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Potential of *Haemonchus contortus* first-stage larvae to characterize anthelmintic resistance through P-glycoprotein gene expression

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ABSTRACT

The high genetic diversity among different geographic isolates of *Haemonchus contortus* is considered a major hurdle to elucidating the mechanisms responsible for parasite multidrug resistance to commercial anthelmintics. Anthelmintic resistance can be assessed by the expression of resistance-related genes. Among them, P-glycoproteins (Pgp) have been extensively associated with anthelmintic resistance due to their role in drug efflux. Therefore, this study aimed to evaluate gene expression levels of nine *Pgp* (*Pgp*-1, *Pgp*-2, *Pgp*-3, *Pgp*-4, *Pgp*-9, *Pgp*-10, *Pgp*-11, *Pgp*-12, and *Pgp*-16) in eggs, first-stage larvae (L_1), third-stage infective larvae (L_3), adult female, and adult male of *H. contortus* from two isolates characterized as anthelmintic-resistant (R) and -susceptible (S). Four worm-free sheep were experimentally infected with approximately 4000 L_3 larvae of *H. contortus*: two animals received L_3 from the R isolate and two animals received L_3 from the S isolate. *Pgp*-9 gene expression in all developmental stages of *H. contortus* was significantly higher ($P < 0.05$) in R isolate compared to S isolate. Higher expression ($P < 0.05$) of *Pgp* genes, except *Pgp*-4, was observed in L_1 stage larvae from the R isolate. Our findings suggest that the L_1 stage can be potentially used for anthelmintic resistance characterization through monitoring of different P-glycoproteins gene expression. These results may be useful in subsequent research to unveil anthelmintic resistance mechanisms in *H. contortus* without slaughtering sheep hosts, because L_1 stages can be directly produced from eggs collected from feces.

1. Introduction

In ruminants, the occurrence of multidrug-resistant isolates in most anthelmintics is an increasingly relevant global problem that raises concerns regarding parasite control programs (Kaplan and Vidyashankar, 2012; Kotze and Prichard, 2016). Small ruminants are affected by several gastrointestinal nematode species, including *Haemonchus contortus*. This parasite presents great biotic potential and high pathogenicity to the host, it fixes to the abomasum mucosa, feeds on blood, and causes anemia and changes in abomasal secretion (Besier et al., 2016). *H. contortus* is the most important gastrointestinal nematode in small ruminant systems worldwide and has developed resistance to several drugs (Pacheco et al., 2022).

Studies have shown an intrinsic relationship between the resistance of *H. contortus* to macrocyclic lactones (especially ivermectin - IVM) and gene expression of P-glycoprotein (*Pgp*) membrane transporters (Alvarez et al., 2015; Godoy et al., 2015a; Godoy et al., 2016; Peachey et al., 2017; David et al., 2018; Maté et al., 2018; Reyes-Guerrero et al., 2020; Pacheco et al., 2022). *Pgp* integrates the ATP-binding cassette transporter, which acts as a physiological barrier through the extrusion of toxins and xenobiotics from cells (Prichard et al., 2012), removing substrates through the lipidic double layer of the extracellular space (Molento and Prichard, 1999). Membrane transporters, such as *Pgp* present in *H. contortus*, exhibited a protective function by promoting

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efflux of substances, such as anthelmintic molecules (Ménez et al., 2012). In this parasite, eleven functional *Pgp* genes have already been characterized (*Pgp* 1, 2, 3, 4, 9, 10, 11, 12, 13, 14 and 16) (Williamson et al., 2011; Issouf et al., 2014; Godoy et al., 2016; David et al., 2018). Macrocyclic-lactones (MLs) resistance may be due to alterations in the *Pgp* gene (Xu et al., 1998), have been described as multigenic origin (Bygarski et al., 2014; Godoy et al., 2016), and still is a complex process not entirely understood (Prichard and Roulet, 2007). Besides a strong relationship with ML-resistance, the Pgp efflux mechanisms have been also implicated in resistance to benzimidazoles (Beugnet et al., 1997; Kerboeuf et al., 2002; Blackhall et al., 2008).

In addition, changes in gene expression of different *Pgps* in *H. contortus* can occur at different development stages (Sarai et al., 2013; Reyes-Guerrero et al., 2020), which could be chosen as biological reference material for the characterization of anthelmintic resistance in gastrointestinal nematodes. Another important fact is the high genetic diversity of parasites (Troell et al., 2006), leading to adaptation to different climatic conditions, host species, and drug-imposed selective pressure (Gilleard and Beech, 2007). Several studies have reported that changes in expression levels of different *Pgps* genes may be associated with *H. contortus* resistance (Blackhall et al., 2008; Williamson et al., 2011; Sarai et al., 2013; Sarai et al., 2014; Alvarez et al., 2015; Godoy et al., 2015a; Godoy et al., 2015b; Godoy et al., 2016; Raza et al., 2016; David et al., 2018; Maté et al., 2018; Sallé et al., 2019). Therefore, the present study aimed to compare gene expression of nine *Pgps* in different stages of *H. contortus* development, including egg, first-stage larvae (L_1), third-stage infective larvae (L_3), and adults (female and male) from two isolates phenotypically characterized as anthelmintic multi-resistant (R) and susceptible (S).

