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# Evaluation of permissible glycoprotein expression in an anthelmintic resistant and susceptible population of *Haemonchus contorts* of sheep and goats

**Om Prakash, Shakarlingam Gomathinayagam, TJ Harikrishnan, M Raman, Velayudham Pandiyan and P Azhahianambi**

**Abstract**
The ABC transporter, P-glycoprotein of parasitic nematodes is one of the reasons for the resistance development mechanism to different, classes of anthelmintic drugs. The gastrointestinal nematodes *H. contortus* is responsible for haemonchosis that causes economic loss in the sheep and goat farming industry. Present research work was conducted in the *H. contortus* of sheep and goats to evaluate the involvement of Permissible glycoprotein (Pgp 2/PgpA) in the susceptible and resistant population by using qPCR. In the present study, the result shows that the Pgp gene expression was elevated in resistance as compared with the susceptible population of *H. contortus.*

**Keywords:** Small ruminants, P glycoprotein, anthelmintic resistance, Haemonchus and ABC transporter

## Introduction
Harmonchosis is a gastrointestinal nematode parasitic disease of sheep and goats caused by the haematophagus, abomasal worm, barber's pole worm *Haemonchus contortus (H. contortus). Haemonchosis* can be an acute, hyperacute or chronic disease and clinical symptoms such as anaemia, bottle jaw and loss of water, thrust lead to death. Over five decades, the broad spectrum of anthelmintics is using to control these parasitic infections in cattle and sheep (McKellar & Jackson, 2004) [1]. The continuous and improper use of these broad anthelmintics resulting emerging of anthelmintic resistance (AR) in intensive farming of sheep, goats, horses, and cattle.

To overcome the anthelmintic resistance developed in *H. contortus* is to recognize various mechanisms for different targets for each anthelmintic class that may be involved. Mainly there are three categories of anthelmintics resistance development mechanisms: change in the binding sites of drugs, detoxification, and membrane transporter involvement in active drug efflux (Kerboeuf *et al.,* 2003) [7]. The detoxification of Pgp to macrocyclic lactones resistance is considered the primary resistance mechanism. The active transport of compounds, including drugs, across membranes by the efflux transporters, P-glycoproteins (Pgps) which belong to the superfamily of ATP binding cassette (ABC). (Sangster and Dobson, 2002) [2]. The protective function of Pgp by actively pumping toxic substances out of organism cells (Sangster, 1994) [10]; Thompson and Geary, 2002 [3]). P-glycoproteins of *H. contortus* have been identified and obtained the full cDNA sequences (Xu *et al.,* 1998) [11]. The mechanism believed that anthelmintic resistance and over-expression of Pgp in *H. contortus* to be associated. Higher frequencies of Pgp alleles in Both benzimidazole and ivermectin-resistant strains of *H. contortus* than susceptible strains have been found.

The concentration of benzimidazole at the target site may be modulated by the Pgp (Kerboeuf *et al.,* 2003) [7]. The drug efflux pump plays a significant role in anthelmintic ML resistance in *H. contortus* and other nematohelminth parasites. Many researchers have found the increased expression of Pgps gene in IVM-resistant isolates (Xu *et al.,* 1998; Williamson *et al.,* 2011; Sarai *et al.,* 2014) [11]. Tariquidar, zosuquidar and elacridar (third-generation Pgp inhibitors) increas the efficacy of IVM, levamisole (LEV) and thiabendazole (Raza *et al.,* 2015) [5]. *In vivo* field study, Xu *et al.,* 1998; Molento and Prichard, 1999) [6, 11] in jirds and Lifschitz *et al.,* 2010[a] [12] in sheep have observed the increased efficacy of ivermectin and moxidectin efficacy on *H. contortus* resistant isolates by using in the P-gp inhibitors.

## 2. Material and Methods

### 2.1 Source of Parasites

Resistant and susceptible *H. contortus* live larvae were provided by Central Sheep and Wool Research Centre, Avikanagar, Rajasthan, India**.** The *H. contortus* larvae were shifted to a 3-well depression (one well per depression) slide with the help of a 100 µl pipette with a bored tip. The live larvae were collected and transferred to 2ml tubes with the help of a 100 µl fine tipped pipette under a stereo zoom microscope (Olympus, Japan) from the depression slide and were labeled. The washing of larvae was done with 500 µl phosphate buffer saline (PBS). RNA later (AM 7021, Ambion, USA) in the ratio of 1:10 was added to the tube containing larvae and stored at -80 °C until RNA isolation.

