Asian-Aust. J. Anim. Sci.
Vol. 20, No. 1 : 7 - 11
January 2007

www.ajas.info

Diagnosis of Benzimidazole Resistance in *Haemonchus contortus* of Sheep by Allele Specific PCR

J. Tiwari[2], A. P. Kolte[1], S. Kumar[1,*], C. P. Swarnkar[3], D. Singh[3] and K. M. L. Pathak[2]

[1] Animal Biotechnology Section, Central Sheep and Wool Research Institute, Avikanagar, Rajasthan 304 501, India

ABSTRACT : The study was conducted on 162 adult male *Haemonchus contortus* of sheep collected from Avikanagar, Jaipur and Bikaner regions to diagnose the benzimidazole (BZ) resistance in *H. contortus*. The BZ resistance is primarily linked with the mutation in β-tubulin isotype 1 gene which substitute phenylalanine (Phe) into tyrosine (Tyr) at the 200 codon of the gene. An allele specific polymerase chain reaction (AS-PCR) technique was used for diagnosis of BZ resistance in *H. contortus*. In AS-PCR, one reverse primer (TGG 312) was used in two separate reactions with each of 2 forward primers (resistant TGG 331 and susceptible CAW 106 primer) that differed only at 3' nucleotide position. Therefore, the amplified products from resistant and susceptible parasites were produced 267 and 266 bp, respectively. A total of 162 parasites were genotyped, of which 130 parasites found homozygous resistant 'rr', 22 heterozygous 'rS' and 10 homozygous susceptible 'SS' type. The prevalence of 'rr' individuals was higher in Jaipur (98%) followed by Avikanagar (93%) and Bikaner (50%) regions. Overall, the prevalence of BZ resistant allele (r) was higher (87%) as compared to 13% of BZ susceptible allele (S). (**Key Words :** *Haemonchus contortus*, Benzimidazole Resistance, β Tubulin, AS-PCR)

INTRODUCTION

In India, *Haemonchus contortus* is one of the major parasite of sheep and goat, which causes production losses and even death in severe cases. However, the production losses are also affected by the genetic and non-genetic factors (Assan and Makuza, 2005; Mandal et al., 2005). The heavy infection of *H. contortus* decreases the packed cell volume (Howlader et al., 1996), red blood cells (Howlader et al., 1997a) and body weight gain in goats (Howlader et al., 1997b). Control of this parasite is mainly based on the use of benzimidazole (BZ) and tetramisole/levamisole. Benzimidazole is the most extensively used anthelmintics for the control of parasite since 1960s. Benzimidazole (albendazole) was found highly effective (>95%) for control of strongyle infection in sheep (Dorny et al., 1995). These drugs are preferred because of their low cost, broad-spectrum activity and high efficacy. But the widespread use of anthelmintics has led to the development of resistance in the parasites (Jackson 1993; Waller 1994).

Several methods have been used for detection of BZ resistance viz. *in vivo* and *in vitro* assays. The fecal egg count reduction test (FECRT), egg hatch assay (EHA) and larval development assay (LDA) have proven to be suitable tests for detecting BZ resistance (Le Jambre, 1976; Coles et al., 1992). However, these tests are time consuming, costly, low in sensitivity and can detect resistance only when 25% of the individuals in the population are already resistant (Martin et al., 1989; Humbert et al., 2001). On the other hand, the molecular assays are highly sensitive and less expensive for diagnosis of anthelmintic resistance. These assays detect mutation at the codon 200 of the β-tubulin isotype 1 gene that is primarily linked with BZ resistance in nematode species. The point mutation (TTC to TAC) leads to a change in the amino acid from phenylalanine to tyrosine (Kwa et al., 1993, 1994; Ross et al., 1995; Elard et al., 1996; Elard and Humbert 1999). Allele specific-PCR (AS-PCR) technique has been used to detect the BZ resistance in nematode species (Lehrer et al., 1995; Elard et al., 1999; Silvestre and Humbert, 2000, 2002; von Samson-Himmelstjerna et al., 2002a, 2002b; Pape et al., 2003; Winterrowd et al., 2003). AS-PCR has an advantage over the existing assays in terms of specificity, sensitivity and give quick results with less input. Recently, Alvarez-Sánchez et al. (2005) used real time PCR for the diagnosis

* Corresponding Author: S. Kumar. Tel: +91-1437-220142, Fax: +91-1437-220163, E-mail: satish_kumar@scientist.com

[2] Department of Veterinary Parasitology, Rajasthan Agricultural University, Bikaner, Rajasthan 334 001, India.