2. Material and methods

2.1. H. contortus isolates and Experimental infections

Two *H. contortus* isolates were used for artificial infections, one susceptible (S) isolate and one multidrug-resistant (R), identified as RsHco1 and SpHco2, respectively (Amarante et al., 2017), both isolates were characterized and maintained at the Universidade Estadual Paulista Júlio de Mesquita Filho, Botucatu campus, State of São Paulo, Brazil. According to anthelmintic efficacy evaluations using worm counts following necropsy, the R isolate is resistant to albendazole, levamisole, ivermectin, and closantel (Almeida et al., 2010), while the S isolate is susceptible to albendazole, levamisole, and ivermectin (Echevarria et al., 1991).

Four lambs were treated with 2.5 mg/kg body weight (BW) monepantel 2.5 % (Zolvix ™ Novartis) and 1 mL/20 kgBW levamisole phosphate 18.8 % (Ripercol® L 150 F Zoetis) to eliminate natural nematode infection. After confirmation of parasite-free status, artificial infection was performed by administration of 4000 L_3 larvae of *H. contortus*. Two lambs were infected with the S isolate and two lambs were infected with the R isolate. Animal procedures and management protocols were approved by the Ethics Committee to the Animal Welfare (CEUA-IZ) in Nova Odessa, São Paulo, Brazil (protocol number 257–18).

2.2. Recovery of egg, L_1, L_3, and adults of H. contortus

Eggs and L_1 larvae were retrieved according to the methodology described by Bizimenyera et al. (Bizimenyera et al., 2006), and L_3 recovery was performed following the methodology of (Roberts and O'Sullivan, 1950). Three aliquots containing approximately 100,000 eggs, 100,000 L_1 larvae, and 100,000 unsheathed L_3 of *H. contortus* from each isolate (S and R) were collected. Females and adult males of *H. contortus* were obtained after euthanasia of infected lambs (*item 2.1*). For this procedure, the abomasum was removed, and the contents were placed in trays for *H. contortus* recovery, which were washed with distilled water at 37 °C and kept in a culture medium (RPMI-1640 Cultilab medium (26.7 g/L) containing 0.8 % of glucose, 20 % of fetal bovine serum, 10 nM of Hepes, and 4 % of penicillin and streptomycin) following (O'Grady and Kotze, 2004) methodology. The use of the culture medium was adopted to ensure increased viability during the identification and sorting of females and males. Three groups containing 10 males (n = 30) and 5 females (n = 15) of *H. contortus* from each S and R isolate were collected. Immediately after recovery, the samples were snap frozen and stored in liquid nitrogen (−196 °C) until RNA extraction.

2.3. RNA extraction and cDNA synthesis

For RNA extraction, samples were homogenized in liquid nitrogen, and 350 μL of TRI Reagent® (Sigma-Aldrich, St. Louis, US, cat. no. T9424) was added, followed by a second homogenization to ensure complete tissue lysis. Then, 650 μL of TRI Reagent® was added to the tube, submitted to vortexing and incubated for 5 min at room temperature (RT). A volume of 200 μL of 1-bromo-3-chloropropane was added, submitted to vortexing, incubated at RT for 5 min, and centrifuged at 12,000 g for 15 min at 4 °C. Upper aqueous phase (500 μL) was transferred to a new microtube containing 500 μL of isopropanol. The solution was mixed by inversion, incubated at RT for 10 min, and centrifuged at 12,000 g for 10 min at 4 °C. The supernatant was removed, 1 mL of ethanol 75 % was added, homogenized by inversion, and centrifuged at 7500 g for 15 min at 4 °C. The supernatant was discharged, and the tube was left to stand for 10 min until dry. The RNA was suspended in 50 μL of ultra-pure water. The concentration and purity rate of RNA samples were estimated using a BioDrop spectrophotometer (BioDrop μLITE, Integrated Scientific Solutions Inc., Walnut Creek, CA, USA) and after electrophoresis on a 2 % agarose gel stained with ethidium bromide visualized on the BioRad Gel DocTM XR+ imager (Bio-Rad Laboratories, Hercules, CA, USA). RNA samples were treated with RQ1 RNase-Free DNase (Promega, Wisconsin, US, cat. # M6101), following the manufacturer's recommendations.

The cDNA synthesis was performed using the High-Capacity cDNA Reverse Transcription kit with RNase inhibitor (ref. No. 4374967, Applied Biosystems, CA, USA) and OligodT primers (IDT, Coralville, IA, USA), following the manufacturer's protocol. In addition to RNase treatments, the absence of genomic DNA amplification in the RNA samples was confirmed by qPCR assays containing treated RNA instead of cDNA.

