EVALUATION OF ALBENDAZOLE AGAINST SHEEP STRONGYLES IN FARMS OF KARNATAKA STATE BY DIFFERENT IN VITRO TESTS

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ABSTRACT

Faecal samples of 264 sheep from 4 different sheep farms belonging to three different districts of Karnataka were screened to note the incidence of gastrointestinal nematodes. It was found that 93% of the sheep harboured strongyle infection. The faecal egg counts were found to be light to moderate. The in vitro egg hatch assay was employed to assess the resistance of strongyles in 4 sheep farms. The ED50 value for albendazole ranged between 2.5µg/ml to 6.9 µg/ml which indicated the resistance of the gastrointestinal nematodes. All the samples were also subjected to another in vitro test, viz., larval development assay. The values ranged between 3-2µg to 4.2µg / ml which also indicated the development of resistance to albendazole. Larval paralysis assay confirmed the development of resistance to albendazole.

Key words : In vitro tests, albendazole, strongylosis, efficacy, resistance

INTRODUCTION

Strongylid and trichostrongylid infections such as haemonchosis, bunostomosis, trichostrongylosis, oesophagostomosis and cooperiosis are the most important disease entities responsible collectively for parasitic gastroenteritis causing considerable morbidity and mortality in sheep.

The use of anthelmintics in India is mainly tactical based on season or expression of disease. High frequency of treatment with improper dosage in conjunction with administration of the same chemical group to young & adult animals and longer persistence at low level of the therapeutic activity of the compound prompt for the development of anthelmintic resistance.

The development of anthelmintic resistance in parasite populations continues to increase in small ruminants in most part of the world and places a serious limitation on the use of available anthelmintics.

The problem becomes even more severe if new generation anthelmintics with unique mode of action are not developed and replaced. It is therefore necessary to know the status of efficacy of a particular commonly used
anthelmintic. Some of the in vitro methods such as egg hatch assay and larval paralysis assay are known to be specific for a particular class of anthelmintic whereas larval development assay can be used with most anthelmintics. Therefore a study was undertaken to observe the efficacy of albendazole a commonly used anthelmintic in organised farms.

MATERIALS AND METHODS

Four farms located in different parts of Karnataka viz., KVAFSU sheep farm, Hebbal, Bangalore; Livestock Research and Training Center (LRIC), Nagamangala; Sheep breeding and training centers at Kudapura and Dhanagur were included in the study. It was ensured that sheep were not dosed with anthelmintics for the past 8-12 weeks. The faecal samples of sheep from these farms were collected and screened and the eggs per gram (EPG) was determined by modified McMaster method as per Coles et al. (1992).

Egg hatch test : The procedure as per Coles et al. (1992) was followed with minor modifications. The faecal samples were stored anaerobically and brought to the laboratory from the farms, since the eggs must be used within 3 hours of being shed from sheep as the sensitivity to benzimidazole decreases when embryonation proceeds.

The samples were taken in a pestle and homogenized after adding water by using a mortar. The homogenized material was poured through a sieve into a bowl. The filtrate was taken in several 15ml centrifuge tubes and centrifuged at 2000 rpm for 3 min. The supernatant from all the tubes was aspirated and the tubes were agitated to loosen the sediment. Then saturated solution of sodium chloride was added to all the tubes. The tubes were then centrifuged at 2000 rpm for 2mins. Tubes were kept in a stand and left undisturbed for 3 mins. One ml of supernatant from all the tubes was transferred into new test tubes and 13ml of tap water was added and centrifuged. Then 0.5ml of sediment from all test tubes was collected in a new tube and centrifuged. The concentration of eggs was adjusted to 100 per ml by adding or removing water from the egg suspension.

Preparation of drug dilutions : Pure form of albendazole was procured (PETCARE, Bangalore) and 50mg of albendazole was dissolved in 50ml of DMSO which gave working albendazole solution with the concentration of 1000 ppm. Working solution was diluted with distilled water to get different drug dilutions 0.05, 0.1, 0.2, 0.5, 1, 5, 10, 20 and 40 µg per ml.

Egg hatch test procedure :100 µl of egg suspension was added to all the wells of 24 multi well plate. 10 µl (micro litre) of albendazole solution was added to the wells and to the control well only 10 µl of DMSO was added. Each concentration of albendazole was added to 3 wells (triplicates) and 1.89 ml of distilled water was added to all the wells. The plate was kept in an incubator at 26°C for 48hrs. After 48 hours 3 drops of Lugol’s iodine was added to all the wells to arrest the further growth of eggs and hatching of larva. All the wells were examined under stereozoom microscope and number of unhatched eggs and L₁ were counted and recorded.

