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Emergence of benzimidazole resistance in nematodes of small ruminants in an organized farm and some smallholder flocks in Phaltan Taluka, Maharashtra

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Abstract

The emergence of resistance to albendazole in the gastro-intestinal nematodes *Haemonchus contortus* and *Trichostrongylus colubriformis* in sheep and goats belonging to the Nimbkar Agricultural Research Institute and some smallholders' goat flocks in Bibi village of Phaltan Taluka in Maharashtra was detected by faecal egg count reduction (FECR) test. The status was further confirmed by detection of the mutation in the β-tubulin gene (isotype I), in the DNA of resistant larvae. The drenching strategy needs to be changed in the light of these findings to prevent further development of resistance.

Keywords: Anthelmintic resistance, Benzimidazole, Faecal egg count reduction, Goat, Sheep.

Introduction

Gastrointestinal nematode (GIN) infection is recognized as a major constraint to profitable sheep and goat production by adversely affecting their health, leading to substantial reduction in production if left untreated. (*Le* Jambre, 1996). The broad spectrum, short acting anthelmintic benzimidazole (BZ) also known as 'white drench' is widely used due to its high efficacy, easy availability and affordability. The widespread indiscriminate use of the BZ class of anthelmintics has led to the development of resistance to it in GIN in sheep and goats all over India, (Easwaran *et al.*, 2009; Maharishi *et al.*, 2011) and in several parts of the world (Howell *et al.*, 2008; Taylor *et al.*, 2009). However, anthelmintic resistance has not been reported from Maharashtra State (Nipane *et al.*, 2008). Benzimidazole resistance in the strongyle worms, *Haemonchus contortus* and *Trichostrongylus colubriformis* is linked primarily with a mutation in the codon 200 of the β-tubulin isotype I gene that leads to a change in the amino acid from phenylalanine to tyrosine (Tiwari *et al.*, 2006). This paper reports emerging resistance to BZ class of anthelmintics in *H. contortus* and *Trichostrongylus* species in sheep and goats in an organized farm and in some smallholder flocks in Bibi village in Phaltan taluka of Satara district in Maharashtra State.

Materials and Methods

Location, Animals and Management: Nimbkar Agricultural Research Institute (NARI) is located at latitude 18°N and longitude 74°E in a drought prone area. NARI maintains flocks of crossbred sheep comprising of Deccani, Garole, Bannur and Awassi breeds and nucleus flocks of pure Garole sheep and Boer goats. The animals belonging to NARI used in this study were partly grazed on pastures within NARI premises and partly stall-fed. Under the All India Coordinated Research Project on Goat Improvement of the Indian Council of Agricultural Research, NARI records the performance and FEC of Osmanabadi goats belonging to village smallholders. Bibi and Wadgaon are such villages near Phaltan. The goats in these villages are grazed on communal pastures and crop residues.

Deworming practices: At NARI, mass drenching or targeted selective treatment is carried out when FEC goes above the level of 1000 eggs per gram of faeces (epg). The anthelmintics albendazole, levamisole and closantel were used effectively for the past 15 years.

Evaluation of anthelmintic efficacy : In November, 2009 the effectiveness of albendazole was suspected to have reduced because out of 14 crossbred rams drenched, 7 were found to have positive FEC on post drench testing. The use of BZ class of anthelmintics was subsequently discontinued. However, in order to assess its effectiveness, in October 2010, out of 47 crossbred rams, Garole sheep and Boer goats screened for FEC and 10 animals with FEC > 1000 epg were drenched with Albomar® (Agrivet Farm Care, Virbac Animal Health, @10 mg/kg body weight. At the same time representative faecal samples of Osmanabadi goats in Bibi

village were screened for FEC and mass drenching of 333 goats in Bibi and 189 goats in Wadgaon was done using Albex® (Indian Genomics Pvt. Ltd.) @ 10mg/kg body weight. Post treatment FEC of a sample of all drenched animals was measured 10 to 14 days after every drenching. The animals drenched once and found to have an FEC > 400 epg were drenched again with the same drug and their post-drench FEC measured in order to confirm drug resistance.

Pre-drench FEC under natural infection was measured for both NARI animals and smallholders' goats. Faecal samples from the rectum were collected from individual sheep or goats and brought as soon as possible to NARI's laboratory for analysis. Quantification of FEC was done by the Modified McMaster Floatation technique. Nematode eggs were identified in this sample as described by (Soulsby, 1982) under a compound microscope. The eggs per gram faeces (EPG) was calculated using the following formula:

$$\text{FEC (epg)} = \frac{\text{No. of eggs counted x 60 ml (total vol)}}{\text{0.3 (vol. of counted chamber)x2 (wt of faeces)}}$$

Post drench FEC was tested 10-14 days after drenching. Coproculture was made at the time of FEC measurement pre- and post-drench using pooled faecal samples. The larvae were harvested using the Roberts and O'Sullivan technique. Gram's iodine was used to kill and stain the larvae to study their morphology. Larval species differentiation was done by referring to the key characteristics used in identification of third-stage larvae of sheep and goats (MAFF, 1986).

