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Assessment of P-glycoprotein gene expression in adult stage of *Haemonchus contortus in vivo* exposed to ivermectin.

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Highlights

- A *Haemonchus contortus* isolate showing high resistance to ivermectin was obtained.
- Ivermectin exposure decreased both P-gp-3 and P-gp-9.1 gene expression.
- P-gp mRNA levels varied considerably among intragroup *H. contortus* samples.
- Changes in P-gp mRNA levels would not be enough to explain ivermectin resistance.

ABSTRACT

The efflux transporter P-glycoprotein (P-gp) has been implicated in multidrug resistance of different nematode parasites affecting livestock species. Increased expression of P-gp in nematodes after their *in vitro* as well as *in vivo* exposure to anthelmintics suggests a role of P-gp in drug resistance. The current work evaluated the P-gp gene expression in a highly-resistant isolate of the sheep nematode *Haemonchus contortus*, selected after exposure to ivermectin (IVM) treatments at 10-fold the therapeutic dose. Four lambs were artificially infected with L_3 (7.000

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L_3/animal) of a previously selected IVM highly resistant *H. contortus* isolate. Forty five (45) days after infection, adult worms were collected at 0 (untreated), 6, 12 and 24 h post-oral IVM (2 mg/kg) administration. The relative transcription levels of different *H. contortus* P-gp genes were studied by quantitative real-time PCR (qPCR) and confirmed by RNA-seq. P-gp1 and P-gp11 gene expressions do not change throughout the experimental sampling period. P-gp3 and P-gp9.1 transcripts decreased significantly at both 12 and 24 h post IVM exposure. P-gp2 expression was progressively increased in a time-dependent manner at 1.81 (6 h), 2.08 (12 h) and 2.49 (24 h) -fold compared to adult worms not exposed (control 0 h) to IVM, although without reaching statistically significant differences ($P>0.05$). P-gp12 was neither detected by qPCR nor by RNA-seq analysis. These relatively modest changes in the P-gp gene expression could not be enough to explain the high level of IVM resistance displayed by the *H. contortus* isolate under assessment. Overexpression of membrane drug transporters including P-gp has been associated with IVM resistance in different nematode parasites. However, some evidences suggest that resistance to IVM and other macrocyclic lactones may develop by multiple mechanisms. Further studies are needed to improve the understanding of resistance mechanisms in adult stages of *H. contortus.*

Key words: Drug resistance; ivermectin; *Haemonchus contortus*; P-glycoprotein.

1. Introduction

Gastrointestinal parasitism constitutes a major health and welfare problem for ruminants. Among the parasites that affect small ruminants, the abomasal sheep nematode *Haemonchus contortus* is one of the most widespread parasites in tropical and temperate farming areas, causing substantial economic losses to livestock worldwide (Lane et al., 2015). The control of these parasites relies on chemotherapy to ease the consequences of parasitism and reduce transmission. Among the chemical groups available against parasitic diseases affecting ruminants, the broad-spectrum macrocyclic lactones (ML) antiparasitic family has been one of the most widely used drug in veterinary medicine over the last 30 years (Chen, 2016; Omura, 2008; Ōmura

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and Crump, 2014). Inadequate and intensive use of these antiparasitic compounds has led to the emergence of high levels of parasite resistance in nematodes of sheep, goats and cattle (Demeler et al., 2009; Lomniczi et al., 2013). The high prevalence of nematode resistant to multiple classes of anthelmintics, including ML, is nowadays an increasingly relevant worldwide problem (Kotze et al., 2018; Molento, 2009; Wolstenholme et al., 2004). However, there are several gaps concerning our understanding of the mechanisms underlying anthelmintic resistance. Changes on drug target sites and the up-regulation of cellular detoxification systems seem to be implicated in this phenomenon (Jani et al., 2011). In that respect, a number of studies have shown that nematode ATP binding cassette transporters (ABC transporters) display a protective function in parasites through their role in the efflux of anthelmintic (Lespine et al., 2012; Prichard and Roulet, 2007; Raza et al., 2016b, 2016a; Xu et al., 1998a).

