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# *Haemonchus contortus* load and anthelmintics resistance in Sheep and Goats in Chennai, Tamil Nadu, India

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**Abstract**
Sheep and goats are the small ruminants which have enormous potential to boost economy and this may be major source of income especially to marginal farmers and landless laborers. Gastrointestinal (GI) parasitism is a very common and economically important condition affecting domestic livestock species worldwide. Among the gastrointestinal parasites *Haemonchus contortus,* commonly known as barber's pole worm, wire worm is an economically important parasite causing a great economic loss in small ruminants industry. The present study was conducted to infection load and anthelmintics resistance status of *Haemonchus contortus* in sheep and goat. A total of 6785 sheep/goats abomasum were examined, out of which 2645 abomasal samples were found to contain *H. contortus* worms. Egg hatch assay (EHA) and larval migration inhibition assay (LMIA) were carried out to assess the status of thiabendazole and ivermectin resistance. In EHA, 36% of the samples were benzimidazole resistant with a mean $ED_{50}$ of 0.247 µg/ml and $ED_{50}$ 0.070 µg/ml in susceptible populations of *H. contortus*. Resistance factor for thiabendazole was 3.5. In LMIA, 42% of the samples were ivermectin resistant with $LM_{50}$ value of 0.149.

**Keywords:** Egg hatch assay, larval migration inhibition assay, *Haemonchu*s, anthelmintics

## Introduction
Small ruminants especially sheep and goats contribute to the livelihoods of millions of rural poor in most of the developing countries of the Asia and Africa. Small ruminants make a very valuable contribution to the poor in the rural areas. Their importance is indicating by various functional contributions (meat, milk, fibre, skin etc.), socio-economic relevance and stability to farming systems [1]. *Haemonchus contortus* (also called barber's pole worms, twisted wireworms or large stomach worms) is a highly pathogenic, blood-feeding nematode of small ruminants and a significant cause of mortalities worldwide. Haemonchosis is a particularly significant threat in tropical, subtropical and warm temperate regions, where warm and moist conditions favour the free-living stages, but periodic outbreaks occur more widely during periods of transient environmental favourability [2]. In the continued absence of commercial vaccines, the use of broad-spectrum anthelmintics has been the primary method to control these pathogens in cattle and sheep for over 50 years [3, 4]. The use of anthelmintics in intensive farming has followed swiftly by the emergence of anthelmintic resistance (AR) which is now an emerging phenomenon in parasitic nematodes of sheep, goats, horses, and cattle [5]. AR is defined as a genetically transmissible trait in which the sensitivity to a particular drug is lost in a population of worms over time [6]. Resistance to the majority of the anthelmintics including the Benzimidazole [7, 8], Salicylanilides [9, 10], Organophosphates and Imidazothiazoles [11], Macrocyclic lactones [12, 8], Amino-acetonitrile derivatives [13, 14] were reported. Extensive use of drug treatments, whether proper or improper, and the ability of *H. contortus* to adapt and to overcome the deleterious effects of the drugs, have led to the development of drug resistance and therefore, to the success of the parasite. The key to control anthelmintic resistance in *H. contortus* is to understand various mechanisms that may be involved, for each class of anthelmintics has a known different target. 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 [15]. A mutation in the β-tubulin was most likely the cause of resistance to the class of benzimidazole [16].

An E198A mutation also confirmed BZ resistance in *H. contortus* [17].

The mechanisms involved with macrocyclic lactone (ML) resistance are not fully understood [6]. GluCl gene mutation in selected ivermectin and moxidectin resistant *H. contortus* isolates associated with resistance to ivermectin [18] Blackhall *et al*., 1998a The mechanism that is primarily considered to be involved in resistance to macrocyclic lactones is the detoxification process of P-glycoproteins. The primary function of PGP is to protect the organism by actively pumping toxic substances out of its cells [19, 20, 21]. 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 benzimidazole concentration at the target site [15]. A relationship between PGP and benzimidazole resistance was indirectly demonstrated through the use of the PGP inhibitor Verapamil [22]. A role for P-glycoprotein (P-GP) drug efflux pumps in ML resistance in *H. contortus* and other parasitic nematodes was reviewed [23]. Third generation PGP inhibitors including tariquidar, zosuquidar and elacridar increased the efficacy of IVM, levamisole (LEV) and thiabendazole [24] (Raza *et al*., 2015). Hence, the present study has been proposed with the objective of infection load and status of anthelmintics resistance in sheep and goat in Tamil Nadu Chennai, India.

