Overcoming of p-glycoprotein-mediated benzimidazole resistance in *Haemonchus contortus* of small ruminants with flavinoids, verapamil and loperamide

Om Prakash, Shankarlingam Gomathinayagam, Tirunelveli Jeyagopal Harikrishnan, Muthusamy Raman, Velayudham Pandiyan, Samapika Sahoo and Namita Shukla

**Abstract**

*In vitro* study was conducted for overcoming of P-glycoprotein mediated benzimidazole resistance in *Haemonchus contortus* of sheep and goats. *In Vitro* egg hatch assay (EHA) was carried out to assess the P-glycoprotein associated benzimidazole resistance and it’s overcome with verapamil, loperamide and flavinoids (quercetin, kaempferol, Phloretin and curcumin) in *Haemonchus contortus*. In EHA, benzimidazole resistant with a mean ED₅₀ of 0.247 μg/ml and ED₅₀ 0.070 μg/ml in susceptible populations of *H. contortus* were recorded. Resistance factor was 3.5. *In vitro* overcomin of benzimidazole resistance in *H. contortus* with co-incubation of thiabendazole and identified plant flavinoids /chemicals were carried out. The reversal/overcoming of thiabendazole resistance was observed with the highest concentration of different chemical and plant flavinoids with reduced ED₅₀ values 0.02; 0.024; 0.03; 0.041; 0.052 and 0.064 μg/ml for verapamil, loperamide, quercetin, kaempferol, Phloretin and curcumin respectively. This result indicates the capacity of these compounds to reverse/overcome the benzimidazole resistance. The data obtained in laboratory studies using *in vitro* tests need to be confirmed with *in vivo* trials performed under field conditions.

**Keywords:** *Haemonchus*, flavinoids, anthelmintic resistance, sheep and Goat, P-glycoprotein

**Introduction**

The gastrointestinal nematodes *Haemonchus contortus*, commonly known as the Barber’s pole worm, wire worm, red stomach worm, is a most economically important endoparasite which infects the small ruminants throughout the world. The use of broad-spectrum anthelmintic for over 50 years is the primary methods to control these parasites [1-2]. Extensive use of broad-spectrum anthelmintics, whether proper or improper, and the ability of *H. contortus* to adapt and to overcome the deleterious effects of the anthelmintics, have led to the emergence of anthelmintic resistance (AR). The definition of resistance is “the ability of parasites to survive doses of drugs that would normally kill parasites of the same species and stage” published by the World Association for the Advancement of Veterinary Parasitology (WAAVP) [3]. There are three main groups of mechanisms: those that change the binding sites of drugs, those that detoxify, and those that involve the active efflux of drugs by membrane transporters [4]. P-glycoproteins (Pgps) that belong to the ATP binding cassette (ABC) superfamily are efflux transporters which actively transport compounds, including drugs, across membranes [5]. The Pgp is to protect the organism by actively pumping toxic substances out of its cells [6-8]. P-glycoproteins have been identified in *H. contortus* and the full cDNA sequence has been obtained [9]. The mechanism believed to be associated with anthelmintic resistance in *H. contortus* is the overexpression of Pgp. Both benzimidazole and ivermectin-resistant strains of *H. contortus* have been found to possess Pgp alleles in higher frequency than susceptible strains. Pgp may modulate the benzimidazole concentration at the target site [10]. A relationship between Pgp and benzimidazole resistance was indirectly demonstrated through the use of the Pgp inhibitor Verapamil [11]. The presence of Verapamil, the toxicity of the drug increased and the benzimidazole resistance could be partially reversed [11]. A number of P-gp inhibitors/modulators have been shown to reverse BZ and ML resistance in *H. contortus*, both *in vitro* and *in vivo*. Third generation Pgp inhibitors including tariquidar, zosuquidar and elacridar increased the efficacy of IVM, levamisole (LEV) and thiabendazole [12]. P-gp inhibitors have been used *in vivo* to increase the efficacy of ivermectin and moxidectin towards resistant *H. contortus* in jirds [9, 13] and in sheep [14].
Materials and methods
Parasites and Eggs
Haemonchus contortus eggs were harvested as per method used [18]. Briefly, Adult live worms with abomasal contents of small ruminants (sheep and goat) were collected in normal saline from the slaughter house, Perambur, Chennai, India. The adult male and female worms were separated by gross morphology and washed twice in normal saline. The female worms were incubated in normal saline at 37°C for 2 hours for the release of eggs normally and male worms were discarded. After incubation, the normal saline was collected in 15 ml centrifuge tubes (Eppendorf) and centrifuged (Centrifuge-5804, Eppendorf AG, Hamburg) at 2000rpm for 5 min to sediment the eggs. The supernatant was poured off and the sediment was examined for the presence of eggs. The concentration of the eggs was adjusted to average 50-60 eggs per 20μl of normal saline and aliquot. The harvested eggs were immediately used for In vitro Egg Hatch Assay.

