISTANT DETECTION METHODS: A BRIEF OVERVIEW

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ABSTRACT

Anthelmintic have been the mainstay drugs for the last five decades to control the Gastro Intestinal (GI) nematodes, have resulted in the co-evolution of resistant parasites across the globe including India. Anthelmintic resistance (AR) in small ruminants has reached alarming proportions throughout the world and threatens the future viability of small ruminant production. The common diagnostic methods for anthelmintic resistance in nematode parasites are *in vitro* and *in vivo* methods. *In vivo* tests, like the Faecal Egg Count Reduction Test (FECRT), Critical anthelmintic test and the controlled anthelmintic efficacy test. Alternative *in vitro* tests have been established, like the Egg Hatch Test (EHT), Larval development assay (LDA), Larval paralysis test, Larval motility test, Adult migration inhibition test, Colorimetric assays, Tubulin binding test and Molecular Techniques. Traditional methods have proved their efficacy but can only detect the resistance in worm populations when more than 25% of the parasites are resistant to anthelmintics. In contrast, Molecular tools like AS-PCR efficiently detect 1% level of resistant within a susceptible worm population, which is emerged tremendously over *in vivo* and *in vitro* tests.

KEY WORDS: Anthelmintic resistant, LDA, FECRT, EHT and AS-PCR.

INTRODUCTION

Resistance to anthelmintic drugs amongst the major nematode parasites of sheep and goats has now reached alarming proportions throughout the world and threatens the future viability of continued small ruminant production in many countries (Fleming *et al.*, 2006; Larsen, 2006). Effective control and management strategies using anthelmintics require a clear appraisal of the resistance or susceptibility of the target worms. It is important to maintain the efficacy of currently available anthelmintics wherever resistance has not emerged and to prevent the further selection of resistance where it has already started to become apparent. This is only possible if adequate means are available for the diagnosis of anthelmintic resistance. Benzimidazole (BZ) resistance may be detected by examining embryonated and hatching of eggs since eggs of resistance strains will embryonated and hatch in higher concentrations of anthelmintics than do eggs of non-resistance strains (Le Jambre, 1976). *In vivo* tests, like the controlled test, which involves necropsy of treated and untreated animals, or the Faecal Egg Count Reduction Test (FECRT) are expensive and laborious. Alternative *in vitro* tests have been established, like the Egg Hatch Test (EHT) and the Larval Development Test (LDT). These are faster, less labour intensive and have found broad acceptance, mainly for surveys of anthelmintic resistance prevalence. The best evaluated and most often used *in vitro* tests are those for the detection of BZ resistance, while so far those developed for use with pyrantal or the ivermectin (IVM) often lack sensitivity or fail to agree with other tests like the FECRT (Craven *et al.*, 1999; Varady and Corba, 1999;

Tandon and Kaplan, 2004; Lind *et al.*, 2005). BZ have been regularly and widely used in India for controlling parasitic gastroenteritis (PGE) in small ruminants (sheep and goat) for the last 2-3 decades (Gill, 1996). There are frequent complaints on these drugs to provide the expected degree of control of PGE. Many reports of anthelmintics resistance particularly in small ruminants are on record mainly from organized farms. These reports are based on the classical methods such as *in vitro* Egg Hatch Assay (EHA) or Larval Development Assay (LDA) and *in vivo* Faecal Egg Count Reduction Test (FECRT) and few reports are based on molecular techniques (Sankar, 2003, 2007; Tiwari *et al.*, 2006; Garg *et al.*, 2009; Chandra *et al.*, 2014, 2015). Traditional methods have proved their efficacy but can only detect the resistance in worm populations when more than 25% of the parasites are resistant to anthelmintics (Roos *et al.*, 1995). However, all the molecular methods are mainly targeted on *H. contortus* with exception of Sankar, 2007, where *T. colubriformis* and *Oesophagostomum columbianum* also evaluated in limited number of samples.

