CHAPTER – III
MATERIALS AND METHODS

The present study was aimed to assess the status of anthelmintic resistance in gastrointestinal nematodes (GINs) of sheep and goats in small holder farmer's flocks at six taluks viz., Chidambaram, Kattumannarkoil, Cuddalore, Panruti, Vridhachalam and Tittakudi taluks of Cuddalore District, Tamil Nadu by in vivo and in vitro assays. A survey on the epidemiology of GINs and the parasite control practices in sheep and goats in Cuddalore district of Tamil Nadu was carried out.

3.1 STUDY AREA

The study was carried out throughout small holder farmer's flock of sheep and goats in six taluks viz., Chidambaram, Kattumannarkoil, Cuddalore, Panruti, Vridhachalam and Tittakudi of Cuddalore district. Cuddalore district is located between 11°11' to 12°35' North latitude and 78°38' to 80° East longitude and is predominately an agricultural district. The average elevation of the district is 1m (3 ft.) above mean sea level. It has a coastline of 57 km with one fishing harbour and five fish landing centres. It is bounded on the north by Villupuram District, east of the Bay of Bengal, south by Nagapattinam District, and west by Perambalur District. The district has a hot tropical climate. The summer season, which is very oppressive, is from March to May. The south-west monsoon, which follows, lasts through September. October to December constitutes northeast monsoon season. January to February is the comparatively cooler period. The annual normal rainfall for the period ranges from 1050 – 1400 mm. The normal annual rainfall in the district varies from about 1050 mm to about 1400 mm. It is the minimum around
Vridhachalam (1051.3 mm). It gradually increases and reaches maximum around Chidambaram (1402.6 mm) and Portonovo (1347.1). The rainfall contributions of individual seasons are as follows: NE-57%, SW-31%, summer-7% and winter 5%.

3.2 STUDY ANIMALS

The small holder sheep and goat flocks in each taluk were selected for the study. The flock size ranged with a minimum of 20 and the maximum of 70. Mecheri was the only sheep breed, Tellichery and Non-descript goat breeds were maintained in the flocks.

Regular and rotational deworming of sheep and goats was undertaken once every 6 months in organised farms. The small holder's flocks were reported to be dewormed once in a year.

3.3 EPIDEMIOLOGY OF GASTROINTESTINAL PARASITES

The study was carried out over one year from October 2013 to September 2014. A total of 180 faecal samples were collected from sheep and goats every month from all over the Cuddalore district. The infection was determined by using sedimentation and floatation techniques for helminth eggs.

The meteorological data viz., temperature, relative humidity (RH) and rainfall during the study period at Annamalai University were recorded from Meteorological Observatory, Department of Agronomy, Faculty of Agriculture, Annamalai University.
3.4 FAECAL SAMPLE ANALYSIS

3.4.1 COLLECTION OF FAECAL SAMPLE

The fresh faecal samples were collected from the rectum of sheep and goats for parasitological examinations. Suitable containers like screw-capped wide-mouthed glass bottles were used for the collection of faecal samples and transported to the laboratory for further analysis. Sometimes, plastic bottles and polythene bags were also used. These were made as airtight as possible in order to prevent the rate of development and hatching of eggs. 10 to 15 g of faecal samples were collected each time for examination of eggs of nematodes.

3.4.2 EXAMINATION OF FAECAL SAMPLE

3.4.2.1 CONCENTRATION METHODS

The parasites were separated from the collected faecal samples by adopting the two concentration methods viz., sedimentation and the floatation techniques as described by Zajac and Conboy (2012).

3.4.2.1.1 CONCENTRATION BY SEDIMENTATION TECHNIQUE

About 4 to 5 g of the faecal sample was taken and thoroughly mixed with 10 to 15ml of water; the emulsion was strained through a sieve to remove the coarse particles. This filtrate was poured into a centrifuge tube, and centrifuged at 1500 rpm for five minutes. After centrifugation, the supernatant was discarded and the sediment was examined microscopically (10X) by placing a drop of sediment on the slide and covered with a coverslip.