The per cent reduction in FEC was calculated by using the following formula (Kemper, 2003; Denwood, 2010).

$$\text{FECR} = 100\left[\frac{\text{Mean treatment final egg count Xt (epg)}}{\text{Mean treatment initial egg count Xi (epg)}}\right]$$

where Xt is the arithmetic mean FEC of the treated group at 10-14 days after drenching and X*i* is the arithmetic mean of the same group before treatment.

Albendazole resistant larvae were obtained by culturing the faeces of only those animals drenched twice with albendazole. The resistant larvae (L_3) obtained from NARI animals and Bibi goats were preserved in separate jars half filled with distilled water to provide air space and maintained at 10°C in the refrigerator. The DNA extraction and PCR-RFLP test were performed using these larvae.

DNA extraction from larvae: Genomic DNA was extracted from the preserved L_3 larvae using the method described by Silvestre and Humbert (2000) and Tiwari *et al.* (2006) with some modifications. DNA was extracted from pooled larvae; ten L_3 were picked up in two separate tubes from NARI and Bibi groups. They were recovered in 20 µl distilled water in 1.5 ml micro-centrifuge tubes and were placed at -20 °C for 1 hour to kill the larvae. DNA was extracted by adding 50 µl PCR buffer (1 mM Tris-HCl, 0.1 mM EDTA, and 5 mg proteinase-K (20 mg/ml), (Bangalore Genei). Tubes were then transferred to a Thermal Cycler (Eppendorf Mastercycler Gradient) and kept at 55°C for 90 minutes and at 95°C for 30 minutes to activate and deactivate the proteinase-K enzyme, respectively. The resultant DNA samples from larvae were stored at 4°C for further use.

PCR-RFLP : The PCR reaction mixture comprised of the following components: 1X PCR buffer (16 mM $(NH_4)_2SO_4$, 67 mM Tris–HCl, 0.01% Tween-20, Genei), 1.5 mM $MgCl_2$, 200 µM dNTP's mixture, 200 nM of each primer (AvikaF and AvikaR), 2 µl of template DNA and 0.5 U *Taq* DNA polymerase (Genei) in 20 µl reaction volume. Primer sequences used were (Tiwari *et al.*, 2006) forward primer AvikaF 5'CTACCCTTTCCGT CCATCAA3' and the reverse primer AvikaR 5'TGAAGACGAGGGAATGGAAC3'.

The amplification was performed in a thermal cycler (Mastercycler Gradient, Eppendorf) with the following amplification conditions; initial denaturation at 95°C for 10 min followed by 50 cycles of denaturation at 95°C for 30s, annealing at 56°C for 30s and extension at 72°C for 60s followed by a final extension at 72°C for 5 min. After amplification, PCR product was digested with *TaaI* (HpyCH4III), MBI Fermantas) that recognizes the nucleotide sequence 5'-ACTGT-3'. Restriction digestion was carried out at 65° C for 1 h and the product was loaded on a 2.0% high resolution blend (Ameresco) agarose gel. Electrophoresis was carried out using Tris Borate EDTA 1X buffer.

Results

The data obtained from pre and post-drench FEC measurements are summarized in Table 1. It was found that albendazole reduced FEC of all animals. However, 33-67% animals were found to have positive FEC after drenching which is an indication of emerging drug resistance.

Coproculture: Prevalence of four different worm species was seen in the pre-drench cultures with the predominance of *H. contortus* followed by *T. coulbriformis, Oesophagostomum spp. and Strongyloides papillosus*. In the post drench cultures, two nematode species *H. contortus and T. coulbriformis* were identified from NARI animals in the proportions of 95% and 5% respectively and in Bibi goat flocks, 97% and 3% respectively.

FECRT: The per cent reduction in FEC of the two groups from NARI and Bibi village was less than 95% which indicates the prevalence of Benzimidazole resistance.

