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Efficacy of closantel against benzimidazole resistant *Haemonchus contortus* infection in sheep

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ABSTRACT

Benzimidazoles are widely used and readily available ovine anthelminthics across the country. However, widespread resistance to this drug class has been documented, primarily in *Haemonchus spp*. The present study was conducted to determine the efficacy of closantel against benzimidazole resistant *Haemonchus contortus* infection in sheep. Naturally infected sheep (n=34) were divided into four groups on the basis of fecal egg counts (FEC) using modified McMaster technique with a lower detection limit of 8.3 eggs per gram of faeces (EPG) *viz*. Group-A (500-10000), Group- B (>10,000-20,000), Group-C (>20,000-30,000) and Group-D (>30,000). Generic differentiation of larvae was carried out by coproculture performed on pooled faeces which showed the presence of *Haemonchus*, *Trichostrongylus*, *Oesophagostomum* and *Strongyloides* larvae. To ascertain the evidence of benzimidazole resistance, Egg Hatch Assay (EHA) was performed. All animals were treated with Closantel @10mg\kg body weight and EPG was determined on 7th and 11th day post treatment. Animals of different groups had mean EPG range of 400-760 at 11th day of treatment. In group A, B, C and D mean per cent efficacy of closantel was 91.24±3.49, 95.15±1.72, 97.73±0.72, 98.23±0.86 respectively. Efficacy of closantel against *Haemonchus* and other gastrointestinal nematodes was further confirmed by performing coproculture 11th day post treatment. Post treatment coproculture revealed presence of *Trichostrongylus*, *Oesophagostomum* and *Strongyloides* larvae and were devoid of *Haemonchus* larvae. To clear the left out infection of *Trichostrongylus*, *Oesophagostomum* and *Strongyloides* animals were further treated with Fenbendazole @5mg/kg body weight and EPG 14 days post treatment became zero. The results of the study suggested that closantel can be used for Targeted Selective Treatment (TST) in sheep primarily infected with *Haemonchus*. Since closantel is highly efficacious against *Haemonchus* its use as an alternative to benzimidazoles group may be helpful to decrease pasture contamination. Overall control of gastrointestinal nematodiasis may therefore be possible by use of closantel along with benzimidazoles.

Key words: Benzimidazole resistance, Closantel, Efficacy, Fenbendazole, Gastro-intestinal nematodes, Sheep.

INTRODUCTION

In India, sheep and goat farming is the main source of income to small and marginal farmers. Of about 37% of the total world's sheep population and 56% of the goat population are bred and reared in Asian countries (FAO, 2015). Parasitic gastroenteritis accounts for heavy production loss in the small ruminant industry. Infestation with these endoparasites is a severe threat to veterinary health with helminthiasis at its top (Hotez *et al.,* 2016). Reduction in growth rates of up to 1/3rd is reported due to infestation with helminth parasites (Faizal and Rajapakse, 2001). Among all gastrointestinal nematodes that infect sheep, Family- *Trichostrongylidea* is predominant with *Haemonchus* and *Trichostrongylus* as major contributors to infection in animals (Rey, 1991). Infection with *Haemonchus contortus* is very severe (Santos *et al.,* 2012) throughout the year with high increase in faecal egg count (FEC) in summer season (Leathwick and Besier, 2014). As a result, control of *H. contortus* is essential for ensuring both animal welfare and reducing economic losses.

Various control measures have been employed against these GI parasites which include grazing/pasture management, chemotherapy, immunoprophylaxis etc. Among these the most common and widely employed method under field conditions is chemotherapy. For decades, the application of broad spectrum anthelmintic has remained as primary strategy for their control. However, resistance to these anthelmintics continues to be documented in nematode populations worldwide, including India (Singh *et al.,* 2002). Widespread resistance is present against ivermectin and fenbendazole, the most commonly available drugs against gastrointestinal nematodes (Falzon *et al.,* 2013). Furthermore among this, most of the resistance was found in *Haemonchus contortus*. Development of resistant strains of *Haemonchus contortus* has been

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reported in Netherlands (Van den Brom *et al.,* 2015) where the most recently introduced amino-acetonitrile derivative (monepantel) had been used. Furthermore, reduced efficacy of the spiroindole/ML combination (derquantel/ abamectin) has been reported in Australia recently by Sales and Love (2016); (Mahieu *et al.,* 2014).

Closantel, a salicylanilide drug specifically targets haematophagus parasite such as *Haemonchus sp*. It acts by decreasing the energy level of the parasite by uncoupling oxidative phosphorylation hence deprives availability of ATP and nicotinamide adenine dinucleotide in the mitochondria (Lanusse *et al.,* 2009).