3.4.2.1.2 CONCENTRATION BY FLOATATION

In the present study, Will's floatation technique was employed. In this technique floatation was done in tubes that were filled up to one-third with a thick
emulsion of faeces, rest of the tubes were filled up to the brim with the saturated solution of common salt till a convex surface was formed at the top. The saturated salt solution was prepared by mixing 400gms of common salt in one litre of distilled water. This floatation tube containing the faeces and the saturated solution was allowed to stand for half an hour undisturbed. A cover glass was placed on to the surface of fluid and the cover glass carrying a drop of solution was placed on a slide and was examined for parasitic eggs initially under low power and then under high power microscope (10x and 40x).

3.4.2.2 FAECAL EGG COUNT

The study was carried out for a period of one year from October' 2013 to September' 2014 in the small holder sheep and goat flocks of all the six taluks of Cuddalore district. Faecal samples were collected directly from the rectum of each animal and the faecal egg load was determined using the modified McMaster technique with saturated sodium chloride solution as the floatation medium. In each case, 1 g of faeces was mixed with 14 ml of water in separate polythene bags. The faecal samples were homogenised, and centrifuged at 1000 rpm for 2 min. The supernatant was discarded and to the sediment, 15 ml of saturated salt solution (Sp. Gr 1.18) was added. After mixing thoroughly, 0.15 ml of the suspension was loaded into each chamber of McMaster slide and kept undisturbed for 10 min. The McMaster slide was examined thoroughly. The total number of eggs per gram of faeces was obtained by multiplying the number of nematode eggs counted in both the chambers of the McMaster slide by a dilution factor of 100 (Coles et al., 1992). The number of eggs per gram (EPG) of faecal sample was noted. The nematode eggs were grouped as Strongyle eggs based on their general morphology.
Eggs per gram (EPG) = Total number of eggs present in 0.15 ml of sample X 100

3.4.2.3 COPROCULTURE AND LARVAL IDENTIFICATION

Coprocultures were made as per MAFF (1971). Pooled faecal samples of 250 g were collected from sheep and goat flocks of all the six taluks of Cuddalore district. The homogenized dung samples were packed up to the neck of a 300 ml wide mouthed jar. The 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 petridish was inverted over the mouth of the jar and entire setup was inverted upside down and kept in a slanting position. About 15 ml of water was poured into the petridish and the setup was allowed to stand overnight.

The water in the petri dish was examined for the presence of L₃ larvae. The L₃ larvae were collected into a 100 ml beaker using sterile pasteur pipette. The fluid was transferred into a centrifuge tube and centrifuged at 2000 rpm for 2 minutes. The supernatant was discarded and 1 ml of the sediment was collected separately into another tube. About 100 μl of the sediment was taken on a slide and the number of larvae was counted under a binocular stereozoom microscope.

Total number of infective larvae in 100 ml was calculated as follows

\[
\text{Number of larvae in 100 μl} = X \\
\text{Number of larvae in 1 ml} = 10X \\
\text{Number of larvae in 100 ml} = 1000 X
\]
3.4.2.4 ENTRAILS EXAMINATION

The GITs of sheep (24) and goats (32) starting from abomasum to the hind end of the rectum were collected during the study period from different abattoirs in all the six taluks of Cuddalore district. Entrails procured were brought to the laboratory for further processing. The whole gastrointestinal tract was dissected using scissors and the contents were washed with saline solution. The washed contents were allowed to remain for 30 min and the worms thus recovered were washed in normal saline twice and preserved in 10 percent formalin solution for identification (Soulsby, 1982).