AR detection methods

The growing importance of AR has led to an increased need for reliable and standardized detection methods (Coles *et al.*, 1992, 2006). Anthelmintic resistance can be detected by a number of *in vivo* and *in vitro* techniques. In general *in vivo* techniques are found to be suitable for all types of anthelmintics. Nevertheless these are time consuming, expensive and labour intensive. On the other hand, *in vitro* techniques are rapid, sensitive and inexpensive. The most widely used method for detecting

and monitoring the presence of AR in nematodes is the faecal egg count reduction test (FECRT), which is suitable for all types of anthelmintics including those that undergo metabolism in the host and *in vitro* Egg hatch test (EHT) suitable for detection of BZs resistance. In addition, a number of *in vitro* assays have been developed as alternative methods of detection. These assays have been a useful tool for research but with limited application for routine testing of isolates from farms. It is expected that anthelmintic chemotherapy will remain the mainstay of helminth control programmes in future. Effective monitoring of AR enables agricultural advisors to detect resistance at an early stage when it is present in only a small proportion of a local worm population. Knowledge of the emergence of this early stage of resistance may allow for management decisions (*e.g.* drug rotation) designed to prevent further selection pressure on that population.

***In vivo* tests**

1) Faecal egg count reduction test

Of all the methods used to determine AR in the field or in research studies, the FECRT, which was the first to be developed, still the most widely used and recommended (Presidente, 1985). This test can be used with ruminants, horses and pigs, with all types of anthelmintic and with all species of nematodes in which eggs are shed in the faeces. This test provides an estimate of anthelmintic efficacy by comparing the faecal worms and egg counts for groups of animals before and after treatment. A standardized protocol for the FECRT is available for the detection of AR in nematodes of veterinary importance (Coles *et al.*, 1992, 2006).

One of the important limitations of FECRT is that test results may not estimate anthelmintic efficacy accurately because nematode egg output does not always correlate well with actual worm numbers, and the test only measures effects on egg production by mature worms. Faecal egg counts are generally high in goats but they do not correlate well with worm counts. If the interval between treatments is less than 10 days, egg production may be suppressed leading to an overestimation of anthelmintic efficacy with the BZ anthelmintic (Martin *et al.*, 1989). For this reason, the recommendation is to collect faecal samples 10–14 days after treatment in case of BZs, 7 days after for Levamisol (LEV) and 14 days after for IVM (Coles *et al.*, 1992, 2006). Larval culture can be used to determine the species involved, but culture conditions may favour the development of one species over another (Presidente, 1985). Parasites with a high biotic potential, *e.g.* *H. contortus*, may exert a disproportionate influence on the results and, therefore, correction factors have to be included. It also appears that the test lacks the sensitivity to detect levels of resistance below 25% (Martin *et al.*, 1989). However, a simple definition of resistance based on faecal egg counts is probably no longer possible (Coles *et al.*, 2005).

2) Critical anthelmintic test

The test is based on the collection parasites from faeces of treated animals for at least 4 days after treatment as well as residual worms of slaughtered animals, and then the percentage of efficacy will be calculated by dividing the number of expelled worms by the residual number and

multiplying by 100. The major advantage of this test is that each animal serves as its own control and thus fewer animals are required for the test. However, it is unsatisfactory for estimating anthelmintic efficacy against abomasal/ stomach parasites because they undergo digestion during their passage through the gut (Reinecke *et al.*, 1962) and also it is time and labour consuming.

3) The controlled anthelmintic efficacy test

This test compares the worm burdens of animals post-treatment which have been artificially infected with susceptible or suspected resistant isolates of nematodes. The test can also be used to determine the anthelmintic activity against all stages of development of parasites by slaughtering at varying times after infection (Reinecke *et al.*, 1962). It is considered to be the most reliable method for assessing AR and has, therefore, been widely used to confirm the results of FECRTs as well as for validating different *in vitro* tests (Presidente, 1985). However, it is the most costly test in terms of labour requirements and animal usage and is now rarely used.

***In vitro* tests**

A variety of different laboratory tests have been described for the detection of AR in livestock helminths (Conder and Campbell, 1995). Those, which are most commonly used and which might be applied to detect AR in helminths are briefly described here.

1) Egg hatch test

The egg hatch test (EHT) is an *in vitro* test, which is used only for the detection of BZ resistance in livestock helminths. Principal of this assay is to incubate eggs in serially diluted BZ with predetermined time and count the proportion, which fail to embryonate and hatch. EHA was found to be inexpensive, sensitive and repeatable when a single species was involved. However, this test has a prerequisite of using fresh eggs (within three hours of collection from animals) (Le Jambre, 1978). If this is not possible, samples must be stored anaerobically (Taylor and Hunt, 1989). A standardized protocol was adopted by the World Association for the Advancement of Parasitology (WAAVP) (Coles *et al.*, 1992, 2006). Unfortunately, the FECRT and the EHT is supposed to detect resistance only when at least 25% of the worm population carries resistance genes (Martin *et al.*, 1989). In spite of the above mentioned problems associated with EHT, it is still widely used to determine AR along with FECRT. With regard to equine nematodes, the egg hatch assay has been claimed to be the only practical definitive test for confirming BZ resistance.

