

ASSESSMENT OF BENZIMIDAZOLE RESISTANCE STATUS IN AN ORGANIZED GOAT FARM BY EGG HATCH ASSAY

Asha Rajagopal^{1*}, Lucy Sabu¹, Devada, K.¹, Radhika, R.¹ and Gleeja V.L.²

¹Department of Veterinary Parasitology, College of Veterinary & Animal Sciences,
Mannuthy, Thrissur, Kerala

²Department of Statistics, College of Veterinary & Animal Sciences, Mannuthy,
Thrissur, Kerala

E-mail: asha@kvasu.ac.in (*Corresponding Author)

Abstract: Regular monitoring of benzimidazole resistance status helps in timely implementation of resistance mitigation strategies in farms. Egg hatch assay (EHA) evaluates the ability of benzimidazoles to inhibit or prevent embryonation and hatching of nematode eggs and is the standard *in vitro* test for detection of benzimidazole resistance. The present study reports detection of benzimidazole resistance in an organized goat farm in Kerala by EHA. Log-probit analysis revealed ED₅₀ of 0.25 µg/ ml thiabendazole which indicated benzimidazole resistance in the farm. The assay was also interpreted using the discriminating dose criterion. Hatching ratio of strongyle eggs (H_{dd}) at the discriminating dose of 0.1 µg/ml was 0.62 indicating benzimidazole resistance. EHA was found to be a rapid, economic and convenient detection method suitable for routine evaluation of benzimidazole resistance. Detection of benzimidazole resistance in the farm warrants implementation of strategies to control further progression of resistance.

Keywords: Egg hatch assay, benzimidazole resistance.

Introduction

Benzimidazoles are extensively used for nematode control in livestock leading to the development of drug resistance which has now evolved as a major problem worldwide. The first report of benzimidazole resistance in India was by Varshney and Singh (1976). Subsequently, there were several reports of benzimidazole resistance in GI nematodes of small ruminants from different states (Yadav and Uppal, 1993; Gill, 1996; Dhanalakshmi *et al.*, 2003; Easwaran *et.al.*, 2009). Previous reports of benzimidazole resistance from Kerala include those of Deepa and Devada (2007) from an organized farm and Asha *et al.* (2013) from small holder farmers' flocks. The growing importance of anthelmintic resistance emphasizes the need for reliable and standardized detection methods (Coles *et al.*, 2006). Use of a simple and reliable technique for detecting resistance helps in routine assessment of the resistance status in farms and in planning optimal use of anthelmintics to slow down the spread of resistance. Egg hatch assay (EHA) has been recommended as standard *in vitro* test

for detection of anthelmintic resistance by World Association for the Advancement of Veterinary Parasitology (WAAVP). The assay evaluates the ability of benzimidazoles to inhibit or prevent embryonation and hatching of nematode eggs. This test was first described by Le Jambre (1976) and has been subsequently used by a number of workers for benzimidazole resistance detection. The present study reports the *in vitro* detection of benzimidazole resistance in an organized goat farm in Kerala. The farm had 140 malabari goats reared semi-intensively and dewormed once in every three months with benzimidazoles.

Materials and methods

Egg hatch assay was done as per the protocol of Coles *et al.* (2006) and von Samson-Himmelstjerna *et al.* (2009) with modifications. Pooled faecal samples collected from goats in the farm were transported to the laboratory under anaerobic storage conditions as described by Hunt and Taylor (1989). The samples were added to 100 ml screw capped plastic bottles containing about ten 8 mm glass beads. The bottles were filled almost fully with water and shaken vigorously. The samples were then stored at room temperature until processing.

Preparation of the egg suspension

Pooled fecal sample was homogenized with mortar and pestle in water and centrifuged at 2000 rpm for 3 minutes. The sediment was loosened and mixed with saturated saline and centrifuged at 2000 rpm for 2 minutes. The tube was left undisturbed in a stand for 3 minutes after which 1 ml of the supernatant was collected. The nematode eggs from the supernatant were washed twice by sedimentation in water. The final concentration of the egg suspension was adjusted to 50 eggs per 100 μ l.