3.5 IN VIVO ASSAY

3.5.1 FAECAL EGG COUNT REDUCTION TEST (FECRT)

The naturally infected animals in the small holder sheep and goat flocks of all the 6 taluks of Cuddalore district were divided into four groups depending on the total number of animals available. Number of animals in each group varied from 12 -15. The first group was treated with benzimidazole (Fenbendazole @ 7.5 mg / Kg BW - Panacur, Intervet India Pvt Ltd), the second with Imidazothiazole (Levamisole hydrochloride @ 7.5 mg / Kg BW - Nilverm, Virbac Animal Health India Pvt. Ltd) and third was drenched with macrocyclic lactone (Ivermectin @ 200 microgram (µg) / Kg BW – Hitek oral solution, Virbac Animal Health India pvt. Ltd). The fourth group was the untreated control group. Fresh faecal samples (5g) were collected from the rectum of animals on day ‘0’ and day ‘10’ post treatment. Faecal egg counts were carried out using a modified McMaster method (Coles et al., 1992). The percentage (%) of faecal egg count reduction was calculated as given below.
FECR % = 100 X (1 - \(X_t / X_c\))

Where ‘X’ = Arithmetic mean epg,

‘t’ is the treated group egg count

‘c’ is the control egg count

The arithmetic mean, per cent reduction and 95% confidence intervals were calculated.

Approximate 95 % confidence limits

Upper confidence limit = 100 \([1 - X_t / X_c \exp (-2.048 \sqrt{Y^2})]\)

Lower confidence limit = 100 \([1 - X_t / X_c \exp (+2.048 \sqrt{Y^2})]\)

Resistance is present if (i) the percentage reduction in egg count is less than 95% and (ii) the 95% confidence level is less than 90%. If only one of the two criteria is met, resistance is suspected (Coles et al., 1992).

3.5.2 PRE AND POST DRENCH COPROCULTURE

Pre treatment pooled faecal samples were collected from all sheep and goat flocks of Cuddalore district, cultured as described by MAFF (1971) and third stage larvae (L3) were identified. Similarly post treatment pooled faecal samples were collected from each group on 10th day and cultured separately according to group and infective larvae were identified as described by Van Wyk and Mayhew (2013).
3.6 *IN VITRO* ASSAYS

3.6.1 EGG HATCH ASSAY (EHA)

The ability of the anthelmintic to inhibit or prevent embryonation and hatching of nematode eggs is evaluated, as described by Le Jambre (1976). Pooled faecal samples were obtained by mixing several faecal samples from the rectum of a number of animals and then stored anaerobically as described by Hunt and Taylor (1989). The method of storage involved adding 10 g of faeces to approximately 90 ml of water in a 100 ml screw cap plastic bottle containing ten glass beads. The bottle was shaken vigorously for one minute to break up the pellets. The EHA was performed following the method described by Coles *et al.*, (1992) and Lourderaj *et al.*, (2005).

3.6.1.1 EXTRACTION OF NEMATODE EGGS FROM FAECES

Eggs were extracted by a slight modification of the method described by Jackson *et al.* (2001). To the anaerobically stored faecal sample, about 40 ml of water was added and knead thoroughly. The macerated faecal material was then suspended in 1 L of tap water and the faecal suspension was washed through a series of sieves of decreasing sizes (500, 120, 75 and 35 µm).

The filtrate in the 35 µm sieve, which contained the nematode eggs was washed and collected in a polyallomer tube and centrifuged at 1,000 rpm for 1 to 2 min. After removing the supernatant, the sediment was resuspended in 10 to 12 ml of saturated sodium chloride solution. After thorough and gentle mixing, the suspension was centrifuged again at 1000 rpm for 1 to 2 min. Using artery forceps, the polyallomer tube was clamped just below the meniscus and the contents above
the clamp transferred into a 15 ml polystyrene tube and washed twice with distilled water. Extracted eggs were pooled and made up to a volume of 10 ml. From this suspension, 100 microlitre (µl) was pipetted, eggs counted and resuspended in such a manner that 100 microlitre (µl) of the suspension contained approximately 100 eggs.

3.6.1.2 PREPARATION OF FENBENDAZOLE (FBZ) STOCK SOLUTION

Fifty milligrams fenbendazole (Sigma-F5396) was transferred into 50 ml beaker and 50 ml of DMSO (99%) added and mixed thoroughly to prepare the stock solution of 1000 ppm of fenbendazole.

3.6.1.3 PREPARATION OF WORKING SOLUTION OF FENBENDAZOLE

Using the stock solution, a wide range of working solution of Fenbendazole with final concentration of 0.05, 0.1, 0.2, 0.3, 0.4, 0.5, 0.6, and 1µg/ml were prepared by dilution with distilled water.