## Material and Methods

### Collection of *Haemonchus contortus*

Abomasal contents of sheep and goat with live worms were collected in normal saline from slaughter house, Perambur, Chennai, India. In the laboratory, the contents with live worms were transferred to a plastic tray containing normal saline. The adult male and female *Haemonchus contortus* worms were separated by gross morphology and washed twice in the normal saline.

### Harvesting of eggs

The female worms were incubated in normal saline at 370C for 2 hours for the release of eggs naturally and male worms were discarded. After incubation, the normal saline was collected in centrifuge tubes and centrifuged at 2000rpm for 5min 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 50 eggs per 20µl of normal saline and aliquoted. These eggs were immediately used for Egg Hatch Assay and Coproculture.

### Harvesting of larvae

The dung pellets from sheep were collected from the slaughter house, Perambur, Chennai and checked for parasite status. The pellets were homogenized and autoclaved [25] to kill larvae, if any, present. Coproculture were made as per MAFF, 1971[26]. Briefly, the harvested eggs were gently mixed with the autoclaved dung and packed up to the 2/3rd of a 300 ml wide mouthed glass jar. The glass jar was covered with a muslin cloth and stored at 27°C with enough moisture for 7 days. After a week, the muslin cloth was removed and the jar was filled with water up to the brim. A Petridis was inverted over the mouth of the jar and the entire set up was inverted upside down and kept in a slanting position. About 5-10 ml of water was poured into the Petri dish and the setup was allowed to stand for 4 hours. The water in the petri dish was examined for the presence of third stage larvae of *H. contortus.* The third stage larvae were collected into a 15 ml centrifuge tube using a sterile Pasteur pipette and were centrifuged at 1000 rpm for 15 minutes. The supernatant was discarded and 1 ml of the sediment was collected separately into another tube. About 100 ml of the sediment was taken to a slide and the number of larvae was counted under a binocular stereo zoom microscope (Olympus SZ40, Japan). The larvae were stored for Larval Migration Inhibition Assay.

### Preparation of Thiabendazole (TBZ) stock solution

Fifty milligrams of pure thiabendazole (TBZ Sigma, USA) were transferred into a 50 ml beaker and 50 ml of dimethyl sulfoxide (DMSO Fisher Scientific, USA) was added and mixed thoroughly to prepare a stock solution of 1000ppm of thiabendazole. Using the stock solution, suitable range of working dilutions was prepared.

### Preparation of TBZ working solution

A wide range of working solutions of thiabendazole with final concentrate ions of 0.05, 0.1, 0.2 0.3, 0.5, and 1.0µg/ml were prepared

### Preparation of ivermectin (IVM) stock solution (2mM)

To prepare a stock solution 8.71mg of ivermectin was weighed and dissolved in 1 ml of dimethyl sulfoxide (DMSO) and mixed thoroughly. Using the stock solution, a suitable range of working solutions was prepared.

### Preparation of IVM working solution

A wide range of working solutions of ivermectin with final concentrations of $10^{-3}$, $10^{-4,}$ $10^{-5}$ and 10-6M were prepared by serially diluting the IVM stock solution

### Egg hatch assay (EHA)

The EHA was performed as per the procedure of World Association for Advancement of Veterinary Parasitology (WAAVP) [27] with slight modification [7, 8] to assess the benzimidazole resistance.

### Larval migration inhibition assay (LMIA)

The assay was based on the paralysing property of the drug ivermectin and the ability of resistant larvae to migrate through sieves at higher concentration of IVM drug than those of susceptible strains. It was carried out as per method [28] with slight modification.

### Statistical analysis

Mean percentage egg hatch, mean percentage of larval migration were statistically analyzed as per the methods [29]. The $ED_{50}$ and $LM_{50}$ of resistant and susceptible populations of *H. contortus* were calculated using probit analysis [30]

## Result

### Infection load of *H. contortus*

A total of 6785 sheep and goat abomasal were examined for load of *H. contortus* infection at the slaughter house, Perambur, Chennai, India. The result showed 2648 abomasums (39% infection load) were found with male and female worms of *H. contortus* on dissection.

### Thiabendazole resistance by EHA

A total of 225 egg hatch assays were performed with pooled egg samples, out of which 35 EHA were not considered for analysis. The assessment of resistance and susceptibility to benzimidazole was based on the ability of eggs to hatch at concentration greater than 0.1µg/ml of thiabendazole (TBZ). In present study reveals, 36.4% were found to be resistant to

TBZ, where larvae could be seen even at 1.0µg/ml of TBZ concentrations. The mean percentage of egg hatch at different concentrations of TBZ is presented in Table 1 Using probit analysis, $ED_{50}$ values were calculated. Mean $ED_{50}$ value for benzimidazole resistance and susceptible population were 0.247 and 0.070 respectively. The mean hatching percentage of resistant and susceptible populations of *H. contortus* were analysed by one way ANOVA (Duncan) and found to be highly significant (P< 0.001). The resistance factor was 3.5.