Table 1. Details of faecal egg counts and reduction of NARI, Bibi and Wadgaon village sheep and goat flocks in 2009 and 2010

FEC	Animals	Pre-drench FEC details				After first drenching			Percentage reduction (%)	After second drenching
		No. of animals sampled	Mean FEC (epg)	Proportion of positive samples (%)	FEC range (epg)	Mean FEC (epg)	Positive proportion (%)	FEC range (epg)		
Nov 2009	Crossbred rams	46	1557	45	0-5700	264	50	0-1600	83	Not carried out
Oct 2010	Garole lambs	28	400	79	0-2400	133	33	0-400	92	600
Oct 2010	NARI goats	19	837	84	0-2500	150	67	0-500		500
Oct 2010	Bibi goats	72	1705	100	200-8200	183	50	0-1300	66	100-600
Oct 2010	Wadgaon	Not performed				0	0	0	-	Not required

PCR-RFLP: Both the wild type and mutated alleles of the mutation at codon 200 of ß tubulin isotype I gene were identified at 305 and 257 base pairs respectively, in both NARI and Bibi sheep and goat flocks. (Fig. 1)

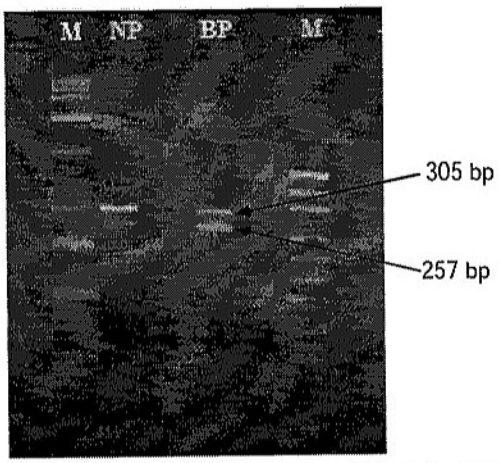

Fig. 1. Detection of the mutation at codon 200 of ß tubulin isotype I gene in DNA of *Haemonchus contortus* and *Trichostrongylus* species larvae
Lane M: DNA molecular weight marker (GeNei™ Ruler) (and GeNei™ pUC19).
Lane NP: DNA from pooled larvae from faecal samples of NARI sheep and goats
Lane BP: DNA from pooled larvae from faecal samples of Bibi village goats

Discussion

Observations of pre- and post- drench FEC of the sheep and goats were recorded as a part of routine monitoring. Therefore, a control group not treated with the anthelmintic could not be recorded.

The FECRT has been used as a reliable, simple qualitative and intuitive method to measure prevalence of anthelmintic resistance. According to the guidelines of World Association for Advancement of Veterinary Parasitology the reduction in FEC indicates that resistance to anthelmintic may be suspected in the nematode species tested against that drug if the anthelmintic has an efficacy of less than 95% (Coles *et al.*, 1992). Both groups of flocks in this study (table 1) met this criterion and therefore showed emerging resistance to Benzimidazole. The resistance was confirmed by detection of the mutation.

In NARI's sheep flock there may be two reasons for development of anthelmintic resistance; long term use of the drug and lack of refugia as the animals always grazed on the pastures within the premises and were not let outside. Studies have pointed out the direct correlation between frequent use of anthelmintics and anthelmintic resistance (Waller, 1987). The size of the population of refugia has a direct bearing on the degree of selection for resistance to a particular anthelmintic (Nari, 2005).

There are several reports on the high prevalence of anthelmintic resistance in organized farms or intensively managed farms in India. One such study was conducted by Swarnakar *et al.* (2001) in sheep flocks maintained at Central Sheep and Wool Research Institute, Avikanagar, Rajasthan. They observed that after eight years of administration of Benzimidazole, resistance had developed. A survey conducted by Kumar and Yadav (1994) in North-West India showed that out of 22 intensively managed farms, 16 farms showed moderate degree and 4 farms showed severe resistance to fenbendazole. A survey done by Singh and Yadav (1997) in Hisar on detection of resistance in sheep and goat farms belonging to Universities and Government farms showed single and multiple anthelmintic resistance in sheep and goats in all the farms. Laha *et al.* (1999) reported resistance in *Haemonchus* spp to benzimidazole in Pashmina goats at Mukteshwar, due to its prolonged use.

In the present study, prevalence of resistance may have been found in Bibi village but not in Wadgaon because of indiscriminate use of anthelmintics usually 3-4 times or more in a year in Bibi and underdosing which is one of the major factors leading to anthelmintic resistance (Waller, 1987). In Bibi village we found in an initial socio-economic survey

that the literacy percentage was 74% which was much higher than 45% among goat keepers of Wadgaon village, so, the Bibi goat owners were more aware and keener to drench their flocks. In Bibi village goats, the resistance developed in flocks of those goat owners who lived on a hill-top away from other owners. Therefore the chances of prevalence of refugia on the pasture after drenching were lower and the selection pressure for resistance probably increased. In Wadgaon village, generally the flocks grazed together or the goats were taken to different fields where the owners were working. This probably led to the presence of refugia in greater numbers and saved their flocks from developing drug resistance. Studies have shown that usually 20-30% of the population in a flock is affected with heavy worm infection but mass administration is carried out in farmers' flocks which greatly reduces the refugia population and also increases the unnecessary expenditure on worm management (Swarnakar *et al.*, 2010). Emerging resistance to Benzimidazole class in nematodes was found in sheep and goats of organized Wadjal farm of NARI and in some smallholders' goat flocks of Bibi village of Phaltan. This is possibly the first anthelmintic resistance report in sheep and goats from Maharashtra.