<table>
<thead>
<tr>
<th>FBZ stock solution (1000ppm/µl)</th>
<th>Distilled water (µl)</th>
<th>Working concentration</th>
<th>FBZ equivalent (µg/ml)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1000 µl</td>
<td>9900 µl</td>
<td>100 ppm</td>
<td>1.0</td>
</tr>
<tr>
<td>900 µl</td>
<td>9100 µl</td>
<td>90 ppm</td>
<td>0.9</td>
</tr>
<tr>
<td>800 µl</td>
<td>9200 µl</td>
<td>80 ppm</td>
<td>0.8</td>
</tr>
<tr>
<td>700 µl</td>
<td>9300 µl</td>
<td>70 ppm</td>
<td>0.7</td>
</tr>
<tr>
<td>600 µl</td>
<td>9400 µl</td>
<td>60 ppm</td>
<td>0.6</td>
</tr>
<tr>
<td>500 µl</td>
<td>9500 µl</td>
<td>50 ppm</td>
<td>0.5</td>
</tr>
<tr>
<td>400 µl</td>
<td>9600 µl</td>
<td>40 ppm</td>
<td>0.4</td>
</tr>
<tr>
<td>300 µl</td>
<td>9700 µl</td>
<td>30 ppm</td>
<td>0.3</td>
</tr>
<tr>
<td>200 µl</td>
<td>9800 µl</td>
<td>20 ppm</td>
<td>0.2</td>
</tr>
<tr>
<td>100 µl</td>
<td>9900 µl</td>
<td>10 ppm</td>
<td>0.1</td>
</tr>
<tr>
<td>50 µl</td>
<td>9950 µl</td>
<td>5 ppm</td>
<td>0.05</td>
</tr>
</tbody>
</table>
3.6.1.4 TEST PROTOCOL

EHA was performed in 24 well plates as per the method described by Jackson et al. (2001). In each well, 20 µl suspension of egg with approximately 50 eggs were added. Further, ten micro liter working solution of fenbendazole was added to each well. Finally, ten micro liter of organic solvent DMSO (99%) was added to the control well. The test was carried out with two replicates for each drug concentration and also for the control well. Then the volume of each well was made up to 2 ml using distilled water. The plates were incubated at 25°C for 48 hrs. After incubation, one drop of Lugol's iodine was added to stop further embryonation of eggs and the number of larvae and unhatched eggs in each well was counted under a microscope. The mean number of eggs and larvae at each concentration was calculated and percentage hatch was derived using the following formula

\[
\text{Percentage hatch} = \frac{\text{Number of larvae}}{\text{Number of eggs} + \text{Number of larvae}} \times 100
\]

The percentage of hatch for each concentration was calculated and the results were subjected to probit analysis to obtain ED\(_{50}\) values. The ED\(_{50}\) values above 0.1 µg FBZ / ml were considered to be resistant.

3.6.2 LARVAL DEVELOPMENT ASSAY (LDA)

The method described by Hubert and Kerboeuf (1992) was followed with some modifications.
3.6.2.1 PREPARATION OF FENBENDAZOLE (FBZ) STOCK SOLUTION

Fenbendazole stock and working solutions were prepared similar to that of EHA.

3.6.2.2 PREPARATION OF LEVAMISOLE (LEV) STOCK SOLUTION

Pure levamisole (Sigma – VETRANAL 31742) of 0.1 g was transferred into a 100 ml volumetric flask through a funnel and washed twice, each with 10 ml of distilled water. The final volume was made up to 100 ml with distilled water, which gave a stock solution of 1 g in 1000 ml or 1000 ppm of LEV in 100 ml.

Using the stock solution, a wide range of working solution of levamisole with final concentration of 0.3, 0.8, 1.0, 1.5, 3, and 6 µg/ml were prepared by dilution with distilled water.

3.6.2.3 PREPARATION OF IVERMECTIN (IVM) WORKING SOLUTION

Five dilutions of working solutions from 0.0001 to 0.03 µg / ml of Ivermectin (Sigma 18898) were prepared from a stock solution of 800 µg / ml as described by Alvarez Sanchez et al. (2005).