In Vitro Assessment of benzimidazole resistance by Egg Hatch Assay
The EHAs were performed as per the procedure of World Association for Advancement of Veterinary Parasitology (WAAVP) with slight modification to assess the benzimidazole resistance and overcome of resistance [19-20]. In this study thiabendazole (TBZ) was used. With the aim to assess resistance to TBZ, 50 mg pure thiabendazole (TBZ-99 percent, Sigma, USA) diluted with 50 ml of dimethyl sulfoxide (DMSO-99 percent, Fisher Scientific, USA) and mixed thoroughly to prepare a stock solution of 1000ppm of thiabendazole. Using the stock solution, ranges of 0.05, 0.1, 0.2, 0.3, 0.5, and 1.0μg/ml of TBZ working solution were prepared. For the EHA, a 24 multiwell plate (Sigma Aldrich, USA) plate was used. In each well, 20 μl of aliquoted egg suspension with approximately 50-60 eggs, and 10 μl each concentration of working solution of TBZ was added. Ten micro litter of DMSO (99 per cent) was added to the control well. The tests were carried out with two replicates for each drug concentration and control well. The volume in each well was made up to 2 ml using distilled water. Plates were incubated at 25°C for 48 hours. After incubation, one drop of Lugol’s iodine (Himedia, India) was added to stop further embryo nation of eggs. The number of larvae and unhatched eggs were counted under a binocular inverted microscope (Olympus SZ40, Japan). The mean number of eggs and larvae at each concentration was determined by counting eggs and larvae in two wells of each concentration and percentage hatch was derived. The percentage hatch was taken as percentage of resistance as the larvae survived anthelmintic treatment.

Overcoming of benzimidazole resistance with flavinoids, verapamil and loperamide
To overcome P-glycoprotein mediated benzimidazole resistance egg hatch assay was performed as above described method. In silico identified P glycoprotein inhibitors/modulators chemicals (verapamil, loperamide) and plant flavinoids (quercetin, kaempferol, Phloretin and curcumin) used as anthelmintic reversal agents [20]. For each agents separately egg hatch assay (n=40 each compound) was performed. All agents were procured from Sigma Aldrich, USA. Verapamil 16.84mg was weighed and dissolved in 10ml of distilled water and mixed thoroughly and 200μL of this solution was added to each well to reach a final concentration of 343μM of verapamil [11]. Loperamide (5.13mg), Curcumin (3.68 mg), Phloretin (2.74 mg), Quercetin (2.74 mg) and Kaempferol (3.68 mg) were weighed and dissolved in 1 ml of DMSO separately to prepare a stock solution of 10^2 M of each compound. Using the stock solutions, a suitable range, 10^3, 10^4, 10^5 and 10^6M of working solutions of each compound (chemosensitizer) was prepared by serially diluting the stock solution. To each well of a 24 multiwell plate (Sigma, USA), 20 μL of egg suspension with approximately 50-60 eggs was added. Ten microliter of different working solution of TBZ as prepared earlier from stock solution was added to each well. Two repetitions per concentration of TBZ were included. Ten microliter of DMSO (99 per cent) in duplicate was added as a negative control in the first two rows (A and B). Two hundred micro litters of Verapamil stock solutions (VRP) without TBZ was added to the last two rows (C and D) only as positive control in two wells. The volume in each well was made to 2ml using distilled water. Similarly, egg suspension, various concentrations of TBZ and DMSO as negative control was taken in another plate in the first two rows (A and B) and in the last two rows (C and D) of this plate, 20μl of one concentration of the plant compounds/Chemicals viz., 10^2M of Curcumin/Loperamide, was added to each well with the same concentration of plant compounds/Chemicals without TBZ as positive control. The volume in each well was made with 2 ml using distilled water. This testing condition was repeated for different concentrations as well as for different compounds/chemicals. The plates were incubated at 25°C for 48 hours. After incubation, the number of larvae and un-hatched eggs were counted under a binocular stereo zoom or inverted microscope (Olympus, Japan) in all four rows and larvae were collected. The mean number of eggs and larvae at each concentration was calculated and percentage hatches were derived using the formula [21].