Larval development assay : The procedure described by Hubert and Kerboef (1992) was followed with some minor modifications. Eggs for the test were collected as detailed in the egg hatch test and 50 µl of amphotericin B was added to 5ml of the egg suspension (100 eggs
in 100µl by adding or removing water from the egg suspension) and thoroughly mixed using a micropipette. Then 100µl of egg suspension was added to each well of a 24 well multiwell plate and 20µl of nutritive media {Composition 1 g of yeast extract (HiMedia) was diluted in 90ml of saline solution. To the 27 ml of this solution 3 ml of Earle’s balanced salt solution (HiMedia) was added. The medium was sterilized just before the use} and 20 µl of lyophilysed E.coli were added to each well and the plate was kept for incubation at 22°C for 48 hours. After 48 hours different concentration of albendazole as prepared for the egg hatch assay was added to the wells. Each concentration was added to 3 wells to avoid errors. Then the plate was kept for incubation at 22°C for 6 days. After 6 days three drops of Lugol’s iodine was added to stop further development of larvae. Then the number of eggs and different stages of larvae were counted by using a stereozoom microscope.

Larval paralysis test : It is the test of choice of nematode anthelmintic resistance. This test was based on the principle that higher levels of acetylcholine esterase will be present in the benzimidazole resistant strains than in the susceptible strains of trichostrongyles. When infective larvae were incubated in serine (acetyl choline esterase inhibitor) paralysis occurs in larvae treated with serine but significant difference was noticed in the percentage of larvae immobilized between benzimidazole resistant and benzimidazole susceptible strains. The procedure described by Sutherland and Lee (1990) was followed with some minor modifications.

The eggs were incubated in 24 well multiwell plate with nutritive medium and lyophilized E.coli culture for 8 days to obtain 3rd stage larvae. On 8th day, 3rd stage larvae were recovered from the wells by using micropipettes. To conduct the test 25-30 infective stage larvae were pipetted into adjacent wells of a 24 multiwell plate. Excess water was removed with a pipette. Then 1ml of a freshly prepared solution of 1mM eserine (sigma) was added to each of the wells. The wells were examined under a low power dissecting microscope and counts of immobile/ mobile larvae were taken at different time intervals (15mins, 30mins, 45mins and 60mins) at room temperature. Each well was observed for approximately 30seconds and the experiment was repeated three times. The numbers of larvae paralysed were counted from the wells at each time interval and expressed as a percentage of the total sample numbers.

RESULTS AND DISCUSSION

In the faecal samples of sheep from all the four farms strongyle infection was found to be most prevalent. The details are depicted in Table 1. The samples were then subjected for coproculture to identify the different species of strongyles, which revealed the presence of Haemonchus sp., Trichostrongylus sp., Oesophagostomum sp. and Cooperia sp. as detailed in Table. 2.

The Egg hatch test was conducted on samples from all the four farms under study. The mean values (number of larvae per number of strongyle eggs in culture) were 87.34, 88.07, 86 and 88.4 per cent in Hebbal, Dhanagur, Kudapura and Nagamangala farms respectively. No significant difference in egg or larval development was observed between the samples from four different farms under study.

The drug albendazole showed a linear relationship between probit values of egg
hatching and the logarithm of the dose rate of albendazole in all four farms (Fig 1-4). LC50 values in microgram per ml obtained for albendazole by egg hatch were 6.9µg/ml, 2.5µg/ml, 3.1µg/ml and 6.6 µg/ml respectively in KVAFSU, Dhanagur, Nagamangala and Kudapura farms respectively. LC50 values more than 0.1 µg/ml (Coles et al., 1992) indicated that the worm population was resistant to the albendazole. In the present study LC50 values in all the farms were higher than 0.1 µg/ml which confirmed the development of resistance to albendazole in all the farms.

Egg hatch test has shown resistance in the present study other two tests like Larval development test & larval paralysis test were done to know the comparision between different in vitro tests. The larval development test was performed on strongyle eggs from all the farms under study. The mean larval development (number of L3 per number of strongyle eggs in culture) was 86.2, 85.1, 81.5 and 84.3 in KVAFSU, Dhanagur, Kudapura and Nagamangala respectively. No significant difference in egg or larval development was observed between the samples from four different farms under study. A linear relationship between probit values of larval development and the logarithm of the dose rate of albendazole was observed (Fig 5-8). LC50 values in microgram per ml obtained for albendazole by means of larval development were 3.5µg/ml, 3.2µg/ml, 3.9µg/ml and 4.2 µg/ml respectively in KVAFSU, Dhanagur, Nagamangala and Kudapura farms respectively. LC50 values more than 0.1 µg/ml (Hubert and Kerboef, 1992) indicated that the worm population was resistant to the drug. The LC50 values in all the farms were higher than 0.1 µg/ml which confirmed the development of resistance to albendazole in all the farms.

