Detection of benzimidazole resistance against naturally occurring gastrointestinal nematodes in different sheep breeds of Odisha

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Abstract
Gastrointestinal nematodes are a major hindrance in sheep husbandry and the efficient management of these parasites is curbed by the development of anthelmintic resistance. The present investigation was carried in different sheep breeds of Odisha and the status of resistance in gastrointestinal nematodes against Fenbendazole was carried out by in vivo faecal egg count reduction test (FECRT) and in vitro Egg Hatch Assay (EHA) test. The results of the anthelmintic resistance study indicated benzimidazole resistance to gastrointestinal nematodes in Kendrapada, Ganjam and Nondescript breed of sheep while Balangir breed of sheep where found susceptible.

Keywords: fenbendazole, gastrointestinal nematodes, sheep, anthelmintic resistance

Introduction
Sheep rearing is one of the oldest occupations adopted by livestock farmers in India as it is a source for providing protein nutrition to the family and also for earning additional income. In Odisha, there are three descript breeds of sheep native to state along with nondescript breeds. The recognized breeds of sheep in Odisha are, Balangir, Ganjam, and Kendrapada. Parasitism is an important global problem and still continue to seriously affect the livestock economy throughout the world. Parasitic gastroenteritis is caused by the gastrointestinal (GI) nematodes of ruminants and is characterized by diarrhoea, anorexia, lethargy, anaemia, sub-mandibular oedema and death particularly in young animals in severe cases (Sargison et al. 2002, Taylor et al. 2007) [19]. The use of anthelmintic drugs has been the common and most reliable practice since decades for effective control of gastro-intestinal nematodes in grazing animals including sheep. Since 1960’s benzimidazoles (BZ) are the most extensively used anthelmintics which were found most effective (>95%) for control of Strongyle nematode infection in sheep and goats (Dorny, et al., 1995). Benzimidazoles get preference for use due to their low cost, broad spectrum activities and high efficacy. But their indiscriminate use over years has led to the development of resistance in the parasites (Waller, 1994) [20]. The first report of occurrence of resistance to BZs (Thiabendazole) in H. contortus was in 1961 and as the time passed this problem became widespread across globe (Wolstenholme et al., 2004) [21]. For detection of BZ resistance, several techniques have been practiced i.e. in vivo and in vitro assays. The faecal egg count reduction test (FECRT), egg hatch assay (EHA) and larval development assay (LDA) have proven to be suitable tests for detecting BZ resistance (Coles et al. 2006) [2]. Studies on the status on Benzimidazole resistance in sheep has been reported by many researcher in different states of the country (Das and Singh, 2005; Rialch et al. 2013; Singh et al. 2015; Lata 2018) [3, 16, 17]. Report on detection of anthelmintic resistance in Odisha is meager (Sahu 2015, Nanda 2016) [14]. Baring these few reports no systematic investigation on the status of anthelmintic resistance among the prevalent population of Haemonchus contortus and other GI nematodes of sheep in Odisha in the native breeds of sheep to Haemonchus contortus has been undertaken till date. The present research was undertaken keeping in view the above facts.
Materials and Methods
The in vivo evaluation of anthelmintic resistance under field condition was done by Faecal egg count reduction test (FECRT) following the standard guidelines of W.A.A.V.P. Sheep belonging to different breeds in their respective native tracts which were detected naturally infected with GI nematodes were included in the study. Flocks which were not dewormed during last three months and found positive for GI nematode eggs with a minimum Egg per gram (EPG) of 150 and above were finally selected, marked with identification number on their fleece and divided into treated and control groups. Ten animals in each groups were selected from the Kendrapada, Ganjam, Balangir and non-descript, sheep breeds. The EPG Pretreatment was done before administration of anthelmintic (Fenbendazole @ 7.5 mg/kg body weight orally as a single dose) and EPG post treatment 10 days after drug administration. Per-rectally collected dung samples from sheep were kept separately in plastic containers with screw cap in an anaerobic condition without adding any preservative. The EPG count of both the treated and control groups were done by Mc. Master’s technique (Soulsby, 1982) [17]. The faecal egg count reduction percentages of sheep naturally infected with gastrointestinal nematodes were calculated by comparing the pre treatment and post treatment EPG.

\[
\text{FECR} \% = \left( \frac{(\text{EPG pre treatment} \times 100)}{\text{EPG pre treatment} - \text{EPG post treatment}} \right) \times 100
\]

A reduction in faecal egg count less than 95% as well as lower 95% confidence level below 90 was taken as criteria to indicate the presence of benzimidazole anthelmintic ‘resistant’ nematodes in the treated population (Coles et al., 1992) [11]. On 11th day the control group animals were also treated with the same drug to make them free from natural infection.