2) Larval development assay (LDA)

The test was originally described by Coles *et al.* (1989) and modified by many workers. In LDA, parasite eggs or L₁ larvae are exposed to different concentrations of anthelmintics incorporated into agar wells in a microtiter plate or in a small test tube containing nutrient medium, and incubated for 7 days at 26°C. The effect of the drugs on the subsequent development into L₃ larvae will be measured. It is claimed that this test is more sensitive than FECRT and EHT and detects AR when about 10% of the worm population carries resistance genes (Dobson *et al.*, 1996). The LDA is more laborious and time-consuming than the EHT but allows the detection of resistance to the

major broad-spectrum anthelmintic classes, including macrocyclic lactones.

3) Larval paralysis test

A larval paralysis test was developed for the detection of LEV and morantel resistance (Martin and Le Jambre, 1979). In the assay, infective third stage larvae are incubated for 24 h in serial dilutions of the anthelmintic. Thereafter, the percentage of paralysed larvae is determined at each concentration and a dose–response line plotted and compared to known reference strains.

4) Larval motility test

In vitro assays to detect resistance to BZ, macrocyclic lactones or LEV and morantel have been described which are based on the motility of larvae (Conder and Campbell, 1995). For the IVM/LEV, a clear cut distinction between susceptible and resistant strains is not always possible (Varady and Corba, 1999).

5) Adult migration inhibition test

A migration assay has been described, using adult stages of the pig nematode, *Oesophagostomum dentatum*, to differentiate between BZ and pyrantel susceptible and resistant strains. In the assay, adult worms removed on post-mortem were incubated in serial concentrations of anthelmintic for 30 min before transfer to the migration chambers. These were equipped with polyamide nets of mesh size 300-500 µm through which the worms are allowed to migrate over a period of 30 min. A dose-response curve was then plotted based on the inhibition of migration by the mesh at the various drug concentrations used (Petersen *et al.*, 2000). This test can also be used in other species of nematodes of livestock including small ruminants but it has a limitation that animals have to be sacrificed for the collection of adult worms.

6) Colorimetric assays

Biochemical assays, comparing non-specific esterases and acetylcholinesterases of BZ-resistant and -susceptible trichostrongylid nematode strains have been described (Sutherland and Lee, 1989). Colorimetric assay has shown that there is significantly more specific esterase in the infective-stage larvae of BZ-resistant strains than in susceptible strains and this may prove to be of use in the detection of resistance to BZ anthelmintics (Sutherland and Lee, 1989).

7) Tubulin binding test

The mechanism of BZ resistance appears to be associated with a reduced affinity of tubulin for the anthelmintic. Tubulin binding test was developed by Lacey and Prichard (1986) based on differential binding of BZ's with tubulin, which was prepared from susceptible and resistant nematodes. In this assay, crude extract of tubulin prepared from different stages of parasites (adult, larval, and eggs) are incubated with tritium labeled benzimidazole until equilibrium is reached. The surplus drug is removed with charcoal and tritium-benzimidazole tubulin complexes are estimated by using liquid scintillation spectrometer.

Molecular Techniques

The molecular basis of AR is well documented only for the BZs where it is caused by mutations in α -tubulin (Kwa *et al.*, 1994). This information can be used to develop tests and study the influence of management on the development of resistance, however, keeping in mind that other changes may also contribute to BZ resistance,

specifically transport of the drug out of the parasite like P-gp and cAMP-450. Tests based on the use of a single point mutation to detect AR also suffer due to that resistance may have resulted from more than one mutation (167th position on beta tubulin) (Prichard, 2001). Molecular tests include conventional PCR (Silvestre and Humbert, 2000; Njue and Prichard, 2003; Winterrowd, 2003), real-time PCR (Alvarez-Sanchez *et al.*, 2005; Walsh *et al.*, 2007) as well as pyrosequencing (Hoglund *et al.*, 2009; Von Samson-Himmelstjerna *et al.*, 2009; Demeler *et al.*, 2013) and are all based on the detection of SNPs in the three above-mentioned codons in the isotype-1 α -tubulin gene.