2.4. Primer design and analysis of the genes

Primers (Table 1) designed in the PrimerQuest software (http://www.idtdna.com/Primerquest/Home/Index) were assessed for specificity and quality using the NetPrimer online tool (http://www.premierbiosoft.com/netprimer/), OligoAnalyzer IDT (https://www.idtdna.com/calc/analyzer), BLAST (https://blast.ncbi.nlm.nih.gov/Blast.cgi?PROGRAM=blastn&PAGETYPE=BlastSearch&LINKLOC=blasthome), and Primer-Blast (https://www.ncbi.nlm.nih.gov/tools/primer-blast/). The following candidate genes: Phosphoglucose isomerase (*PGI*), Fatty acid and retinol-binding protein (*FAR*), Glyceraldehyde 3-phosphate dehydrogenase (*GAPDH*), Actin (*ACTIN*), and 18 S ribosomal (*18S*) were tested as reference using geNorm (Vandesompele et al., 2002), NormFinder (Andersen et al., 2004), and BestKeeper (Pfaffl et al., 2004). In addition, a final classification of the most stable gene was assessed by the RankAggregated tool, which applied a Monte Carlo cross-entropy algorithm (Pihur et al., 2007).

2.5. qPCR assays

RT-qPCR reactions were performed using cDNA from RNA extracted from *H. contortus* of different life stages (egg, L_1, L_3, and adult female and male). For each stage, three replicates were evaluated (*item 2.2*). The

Table 1
Information about the genes and its corresponding primers, designed for the RT-qPCR assays.

Target	Sequence 5' → 3'	Base pairs (pb)	Efficiency (%)	Access Genbank
	Glycoprotein-P (*Pgp*)			
Pgp-1	FW: AGGAGACTTCCAGACTGCG	102	100.5	U94401.1
	RV: ATGGCTATACGCTGCTTCTG			
Pgp-2		100		AF003908.1
	FW: CGTGAAGGACTTGGTGATA		98.4	
	RV: GCGTCATTGACCAGCTATA			
Pgp-3		103		JX430936.1
	FW: TGGTGGCGTTCAGTTGTTG		99.4	
	RV: CCTCATCAGTAAGGCTGTCATC			
Pgp-4		101		HM635766.1
	FW: CCTGGTCAGAAACCCTCAA		100.8	
	RV: CACGAGCCTTGTCAAGTG			
Pgp-9		131		CP035804.1
	FW: GTTGGAGTCGTCTCTCAAGAG		99.9	
	RV: GTGACATTCTCTCGTCCGTAG			
Pgp-10		143		HM635769.1
	FW: AGCCGAAGAACGCAATGA		101.2	
	RV: TGATGCTTCTCATACACGAACA			
Pgp-11		95		JX430940.1
	FW: GTGACAAGATCGGAATGCTG		101.0	
	RV: GCACACCAGAGTGATACG			
Pgp-12		108		HM635773.1
	FW: TGAGTTAGCCAACGCCAA		99.3	
	RV: GCGATACGCTGCTTCTGTC			
Pgp-16		114		JX430941.1
	FW: CGAGGAAGAAGCAGAAGAGAAA		100.0	
	RV: CGGGCTACAGCAAGGATAAG			
	Reference genes			
GAPDH	FW: GGGATACACTGAAGACCAAG	97	98.4	HM145749.2
	RV: GTTCGGGTTGAGAGAGATG			
ACTIN		91		DQ080917.1
	FW: GCTCCCAGCACGATGAAA		98.9	
	RV: TGGACAGAGAGGCAAGGATA			
18 S		116		HQ844231.1
	FW: CATTGATGGTTGAGCTTGAG		100.7	
	RV: GGGTACAGTACACCACATAC			
PGI	FW: TTGAAGGGAACCGTCCTACAAC	141	99.99	HCON_00021850
	RV: CTCCCCACTGGTCATAGCTGTT			
*FAR	FW: TGCCAAGGACTATGCCAAGT	128	99.9	FJ985261.1
	RV: TGAGTGCGTCGATCTTTCCC			

* Lecová et al. (Lecová et al., 2015); FW: forward; RV: reverse.

qPCR was carried out on the Rotor gene Q thermocycler (Qiagen, Hilden, Germany), in a final volume of 10 μL, containing: 5 μL of the QuantiNova SYBR® Green RT-PCR Kit (Qiagen) master mix, 0.3 μL of each primer (10 μM), 2.4 μL of ultra-pure water (Sigma-Aldrich), and 2 μL of cDNA (approximately 50 ng). Thermocycling conditions consisted of one cycle of enzymatic activation at 95 °C for 2 min followed by 35 cycles of 95 °C for 10 s (denaturation) and 60 °C for 30 s (annealing/extension). After amplification, a melting-temperature analysis was performed by raising the incubation temperature from 60 °C to 95 °C in 0.5 °C increments with a holding step of 5 s at each increment. Positive and negative controls were included in each run, and all samples were tested using technical duplicates.