Mean number of larvae hatched
Hatching Percentage = -------------------------- X 100
Mean number of eggs added
The hatching percentage of the first two rows (A and B) of a plate was compared to the hatching percentage of the last two rows (C and D) of the plate to know the efficacy of the particular compounds/chemicals in reversing/overcoming the benzimidazole resistance.

**Results**

A numbers of egg hatch assays (n=250) were performed with pooled egg samples to assess the resistance and susceptibility to benzimidazole. Egg hatch assay is based on the ability of eggs to hatch at concentration greater than 0.1μg/ml of thiabendazole (TBZ). In the present study, 36.4% were found to be resistant to TBZ, where larvae could be seen even at 1.0μg/ml of TBZ concentrations. The mean hatching percentage of resistant eggs to hatch at concentration greater than 0.1μg/ml of benzimidazole. Egg hatch assay is based on the ability of pooled egg samples to as

Table 1: Mean percentage of egg hatch in tbzr and tbzs population of *H. contortus* (N=250)

<table>
<thead>
<tr>
<th>H.contortus</th>
<th>SEM of eggs hatch percentage at different TBZ concentrations</th>
<th>ED50</th>
<th>RF</th>
</tr>
</thead>
<tbody>
<tr>
<td>TBZR</td>
<td>87.0±0.55 DMSO</td>
<td>51.02±0.27</td>
<td>0.274</td>
</tr>
<tr>
<td>TBZS</td>
<td>83.08±1.64</td>
<td>20.44±0.63</td>
<td>0.070</td>
</tr>
</tbody>
</table>

BZR - Thiabendazole Resistant; TBZS- Thiabendazole Susceptible; RF: Resistant Factor; SEM: Standard error of the mean

Level of significance P< 0.001 between and within groups

Table 2: Mean percentage of egg hatch and ed50 in tbz or in combination with verapamil, loperamide, quercetin, kaempferol, phloretin and curcumin (N=40)

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TBZ- Thiabendazole, VRP: Verapamil L-Loperamide, Q- Quercetin, K- Kaempferol, P- Phloretin, Level of significance P< 0.001; SEM- Standard error for the mean, Level of significance P< 0.001 between and within groups.
Discussion

In vitro assessment of benzimidazole resistance

In the present study, egg hatch assay was used to assess the P-glycoprotein associated thiabendazole resistance. The egg hatch assay was fast, inexpensive, sensitive and repeatable [21]. In this study, TBZ was dissolved in DMSO to prepare stock solutions of 1000 ppm and different concentrations of TBZ were made by diluting the stock solution with appropriate quantity of distilled water. The use of deionized water for making dilutions of thiabendazole in DMSO and the addition of DMSO- dissolved thiabendazole to water before the addition of eggs to avoid eggs encountering localized high concentration of the solvent [22]. The eggs hatched above the discriminating dose of 0.1 μg per ml of TBZ indicating the resistance status and larvae hatched even at higher concentration of TBZ. This was similar to the report showed that TBZ resistant strains hatched in higher concentration of TBZ than non-resistant strains [24]. The first time reported that eggs with an ED50 value in excess of 0.1 μg TBZ per ml was indicative of benzimidazole resistance [19]. The some report showed that eggs from susceptible individuals rarely hatched at concentrations greater than 0.1μg / ml of thiabendazole [25]. Thus, thirty six percent of samples were considered as resistant to thiabendazole. The total percentage of thiabendazole resistance as detected by EHA was 36 in this study and The ED50 value obtained for resistant populations of H. contortus in this study was 0.247 and was different from the susceptible population (ED50 0.070) by a resistance factor (RF) 3.53. Many scientists from different part of world also reported. The ED50 in resistant strain was 0.668 and LD50 values of resistant strains differed from the susceptible strains by an RF of 13.5±8.7 in the EHA [26]. Resistance to thiabendazole had been reported in H. contortus by EHA in Southeast East with ED50 values of 0.182 to 0.322 μg TBZ per ml; in Kenya with ED50 values of 0.26 μg TBZ per ml; in Big Island of Hawaii with LD50 values 0.67 ppm and 1.6 ppm and in Switzerland with ED50 values of 0.72 μg of TBZ per ml in egg hatch assay [27-30]. In India, benzimidazole resistance in strongyles of sheep in ten farms in Karnataka State with ED50 values ranging between 1.45 to 6.53 μg/ml in EHA [31]. It was observed that the percentage of hatch was variable in increasing concentrations of thiabendazole. This could be attributed to the fact that pooled eggs were used in the test which might contain eggs from susceptible and heterozygous population of H. contortus which might have contributed to difference in percentage of hatch. However the samples were considered resistant based on the egg hatch above the discriminating dose of 0.1μg/ml of TBZ. Our results concur with the findings of earlier reports that benzimidazole resistance is an emerging problem and more studies with samples need to be carried out to know the magnitude of the problem in the control of GI parasites.

