

ANTHELMINTIC RESISTANCE STATUS IN STRONGYLES OF MADRAS RED SHEEP IN AN ORGANIZED FARM

Vijayasarithi, M.K.*, Sreekumar, C¹, Venkataramanan R.¹ and Raman, M²

*Department of Veterinary Parasitology,
Madras Veterinary College, Vepery, Chennai 600 007, Tamil Nadu, India

ABSTRACT

The present study was carried out to detect the status of anthelmintic resistance in strongyle helminths of Madras Red sheep in an organized farm. By using faecal egg count reduction test (FECRT), coproculture, larval paralysis assay (LPA) and allele specific PCR (AS-PCR). FECR values for December 2013 and March 2014 revealed that resistance to fenbendazole (60.96 and 76.96 per cent), susceptibility to tetramisole (98.58 and 98.41 per cent) and ivermectin (99.87 and 99.30 per cent). Coproculture indicated that anthelmintic resistance was established among Haemonchus contortus populations. The LD50 values for levamisole (0.0156µg/ml) obtained larval paralysis assay provided further evidence for the susceptibility to tetramisole. AS-PCR revealed the predominance of heterozygous susceptible genotype (rS) frequency from both pre and post-treatment samples. This study distinctly indicated that use of benzimidazole should be discontinued to prevent fixing of resistance.

Key words: Strongyle helminths, Anthelmintic resistance, Madras Red sheep

INTRODUCTION

Small ruminants are of great importance as a major source of income for small scale and the landless farmers in rural communities of developing countries, including India (Bukhari and Sanyal, 2011). Sheep is called as a museum of parasites and is susceptible to numerous ecto and endoparasites. Gastrointestinal (GI) nematodes are the most important cause for reducing productivity in sheep throughout the world. Financial losses due to parasitic gastroenteritis (PGE) can lead to poor weight gain, mortality, decreased milk yield, higher lamb mortality and reduction in wool quality and quantity. Among the GI parasites, strongyle nematodes that are most pathogenic and responsible

for PGE are Haemonchus contortus, Ostertagia spp. and Trichostrongylus spp. (Trichostrongylus colubriformis, T. vitrinus) (Bal et al., 2007).

Anthelmintic treatment is the most effective means for the control of helminth infections in livestock, including sheep. The prolonged and indiscriminate use of these anthelmintics has led to the emergence of anthelmintic resistance (AR), which is a major constraint for nematode control throughout the world, including India (Butter et al., 2012).

This paper investigates the presence of anthelmintic resistance if any, against common anthelmintic drugs in an organized farm.

1Postgraduate Research Institute in Animal Sciences, Kattupakkam, Kancheepuram 603 203, Tamil Nadu, India; 2Programme Director, Translational Research Platform for Veterinary Biologicals, Madhavaram Milk Colony, Chennai 600 051, Tamil Nadu, India

*Corresponding author: sarathivet@gmail.com (09543860957)

MATERIALS AND METHODS
STUDY ANIMALS

Madras Red sheep maintained at the Sheep and Goat Breeding Unit, Postgraduate Research Institute in Animal Sciences (PGRIAS), TANUVAS were used for the study. These animals were allowed to graze for seven to eight hours a day within farm boundaries and supplemented with 100 - 300 g of concentrate feed daily in the evening. They were provided with adequate veterinary health cover including regular deworming four times a year. Deworming data for the past 10 years were analysed. The animals were divided into four groups, which included three treated and one untreated control group, each with eight animals for the study.

Anthelmintic resistance status was evaluated against commonly used anthelmintics including fenbendazole, tetramisole and ivermectin at recommended doses, from December 2013 to March 2014 against Fenbendazole (F-Zole, Vetindia), ivermectin (Vermact-Vet, Vet Mankind) and tetramisole (Nilverm, Virbac Animal Health).

Faecal egg count reduction test (FECRT) and coproculture

About 5-10g of faecal samples were collected from the rectum from all the study animals on day 0 (pre-treatment sample) and then on the 14th day after treatment (post-treatment sample) for assessing the gastrointestinal strongyle nematode infections in terms of eggs per gram (EPG). A modified McMaster method was used to count the EPG (Coles et al. 1992). Faecal egg count reduction (FECR) was calculated by using resistance software (RESO).

The pooled pre-treatment and post-treatment dung samples were cultured for the identification of the third stage infective larvae (L3) of strongyles as per the procedures adopted by Van Wyk and Mathew, 2013.

Larval paralysis assay

The LPA was as per Martin and Le Jambre (1979). Third stage *H. contortus* larvae from pooled pre and post-treatment dung samples were tested by LPA. A serial aqueous dilution of commercially available levamisole hydrochloride was prepared in the concentration range from 32µg/ml to 0.0039µg/ml. About 0.1 ml of a suspension containing 10-20 larvae was mixed with 0.1 ml of levamisole solution, in a clean cavity microscopic slide. The mixture of L3 and anthelmintic was incubated for 15 min at room temperature. After 15 min, the slide was focused under low power (10X) of the microscope and the larvae were classified as normal (moving) or paralyzed (no observable motion during 5 seconds).