2.6. Statistical analysis

The analysis was performed using a mixed model described by Steibel et al. (Steibel et al., 2009), with some modifications, to jointly analyze the expression of target and reference genes: [$Y_{gikr} = T_{gi} + D_{ik} + e_{gikr}$], where Y_{gikr} is the Cq obtained for the *g*th gene (target genes: all the nine *Pgps* (*Pgp*-1, *Pgp*-2, *Pgp*-3, *Pgp*-4, *Pgp*-9, *Pgp*-10, *Pgp*-11, *Pgp*-12, and *Pgp*-16) and the geometric mean of the Cq of the three most stable reference genes: *FAR*, *PGI* and *GAPDH*) from the *r*th well (referring to the technical replicate), in a sample obtained from group *k* of treatment *i* (R and S). T_{gi} is the effect of treatment *i* in the expression of gene g, D_{ik} is a random sample-specific effect (common to both genes), and e_{gikr} is a residual term. The sample-specific effect, D_{ik}, captures differences among samples that are common to both genes, particularly those that affect total mRNA concentration, such as differential extraction or amplification efficiencies among samples. The model implemented in this study was adjusted using the "Mixed" procedure of SAS (SAS Institute, Cary, NC). The comparisons between treatments were made by the assembly of contrasts according to Steibel et al. (Steibel et al., 2009), which allowed the hypotheses to be tested at values equivalent to fold change ($2^{-\Delta\Delta Cq}$) (Livak and Schmittgen, 2001). Comparisons were performed for each *Pgp* gene and development stage, aiming to evaluate the differences between R and S isolates.

3. Results

3.1. Stability of the reference gene

The quantitative cycle (Cq) standard deviations for the *GAPDH*, *PGI*, *FAR*, *18 S,* and *ACTIN* candidate genes were 0.98, 1.43, 1.04, 2.72, and 1.92, respectively. Agreement of results was obtained for all softwares used to assess reference-gene stability, pointing out that *GAPDH*, *PGI*, and *FAR* are the most stable genes. These genes were then selected for data normalization, while *18S* and *ACTIN* were considered the least stable genes (Table 2).

3.2. Relative expression of Pgp genes

Significant expression differences ($P < 0.05$) for all *Pgp* genes were

Table 2
Stability values and ranking of all evaluated candidates reference genes by different softwares.

Gene	Bestkeeper Power of the gene	NormFinder S-value	geNorm M-value	RankAggreg
GAPDH	1.405 (1)	1.08 (3)	0.689 (1)	1
PGI	1.785 (3)	1.04 (1)	0.689 (1)	2
FAR	1.477 (2)	1.06 (2)	0.879 (3)	3
18S	2.746 (4)	1.10 (4)	1.586 (4)	4
ACTIN	3.447 (5)	1.74 (5)	1.904 (5)	5

observed between R and S isolates, in at least one development stage (Fig. 1). The R isolate was significantly ($p < 0.05$) up-regulated compared to the S isolate in 17 comparative expressions: *Pgp*-1 (2), *pgp*-2 (2), *Pgp*-3 (1), *Pgp*-4 (1), *Pgp*-9 (5), *Pgp*-10 (1), *Pgp*-11 (1), *Pgp*-12 (1) and *Pgp*-16 (1), while inverted regulation was found in two comparisons (*Pgp*-3 (1), and *Pgp*-12 (1)) (Fig. 1). Regarding the general average, considering all stages of development, a significant difference ($P < 0.05$) was found only in *Pgps* 2 and 9 gene expression (Fig. 1). For the L_1 stage, except for *Pgp*-4, for the other *Pgps* genes, the R isolate showed higher gene expression than the S isolate (Fig. 1). *Pgp*-9 transcripts were up-regulated in all life stages of *H. contortus* from the R isolate compared to S isolate. Additionally, this gene presented the widest difference between those groups (Fig. 1).

4. Discussion

Among the various non-drug-specific target resistance mechanisms investigated in nematodes, the main one is the expression of P-glycoprotein (Pgp) in cell membranes, whose role is to protect cells by actively pumping out potentially toxic substances (Prichard et. al., 2012). Several studies have reported that Pgps from *H. contortus* have been associated with resistance to benzimidazoles and macrocyclic lactones (Xu et al., 1998; Lespine et al., 2007; Prichard and Roulet, 2007; Blackhall et al., 2008; Lifschitz et al., 2010; Sarai et al., 2013; Sarai et al., 2014; Reyes-Guerrero et al., 2020). Therefore, the present study aimed to investigate gene expression levels of nine *Pgp* genes (*Pgp*-1, *Pgp*-2, *Pgp*-3, *Pgp*-4, *Pgp*-9, *Pgp*-10, *Pgp*-11, *Pgp*-12, and *Pgp*-16) in different life stages from one susceptible (S) (Echevarria et al., 1991) and one multi-drug resistant (R) *H. contortus* isolate (Almeida et al., 2010).

Treatment challenges was not performed in the present study, aiming to evaluate if the two phenotypes used (R and S) could show variations of the relative expression levels in the different *Pgps* and in the different stages of development, regardless of the challenge. We verified variations in the relative gene expression levels of the different *Pgps* between the two isolates studied, with significantly higher (17 fold) levels of gene expression for the isolate R when compared to the isolate S (2 fold). Under the conditions of the present study, the R isolate kept significantly higher *Pgps* gene expression levels than the S isolate, without the challenge of treatment (in vitro and in vivo). These findings may contribute to the design of new strategies for a better understanding and characterization of transcriptional levels and/or genetic markers of different geographic isolates of *H. contortus* and different stages of development in the identification of anthelmintic resistance.