Hence, following study was carried out with the objective of determining the efficacy of closantel against GI parasites, especially *Haemonchus sp*. Moreover it was also intended to determine whether resistance was prevalent in *Haemonchus* population only or other gastrointestinal parasites too.

MATERIALS AND METHODS

Location of animals

Naturally infected sheep (n=34), with average age of 5-7 months were used. Animals were raised on seasonal green pastures with ad-libitum drinking water. After procurement animals were kept in semi intensive loose housing system in Division of Parasitology, Indian Veterinary Research Institute, Izatnagar, Uttar Pradesh, India (28°23'34.8°N 79°25'59.9°E). Flock was not treated with any anthelmintic expect for benzimidazoles, earlier in their life.

Collection of faecal samples and concentration of eggs

Faecal samples were collected from each sheep on day zero (pretreatment) and again on days 7 and 11 post closantel treatment and day 14 for fenbendazole. Samples were collected per rectally and were stored in air tight sealable plastic bags. Samples were kept in ice packs and were shifted to 4°C refrigeration in Lab until further analysis. Qualitative assessment for the presence of parasitic eggs/ ova was done by flotation method using saturated salt solution, within 48 hours.

Eggs per gram (EPG) of faeces by McMaster method

FEC is efficient and a cost effective method to determine parasitic burdens. Faeces were subjected to modified McMaster technique (Zajac and Conboy, 2012) and the FEC value is presented as eggs per gram (EPG). Based on the pre-treatment EPG, animals were divided into four groups: A, B, C and D.

Coproculture of faeces

Generic differentiation of larval types was done using Coproculture. Fresh faeces from the entire sheep flock were collected and pooled. Inverted petridish method was used along with humid conditions at 27°C for 7 days. Harvesting of L_3 larvae was done 7 days post incubation. Generic compositions of harvested larvae were determined as per standard keys (VanWyk and Mayhew, 2013).

Determination of benzimidazole resistance by Egg hatch assay (EHA)

EHA was performed as per standard protocol described by Zhu *et al.* (2013). Eggs were exposed to different concentrations of albendazole 0.1 µg/ml, 0.3 µg/ml, 0.5 µg/ml, 0.7 µg/ml, 1.0 µg/ml) and inhibition of hatching of larvae was determined. 0.1 µg/ml concentration of albendazole was used as discriminating dose (Coles *et al.,* 1992). In the assay, 100 µl of egg suspension containing about 100-150 fresh eggs were dispersed into individual wells of flat bottom cell culture plates. Entire volume was made up to 500 µl with distilled water. The egg suspension in each treated well was mixed with stock albendazole to obtain the final concentration of 0.1 µg/ml, 0.3 µg/ml, 0.5 µg/ml, 0.7 µg/ml, 1.0 µg/ml. Untreated eggs in distilled water served as negative control and 1% DMSO used as emulsifier as solvent control. Plates were incubated at 27°C for 48 hours under humidified conditions. A total of hundred eggs and hatched larvae were counted under 10X of microscope. The efficacy was determined by the following equation:

$$\text{Inhibition \%} = \frac{\text{Number of unhatched eggs}}{\text{Number of hatched larvae} + \text{Number of unhatched eggs}} \times 100$$

Anthelmintic treatment

Treatment-1- Closantel (ZenVet™) was given orally @10mg/ kg body to sheep having mixed infection with *Haemonchus sp*, *Trichostrongylus sp* and *Oesophagostomum sp.* as made evident by results of coproculture.
Treatment-2- Fenbendazole was given orally @5mg/kg body weight on 25th day of experiment.

Faecal samples were collected per rectally on 7th and 11th day post treatment -1 (T1) and 14th days post treatment-2 (T2) for FEC estimation. Efficacy of the drug was determined by using formula:

$$\text{FECRT\%} = \frac{\text{(Arithmetic mean of pre-treatment EPG – Arithmetic mean of post treatment EPG)}}{\text{Arithmetic mean of pre-treatment EPG}} \times 100$$

(Dash *et al*., 1988).

RESULTS AND DISCUSSION

Haemonchus contrortus, a blood feeding ovine nematode is responsible for hyper acute outbreaks with FEC ranging up to >30,000 (Selemon, 2018). In the present study, based up on FEC severity, grouping of animals was done; *viz* Group-A (500-10000), Group- B (>10,000-20,000), Group-C (>20,000-30,000) and Group-D (>30,000) (Table 1). A wide variation in the range from 500-30,000 was observed. Such a high FEC ≥30,000 has been earlier reported in infection with *H. contortus* (Besier *et al.,* 2016).

Morphometric identification of the larvae through coproculture to ascertain the larval types present before

Table 1: Mean FEC with FECR of various groups.