Egg Hatch Test (EHT)
Collection of Eggs
Freshly collected and anaerobic stored dung samples were homogenized using pestle and mortar. The homogenized dung sample was filtered, filtrate was centrifuged at 2000 rpm for 2 minutes and the sediment was cleaned with distilled water by centrifugation. Then saturated salt (NaCI) solution was mixed with the sediment and centrifuged. The supernatant solution containing eggs was collected in centrifuge tube and washed in distilled water for three times by centrifugation. Finally, the supernatant was discarded and the sediment was resuspended with distilled water. Finally, the number of eggs was estimated and diluted to 100-150 eggs/ml.

Preparation of Thiabendazole (TBZ) stock solution
A stock solution was prepared as described by Himmelstjerna et al. (2009) [5]. TBZ 50 mg powder was dissolved in 5 ml of dimethylsulphoxide (DMSO) in a test tube (Solution A). Then 1 ml from Solution A was added to 9 ml of DMSO (Solution B) having a concentration of 1 mg TBZ per ml. Different concentration of working solution were prepared.

The working dilution were made for 24 well culture plate. In each well, 1 ml of egg suspension were added to 10 µl of thiabendazole in different concentrations and mixed well. In control well, 10 µl of dimethylsulphoxide (DMSO) was added with 1 ml of egg suspension. The 24-well culture plate were placed in the incubator for 48 hours at 26°C. The incubation was terminated by adding 2- drops of Lugol’s iodine to each well and embryosynated eggs, unhatched eggs and hatched first stage larva from each well were counted and proportion of egg hatch was determined. The trial was conducted with two replicates and result was expressed as ED50 values.

\[
\text{EHA} \% = \frac{\text{No. of hatched larvae counted}}{\text{Total no of eggs and larvae}} \times 100
\]

Effective Dose ED50 (ED50) value was calculated for the eggs by log probit analysis. Eggs having ED50 value exceeding 0.1 µg BZ anthelmintic per ml was indicative of resistance against Benzimidazole (Coles et al. 2006) [2].

Statistical Analysis
A log probit model was used to estimate the ED50 of the concentration of the drug based on the hatching percentage in EHA by using SPSS 21.

Results and Discussion
The efficacy of fenbendazole was assessed by faecal egg count reduction test (FECRT) in different sheep breeds of Odisha naturally infected with GI nematode. Faecal egg count reduction was recorded based on pretreatment egg per gram (0-day) and post treatment egg per gram (on 10th day of oral administration of Fenbendazole @ 7.5mg/kg) and compared with the untreated control group. The efficacy of Fenbendazole in Kendrapada, Ganjam, Balangir and Nondescript breeds of sheep were found to be 93.55%, 91.08%, 97.10% and 90.98 % respectively (Table 1). The results reflected the resistance to gastrointestinal nematodes in Kendrapada, Ganjam and Nondescript breed of sheep, where efficacy were recorded lower (<90%) 95% confidence interval. Gastrointestinal nematodes in Balangir breed of sheep where recorded (>92%) 95% confidence interval was found susceptible.

Table 1: Efficacy of Benzimidazole (Fenbendazole) against naturally occurring gastrointestinal nematodes in different breeds of sheep

| Breed       | Pre-treatment EPG | Post-treatment EPG | FECR % | Confidence interval | Remarks   |
|-------------|-------------------|--------------------|--------|---------------------|-----------|
|             | EPG               | EPG                |        | Lower               | Higher    |          |
| Kendrapada  | 475 ± 71.59       | 30 ± 15.28         | 93.55% | 87                  | -         | Resistant|
| Ganjam      | 785 ± 52.73       | 70 ± 26.03         | 91.08% | 82                  | 99        | Resistant|
| Non descript| 610 ± 65.74       | 55 ± 26.30         | 90.98% | 86                  | -         | Resistant|
| Balangir    | 690 ± 70.91       | 20 ± 9.51          | 97.10% | 92                  | 99        | Susceptible|