Expression of all evaluated *Pgp* genes were higher in the R compared to the S isolate for the L_1 larvae stage, except for *Pgp*-4. In this context, another previous study compared gene expression of different *Pgps* from three resistant isolates (LW, LV and WAL) and from different stages of development (L_1, L_3, and adults) of a susceptible isolate of *H. contortus* (Sarai et al., 2013). In this study, increased gene expression of *Pgp*-3 (LW, LV and WAL), *Pgp*-10 (LV and WAL), *Pgp*-11 (WAL), and *Pgp*-12 (LV) were observed in the L1 stage of the R isolate. Since up-regulated levels of *Pgp-3* have been demonstrated in four different resistant isolates, including one in our study, and three in the study cited above, we suggested that *Pgp-3* gene could be potentially applied as a genetic marker of resistance. The advantages of using the L1 larvae stage compared to the other stages of development are: confirmation of L_1 viability before RNA extraction, which is not possible in eggs; presence of double cuticle in L_3, which may hamper RNA extraction (an additional step in the RNA extraction process); and no need of animal necropsy, which is required for the recovery of adults.

In addition, our findings showed that, regardless of the parasite life stage, the R isolate presented an increased *Pgp*-9 gene expression compared to the S isolate. Godoy et al. (2016) reported a strong interaction of avermectins and *Pgp*-9.1, suggesting that over-expression of this gene in the female reproductive systems of resistant worms could reduce uterus paralysis promoted by macrocyclic lactones, allowing continued egg release in drug-challenged resistant worms. In that study, only adult female *H. contortus* were used. Ardelli and Prichard (2013) verified that the *Pgp*-9 expression was significantly higher in ivermectin-resistant isolates compared to the ivermectin-susceptible wild-type isolates after 0.5 h of treatment. Sarai et al. (2013) observed greater gene expression of *Pgp*-9 in L_3 of two *H. contortus* resistant isolates, one resistant to macrocyclic lactones and the other resistant to levamisole and benzimidazoles. However, in that same study, for the isolate resistant to benzimidazoles, monepantel tartrate, and levamisole, this difference was not found in L_3. In a similar study, gene expression levels of *Pgp*-9 in the L_3 stage were significantly higher in ivermectin-resistant isolates compared to susceptible isolates (Raza et al., 2016). Williamson and Wolstenholme (2012) evaluated gene expression levels of nine *Pgps* genes in the L_3 larvae stage of *H. contortus* and verified significant differences between susceptible and resistant isolates for *Pgps* 1, 2, and 9. According to the authors, these findings could be due to the use of L_3 stage worms, suggesting that different results could be obtained from adult worms. In our study, in the L_3 stage, the R isolate showed lower gene expression than the S isolate for *Pgp*-3 and *Pgp*-12 genes, which did not occur at any other stage of development. Sarai et al. (2014) verified higher *Pgp*-3 gene expression in L_3 larvae treated with 0.31 μg/mL of levamisole, and lower gene expression in larvae treated with 2.5 μg/mL of levamisole, suggesting the presence of a biphasic pattern of gene expression according to the level of resistance in larvae. Despite divergences observed in previous studies evaluating *Pgp*-9 gene expression, our findings highlight the importance of better elucidation of the association between *Pgp*-9 gene dynamics and parasite resistance, especially for macrocyclic lactones.

5. Conclusion

Our results suggest that *Pgp*-9 mRNA loads of *H. contortus* may be employed as a potential genetic marker to establish multidrug resistance in every life stage of this important parasitic nematode and should be better investigated to characterize parasite resistance in different populations. In addition, our findings highlight the importance of further investigation of the role of L_1 stage as a target for anthelmintic resistance characterization through monitoring of different P-glycoproteins gene expression. The advantage is that the early stages of development of *H. contortus*, such as eggs, L_1, and L_3 larvae can be evaluated without the requirement of an animal necropsy, currently needed to obtain adult parasites. Thus, this method reduces time, and expenses, and eliminates ethical concerns.

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Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence

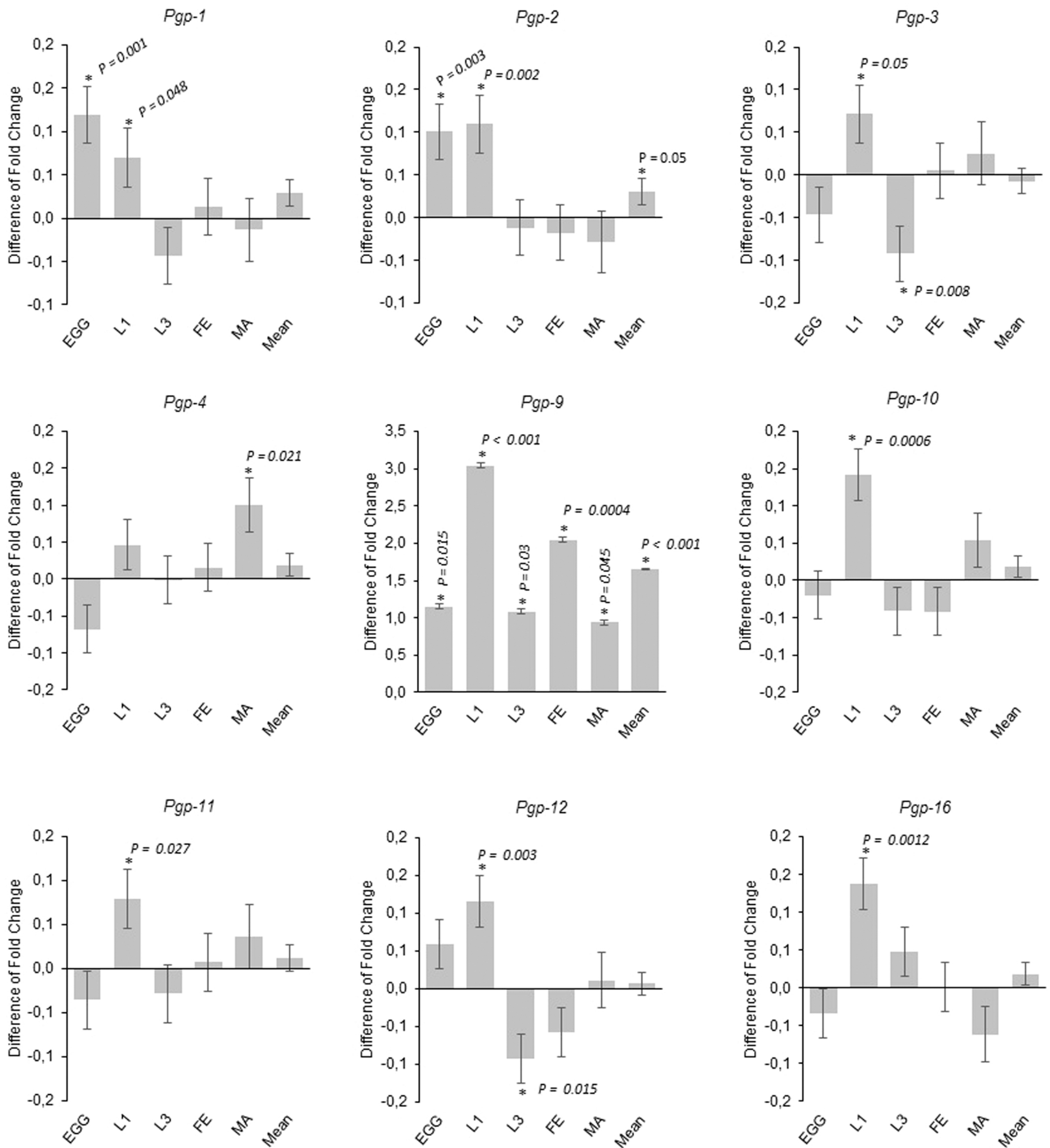

Fig. 1. Differences of gene expression levels (fold change) for nine *Pgp* genes (1, 2, 3, 4, 9, 10, 11, 12, and 16) in resistant (R) and susceptible (S) isolates and different development stages of *H. contortus* including eggs, L_1 first-stage larvae, L_3 infective larvae, FE (adult female), and MA (adult male). Positive and significant values (*, $P < 0.05$) represent higher gene expression in the R isolate, while significant negative values (*, $P < 0.05$) represent greater gene expression in the S isolate.

the work reported in this paper.

References

Almeida, F.A., Garcia, K.C.O.D., Torgerson, P.R., Amarante, A.F.T., 2010. Multiple resistance to anthelmintics by *Haemonchus contortus* and *Trichostrongylus colubriformis* in sheep in Brazil. Parasitol. Int. 59, 622–625.

Alvarez, L., Suarez, G., Ceballos, L., Moreno, L., Canton, C., Lifschitz, A., Lanusse, C., 2015. Integrated assessment of ivermectin pharmacokinetics, efficacy against resistant *Haemonchus contortus* and P-glycoprotein expression in lambs treated at three different dosage levels. Vet. Parasitol. 210 (1–2), 53–63.

Amarante, M.R.V., Santos, M.C., Bassetto, C.C., Amarante, A.F.T., 2017. PCR primers for straightforward differentiation of *Haemonchus contortus, Haemonchus placei* and their hybrids. J. Helminthol. 91 (6), 757–761.

Andersen, C.L., Jensen, J.L., Ørntoft, T.F., 2004. Normalization of real-time quantitative reverse transcription-pcr data: a model-based variance estimation approach to identify genes suited for normalization, applied to bladder and colon cancer data sets. Cancer Res. 64, 5245–5250.