Parameters	Group A	Group B	Group C	Group D
Number of sheep	8	12	9	5
Mean FEC on Day 0	500-10,000	>10,000-20,000	>20,000-30,000	>30,000
Mean FEC 7 days post closantel treatment	0-1900	100-1700	0-2100	1000-2300
Mean FEC 11 days post closantel treatment	0-1100	0-3400	100-1800	100-2000
FECR, 7 days post closantel treatment	77.87 ± 10.43	94.37 ± 0.88	96.45 ± 1.11	97.68 ± 0.87
FECR, 11 days post closantel treatment	91.24 ± 3.49	95.15 ± 1.72	97.73 ± 0.72	98.23 ± 0.86
Resistance status of *H. contortous*	Susceptible	Susceptible	Susceptible	Susceptible
Mean FEC 14 days post fenbendazole treatment	0	0	0	0
FECR, 14 days post fenbendazole treatment	100	100	100	100

Table 2: Generic composition of different larvae as determined by coproculture.

Anthelmintic treatment	Generic composition of larvae		
	Haemon -chus	*Oesopha -gostomum*	*Trichost rongylus*
Pre treatment	+++	+	++
Closantel @ 10mg/kg B.W	Nil	+	++
Fenbendazole @5mg/kg B.W	Nil	Nil	Nil

(+++ = >90%; ++ = 5-7%; + = 1-3%)

Table 3: Egg hatch assay (EHA) for determination of benzimidazole resistance.

Albendazole concen -tration (µg/ml)	Inhibition of L_1 hatching (%)	Hatching of L_1 (%)
0.1	36	64
0.3	52.4	47.6
0.5	59.4	40.6
0.7	64.9	35.1
1.0	70.1	29.1

undergoing treatment showed initial mixed infection with *Haemonchus spp., Oesophagostomum spp.*, *Trichostrongylus spp*. and *Strongyliodes spp*. with the predominance of *H. contortus*. Since majority of L_3 were of *H. contortus,* this led to the formulation of hypothesis for selection of an effective drug against the blood feeding *H. contortus*.

Simultaneously resistance to benzimidazole, a broader, widely available and most commonly used anthelmintic against gastrointestinal parasites, was checked by performing Egg Hatch Assay (EHA). The results of EHA are presented in (Table 3), which showed presence of resistance at different concentrations of albendazole. In the recent times, parasite resistant strains to benzimidazoles, macrolide lactone, monepantel, derquantel have emerged making the problem of resistance a global issue (Keane *et al.,* 2014; Cazajous *et al.,* 2018; Sales and Love (2016). Experimental design along with treatment regimen followed is presented in Fig 1 (Table 4).

Following the WAAVP guidelines which are based on FECRT, GIN infection was found susceptible to the combined treatment with closantel and albendazole (Alcalá Canto *et al.,* 2017). Therefore, treatment with a narrow spectrum drug like closantel (ZenVet™ Oral suspension, Intas Pharmaceutical Ltd. Ahmedabad, India) @10mg/kg. particularly aiming *H. contortus* was undertaken. FEC post 7 and 11 days of treatment were compared which lowered significantly with a mean value of 1100, 3400,1800, 2000 in Group A,B,C,D (Table 1). A slightly higher range of FEC was noticed in Group B when compared at Day 7 and Day 11 which may be due to some concurrent infection. Similar findings have been reported in the studies of certain workers (Westers *et al.,* 2016). Overall mean efficacy varied from 91.24% to 98.23% among different groups, after treatment. These results can be well correlated to similar observation made by previous workers (Sivajothi and Reddy, 2017), citing a 90.6% reduction in FECRT following closantel treatment in sheep.

Coproculture post closantel treatment to check reduction in infection due to *H. contortus* showed absence of *H. contortus* larvae with presence of *Oesophagostomum spp* and *Trichostrongylus spp*. Larvae were differentiated

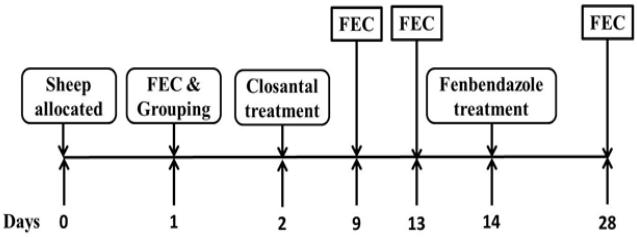

Fig 1: Experimental design.

Table 4: Treatment regimen followed.