**Ivermectin resistance by LMIA**

In this study, 290 pooled larval samples were subjected to LMIA to assess the IVM resistance out of which 119 (41.0 percent) were found to be resistant where migration of larvae could be seen even at concentrations as high as $10^{-2M}$ of IVM. The mean percentage of larval migration at different concentrations was presented in Table 2. Using probit analysis, $LM_{50}$ values were calculated. The resistance factor was 1492. Mean LM50 value for Ivermectin resistance and susceptible population were 0.1492 and 0.0001 respectively. The mean migration percentage of resistant and susceptible populations of *H. contortus* larvae at different concentration were analysed by one way ANOVA (Duncan) and found to be highly significant (P< 0.001).

**Table 1:** Mean Percentage of Egg Hatch in TBZR and TBZS Population of *H. contortus* (N=190)

| *H. contortus* | SEM of eggs hatch percentage at different TBZ concentrations | | | | | | $ED_{50}$ | RF |
|---|---|---|---|---|---|---|---|---|
| | Control DMSO | 0.05µg/ml | 0.1µg/ml | 0.3µg/ml | 0.5µg/ml | 1.0µg/ml | | |
| TBZR | 87.0±.0.55 | 81.7±.0.05 | 74.79±.024 | 67.73±.00 | 44.25±.015 | 3.5102±.27 | 0.274 | 3.5 |
| TBZS | 83.08±1.64 | 54.97±2.15 | 38.34±2.22 | 29.25±2.17 | 14.4±2.11 | 2.62±0.58 | 0.070 | |

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 Larval Migration in IVMR and IVMS Population of *H. contortus* (N= 290)

| *H. contortus* | SEM of Larval migration percentage at different IVM concentration | | | | | | LM50 | RF |
|---|---|---|---|---|---|---|---|---|
| | Control DMSO | $10^{-2}$M | $10^{-3}$M | $10^{-4}$M | $10^{-5}$M | $10^{-6}$M | | |
| IVMR | 90.06±0.21 | 57.86±1.23 | 64.53±3.21 | 72.93±1.43 | 79.93±3.21 | 84.73±0.91 | 0.1492 | 1492 |
| IVMS | 87.06±1.21 | 16.46±2.32 | 22.26±1.21 | 36.73±3.23 | 50.66±3.02 | 69.13±0.6 | 0.0001 | |

IVMR -Ivermectin Resistance; IVMS- Ivermectin Susceptible; SEM- Standard error for the mean
$LM_{50}$-50 Percent Migration inhibition; RF- Resistant Factor
Level of significance P< 0.001 between and within groups

**Discussion**

The previous studied about prevalence of *H. contortus* reported by scientist time to time. The seasonal variation throughout the year and was highest during the rainy season (88.54%) followed by summer (83.15%) and winter (76.01%) [31]. Al-shaibani *et al*. (2008) Observation for 12 months and *H. contortus* (24.6%) were found to be predominantly of gastrointestinal nematode parasites [32]. The haemonchosis in small ruminants through examination of 613 abomasums of small ruminants, 355 sheep and 258 goats. The overall prevalence in this study was 38.6%, with a prevalence of 22.8%, and 15.8% were recorded for sheep and goats respectively [33]. Similarly, number of studied about prevalence of haemonchosis in sheep and goat conducted time to time with 57.8% [34], 9.18% [35], 40.9% [36]; in sheep and goats 67.2% and 56.6% respectively [37] (Bulbul *et al*. 2015), 12.1% [38] and 38.0% [39].