Ardelli, B.F., Prichard, R.K., 2013. Inhibition of P-glycoprotein enhances sensitivity of *Caenorhabditis elegans* to ivermectin. Vet. Parasitol. 191, 264–275.
Besier, R.B., Kahn, L.P., Sargison, N.D., Van Wyk, J.A., 2016. The pathophysiology, ecology and epidemiology of *Haemonchus contortus* infection in small ruminants. Adv. Parasitol. 93, 96–136.
Beugnet, F., Gauthey, M., Kerboeuf, D., 1997. Partial in vitro reversal of benzimidazole resistance by the free-living stages of *Haemonchus contortus* with verapamil. Vet. Rec. 141, 575–576.
Bizimenyera, E.S., Githiori, J.B., Eloff, J.N., Swan, G.E., 2006. *In vitro* activity of *Peltophorum africanum* Sond. (Fabaceae) extracts on the egg hatching and larval development of the parasitic nematode *Trichostrongylus colubriformis*. Vet. Parasitol. 142, 336–343.
Blackhall, W.J., Prichard, R.K., Beech, R.N., 2008. P-glycoprotein selection in strains of *Haemonchus contortus* resistant to benzimidazoles. Vet. Parasitol. 152, 101–107.
Bygarski, E.E., Prichard, R.K., Ardelli, B.F., 2014. Resistance to the macrocyclic lactone moxidectin is mediated in part by membrane transporter P-glycoproteins: Implications for control of drug resistant parasitic nematodes. Int. J. Parasitol. Drugs Drug Resist 4, 143–151.
David, M., Lebruna, C., Duguet, T., Talmont, F., Beech, R., Orlowski, S., André, F., Prichard, R.K., Lespine, A., 2018. Structural model, functional modulation by ivermectin and tissue localization of *Haemonchus contortus* P-glycoprotein-13. Int. J. Parasitol. Drugs Drug Res. 8, 145–157.
Echevarria, F.A., Armour, J., Duncan, J.L., 1991. Efficacy of some anthelmintics on an ivermectin-resistant strain of *Haemonchus contortus* in sheep. Vet. Parasitol. 39, 279–284.
Gilleard, J.S., Beech, R.N., 2007. Population genetics of anthelmintic resistance in parasitic nematodes. Parasitology 134, 1133–1147.
Godoy, P., Lian, J., Beech, R.N., Prichard, R.K., 2015a. *Haemonchus contortus* P-glycoprotein-2: in situ localisation and characterisation of macrocyclic lactone transport. Int. J. Parasitol. 45, 85–93.
Godoy, P., Che, H., Beech, R.N., Prichard, R.K., 2015b. Characterization of *Haemonchus contortus* P-glycoprotein-16 and its interaction with the macrocyclic lactone anthelmintics. Mol. Biochem. Parasitol. 204, 11–15.
Godoy, P., Che, H., Beech, R.N., Prichard, R.K., 2016. Characterisation of P-glycoprotein-9.1 in *Haemonchus contortus*. Parasit. Vectors 9, 1–11.
Issouf, M., Guégnard, F., Koch, C., Vern, I.L., Blanchard-Letort, Che, H., Beech, R.N., Kerboeuf, D., Neveu, C., 2014. *Haemonchus contortus* P-Glycoproteins Interact with Host Eosinophil Granules: A Novel Insight into the Role of ABC Transporters in Host-Parasite Interaction. Plos One 9 (2), e87802.
Kaplan, R.M., Vidyashankar, A.N., 2012. An inconvenient truth: global worming and anthelmintic resistance. Vet. Parasitol. 186, 70–78.
Kerboeuf, D., Guégnard, F., Le Vern, Y., 2002. Analysis and partial reversal of multidrug resistance to anthelmintics due to P-glycoprotein in *Haemonchus contortus* eggs using *Lens culinaris* lectin. Parasitol. Res. 88, 816–821.
Kotze, A.C., Prichard. R.K., 2016. Anthelmintic resistance in Haemonchus contortus: history, mechanisms and diagnosis. Adv. Parasitol. 93, 397–428.´
Lecová, L., Růžičková, M., Laing, R., Vogel, H., Szotáková, B., Prchal, L., Lamka, J., Vokřál, I., Skálová, L., Matoušková, P., 2015. Reliable reference gene selection for quantitative real time PCR in Haemonchus contortus. Mol. Biochem. Parasitol. 201 (2), 123–127.
Lespine, A., Martin, S., Dupuy, J., Roulet, A., Pineau, T., Orlowski, S., Alvineri, M., 2007. Interaction of macrocyclic lactones with P-glycoprotein: structure–affinity relationship. Eur. J. Pharm. Sci. 30, 84–94.
Lifschitz, A., Entrocasso, C., Alvarez, L., Lloberas, M., Ballent, M., Manazza, G., Virkela, G., Borda, B., Lanusse, C., 2010. Interference with P-glycoprotein improves ivermectin activity against adult resistant nematodes in sheep. Vet. Parasitol. 172, 291–298.
Livak, K.J., Schmittgen, T.D., 2001. Analysis of relative gene expression data using real-time quantitative PCR and the 2− ΔΔCT method. Methods 25 (4), 402–408.
Maté, L., Ballent, M., Cantón, C., Ceballos, L., Lifschitz, A., Lanusse, A., Alvarez, L., Liron, J.P., 2018. Assessment of P-glycoprotein gene expression in adult stage of *Haemonchus contortus* in vivo exposed to ivermectin. Vet. Parasitol. 264, 1–7.
Ménez, C., Mselli-Lakhal, L., Foucaud-Vignault, M., Balaguer, P., Alvinerie, M., Lespine, A., 2012. Ivermectin induces P-glycoprotein expression and function through mRNA stabilization in murine hepatocyte cell line. Bioch. Pharmacol. 83, 269–278.