[3] Division of Animal Health, Central Sheep and Wool Research Institute, Avikanagar, Rajasthan 304 501, India.

Received September 21, 2005; Accepted February 1, 2006

of BZ resistance in Trichostrongylids of sheep, which is found to be more sensitive, rapid and inexpensive.

In India, the first occurrence of anthelmintic resistance in *H. contortus* of sheep was reported by Varshney and Singh (1976). Anthelmintic resistance in *H. contortus* has been detected through FECRT, EHA, LDA tests (Singh et al., 1992; Yadav et al., 1993; Singh et al., 1995, 1996), but information on molecular assays for BZ resistance is not available. In the present study, the aim of the study was to use an allele specific-PCR for the detection of BZ resistance in *H. contortus* of sheep.

MATERIALS AND METHODS

Collection of parasite

Adult male *H. contortus* were collected directly from the abomasum of sheep from three different locations of Rajasthan, India viz. local abattoir of Bikaner (arid region) and Jaipur (semi-arid region) as well as sheep necropsied at Central Sheep and Wool Research Institute (CSWRI) Avikanagar.

The individual abomasum was collected and put in to the polythene bag. The abomasum was opened by incising along the greater curvature and the whole contents along with mucosal surface of abomasum were washed several times with distilled water. The washings were added in to the bucket and mixed thoroughly. The contents were sieved to remove the feed particles. Remaining contents were repeatedly washed and sieved unless the remnant having parasites become clear. Individual adult male *H. contortus* was picked with the help of needle and the species was confirmed by studying their characteristic morphological features (Soulsby, 1965). A total of 162 parasites (54 from each location) were collected in PBS and incubated at 37°C for five hours. The worms were stored at 4°C till further use.

DNA extraction

The genomic DNA from each adult male *H. contortus* was isolated using standard protocol of Beech et al. (1994) with minor modifications. Individual worm was mixed in 224 µl of digestion mixture (comprised of 200 µl STE, 10 µl sodium dodecyl sulphate (10%), 10 µl β-mercaptoethanol and 4 µl Proteinase-K (10 mg/ml) and incubated at 37°C for 4 h. After incubation, 200 µl of Phenol: Chloroform: *Iso*-amyl alcohol (25:24:1) was added and centrifuged at 12,000 rpm for 1 min. The aqueous phase was collected in a fresh tube and 4 µl of linear acrylamide (2.5 mg/ml), 270 µl of absolute *iso*-propanol and 130 µl of 7.5 M Ammonium acetate were added. DNA was recovered by centrifugation at 14,000 rpm for 20 min and pellet was dissolved in 30 µl TE buffer (pH 8.0).

Detection of BZ resistance through AS-PCR assay

BZ resistance, which is linked to the mutation at the 200 codon of β-tubulin isotype 1 gene, was detected by allele specific-PCR. Two sets of primers were used for amplifications; same reverse primer TGG 312 (5'-GGA ACC ATG TTC ACG GCT AAC-3') was used in two separate reactions with CAW 106 (5'-TAG AGA ACA CCG ATG AAA CAT T-3') as susceptible primer and TGG 331 (5'-G TAG AGA ACA CCG ATG AAA CAT A-3') as resistant primer. Primer CAW 106 annealed with complementary sequence with phenylalanine (TTC) codon, whereas primer TGG 331 annealed with complimentary sequence of tyrosine (TAC) at codon 200 of β-tubulin gene. Only the final base at the 3' end of each forward primer confers the specificity.

The PCR reaction mixture was comprised in 20 µl volume as follows; 1.5 mM $MgCl_2$, 200 µM dNTP's mixture, 1×Taq polymerase buffer (16 mM $(NH_4)_2SO_4$, 67 mM Tris-HCl (pH 8.8), 0.01% Tween-20, Bio-line), 200 nM of each primer, 2 µl of template DNA and 0.5 U Taq DNA polymerase. PCR reaction conditions were optimized using initial denaturation at 95°C for 10 minutes followed by 50 cycles of denaturation at 95°C for 30 s, annealing at 61°C for 30 s and extension at 72°C for 60 s followed by final extension at 72°C for 5 min. All reactions were carried out in Thermal Cycler (Mastercycler Gradient, Eppendorf). Amplified products were analyzed on 2% agarose gel stained with ethidium bromide. The susceptible primer (CAW 106) and resistant primer (TGG 331) amplify a product of 266 bp and 267 bp, respectively. Worms that gave amplification only with BZ-susceptible primer were designated as homozygous susceptible (SS), the individuals that amplify only with BZ-resistant primer were designated as homozygous resistant (rr), the individuals gave amplification with both the primers were designated as heterozygous (rS) for BZ resistance.