The larval paralysis test was performed on strongyle larvae obtained by culturing of eggs from all the four farms under study. The percentage of infective stage larvae immobile in 1mM serine with 15 min time interval upto 60 minutes is represented in the Table 3. Although there was a significant difference in the percentage of immobile larvae between the four farms, all the values indicated the existence of resistance (Table 3).

Katoch et al., (1997) reported the predominance of Haemonchus followed by Strongyloides and Trichostrongylus spp in Himachal Pradesh. Mixed infection of Nematodirus, Chabertia and H. contortus from Karnataka were reported by Abdul Rehman and Krishna Rao (1969).

In this study a mixed infection of Strongyle along with Eimeria and Moniezia sps was found to be 28 per cent and 25 per cent respectively whereas 15 per cent animals showed only Eimeria infection. Bali (1973) reported a mixed infection of Strongyle (25 percent) with Moneizia expansa (6.8 percent) and with Moniezia benedini (2.7%)

In the present study the faecal samples from different sheep farms were screened from the month of February to June (summer and rainy season) and the EPG count was found to be light to moderate in three farms and heavy in Dhanagur farm which was similar to the finding of Mishra et al., (1974) who reported that the intensity of strongyle infection was highest in rainy season and lowest in summer season. Similarly Vasudevan and Basuthakur (1986) opined that the rainfall was prime limiting factor for strongyle infection.
Egg hatch test: The in vitro techniques may be applied in surveys of field population of unknown resistant status or as a confirmatory diagnostic test in situations where anthelmintic treatment failed to reduce the egg count. The egg hatch assay has been used for field screening of benzimidazole resistance in sheep nematodes as per Hong et al. (1992) and was recommended by the World Association for the Advancement of Veterinary Parasitology as the most reliable in vitro test for detection of benzimidazole resistance.

LC$_{50}$ values for albendazole ranged from 2.5µg per ml to 6.3µg albendazole per ml in 4 different farms by egg hatch assay which confirmed the existence of resistance as the value was more than 0.1 µg per ml. Coles et al., 1992 reported for the first time that eggs with ED$_{50}$ values in excess of 0.1 µg per ml was indicative of benzimidazole resistance. Dhanalakshmi et al., (2000) conducted EHT in different sheep farms in Karnataka and found ED50 values ranging from 3.02 to 6.3µg albendazole/ml. Arunachalam et al.,(2005) reported an ED 50 values of 0.586 µg per ml confirming the existence of benzimidazole resistance in an organised farm in Tamil nadu. All these observations are in agreement with the present study.

In the present study, the anaerobic storage of eggs had helped to prevent the embryonation of eggs. This overcame the need for fresh eggs for assessment of benzimidazole resistance by egg hatch assay. Hunt and Taylor (1989) demonstrated that the anaerobic storage of nematode eggs upto seven days had no significant effect on ED50 values when compared with the values of fresh eggs. Coles et al., (1992) opined that anaerobic storage of eggs could overcome the need for fresh eggs for egg hatch test, as sensitivity to thiabendazole decreases as embryonation proceeded.

Larval development test: In the present study the larval development in the control wells of all the four farms was more than 84 %. The rate of development of third stage larvae in control wells is similar to the findings of Hubert and Kerboef (1992) in which they obtained 85% of L3 in control wells.

Obendorf et al., (1986) observed fungal growth after 48 hours of incubation which stopped further larval development and also gave an opinion that amphotericin B could prevent impairment of larval development by the developing mycelium. In the present study 50 µg amphotericin B per ml prevented fungal growth which allowed the larval growth and they were alive in control wells even on tenth day of incubation.

In the present study the minimum inhibitory concentration values for albendazole required to inhibit the development of third stage larvae of strongyles ranged from 3.2µg per ml to 4.2 µg of albendazole per ml in four different farms under study. The results of current study correlated with observations of Coles (1988), Taylor (1990), Hubert and Kerboef (1992) and Varady and Corba (1999) who reported the MIC values as 0.43, 0.5, 0.24 and 1.43 µg per ml.

Larval paralysis test: It was observed that there was no significant difference in between the percentage of larvae from different farms paralysed at different time intervals viz., 15, 30 and 45mins. But there was significant difference in the per cent of larva paralysed after 60 minutes between samples from Dhanagur, Nagamangala, K.V.A.F.S.U and Chitradurga. Though difference in resistance was observed in all the farms under study.
Sutherland and Lee (1990) observed significant differences in the paralysis between resistant and susceptible strain after 15 mins. In the present study, L3 larvae of mixed infection with more than 70% of *Haemonchus contortus* population was assayed. The lower significance of paralysis between resistant and susceptible population as observed during the early 15 min could be due to differential uptake of serine by resistant and susceptible population as opined by Sutherland and Lee (1990).