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References

Coles, G.C., Bauer, C., Borgsteede, F.H.M., Geerts, S., Klei, T.R., Taylor, M.A. and Walter, P.J., 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.

Denwood, M.J., 2010. A quantitative approach to improving the analysis of faecal worm egg count data. Ph.D. Thesis, University of Glasgow, U.K.

Easwaran, C., Harikrishnan, T.J., and Raman, M., 2009. Multiple anthelmintic resistance in gastrointestinal nematodes of sheep in Southern India. Vet. Arhiv., 79: 611-620.

Howell, S.B., Burke, J.M., Miller, J.E., Terrill, T.H., Valencia, E., Williams, M.J., Williamson, L.H., Zajac, A.M. and Kaplan, R.M., 2008. Prevalence of anthelmintic resistance on sheep and goat farms in the Southeastern United States. J. Am. Vet. Med. Assoc., 233: 1913-1919.

Kemper, K., 2003. Detection and Management of Anthelmintic Resistance at Kirby Research Station. Thesis, University of New England, Armidale NSW, Australia.

Kumar, R. and Yadav C.L., 1994. Prevalence of Fenbendazole resistance in ovine nematodes in North West India. Trop. Anim. Hlth. Prod., 26: 230-234.

Laha, R., Hemaprasanth and Harbola, P.C. 1999. Anthelmintic Resistance in Pashmina (Cashmere) producing goats in India. Vet. Res. Comm., 23: 187-189.

Le Jambre, L.F., Knox, M.R. and Gray, G.D. 1996. Sustainable parasite control in small ruminants. Introduction. In Proceedings of the International workshop Bogor, Indonesia, ACIAR Proceedings No. 74: 3-4.

Maharishi, A.K., Swarnakar, C.P., Singh, D., Manohar, G.S. and Ayub, M., 2011. Status of anthelmintic resistance in gastrointestinal nematodes of sheep in Rajasthan. Indian J. of Anim. Sci., 81: 105-109.

Ministry of Agriculture, Fisheries and Food (MAFF). 1986. Manual of veterinary parasitological laboratory techniques. Reference book 418. Her Majesty's Stationery Office, pp. 160.

Nari, A., 2005. The FAMACHA© Assay as a component of integrated pest management. Integrated animal parasite management: From academic interest to reality. FAO symposium. Alongside XVI National Congress of Veterinary Parasitology. Anjora, Durg, Chhattisgarh., pp 95-101.

Nipane, S.F., Mishra, B. and Panchbuddhe, A.N., 2008. Anthelmintic resistance -clinician's present concern. Vet. World., Vol 1: 281-284. www.scopemed.org/mnstemps/2/2-1295628818.pdf

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.

Singh, D. and Swarnakar, C.P., 2008. Role of refugia in management of anthelmintic resistance in nematodes of small ruminants- A review. Indian J. Small Rumi., 14: 141-180.

Singh, S. and Yadav, C.L., 1997. A survey of anthelmintic resistance by nematodes on three sheep and two goat farms in Hisar (India). Vet. Res. Comm., 21: 447-451.

Soulsby, E.J.L., 1982. Helminths, Arthropods and Protozoa of Domesticated Animals. 7th Edition, Bailliere Tindall (ELBS) pp. 809.

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

Swarnakar, C.P., Singh, D. and Solanki, V.K., 2010. Prevalence of Gastrointestinal parasites of sheep in semi-arid Rajasthan: A field study. Indian J. Small Rumi., 16: 221-227.

Taylor, M.A., Learmount, J., Lunn, E., Morgan, C. and Craig, B.H., 2009. Multiple resistance to anthelmintics in sheep nematodes and comparison of methods used for their detection. Small Rumi. Res., 86: 67-70

Tiwari, J., Kolte, A.P., Kumar, S., Swarnkar, C.P., Singh, D., and Pathak, K.M.L., 2007. Comparative efficacy of AS-PCR and RFLP-PCR techniques for detection of benzimidazole resistance in *Haemonchus contortus* of sheep. J. Vet. Parasitol., 21: 101-104.

Waller, P.J., 1987. Anthelmintic resistance and the future for roundworm control. Vet. Parasitol., 25: 177-191.