### 2.2 RNA extraction

*H. contortus* larvae were homogenized in 1000 µl Trizol® reagent (Invitrogen Life Technologies, Burlington). Incubation of larval suspension (LS) was done at 27-28 °C for 10 minutes and Thereafter, 200 µl cold chloroform per 1000 µl of Trizol was added and the tube was vibrated vigorously for 15 seconds. The larval suspension incubation was performed at 27-28 °C for 10 minutes. The centrifugation of larval suspension was done at 12,000 x g for 15 minutes at refrigerator temperature (4 °C). The colourless upper phase was taken and transferred to a new tube. Then, the LS are incubated at room temperature for 10 minutes after adding the 500 µl of isopropyl alcohol. And centrifugation of LS was done at 12,000 x g, at 4 °C temperature for 15 minutes. The supernatant was drained out and the RNA pellet was cleansed with ethanol (70%) by vortexing and centrifugation at 7,500 x g and 4 °C for 5 minutes. The cleansing was repeated. Now, the RNA clump has been air-dried for 5-10 minutes. The RNA clump was preserved at -80 °C in 50 µl RNase-free water treated with DEPC (Thermo Scientific, USA). All the above procedures were strictly carried out in cold conditions (on ice).

### 2.3 cDNA synthesis

The reverse transcription (RT) was executed following the Omniscript® Reverse Transcription protocol, using a DNA wipe-out buffer (Qiagen, Germany). In a fresh 0.5ml Eppendorf tube, 4µl of total RNA, 1µl oligo-primer and 7.5µl nuclease-free water was added to obtain a single strand of complementary DNA (cDNA), Then incubate the mixture at 65˚C for 5 minutes. After that, 4µl of first strand reaction buffer (5X), 2µl of dNTP mix (10 µM), 1µl 200 unit reverse transcriptase enzyme and 0.5µl RNase inhibitors were added and incubated for 90 minutes at 37 °C and then for 10 minutes at 70 °C.

### 2.4 Quantitative Polymerase Chain Reaction (qPCR) for Pgp expression

Xu *et al*., 1998 [11] described the *H. contorus* P-gp primer for quantitative PCR. For relative Pg-p gene expression, Actin and GAPDH (Housekeeping genes) were employed as references. The PCR reactions were set up in 20µl volumes on 96-well PCR plates (Bio-Rad, India). The reaction mixture consisted as follows:

The reaction mixture consisted as follows

| Reagent | Volume/ reaction |
|---|---|
| Sso AdvancedTM SYBER® Green Supermix | 10 µl |
| PGP2 F(Forward)- 5‟ TGTAGCGCATCGTCTGTCTAC3‟ | 1.0 µl |
| PGP2 R (Reverse)- 5‟ GGTGACACGACATCGGATTC3‟ | 1.0 µl |
| Or | |
| ACTIN-F (Forward)- 5‟ ACAGGATGCAGAAAGAAATCAC3‟ | 1.0 µl |
| ACTIN -R (Reverse)- 5‟TGGACAGAGAGGCAAGGATAG 3‟ | 1.0 µl |
| Or | |
| GAPDH-F (Farward)- 5‟GTGTGAACCACGAGACCTACA3‟ | 1.0 µl |
| GAPDH-R (Reverse) -5‟TATCGTCCATGCTAGCTGGTT3‟ | 1.0 µl |
| cDNA template | 1.0 µl |
| Nuclease-free water | 7.0 µl |
| Total | 20 µl |

The qPCR reaction is performed in a CFX96TM Real-Time System, C1000TM Thermal cycler, Bio-Rad, India with cyclic conditions for 2 min at 50 °C and 10 minutes at 95 °C, followed by 40 cycles for 30 sec, 1 minute, 2 minutes and 15 seconds at 95 °C, 60 °C, 95 °C and 60 °C respectively (Sarai, *et al*., 2013) [18]. In Both resistant and susceptible populations of *H. Contort* Ct values were observed and calculated the fold changes.

## 3. Results and Discussion

In the susceptible and resistant populations of *H. contortus* the expression of the permissible glycoprotein (Pgp 2/PgpA) gene was assessed through qPCR. The Ct values of resistant, susceptible β actin and GAPDH are presented in Table.1. The present study demonstrated higher relative Pgp gene expression 2.37 fold with β actin and 2.18 fold with GAPDH in the resistant population when compared with the susceptible population susceptible of *H. contortus.* Previous studies, clearly indicated that *H. contortus* p-glycoprotein in both ivermectin and moxidectin resistance might be involved (Sangster, 1994; Xu *et al*., 1998; Kerboeuf *et al.*, 2003; Bygarski *et al.*, 2014; Heckler *et al.*, 2014) [10, 7, 11, 9]. Recently, the involvement of P-gps in the development of IVM resistance in *C. elegans* was recorded. In the presence of IVM, increased Pgps expression was noticed in resistant and susceptible strains of different nematodes (Ardelli and Prichard, 2013). Sangster *et al.*, 1994 [10] explained the Pgp of both mammals and parasites. An intensified P-gp-mediated drug efflux as a potential IVM resistance mechanism has been expected in the target parasite, (Xu *et al.*, 1998) [11]. In the field, MLs efficacy against resistance population of parasitic nematodes in sheep and cattle enhanced modulation of Pgp for the systemic availability of MLs has been recorded (Lifschitz *et al*., 2010a, b; Bartley *et al.*, 2012) [12-13]. However, to appreciate and apprehend the potential interrelate between MLs and the nematode P-gps are further required. Garretson (2007) performed qPCR and the result demonstrated that increased level of Pgp was not involved in