Days	Treatment	Dose rate	Average body weight	Dose volume	Route
Day 0	-	-	18.7 kg	-	-
Day 1 post 1st FEC	Closantel (ZenVet™)	10 mg/kg Body weight	18.7 kg	5.5 ml	Oral
Day 12 post 1st FEC	Fenbendazole (Panacur®)	5 mg/ kg Body weight	20.2 kg	4 ml	Oral

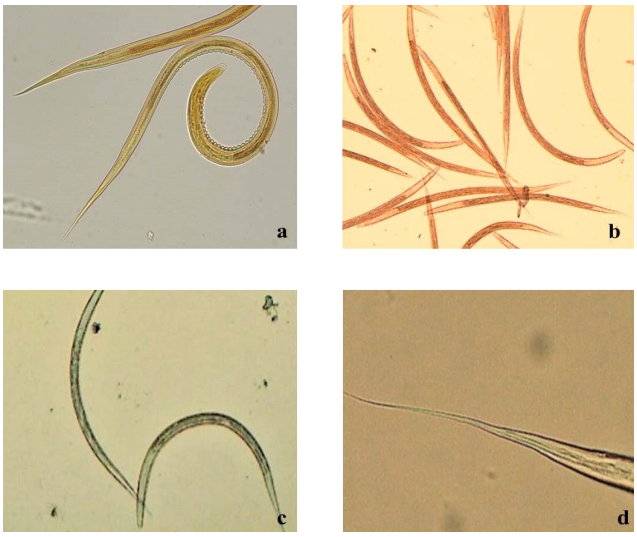

Fig 2: Morphometric larval examination through coproculture. (a) *Haemonchus* larvae in the coproculture (40X), (b) Predominance of *Haemonchus* in coproculture (40X), (c) Larvae of *Trichostrongylus spp.* showing pencil tail, (d) - Larvae of *Oesophagostomum spp.* with long filamentous tail.

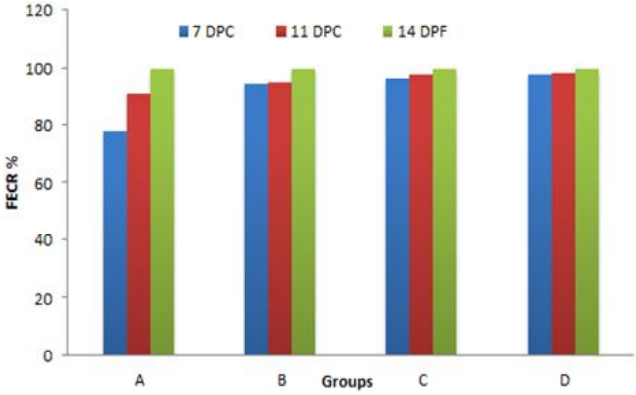

Fig 3: Percentage reductions in FEC with days.DPC: Number of days post closantel treatment; DPF: Number of days post fenbendazole treatment.

up to generic level based on morphological characteristics (Fig 2). Larval pattern during the entire study period is presented in (Table 2). To clear up the residual infection, treatment with Fenbendazole (Panacur® Vet Suspension, MSD Animal Health, Mumbai, India) @ 5mg/kg, orally was done and FEC after 14 days was determined. Post fenbendazole treatment, no parasitic eggs could be seen with 100% reduction in FEC. The combined overall efficacy of both the drugs was 100% (Fig 3). Combination of different anthelmintic classes continues to be the best approach for the efficient control of GIN as the survival of resistant genotypes is minimized (Leathwick *et al.,* 2015).

Evidently, the strain of *H. contortus* present in Indian sheep from Northern region was found susceptible to closantel with resistance to Benzimidazole group. Considering above fact, it is observed that closantel may be used to clear up very heavy infection due to *Haemonchus* in case of reported benzimidazole resistance. It is further suggested that we can use closantel in the areas where *Haemonchus* is endemic in the view of its residual effect (Anonymous, 1990). Moreover, prolonged activity of this salicylanilide has an added advantage in lowering pasture contamination, hence achieving better control (Dash, 1986). Closantel has efficacy against nematodes, trematodes and arthropods adding to its advantage for use in sheep (Maes *et al.,* 1988).

It should be kept in mind that frequent use of closantel may lead to its resistance and such an efficacious anthelmintic against *Haemonchus* may be lost in future due to indiscriminate use. Thus, to decrease the selection pressure that leads to the development of anthelmintic resistance in the animal, approach to control of nematode in the flocks must be based on diagnosis through fecal examinations, epidemiological studies and prophylaxis via nutritional strategies and targeted selective treatments (Torres Acosta *et al.,* 2012).

CONCLUSION

Results from the present study showed that closantel was highly effective in eliminating infection with *Haemonchus contrortus.* Therefore, in animals with mixed gastrointestinal nematodes infection, not showing response to benzimidazole treatment, infection with *Haemonchus spp.* should be confirmed and further treatment with closantel should be taken up. However, to slow down the development of anthelmintic resistance among animals, closantel should only be considered in the farms where heavy *Haemonchus* burdens are reported with documented resistance to benzimidazoles. The drug can be incorporated as a part of target strategic treatment for improved results.

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