Data analysis

Genotypic and allele frequencies were calculated as per the method of Pierce (2003). The genotypic and allelic frequency data were compared using χ^2 test (Snedecor and Cochran, 1967).

RESULTS AND DISCUSSION

Adult male *H. contortus* were genotyped for detection of mutation in the β-tubulin isotype 1 gene using AS-PCR technique. A total of 162 parasites consisted of 54 from each location viz. Bikaner, Avikanagar and Jaipur were used in the study. The genotypic frequencies of three genotypes (rr, rS and SS) for BZ resistance were significantly ($p<0.001$) different among three locations shown in Table 1.

Table 1. Genotypic and allelic frequencies of BZ resistance in *H. contortus* by AS-PCR technique

Location	Genotypes frequency			Allele frequency	
	Homozygous resistant (rr)	Heterozygous (rS)	Homozygous susceptible (SS)	Resistant (r)	Susceptible (S)
Bikaner	0.50** (27)	0.33** (18)	0.17** (9)	0.67**	0.33**
Avikanagar	0.93** (50)	0.05** (3)	0.02** (1)	0.95**	0.05**
Jaipur	0.98** (53)	0.02** (1)	0.00** (0)	0.99**	0.01**
Overall	0.80** (130)	0.14** (22)	0.06** (10)	0.87**	0.13**

** Significant, p<0.001, Values in parenthesis are the numbers of individuals.

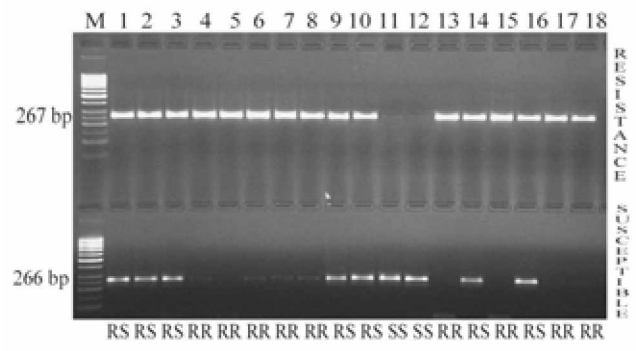

Figure 1. AS-PCR profile of β-tubulin isotype 1 gene of *H. contortus*. Upper lane: resistant individuals, Lower lane: susceptible individuals, M: 50 bp ladder.

Allele frequency was also revealed similar trends as found with genotypic frequency in all the locations. Overall, proportion of BZ resistant (r) allele was significantly (p<0.001) higher (87%) as compared to 13% prevalence of BZ susceptible (S) alleles (Table 1).

From Bikaner region 54 parasites were analyzed, of that 27 'rr' type, 18 rS and 9 SS types. The AS-PCR profile of β-tubulin isotype 1 gene has shown in Figure 1. The prevalence of different genotypes varied significantly (p<0.001) with 50% 'rr' type, 33% rS and 17% SS types. The prevalence of BZ resistance in Bikaner was found less as compared to Avikanagar and Jaipur regions. This might be due to the harsh climate of this region, where the average annual rainfall is very less and the temperature exceeds up to 45°C. Such environmental conditions are detrimental for survival of infective stages of the parasites, causes low infection rate thereby low use of anthelmintics. The drenching of animals is practiced only once in a year in Bikaner and adjoining areas that causes the possibility of low BZ resistance in the flocks. The results also support the findings of Annon (2004), who found the low level of BZ resistance in *H. contortus* in arid regions by FECRT and EHA tests.