the drug resistance development in *H. contortus* L3 stages. Similarly, Williamson and Wolstenholme (2012) conducted the study and they did not find differences in P-gp expression between resistant and susceptible isolates of *H. contortus*. As that comparison was performed using *H. contortus* larvae (L3 stage), the changes in the nematode. P-gp expression occurring throughout the life cycle could be a confounding factor.

In the present study, we have optimized a qPCR to assist the Pgp 2 gene expression in resistant and susceptible isolates received from CSWRI, Avikanagar, Rajasthan, India and found that the relative expression was 2.37 folds with β Actin and 2.18 folds with GAPDH in *H contortus* resistant population when correlate with susceptible population. (Prichard and Roulet, 2007 reported that P-gp up-regulation in *H. contortus* recovered before and after 1- day administration of IVM treatment whereas, a lower degree than after Moxidectin administration. The IVM treatment remarkable elevation of the P-gp2 expression in resistant *H. contortus* improved from treated lambs 0.5 and 1-day of post-treatment about parasites recovered from controlled animals. However, did not activate any exceptional modification in the pattern of the drug transporter expression in the nematode treatments when treated either with Moxidectin or Abamectin. The association between expression of P-gp and the concentration of the MLs in both susceptible and resistant nematodes is not clear. and *In vitro* assays would be developed to understand the possibility of P-gps mediating ML resistance in the nematode (Lloberas, *et al*., 2013) [15]. Sarai *et al.* (2013) [18] completely not find any significant role for P-gps in the LEV resistances. However, Hco-pgp-3 and -4 were increased in few life stages. However, these increases were not consistent across all life stages for either gene. They did not find any increase in Hco-pgp-2 in the multidrug resistant WAL isolate. Issouf *et al*. 2014 conducted the quantitative RT-PCR and concluded that, *16* among nine different Pgp genes of *H. contortus Hco-pgp-3, Hco-pgp-9.2, Hco-pgp- 11* and, *Hco-pgp-* were up-regulated in life stages of parasitic recommended that possibilities of Pgps involvement in the detoxification of eosinophil granule products. They demonstrated the first evidence that a subgroup of helminth Pgps interacted with the detoxification of host products.

Quantitative PCR was performed by Raza *et al.* (2016) [17] using primer sequences of the ABC-transporters (GAPDH, actin, pgp-1. pgp-2, pgp-3, pgp-10, pgp-12 and pgp-14, pgp-9.2 and pgp-16). Three reference housekeeping genes, GAPDH, actin and b-tubulin, were used for the qPCR analyses. The ABC transporter genes transcription was significantly elevated after subsequent exposure for 3 hours to in both IVM and LEV in the resistant isolate only. this research finding suggested that an ability to rapidly regulated protective pathways in response to drugs might be a component of the resistance displayed by the isolate.

In the present work, we performed a qPCR with two housekeeper genes as reference genes *viz*., β actin and GAPDH and evaluated the role of Pgp2 in the resistance to anthelmintic. We could find regulation of Pgp2 expression in the larval stages (L3) of *H. contortus.* The reasearch findings confirmed the involvement of Pgp in the anthelmintic resistance developement mechanism in *H. contortus* of small ruminants.

**Table 1:** Quantitative PGP expression in anthelmintic resistance and anthelmintic susceptible populations of *H. contortus*

| CT values | | ΔCt- resistant | | ΔCt- susceptible | | ΔΔCt value | | Fold change $2^{-\Delta\Delta Ct}$ | |
|---|---|---|---|---|---|---|---|---|---|
| | | βactin (A-C) | GAPDH (A-D) | βactin (B-E) | GAPDH (B-F) | β actin (A-C)- (B-E) | GAPDH (A-D)- (B-F) | β actin | GAPDH |
| Pgp2- R (A) | 17.85224528 | -14.1041 | -14.5678 | -15.3517 | -13.4404 | -1.2476 | -1.1274 | 2.37 | 2.18 |
| Pgp2- S (B) | 22.09962042 | | | | | | | | |
| βactin-R (C) | 31.95632373 | | | | | | | | |
| βactin-S (D) | 37.45127603 | | | | | | | | |
| GAPDH-R (E) | 32.42 | | | | | | | | |
| GAPDH-S (F) | 35.54 | | | | | | | | |

R- resistant; S- susceptible

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