<table>
<thead>
<tr>
<th>Working concentration (ng / ml)</th>
<th>Stock solution (µl)</th>
<th>Distilled water (ml)</th>
</tr>
</thead>
<tbody>
<tr>
<td>0. 0001</td>
<td>0.0014</td>
<td>9.9986</td>
</tr>
<tr>
<td>0.001</td>
<td>0.0126</td>
<td>9.9840</td>
</tr>
<tr>
<td>0.01</td>
<td>125.0</td>
<td>9.8750</td>
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<tr>
<td>0.02</td>
<td>250.0</td>
<td>9.7500</td>
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<tr>
<td>0.03</td>
<td>375.0</td>
<td>9.6250</td>
</tr>
<tr>
<td>0</td>
<td>0</td>
<td>10.0</td>
</tr>
</tbody>
</table>

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3.6.2.4 COLLECTION OF SAMPLES AND TEST PROTOCOL

Faecal samples collected from sheep and goats in small holder farmer flocks in all the six taluks of Cuddalore district were pooled and samples weighing between 100 to 200 g were transported in anaerobic condition to the laboratory. Eggs were harvested from pooled faecal samples as described under EHA. The concentration of eggs was estimated and adjusted to 100 eggs per 100 µl of sample. The egg suspension (100 eggs) in 100 µl was mixed with 20 ml nutritive medium in 24 well plate. The nutritive medium composed of Earle's balanced salt solution (Himedia) plus yeast extract (Himedia) diluted in saline solution, (1g of yeast / 60 ml of saline solution) in the proportion of 1:5 (v/v) (Hubert and Kerboeuf, 1992). Amphotericin B (5 mg) was added per ml of egg suspension to avoid the proliferation of fungi. The anthelmintics were used at the following concentrations: FBZ (Sigma-F5396), 0, 0.05, 0.1, 0.3, 0.7 and 0.9 µg / ml; LEV (Sigma – VETRANAL 31742), 0, 0.3, 0.8, 1.0, 3 and 6 µg / ml and IVM (Sigma 18898) 0, 0.0001, 0.001, 0.01, 0.02 and 0.03 ng / ml. FBZ and IVM were dissolved in 1% DMSO while LEV was dissolved in distilled water. A 20 µl of FBZ, LEV and IVM were added to each of treatment wells and compared with control wells containing DMSO without anthelmintics for FBZ and IVM and distilled water for LEV. The well plates were covered with a lid and incubated in a 100 per cent relative humidity chamber at 26 °C for seven days. After seven days, one drop of Lugol's iodine was added to each well and the number of undeveloped eggs, L3 larvae and other larvae were counted using an inverted binocular stereo zoom microscope. The mean larval development for each drug concentration was calculated using the standard formula
Per cent mean larval development = \[ \frac{\text{No. of live } L_3/ \text{Total larvae in treated wells}}{\text{No. of live } L_3/ \text{Total larvae in control wells}} \times 100 \]

\[ L_3 = \text{Third stage larvae} \]

The LD\textsubscript{50} value was determined by plotting the percentage larval development and drug concentration.

### 3.7 DOCUMENTATION OF PARASITE CONTROL PRACTICES

A questionnaire was prepared as described by Swarnkar and Singh, (2010a) with slight modification. Information on small ruminants' management, worm control measures, anthelmintic use pattern and other prophylactic health measures were collected by interviewing sheep and goat farmers from all taluks of Cuddalore district using a questionnaire. The questionnaire broadly contained two sections. One section of the questionnaire with management practices for sheep and goats and the other section with worm control practices adopted by the farmers. This included frequency and timing of drenching, criteria used for selection of anthelmintics, types of anthelmintics used in past, and determination of drug dosage etc.

### 3.8 STATISTICAL ANALYSIS

Results of \textit{in vivo} and \textit{in vitro} assays were analysed by a RESO software, PROBIT and Statistical Package for Social Sciences (SPSS - 19).