The results of the present study also confirms the reports of Sutherland and Lee (1990), who opined that this bioassay offered rapid diagnosis for presence of resistance to thiabendazole and enabled their detection of resistance in larvae for a particular species, in samples.

**Table 1. Results of faecal samples screened from different sheep farms.**

<table>
<thead>
<tr>
<th>Name of the farm</th>
<th>No. of animals screened</th>
<th>No. Of Positive* cases</th>
<th>*Strongyle eggs</th>
<th>*Eimeria oocyst</th>
<th>*Moniezia indicum</th>
<th>Schistosoma</th>
<th>*Trichuris</th>
</tr>
</thead>
<tbody>
<tr>
<td>KVAFSU farm Hebbal</td>
<td>69</td>
<td>69</td>
<td>61</td>
<td>43</td>
<td>6</td>
<td>13</td>
<td>12</td>
</tr>
<tr>
<td>SBTC- Dhanagur</td>
<td>67</td>
<td>67</td>
<td>67</td>
<td>53</td>
<td>2</td>
<td>-</td>
<td>5</td>
</tr>
<tr>
<td>SBTC – Kudapura</td>
<td>63</td>
<td>59</td>
<td>57</td>
<td>15</td>
<td>2</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>LRIC - Nagamangala</td>
<td>65</td>
<td>60</td>
<td>60</td>
<td>20</td>
<td>4</td>
<td>-</td>
<td>-</td>
</tr>
</tbody>
</table>

*No of animals positive for gastrointestinal parasitic infections

**Table 2: Species of larvae in per cent obtained in different farms by copro culture**

<table>
<thead>
<tr>
<th>Species of larvae</th>
<th>KVAFSU farm Hebbal</th>
<th>SBTC - Dhanagur</th>
<th>L.R.I.C- Nagamangala</th>
<th>SBTC - Kudapura</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>Haemonchus</em></td>
<td>79%</td>
<td>62%</td>
<td>69%</td>
<td>73%</td>
</tr>
<tr>
<td><em>Trichostrongylus</em></td>
<td>12%</td>
<td>23%</td>
<td>20%</td>
<td>15%</td>
</tr>
<tr>
<td><em>Oesophagostomum</em></td>
<td>7%</td>
<td>12%</td>
<td>9%</td>
<td>12%</td>
</tr>
<tr>
<td><em>Cooperia</em></td>
<td>2%</td>
<td>3%</td>
<td>2%</td>
<td>0%</td>
</tr>
</tbody>
</table>
Table 3: Percentage of infective stage larvae immobile or paralysed in 1mM eserine (Physostigmine) with different time intervals.

<table>
<thead>
<tr>
<th>FARM NAME</th>
<th>Per cent paralysis at different time interval</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>15 min</td>
</tr>
<tr>
<td>KVAFSU sheep farm Hebbal</td>
<td>0</td>
</tr>
<tr>
<td>SBTC- Dhanagur</td>
<td>7.6</td>
</tr>
<tr>
<td>SBTC- Kudapura</td>
<td>6.45</td>
</tr>
<tr>
<td>LRIC Nagamangala</td>
<td>11.2</td>
</tr>
</tbody>
</table>

* indicates the existence of higher number of resistant worm population (Sutherland and Lee 1990)

** indicates the existence of less number of resistant worm population

Fig. 1: Log dose-probit lines indicating hatching percentage of fresh strongyle eggs from KVAFSU sheep farm, Hebbal, in solution of albendazole in egg hatch assay.

Fig. 2: Log dose-probit lines indicating hatching percentage of fresh strongyle eggs from SBTC-Dhanagur, in solution of albendazole in egg hatch assay.
Evaluation of albendazole against sheep strongyles in some farms of in vitro tests

**Fig. 3**

![Log dose-probit lines indicating hatching percentage of fresh strongyle eggs from L.R.I.C Nagamangala, in solution of albendazole in egg hatch assay.](image)

**Fig. 4**

![Log dose-probit lines indicating hatching percentage of fresh strongyle eggs from SBTC-Kudapura, in solution of albendazole in egg hatch assay.](image)

**Fig. 5**

![Log dose-probit lines indicating the percentage of live L3 obtained from strongyle eggs incubated from KVAFSU sheep farm, Hebbal, in solution of albendazole in larval development assay.](image)

**Fig. 6**

![Log dose-probit lines indicating the percentage of live L3 obtained from incubated strongyle eggs from SBTC-Dhanagur, in solution of albendazole in larval development assay.](image)
Fig. 7: Log dose-probit lines indicating the percentage of live L_3 obtained from incubated strongyle eggs from L.R.I.C. Nagamangala, in solution of albendazole in larval development assay.

Fig. 8: Log dose-probit lines indicating the percentage of live L_3 obtained from incubated strongyle eggs from SBTC-Kudapura, in solution of albendazole in larval development assay.

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REFERENCES


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