Overcoming of benzimidazole resistance with chemicals and flavinoids

In Silico identified reversal agents/chemosensitizers viz., verapamil, loperamide, qercetin, kaempferol, phloretin and curcumin were subjected to EHA and the overcoming of thiabendazole resistant H. contortus of sheep/goats. The result showed that all compounds were able to overcome the BZ resistance, but it was dose dependent. After four decades of intensive use with a high selection pressure on parasite population, the development of resistance of H. contortus to TBZ is a seriously increasing problem in small ruminants. The ED50 value of resistant H. contortus was 0.247 and the ED50 of TBZ resistant population after Co- incubation with four plant compounds and two chemicals as Pgp modulators/ inhibitors was as follows; 0.02 (342μ M VRP); 0.079 (10-3M loperamide); 0.094 (10-4M quercetin); 0.068 (10-3M kaempferol); 0.088 (10-3M phloretin) and 0.095 (10-3M curcumin). It indicated that there was reversal/overcoming of resistance to susceptibility as the ED50 values had gone below the discriminating level of 0.1μg/ml after the addition of these compounds. The in vitro activity of TBZ against eggs of H. contortus was increased 3-10 folds after Co-incubation with Pgp modulators/ inhibitors. Higher exposure to TBZ obtained after co-incubation with P-gp modulators/ inhibitors accounted for a higher efficacy i.e. egg hatch inhibition resistant against H. contortus. It could be interpreted that these compounds at the given concentration inhibited Pgp mediated resistance by preventing the efflux of the drug or potentiating the toxicity of TBZ resulting in the inhibition of embryonation of eggs/ egg hatch. Many researchers demonstrated the relationship between Pgp and benzimidazole resistance through the use of the p-gp inhibitors/ modulators such as Verapamil[11, 20, 32]; Buthionine sulfoximine [18]; lectin [10]; cholesteryl [33]. The combination of anthelmintic targeting nematodes with an inhibitor of p-gp efflux pumps had a significant effect on both egg excretion and the number of worms coming from resistant nematodes to anthelmintics [34]. From this study, it had been demonstrated that it was possible to modulate the sensitivity of TBZ resistance of H. contortus with a Pgp inhibitor/ modulators like verapamil, loperamide, quercetin, kaempferol, phloretin and curcumin and rendered it more susceptible to TBZ action. All these compounds have been tested for the first time in EHA and reversal to susceptibility is confirmed in vitro. Pgp modulation may be useful to prolong the effectiveness of some of the antiparasitic compounds at least in the early stages of resistance development. The data obtained in laboratory studies using in vitro tests need to be confirmed with in vivo trials performed under field conditions. Many researchers used verapamil as Pgp inhibitors. Verapamil were a calcium channel blocker, which might be contraindicated in certain hosts when administered in vivo. Use of plant compounds in place of chemicals like flavinoids i.e. Verapamil and Loperamide to bring about a overcoming of resistance could be viewed as safe alternatives.

Acknowledgement

The authors would like to acknowledge The Dean, Madras Veterinary College, Tamil Nadu Veterinary and animal Science University, Chennai, India, 600 007, acknowledged for providing facilities to conduct research work.

References