Preparation of thiabendazole stock solution and working dilutions

Stock solution of thiabendazole (Sigma Aldrich, Bangalore) was made in DMSO by dissolving 0.1 gram of pure thiabendazole in 100 ml DMSO. Using the stock solution (1000 ppm), a range of working dilutions were prepared in DMSO to get final concentrations of 1.5, 1.0, 0.5, 0.3, 0.2, 0.1 and 0.01 μ g / ml of thiabendazole in the assay.

Test protocol

Egg hatch assay was performed in 24 well plates (Tarsons, Kolkota). Ten microlitre of thiabendazole working solution was added to each well followed by the addition of 1890 μ l of distilled water. Hundred microlitre of egg suspension (with approximately 50 eggs) was added to the wells in the test plate to make a total volume of 2 ml in each well. In the control wells 10 μ l of DMSO was added instead of the drug solution. Each drug concentration was

tested in triplicate. The plate was then incubated at 25°C for 48 hrs in a BOD incubator following which one drop of 1 % iodine was added to each well. The contents of each well were transferred into a Petri-dish marked with grid and larvae and unhatched eggs counted using binocular microscope.

The mean number of eggs and larvae at each concentration was calculated and percentage hatch was derived using the following formula

$$\text{Percentage hatch} = \left(\frac{\text{Number of larvae}}{\text{Number of eggs} + \text{Number of larvae}} \right) \times 100$$

From the percent egg hatch at each drug concentration, ED₅₀ (dose required to prevent 50 per cent of the viable eggs from hatching) values were calculated by log-probit analysis using SPSS version 24.0. Benzimidazole resistance was confirmed if the ED₅₀ value was above 0.1 µg thiabendazole / ml.

The result of EHA was also interpreted using the discriminating dose criterion (dose that prevent hatching of 99 per cent of the eggs). The proportion of eggs that hatched at the discriminating dose of 0.1 µg/ml thiabendazole (H_{dd}) was counted which indicated the proportion of benzimidazole resistant worms in the population (Coles *et al.*, 2006).

Results & discussion

The probit values of the hatching percentage were plotted against the logarithm of thiabendazole concentrations from which ED₅₀ was calculated. Log-probit analysis revealed an ED₅₀ of 0.25 µg/ ml thiabendazole (Fig.1). An ED₅₀ above 0.1µg/ ml indicated benzimidazole resistance in the farm.

The assay was also interpreted by considering the hatching ratio of strongyle eggs (H_{dd}) at the discriminating dose of 0.1 µg/ml (Coles *et al.*, 2006). In this study the H_{dd} at the discriminating dose was 0.62. Calvete *et al.* (2012) reported that H_{dd} over 0.5 in EHA indicated resistance. Thus by both the criteria benzimidazole resistance was detected in the farm.

Egg hatch assay is an *in vitro* diagnostic test that can be used in surveys for anthelmintic resistance detection in farms. The assay is less time consuming, easier and results are obtained faster when compared with other detection tests such as faecal egg count reduction test (FECRT) and larval development assay (LDA). However, conventional tests for resistance detection like EHA have low sensitivity and detect resistance only when the resistance alleles are more than 25% in the population (Taylor *et al.*, 2002). Modification of EHA by using discriminating doses instead of ED₅₀ greatly increases its sensitivity and give

results comparable to molecular detection (Varady *et al.*, 2007, Cudecova *et al.*, 2010). EHA based on discriminating doses will also be useful in situations when there are no sufficient nematode eggs available for assaying all the drug concentrations (Calvete *et al.*, 2012).

The continuous use of benzimidazoles in the farm for years might have contributed to the emergence of resistance. Detection of benzimidazole resistance in the farm warrants implementation of strategies to control further progression of resistance. Under dosing is an important factor that may lead to the development of resistance. Goats rapidly metabolize the drugs resulting lower bioavailability and therefore require twice the dosage of benzimidazoles compared to sheep. Adopting targeted selective treatment instead of whole flock treatment is another practice that can greatly control the spread of benzimidazole resistance in farms.

Acknowledgement

The authors are thankful to state plan 2015-16 for the financial assistance provided for carrying out this work.

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Figure 1
Probit values of hatching percentage plotted against logarithm of thiabendazole concentrations