From Avikanagar, 54 parasites were genotyped, 50 individuals were 'rr' type, 3 rS and one SS type. The types of genotypes varied significantly (p<0.001) with 93% individuals 'rr' type, 5% rS and 2% SS type. The results indicated that high prevalence of BZ resistance found at Avikanagar, as the 98.0% individuals carry TAC allele. The high level of BZ resistance at this location is due to the frequent use of anthelmintic treatments thereby parasites have become resistant to benzimidazoles (Swarnkar et al., 1999). These results are in agreement with the study of Singh et al. (1995), who recorded 0% efficacy of benzimidazole using FECRT, and further confirmed by EHA and LDA tests (Swarnkar et al., 2001).

Fifty-four parasites from Jaipur region showed 53 'rr' type, and one parasite 'rS' type for BZ resistance. In this region, almost all individuals (100%) carry mutated allele (TAC) and due to high frequency of TAC allele, parasites have become resistant to benzimidazoles. The high prevalence of resistant worms may be due to drenching of animals prior to slaughter and indiscriminate use of the drugs, which favours the selection of resistant individuals. Similar trends was also reported by Pape et al. (2003), who reported that the increasing dose of fenbendazole decreases homozygous (TTC/TTC) individuals, and increases heterozygous (TTC/TAC) and homozygous (TAC/TAC) individuals of cyathostomes parasites. In the present study, the 'rr' individuals were higher in the semi-arid areas (Jaipur and Avikanagar) as compared to arid area (Bikaner). This might be because of Avikanagar and Jaipur regions have almost same agro-climatic conditions, which are more favourable for development of *H. contortus* thereby more use of anthelmintics. Another possibility may be collection of parasites in the monsoon season when drenching practices are highest in the flocks, which may also cause the incidences of resistance in the parasites (Singh et al., 1999; Swarnkar et al., 2004).

Several *in vivo* and *in vitro* methods have been used for detection of BZ resistance in *H. contortus* like FECRT, EHA and LDA tests (Singh et al., 2002), however *in vitro* tests have low sensitivity, relatively expensive and detect the resistance only when at least 25% of the individuals within the population are already resistant (Martin et al.,

1989). In contrast, AS-PCR technique is quick for detection of BZ resistance (Wheeler et al., 1995; Elard et al., 1999) and discriminates as little as 1% resistant individuals in samples of susceptible population (Pape et al., 2003). Although, AS-PCR can be influenced by many factors like annealing temperature, magnesium concentration, and concentration of *Taq* DNA polymerase (Silvestre and Humbert, 2000), but technique was found more sensitive, rapid and specific than *in vivo* and *in vitro* tests. AS-PCR determines the resistance status in the parasites based on their genotypes (rr, rS and SS), while *in vivo* and *in vitro* tests provide information either parasite pheotypically resistant or susceptible to benzimidazoles. In summary, AS-PCR technique is rapid and gave the reproducible results for detecting BZ resistance in *H. contortus*.

ACKNOWLEDGEMENT

The authors are highly thankful to the Director CSWRI, Avikanagar for providing the necessary facilities for carrying out the study.

REFERENCES

Álvarej-Sánchez, M. A., J. Pérez-García, M. A. Cruz-Rojo and F. A. Rojo-Vázquez. 2004. Real time PCR for the diagnosis of benzimidazole resistance in trichostrongylids of sheep. Vet. Parasitol. 129:291-298.

Annon. 2004. Annual Progress Report: All India Network Program on Gastrointestinal Parasitism. CSWRI, Avikanagar, India, p. 1-45.

Assan, N. and S. M. Makuza, 2005. The effect on non-genetic factors on birth weight and weaning weight in three sheep breeds of Zimbabwe. Asian-Aust. J. Anim. Sci. 18(2):151-157.

Beech, R. N., R. K. Prichard and M. E. Scott. 1994. Genetic variability of the beta-tubulin genes in benzimidazole susceptible and resistant strains of *Haemonchus contortus*. Genet. 138:103-110.

Coles, G. C., C. Bauer, F. H. Borgsteede, S. Geerts, T. R. Klei, M. A. Taylor and M. J. Waller. 1992. World Association for the Advancement of Veterinary Parasitology (W.A.A.V.P.) methods for the detection of anthelmintic resistance in nematodes of veterinary importance. Vet. Parasitol. 44:35-44.

Dorny, P., E. Romjali, K. Feldman, A. Barubara and V. C. Pandey. 1995. Studies on the efficacy of four anthelmintics against strongyle infections of sheep in North Sumatra, Indonesia. Asian-Aust. J. Anim. Sci. 8:347-352.