Molento, M.B., Prichard, R.K., 1999. Effects of the multidrug-resistance-reversing agents verapamil and CL 347,099 on the efficacy of ivermectin or moxidectin against unselected and drug-selected strains of *Haemonchus contortus* in jirds (*Meriones unguiculatus*). Parasitol. Res. 85, 1007–1011.
O'Grady, J., Kotze, A.C., 2004. *Haemonchus contortus*: in vitro drug screening assays with the adult life stage. Exp. Parasitol. 106, 164–172.
Pacheco, P.A., Louvandini, H., Giglioti, R., Wedy, B.C.R., Ribeiro, J.C., Verissimo, C.J., Ferreira, J.F.S., Amarante, A.F.T., Katiki, M.K., 2022. Phytochemical modulation of P-Glycoprotein and its gene expression in an ivermectin-resistant *Haemonchus contortus* isolate in vitro. Vet. Parasitol. 305, 109713.
Peachey, L.A., Pinchbeck, G.L., Matthews, J.B., Burden, F.A., Lespine, A., Samson-Himmelstjerna, G., von, Krücken, J., Hodgkinson, J.E., 2017. P-glycoproteins play a role in ivermectin resistance in cyathostomins. Int. J. Parasitol. Drugs Drug Resist. 7, 388–398.
Pfaffl, M.W., Tichopad, A., Prgomet, C., Neuvians, T.P., 2004. Determination of stable housekeeping genes, differentially regulated target genes and sample integrity: BestKeeper–excel-based tool using pair-wise correlations. Biotechnol. Lett. 26, 509–515.
Pihur, V., Datta, S., Datta, S., 2007. Weighted rank aggregation of cluster validation measures: a Monte Carlo cross-entropy approach. Bioinformatics 23, 1607–1615.
Prichard, R., Ménez, C., Lespine, A., 2012. Moxidectin and avermectins: consanguinity but not identity. Int. J. Parasitol.: Drugs Drug Res. 2, 134–153.
Prichard, R.K., Roulet, A., 2007. ABC transporters and β-tubulin in macrocyclic lactone resistance: prospects for marker development. Parasitology 134 (1123–32), 24.
Raza, A., Bagnall, N.H., Jabbar, A., Kopp, S.R., Kotze, A.C., 2016. Increased expression of ATP binding cassette transporter genes following exposure of *Haemonchus contortus* larvae to a high concentration of monepantel in vitro. Paras. Vectors 9, 1–13.
Reyes-Guerrero, D.E., Cedillo-Borda, M., Alonso-Morales, R.A., Alonso-Díaz, M.A., Olmedo-Juárez, A., Mendoza-de-Gives, P., López-Arellano, M.E., 2020. Comparative study of transcription profiles of the P-glycoprotein transporters of two *Haemonchus contortus* isolates: Susceptible and resistant to ivermectin. Mol. Biochem. Parasitol. 238, 111281.
Roberts, F.H., O'Sullivan, P.J., 1950. Methods for egg counts and larval cultures from strongyles infesting the gastrointestinal tract of cattle. Austr. J. Agric. Res. 1, 99–102.
Sallé, G., Doyle, S.R., Cortet, J., Cabaret, J., Berriman, M., Holroyd, N., Cotton, J.A., 2019. The global diversity of *Haemonchus contortus* is shaped by human intervention and climate. Nat. Commun 10, 4811.
Sarai, R.S., Kopp, S.R., Coleman, G.T., Kotze, A.C., 2013. Acetylcholine receptor subunit and P-glycoprotein transcription patterns in levamisole-susceptible and -resistant *Haemonchus contortus*. Int. J. Parasitol: Drugs Drug Res. 3, 51–58.
Sarai, R.S., Kopp, S.R., Coleman, G.T., Kotze, A.C., 2014. Drug-efflux and target-site gene expression patterns in *Haemonchus contortus* larvae able to survive increasing concentrations of levamisole in vitro. Int. J. Parasitol.: Drugs Drug Res. 4, 77–84.
Steibel, J.O., Poletto, R., Coussens, P.M., Rosa, G.J.M., 2009. A powerful and flexible linear mixed model framework for the analysis of relative quantification RT-PCR data. Genomics 94, 146–152.
Troell, K., Engstrom, A., Morrison, D.A., Mattsson, J.G., Hoglund, J., 2006. Global patterns reveal strong population structure in *Haemonchus contortus*, a nematode parasite of domesticated ruminants. Int. J. Parasitol. 36, 1305–1316.
Vandesompele, J., Preter, De, Pattyn, K., Poppe, F., Van Roy, B., De Paepe, N., Speleman, F, A., 2002. Accurate normalization of real-time quantitative RT-PCR data by geometric averaging of multiple internal control genes. Genome Biol. 3, 1–12.
Williamson, S.M., Wolstenholme, A.J., 2012. P-glycoproteins of *Haemonchus contortus*: development of real-time PCR assays for gene expression studies. J. Helminthol. 86, 202–208.
Williamson, S.M., Storey, B., Howell, S., Harper, K.M., Kaplan, R.M., Wolstenholme, A.J., 2011. Candidate anthelmintic resistance-associated gene expression and sequence polymorphisms in a triple-resistant field isolate of *Haemonchus contortus*. Mol. Biochem. Parasitol. 180, 99–105.
Xu, M., Molento, M.B., Blackhall, W., Ribeiro, P., Beech, R., Prichard, R., 1998. Ivermectin resistance in nematodes may be caused by alteration of P-glycoprotein homolog. Mol. Biochem. Parasitol. 91, 327–335.