The first specific primers to detect drug-resistant parasitic nematodes were developed by Kwa *et al.* (1994). These primers discriminated between heterozygous and homozygous BZ-resistant *H. contortus* for the alleles in question (α -tubulin isotype 1), even when these genotypes are phenotypically indistinguishable. With PCR, 1% level of resistant individuals can be detected within a susceptible worm population, which is a tremendous improvement over *in vivo* and *in vitro* tests (Roos *et al.*, 1995). Using four primers (two allele-specific and two non-allele-specific ones) in the same PCR, adult worms can be characterized for the mutation of residue 200 of isotype 1 α -tubulin, called allele specific PCR (AS-PCR). The technique has now been refined for use on a single worm, egg, or larva in field level (Silvestre and Humbert, 2000; Sankar, 2007; Garg and Yadav, 2009). PCR-RFLP along with AS-PCR on single larvae or eggs might be a good technique for determining the accurate generic composition of trichostrongylid larval communities of ruminants and small strongyles of horses in the field as well as the resistance of each species to BZ. This method also overcome morphological identification of larval stages of trichostrongylids and cyathostomines and is suitable for epidemiological studies. Since the same mutation is responsible for BZ resistance in many parasitic nematodes, this method may provide a means of investigating the frequencies of alleles bearing it in a wide range of animal and human intestinal nematodes.

However, a much more rapid test is the use of real time PCR (Alvarez-Sanchez *et al.*, 2005) or pyrosequencing that will determine the ratio of susceptible to resistance genes in a population of worms. If probes for the speciation of the nematodes were available, the ratio of species involved in resistance could also be determined at the egg stage. The real time PCR, for the first time, offers the opportunity to analyse allele frequencies based on samples of pooled larvae, thus, allowing the inexpensive, sensitive and rapid molecular diagnosis of BZ-resistance in trichostrongyles. However, this method has not yet been used on mixed species or field samples and thus it remains to be seen to what extent further optimisation will be required. Also, it is probably too late to use real time PCR for detecting BZ resistance in sheep and goats in many parts of the world because it is so common.

CONCLUSION

Currently available *in vivo* and *in vitro* tests for benzimidazole resistance in trichostrongylid nematodes tend to be time-consuming, unreliable and tedious to do. As a result, now attention is turning to molecular tools and

its technically demanding. It is appeared as a good alternative to classical techniques. Molecular tools offer the opportunity to overcome at least some of these shortcomings. As in numerous other fields of Veterinary Parasitology, the polymerase chain reaction technique has opened new perspectives for anthelmintic resistance research. Several PCR protocols have been developed which provide high accuracy and sensitivity when used to investigate single worms. However, meaningful results depend on testing representative number of individuals from the isolate under investigation.