Elard, L., J. Cabaret and J. F. Humbert. 1999. PCR diagnosis of benzimidazole-susceptibility or -resistance in natural populations of the small ruminant parasite, *Teladorsagia circumcincta*. Vet. Parasitol. 80:231-237.

Elard, L., A. M. Comes and J. F. Humbert. 1996. Sequences of beta-tubulin cDNA from benzimidazole -susceptible and -resistant strains of *Teladorsagia circumcincta*, a nematode parasite of small ruminants. Mol. Biochem. Parasitol. 79:249-253.

Elard, L. and J. F. Humbert. 1999. Importance of the mutation of amino acid 200 of the isotype 1 beta tubulin gene in the benzimidazole resistance of the small ruminant parasite *Teladorsagia circumcincta*. Parasitol. Res. 85:452-456.

Howlader, M. M. R., S. S. Capitan, S. L. Eduardo, N. P. Roxas and C. C. Sevilla. 1996. Effect of experimental *Haemonchus contortus* infection on haemoglobin concentration and packed cell volume of does. Asian-Aust. J. Anim. Sci. 9:597-601.

Howlader, M. M. R., S. S. Capitan, S. L. Eduardo and N. P. Roxas. 1997a. Effect of experimental *Haemonchus contortus* infection on red blood cells and white blood cells of growing goats. Asian-Aust. J. Anim. Sci. 10:679-682.

Howlader, M. M. R., S. S. Capitan, S. L. Eduardo, N. P. Roxas and C. C. Sevilla. 1997b. Performance of growing goats experimentally infected with stomach worm (*Haemonchus contortus*). Asian-Aust. J. Anim. Sci. 10:534-539.

Humbert, J. F., J. Cabaret, L. Elard, V. Leignel and A. Silvestre. 2001. Molecular approaches to studying benzimidazole resistance in trichostrongylid nematode parasites of small ruminants. Vet. Parasitol. 101:405-414.

Jackson, F. 1993. Anthelmintic resistance- the state of play. Br. Vet. J. 149:123-138.

Kwa, M. S., F. N. Kooyman, J. H. Boersema and M. H. Roos. 1993. Effect of selection for benzimidazoles resistance in *Haemonchus contortus* on beta tubulin isotype 1 and isotype 2 genes. Biochem. Biophys. Res. Commun. 191:413-419.

Kwa, M. S., J. G. Veenstra and M. H. Roos. 1994. Benzimidazole resistance in *Haemonchus contortus* is correlated with a conserved mutation at amino acid 200 in beta-tubulin isotype 1. Mol. Biochem. Parasitol. 63:299-303.

Le Jambre, L. F. 1976. Egg hatch as an *in vitro* assay of thiabendazole resistance in nematodes. Vet. Parasitol. 2:385-391.

Lehrer, S., H. Davey, T. Watson and R. J. Wilkins. 1995. Sensitive PCR for detecting benzimidazole resistant sub populations of ovine nematodes in the Waikato. In Proceedings of the New Zealand Society of Animal Production, 55:209-210.

Mandal, A., K. P. Pant, D. R. Notter, P. K. Rout, R. Roy, N. K. Sinha and N. Sharma. 2005. Studies on inbreeding and its effects on growth and fleece traits of Muzaffarnagri sheep. Asian-Aust. J. Anim. Sci. 18(10):1363-1367.

Martin, P. J., N. Anderson and R. G. Jarrett. 1989. Detecting benzimidazole resistance with faecal egg count reduction tests and *in vitro* assays. Aust. Vet. J. 66:236-240.

Pape, M., J. Posedi, K. Failing, T. Schnieder and G. Von Samson-Himmelstjerna. 2003. Analysis of the beta-tubulin codon 200 genotype distribution in a benzimidazole- susceptible and resistant cyathostome population. Parasitol. 127:53-59.

Pierce, B. A. 2003. Genetics, A Conceptual Approach. W.H. Freeman and Company, NY, USA, pp. 671-672.

Roos, M. H., M. S. Kwa and W. N. Grant. 1995. New genetic and practical implications of selection for anthelmintic resistance in parasitic nematodes. Parasitol. Today. 11:148-150.