The percentage of gastrointestinal nematode larval composition in pre and post treatment faecal samples (Table 2) reflected the dominance of Haemonchus sp over Trichostrongylus sp, Oesophagostomum sp and Strongyloides sp in Kendrapada, Ganjam and Nondescript breed of sheep. While in Balangir sheep, post treated faecal culture showed absence of larvae. However, post treatment faecal culture revealed absence of Strongyloides sp. in all sheep breeds. The above pattern of survived larvae and predominance of population of Haemonchus contortus among them revealed that there were existence of population of H. contortus which were resistant to Fenbendazole and thus to BZ.
Different in-vivo techniques have been employed for detection of anthelmintic resistance of which most widely used technique recommended by the WAAVP is Faecal Egg Count Reduction Test (FECRT). It is the most suitable method for field level diagnosis of anthelmintic resistance. Quantitative reduction in the faecal egg output post treatment in comparison to pre-treatment is the basis of detection of resistance. A population of worms is declared to be resistant if the percentage reduction is less than 95 % and lower limit of 95% confidence interval is below 90. If one of the two criteria is met, resistance is suspected (Coles et al., 1992) [1]. But FECRT has some limitations due to lack of its analytical sensitivity. It was demonstrated that the FECRT can detect BZ-resistance if the frequency of the resistance alleles is greater than 25% in the parasitic population under test. Therefore, FECRT is not a sensitive test to detect early emergence of anthelmintic resistance (Martin et al., 1989; Levecke et al. 2009) [10].

Egg Hatch Assay (EHT) was conducted using the method recommended by WAAVP and ED_{50} value (concentration of drug required to kill 50% of eggs) was calculated basing on egg hatch percentage. The results of EHT carried out with respect to different sheep breeds have been tabulated in Table 3. As per the recorded results of regression, Anthelmintic Resistance were found in the Kendrapada, Ganjam and Nondescript breed of sheep with 0.117102µg/ml, 0.168242µg/ml, and 0.232582µg/ml, ED_{50} value of Thiabendazole while Balangir sheep showed no evidence of anthelmintic resistance as the regression values recorded 0.041137µg/ml (the ED_{50} values were found less than 0.1 µg/ml).

### Table 2: Gastrointestinal larval population (%) in pre and post treatment faecal culture of different sheep breeds of Odisha

| Breeds      | Nematode larva          | Pre treatment (%) | Post treatment (%) | Control (%) |
|-------------|-------------------------|-------------------|--------------------|-------------|
| Kendrapada  | Haemonchus sp           | 53                | 75.5               | 54          |
|             | Oesophagostomum sp      | 19                | 9                  | 14.5        |
|             | Trichostrongylus sp     | 21                | 15.5               | 22          |
|             | Strongyloides sp        | 7                 | 0                  | 9.5         |
| Ganjam      | Haemonchus sp           | 55.67             | 81.0               | 56          |
|             | Oesophagostomum sp      | 15                | 8                  | 17          |
|             | Trichostrongylus sp     | 22.67             | 11.0               | 21          |
|             | Strongyloides sp        | 6.67              | 0                  | 6           |
| Balangir    | Haemonchus sp           | 60                | 0                  | 63.67       |
|             | Oesophagostomum sp      | 15.0              | 0                  | 11.67       |
|             | Trichostrongylus sp     | 17.33             | 0                  | 15.33       |
|             | Strongyloides sp        | 7.67              | 0                  | 9.33        |
| Non-descript| Haemonchus sp           | 65.30             | 85                 | 64.71       |
|             | Oesophagostomum sp      | 13.86             | 5.0                | 10.57       |
|             | Trichostrongylus sp     | 15.43             | 10                 | 18.4        |
|             | Strongyloides sp        | 5.41              | 0                  | 6.32        |

In increasing drug concentrations is determined. Therefore, this test is only suitable for detection of BZ resistance (Swarnakar and Singh, 2017) [18]. The detection of benzimidazole resistance by FECRT and EHT has been previously reported from three sheep farm in Tamil Nadu (Easwaran, et al. 2009) [4], four sheep farm in Karnataka (Kumar et al. 2014) [7] and sheep in unorganized sector in Haryana (Priyanka 2019) [12]. In the present study, while two describe breeds and non-describe breed showed resistance, Balangir breed showed susceptibility to fenbendazole anthelmintic. The higher efficacy of fenbendazole in the breed might be due to less number of treatments with the right dosage as well as good managemental practices.

The presence of anthelmintic resistance may be due to the selection of resistant genotypes within the parasitic population or reselection of resistant individuals already present in the population at a lower frequency. The use of anthelmintics increases the frequency of these resistant worms, so efforts should be aimed at reducing the further multiplication of these resistant worms.

### Conclusion

The detection of anthelmintic resistance against fenbendazole in different sheep breed necessitates the urgency to acquire strategies that impede the development of anthelmintic resistance. There should be clear focus on the importance of correct dosage, drug rotation and proper management, as these practices are pivotal to delay the onset of benzimidazole resistance in different sheep breeds.

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