REFERENCES

- Alvarez-Sanchez, M.A., Perez-Garcia, J., Cruz-Rojo, M.A. and Rojo-Vazquez, F.A. (2005) Real time PCR for the diagnosis of benzimidazole resistance in trichostrongylids of sheep. *Vet. Parasitol.* **129**(3): 291-298.
- Chandra, S., Prasad, A., Sankar, M., Yadav, N. and Dalal, S. (2014) Molecular diagnosis of benzimidazole resistance in *Haemonchus contortus* in sheep from different geographic regions of North India. *Vet. World*, **7**(5): 337-341.
- Chandra, S., Prasad, A., Yadav, N., Latchumi kanthan, A., Rakesh, R.L., Praveen, K., Khobra, V., Subramani, K.V., Misri, J. and Sankar, M. (2015) Status of benzimidazole resistance in *Haemonchus contortus* of goats from different geographic regions of Uttar Pradesh, India. *Vet. Parasitol.* **208**(3): 263-267.
- Coles, G.C., Bauer, C., Borgsteede, F.H.M. and Geerts, S. (1992) World association for the advancement of Veterinary Parasitology (WAAVP) methods for the detection of anthelmintic resistance in nematode of veterinary importance. *Vet. Parasitol.* **44**: 35-44.
- Coles, G.C., Tritschler, J.P. and Giordano, D.L. (1989) Efficacy of levamisole against immature and mature nematodes in goats with induced infections. *Am. J. Vet. Res.* **50**: 1074-1075.
- Coles, G.C., Jackson, F., Pomroy, W.E., Prichard, R.K., Von Samson-Himmelstjerna, G., Silvestre, A., Taylor, M.A. and Vercruyse, J. (2006) The detection of anthelmintic resistance in nematodes of veterinary importance. *Vet. Parasitol.* **136**(3): 167-185.
- Coles, G.C., Rhodes, A.C. & Wolstenholme, A.J. (2005) Rapid selection for ivermectin resistance in *Haemonchus contortus*. *Vet. parasitol.* **129**(3): 345-347.
- Conder, G.A. and Campbell, W.C. (1995) Chemotherapy of nematode infections of veterinary importance, with special reference to drug resistance. *Adv in Parasitol.* **35**: 1-84.
- Craven, J., Bjorn, H., Barnes, E.H., Henriksen, S.A. and Nansen, P. (1999) A comparison of in vitro tests and a faecal egg count reduction test in detection of anthelmintic resistance in horse strongyles. *Vet. Parasitol.* **85**(1): 49-59.
- Demeler, J., Krüger, N., Krücken, J., Vera, C., Ramünke, S., Küttler, U., Miltsch, S., Cepeda, M.L., Knox, M., Vercruyse, J. and Geldhof, P. (2013) Phylogenetic characterization of β -tubulins and development of pyrosequencing assays for benzimidazole resistance in cattle nematodes. *PLoS one*, **8**(8): 70-212.
- Dobson, R.J., Lejambre, L. and Gill, J.H. (1996) Management of anthelmintic resistance: inheritance of resistance and selection with persistent drugs. *Int. J. Parasitol.* **26**(8): 993-1000.
- Fleming, S.A., Craig, T., Kaplan, R.M., Miller, J.E., Navarre, C. and Rings, M. (2006) Anthelmintic resistance of gastrointestinal parasites in small ruminants. *J. Vet. Internal Med.* **20**(2): 435-444.
- Garg, and Yadav (2009) Genotyping of benzimidazole susceptible and resistant alleles in different populations of *Haemonchus contortus* from Himalayan and sub-Himalayan regions of North West India *Trop. Anim. Health Prod.* **41** (7): 1127-1131.
- Garg, R., Yadav, C.L., Kumar, R.R., Banerjee, P.S., Vatsya, S. and Godara, R. (2009) The epidemiology of fasciolosis in ruminants in different geo-climatic regions of north India. *Trop. Anim. Health Prod.* **41**(8): 1695-17.
- Gill, B.S. (1996) Anthelmintic resistance in India. *Vet. Parasitol.* **63**: 173-176.
- Höglund, J., Gustafsson, K., Ljungström, B.L., Engström, A., Donnan, A. and Skuce, P. (2009) Anthelmintic resistance in Swedish sheep flocks based on a comparison of the results from the faecal egg count reduction test and resistant allele frequencies of the β -tubulin gene. *Vet. Parasitol.* **161**(1): 60-68.
- Kwa, M.S., Veenstra, J.G. and Roos, M.H. (1994) Benzimidazole resistance in *Haemonchus contortus* is correlated with a conserved mutation at amino acid 200 in β -tubulin isotype-1. *Mol. Biochem. Parasitol.* **63**: 299-303.
- Lacey, E. and Prichard, R.K. (1986) Interactions of benzimidazole (BZ) with tubulin from BZ-sensitive and BZ-resistant isolates of *Haemonchus contortus*. *Mol. Biochem. Parasitol.* **19**: 171-181.
- Larsen M. (2006) Biological control of nematodes in sheep. *J. Anim. Sci.* **84**(18): E133-E139.
- Le Jambre, L.F. (1976) Egg hatch as an in vitro assay of thiabendazole resistance in nematodes. *Vet. Parasitol.* **2**(4): 385-391.
- Le Jambre, L.F. (1978) Anthelmintic resistance in gastrointestinal nematodes of sheep. In "the Epidemiology and control of Gastrointestinal parasites of sheep in Australia. (A.D Donald, W.H Southcott and J.K Dineen, eds.). CSIRO, Melbourne: pp109-120.
- Lind, E.O., Ugglå, A., Waller, P. and Höglund, J. (2005) Larval development assay for detection of anthelmintic