Allele specific-PCR (AS-PCR)

Haemonchus contortus larvae from pre-treatment and post-treatment samples (10 larvae from each) were used for AS-PCR. The method adopted by Silvestre and Humbert (2000) for DNA extraction from single larva was followed and larval digest containing DNA was directly used as template. The method adopted by Tiwari et al. (2007) was followed for AS-PCR using the following primers.

Hc Sus Forward	: 5'-TAG AGA ACA CCG ATG AAA CAT T -3'
Hc Res Forward	: 5'-G TAG AGA ACA CCG ATG AAA CAT A -3'
Hc common Reverse	: 5'-GGA ACC ATG TTC ACG GCT AAC -3'

PCR reactions for susceptible and resistant alleles were carried out separately at total reaction volume of 10µl PCR products were visualized by submarine gel electrophoresis and the products were visualized using UV transilluminator and photographed using video gel documentation system (Bio-Rad Gel Documentation System with Image Lab Software).

Descriptive statistics was calculated for all the parameters. The EPG data was analysed using univariate general linear model. The per cent faecal egg count reduction (FECR) was calculated using RESO computer programme (Martin and Wursthorn, 1991).

RESULTS AND DISCUSSION

FECR for fenbendazole during the month of December 2013 and March 2014 was 60.96, 76.96 per cent, and for tetramisole 98.58, 98.41 per cent and for ivermectin 99.87, 99.30 per cent, respectively. These results indicated that strongyle nematodes were resistant to fenbendazole and susceptible to tetramisole and ivermectin.

Records of the farm revealed that fenbendazole was continuously used in the previous nine years (one or two times in a year). The persistent use of the same group of anthelmintics could explain the poor FECR obtained using fenbendazole as the nematodes appear to have developed resistance against it.

Tetramisole and ivermectin were still effective in producing FECR higher than 95 per cent, as these drugs have been used minimally (once and twice, respectively) during the past ten years.

The results of the survey conducted by Easwaran et al. (2009) indicated multiple resistance in *H. contortus* and *Teladorsagia* spp. to benzimidazoles in three farms. In all the three farms, albendazole and fenbendazole had been used over the past 20 years. Similar results were reported by Jaiswal et al. (2013) in Mathura district of India where levamisole was effective and FECR was 97.96 per cent.

Pre-treatment coproculture revealed presence of *H. contortus* and *Strongyloides papillosus*. Among these, *H. contortus* was found to be predominant. Post-treatment coproculture

revealed that *H. contortus* was the only species present, indicating that *S. papillosus* was susceptible and *H. contortus* was resistant in the farm flock. Manikkavasagan et al. (2013) also reported that *H. contortus* was the most predominant species of GI nematode identified in the farm flocks followed by *S. papillosus*.

Das and Singh (2005) also recorded that the predominant larvae were *H. contortus* followed by *Trichostrongylus* spp., *Oesophagostomum* spp., *S. papillosus* and *Bunostomum* spp. from coproculture pre and post-treatment with fenbendazole.

Jaiswal et al. (2013) recorded *H. contortus* to be predominantly present (80 per cent) followed by *Trichostrongylus* spp., *Oesophagostomum* spp., *Bunostomum* spp. and *S. papillosus* in pre-treatment coproculture

Results of LPA revealed that fifty per cent of the *H. contortus* larvae were paralyzed at the concentration of 0.0156µg/ml of larvae against levamisole. This indicated that the larvae were fully susceptible to imidazothiazoles and corroborated the results of FECRT.

Only heterozygous susceptible (rS) population of *H. contortus* were present in both pre and post-treatment samples (Figure 1) on AS-PCR. This can be attributed to the selection pressure exerted by continuous use of same anthelmintics at regular intervals over a period of nine years. Under such conditions, the equilibrium shifts towards heterozygous susceptible and homozygous resistant populations, which can tolerate fenbendazole. In general, increasing dose of fenbendazole decreases homozygous susceptible (TTC/TTC) individuals, and increases heterozygous susceptible (TTC/TAC) and homozygous (TAC/TAC) resistance (Pape et al., 2003).

To conclude, resistance of *H. contortus* to fenbendazole was confirmed in the farm animals

and this could be attributed to frequent use of BZs as seen from the deworming records. Tetramisole and ivermectin were found to be effective anthelmintic drugs. It is recommended that usage of BZ is to be withheld from the farm. Tetramisole and ivermectin can be continued in rotation with other deworming drugs such as salicylanilide groups like closantel and tetrahydropyrimidine group like morantel in order to prevent occurrence of resistance. It is also recommended that the frequency of deworming to be reduced to not more than twice per year.

ACKNOWLEDGEMENT

Thanks to Director for Centre for Animal Production Studies, TANUVAS, Chennai for granting permission to carry out the research.

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Fig.1

AS-PCR of susceptible (266 bp) and resistant (267 bp) alleles of *Haemonchus contortus* β -tubulin gene. A predominance of heterozygous susceptible alleles is seen. M- 100 bp DNA ladder