Silvestre, A. and J. F. Humbert. 2000. A molecular tool for species identification and benzimidazole resistance diagnosis in larval communities of small ruminant parasites. Exp. Parasitol. 95:271-276.

Silvestre, A. and J. F. Humbert. 2002. Diversity of benzimidazole resistance alleles in populations of small ruminants parasites. Int. J. Parasitol. 32:921-928.

Singh, D., R. Gulyani and V. Bhasin. 1992. Occurrence of thiabendazole resistant strains of *Haemonchus contortus* in sheep. Ind. Vet. Med. J. 16:139-141.

Singh, D., P. K. Sanyal, C. P. Swarnkar, F. A. Khan and P. S. K. Bhagwan. 1999. Influence of diet type and pre-treatment fasting on the disposition kinetics of albendazole in sheep. Vet. Res. Commun. 23:229-240.

Singh, D., C. P. Swarnkar and F. A. Khan. 2002. Anthelmintic resistance in gastrointestinal nematodes of livestock in India. J. Vet. Parasitol. 16:115-130.

Singh, D., C. P. Swarnkar, F. A. Khan, C. P. Srivastava and P. S. K. Bhagwan. 1995. Resistance to albendazole in gastrointestinal nematodes of sheep. J. Vet. Parasitol. 9:95-98.

Singh, D., C. P. Swarnkar, C. P. Srivastava, P. S. K. Bhagwan and U. Dimri. 1996. *Haemonchus contortus* resistant to rafoxanide in sheep. J. Vet. Parasitol. 10:53-56.

Snedecor, G. W. and W. G. Cochran. 1967. Statistical Methods. Oxfords and IBH publishing company, Culcatta, India.

Soulsby, E. J. L. 1965. Textbook of veterinary clinical parasitology, Vol. 1, helminths. Blackwell Scientific Publication. Great Britain, pp. 297-310.

Swarnkar, C. P., F. A. Khan, D. Singh and P. S. K. Bhagwan. 1999. Further studies on anthelmintic resistance in sheep at an organized farm in arid region of Rajasthan. Vet. Parasitol. 82:81-84.

Swarnkar, C. P., P. K. Sanyal, D. Singh, F. A. Khan and P. S. K. Bhagwan. 2001. Anthelmintic resistance on an organized sheep farm in India. Trop. Anim. Health. Prod. 33:305-312.

Swarnkar, C. P., D. Singh, F. A. Khan, R. Tiwari and S. C. Dubey. 2004. Prevalence of anthelmintic resistance and seasonal variation in efficacy of anthelmintics in sheep. In XV National Congress of Indian Association for the Advancement of Veterinary Parasitology, GBPUAT, Pantnagar, India, p. 72.

Varshney, T. R. and Y. P. Singh. 1976. A note on development of resistance of *Haemonchus contortus* worms against phenothiazine and thiabendazole in sheep. Indian J. Anim. Sci. 46:666-668.

Von Samson-Himmelstjerna, G., M. Pape, C. Von Witzendorff and T. Schnieder. 2002a. Allele specific PCR for the beta tubulin codon 200 TTC/TAC polymorphism using single adult and larval small strongyle (Cyathostominae) stages. J. Parasitol. 88:254-257.

Von Samson-Himmelstjerna, G., C. Von Witzendorff, G. Sievers and T. Schnieder. 2002b. Comparative use of faecal egg count reduction test, egg hatch assay and beta tubulin codon 200 genotyping in small strongyles (Cyathostominae) before and after benzimidazole treatment. Vet. Parasitol. 108:227-235.

Waller, P. J. 1994. The development of anthelmintic resistance in ruminant livestock. Acta Tropica 56:233-243.

Wheeler, I. E., S. J. Kendall, J. Butters, D. W. Hollomon and L. Hall. 1995. Using allele specific oligonucleotide probes to characterize benzimidazole resistance in *Rhynchosporium secalis*. Pestic. Sci. 43:201-209.

Winterrowd, C. A., W. E. Pomroy, N. C. Sangster, S. S. Johnson and T. G. Geary. 2003. Benzimidazole resistant beta tubulin alleles in a population of parasitic nematode (*Cooperia oncophora*) of cattle. Vet. Parasitol. 117:161-172.

Yadav, C. L., R. P. Uppal and S. Kalra. 1993. An outbreak of haemonchosis associated with anthelmintic resistance in sheep. J. Vet. Parasitol. 23:411-413.