In present study, egg hatch assay was used to assess the thiabendazole resistance. The egg hatch assay was fast, inexpensive, sensitive and repeatable [40]. The mean egg hatch percentage of thirty nine percent of samples was 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 [41]. The first time reported that eggs with an $ED_{50}$ value in excess of 0.1 µg TBZ per ml was indicative of benzimidazole resistance [27]. Thus thirty nine percent of samples were considered as resistant to thiabendazole. The total percentage of thiabendazole resistance as detected by EHA was 39 in this study whereas Calvete *et al*. (2012) found 11 percent of TBZ resistant nematodes in sheep farms in Northeast Spain using egg hatch assay [42]. Thiabendazole resistance in Tamil Nadu was 16.8 percent [8]. The $ED_{50}$ value obtained for resistant populations of *H. contortus* in this study was 0.247 and 0.070 was different from susceptible population by a resistant factor (RF) 3.53. Varady and Corba (1999) the $ED_{50}$ in resistant strain was 0.668 and $LD_{50}$ values of resistant strains differed from the susceptible strains by an RF of 13.5±8.7 in the EHA [43]. Resistance to thiabendazole had been reported in *H. contortus* by EHA in Southeast England with ED50 values of 0.182 to 0.322 µg TBZ per ml [44]; Kenya by Maingi (1991) with $ED_{50}$ values of 0.26 µg TBZ per ml [45]. In India, benzimidazole resistance in strongyles of sheep in ten farms of Karnataka State with $ED_{50}$ values ranging between 1.45 to 6.53 µg/mL in EHA [46]. In Jashpur District of Chhattisgarh, India with $ED_{50}$ 0.196 [47]. In Tamil Nadu, there were earlier reports of benzimidazole resistance with an ED50 value of 0.586 µg albendazole per ml [48]; with ED50 of 0.8 and 0.6 µg TBZ per ml in worm population of Erode and Othiawakam [7]; with ED50 values of 0.627, 0.678 and 0.388 µg/ml of TBZ in three farms of southern India [49]. Our results confirmed the earlier findings of development of benzimidazole resistance in *H. contortus* in Tamil Nadu with $ED_{50}$ value 0.247. 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 in Tamil Nadu and more studies with samples throughout Tamil Nadu need to be carried out to know the magnitude of the problem in the control of GI parasites.

Larval migration inhibition assay was successfully performed to assess the ivermectin resistance. The migration assay is a rapid and cost effective tool for the determination of the effects of drugs that paralyze nematodes [50]. The method

adopted in this study with modification who evaluated the ability of the larval migration inhibition test (LMIT) to detect ivermectin resistance in cattle and sheep nematodes like *Ostertagia ostertagi, Cooperia oncophora, and H. contortus* in Germany [28]. IVM resistance was confirmed when larvae migrated through at the highest concentration of $10^{-2M}$. In this study, those larvae migrated at the concentration of $10^{-2M}$ were considered as resistant larvae. In present study, forty one percent of pooled larval samples were found to be ivermectin resistant through LMIA. The work reported here contributes with further evidence on the high degree of resistance to IVM in *H. contortus* under field conditions. The percentage of larval migration at different concentration of IVM ($10^{-2M}$ to $10^{-6M}$) was 57.86, 64.53, 72.93, 79.93 and 84.73 and $LC_{50}$ was 0.142. In Tamil Nadu, infective larvae had 22.0, 18.0 and 25.0 percent migration even at the maximum concentration (0.4 µg) of levamisole and the $LC_{50}$ values were 0.0156, 0.0286 and 0.0227 µg / ml, respectively [51]. LMIA was conducted to detect the ivermectin resistance in *H. contortus* of sheep and goat and found that the mean percentage of larval migration at different concentrations ($10^{-2}$M to$10^{-8}$M) was 32.1, 38.8, 47.8, 68.4, 83.3, 85.6 and 87.4 indicating resistance [8]. The susceptible and resistant isolates of *H. contortus* had an $EC_{50}$ of 0.7224 and an $EC_{50}$ of 7.0778, respectively [52]. The findings of this study are in conformity with earlier reports and confirm that there is emergence of IVM resistance also in *H. contortus* in Tamil Nadu. In this study, IVM resistance was successfully detected in forty one percent of the samples tested (N=290). On the contrary, Micro motility test was more sensitive for quantitatively measuring the degree of resistance between susceptible and resistant isolates of *H. contortus* compared larval migration inhibition test. The RFs for this test for IVM and eprinomectin ranged from 1.00 to 108.05 and from 3.87 to 32.32, respectively [53]. But they suggested that LMIT might be a superior tool to monitor resistance to moxidectin.

## Conclusion

A study was conducted to study about the infection load of *H. contortus* and status of anthelmintics resistance in sheep and goat. A total of 6785 sheep/goats abomasum were examined, out of which 2645 abomasal samples were found to contain *H. contortus* worms. Egg hatch assay (EHA) and larval migration inhibition assay (LMIA) were carried out to assess the benzimidazole and Ivermectin resistance. In EHA, 36% of the samples were benzimidazole resistant with a mean $ED_{50}$ of 0.247 µg/ml and $ED_{50}$ 0.070 µg/ml in susceptible populations of *H. contortus*. Resistance factor was 3.5. In LMIA, 42% of the samples were ivermectin resistant with $LM_{50}$ value of 0.149.

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