- resistance in *Cyathostomins* of Swedish horses. *Vet. Parasitol.* **128**(3): 261–269.
- Martin, P. J., Anderson, N., and Jarett, R. G. (1989). Detecting benzimidazole resistance with faecal egg count reduction test and *in vitro* assays. *Aus. Vet. J.* **66**: 236-240.
- Martin, P.J. and Le Jambre, L.F. (1979). Larval paralysis as an *in vitro* assay of levamisole and morantel tartrate resistance in *Ostertagia*. *Vet. Res. Com.* **3**(1): 159-164.
- Njue, A. I and Prichard, R. K. (2003). Cloning two full length beta tubulin isotype cDNAs from *Cooperia oncophora*, and screening for benzimidazole resistance associated mutations in two isolates. *Parasitol.* **127**: 579-588.
- Petersen, M.B., Craven, J., Bjørn, H. and Nansen, P. 2000. Use of a migration assay for the separation of adult pyrantel susceptible and-resistant *Oesophagostomum dentatum*. *Vet. Parasitol.* **91**(1): 141-145.
- Presidente, P. J. A. (1985). Methods for detection of nematode resistance to anthelmintics. In: N Anderson and PJ Waller (Eds.), Resistance in Nematodes to Anthelmintic Drugs, CSIRO, Melbourne: pp13-28.
- Prichard, R.K. (2001) Genetic variability following selection of *Haemonchus contortus* with anthelmintics. *Trends in Parasitol.* **17**: 445-453
- Reinecke, R.K., Snijders, A. J. and Horak, I.G. (1962). A modification of standard procedures for evaluating the relative efficacy of anthelmintics. Onderstepoort. *J. Vet. Res.* **29**: 241-257.
- Roos, M.H., Kwa, M.S.G. and Grant, W.N. (1995) New genetic and practical implications of selection for anthelmintic resistance in parasitic nematodes. *Parasitol Today.* **11**: 148-150.
- Sankar, M. (2003) Studies on benzimidazole resistance in *H. contortus* in sheep. Thesis, M.V.Sc. Deemed University, Indian Veterinary Research Institute, Izatnagar, India.
- Sankar, M. (2007) Molecular characterization of β -tubulin gene in benzimidazole resistant and susceptible populations of common gastrointestinal nematodes in small ruminants. Thesis, Ph.D. Deemed University, Indian Veterinary Research Institute, Izatnagar, India.
- Silvestre, A. and Humbert, J.F. (2000) A molecular tool for species identification and benzimidazole resistance diagnosis in larval communities of small ruminant parasites. *Exp. Parasitol.* **95**: 271-276.
- Sutherland, I.A., and Lee, D.L. (1989) Colorimetric assay for detection of benzimidazole resistance in trichostrongylides. *Res. Vet. Sci.* **46**: 363-366.
- Tandon, R. and Kaplan, R.M. (2004) Evaluation of a larval development assay (DrenchRite) for the detection of anthelmintic resistance in cyathostomin nematodes of horses. *Vet. Parasitol.* **121**(1): 125-142.
- Taylor, M.A. and Hunt, K.R. (1989) Anthelmintic drug resistance in the UK. *Ve. Rec.* **125**: 143-147.
- Tiwari, J., Kumar, S., Kolte, A.P., Swarnkar, C.P., Singh, D. and Pathak, K.M.L. (2006) Detection of benzimidazole resistance in *Haemonchus contortus* using RFLP-PCR technique. *Vet. Parasitol.* **138**(3): 301-307.
- Varady, M. and Corba, J. (1999) Comparison of six *in vitro* test in determining benzimidazole and levamisole resistance in *Haemonchus contortus* and *Ostertagia circumcincta* of sheep. *Vet Parasitol.* **80**(3): 239-249.
- Von Samson-Himmelstjerna, G., Walsh, T.K., Donnan, A.A., Carriere, S., Jackson, F., Skuce, P.J., Rohn, K. and Wolstenholme, A.J. (2009) Molecular detection of benzimidazole resistance in *Haemonchus contortus* using real-time PCR and pyrosequencing. *Parasitol.* **136**(03): 349-358.
- Walsh, T.K., Donnan, A.A., Jackson, F., Skuce, P. and Wolstenholme, A.J. (2007) Detection and measurement of benzimidazole resistance alleles in *Haemonchus contortus* using real time PCR with locked nucleic acid Taqman probes. *Vet. parasitol.* **144**(3): 304-312.
- Winterrowd, C.A. (2003) Benzimidazole resistant beta tubulin alleles in a population of parasitic nematodes (*Cooperia oncophora*) of cattle. *Vet. Parasitol.* **117**: 161-172.
- Winterrowd, C.A. (2003) Benzimidazole resistant beta tubulin alleles in a population of parasitic nematodes (*Cooperia oncophora*) of cattle. *Vet. Parasitol.* **117**: 161-172.