Compared to mammals, a greater diversity of ABC transporters has been described in nematodes. In *H. contortus*, at least ten P-glycoprotein (P-gp), one Haf transporter and two multidrug resistance protein (MRP) genes have been reported (Laing et al., 2013; Williamson and Wolstenholme, 2012). The diversity of nematode P-gps suggests that they may play a protective role in the efflux of a wide range of xenobiotics and endogenous metabolites (Prichard and Roulet, 2007). In fact, there is consistent data describing a link between the efflux transporter P-gp and nematode resistance to the major anthelmintic groups, particularly against the ML class (Ardelli and Prichard, 2004; Blackhall et al., 1998; De Graef et al., 2013). Indeed, the increased expression of P-gp in larvae 3 (L_3) of H. *contortus* has been confirmed after their exposure to the ML ivermectin (IVM) (Dicker et al., 2011; Raza et al., 2016b, 2016a; Xu et al., 1998a). Whereas most of the studies have been focused on the *in vitro* exposure of L_3 to a variety of anthelmintics, there is only limited information on the potential association between drug exposure to these molecules and P-gp expression in adult parasites after *in vivo* anthelmintic treatments in the field (Lloberas et al., 2012; Prichard and Roulet, 2007). It has been shown that the transcriptomic profile of *H. contortus*, including that of ABC transporters, changes throughout its life cycle (Issouf

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et al., 2014; Schwarz et al., 2013). Consequently, it is expected that response of these transporter proteins to anthelmintic drugs could differ between infective L_3 and adult stages.

Thus, the aim of the current work was to assess the effect of *in vivo* IVM exposure on the P-gp gene expression in adult *H. contortus* specimens highly resistant to this compound. The transcription levels of P-gp genes in adult worms from treated sheep at recovered at 6, 12, and 24 hours after IVM administration were assessed by quantitative real-time PCR (qPCR) and confirmed by transcriptomic analysis by Next Generation Sequencing (NGS), in comparison to those collected from untreated control infected sheep.

2. Material and methods

2.1. Laboratory-selected isolate

In order to select an IVM-highly resistant isolate, two consecutive efficacy trials involving *H. contortus* artificial infections were carried out in sheep (Figure 1). In the first efficacy trial, parasite free Corriedale lambs (21.2 ± 3.1 kg, n= 6) were infected with an IVM-resistant *H. contortus* isolate (7000 L_3/lamb) (artificial infection I). It has been previously demonstrated that IVM (0.2 mg/kg) failed to control this isolate, with reported efficacies of 42 to 50% (Alvarez et al., 2015). Thirty days after infection, lambs were orally treated with IVM (Ivomec®, Merial, Argentina) at the dose of 2 mg/kg (dose x 10) (Treatment I). Fourteen days after treatment, lambs were checked for faecal egg counts (EPG) and the anthelmintic efficacy of the treatment was evaluated by the faecal egg count reduction test (FECRT), according to McKenna (1990). Faecal cultures (MAFF, 1986) were performed with faecal material obtained from treated animals. The collected L_3 were used for a second efficacy trial, in which parasite free lambs (27.8 ± 7.2 kg, n=6) were infected with the previously IVM treatment survivors *H. contortus* L_3 (7000 L_3/animal) (artificial infection II). Once again, thirty days after infection, lambs were orally treated with IVM at the dose of 2 mg/kg (dose x 10) (Treatment II). Fourteen days after treatment, lambs were checked for EPG and the anthelmintic efficacy was evaluated as previously described. Finally, a highly resistant isolate of *H.*

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contortus was obtained from faecal cultures performed with feces from treated animals. This isolate is maintained under laboratory conditions trough successive passages in parasite free lambs until now.

All experimental animals used in the current studies were housed in a stall without access to grass and fed with a balanced commercial feed (Ovino® TandilCoop, Tandil, Argentina). Water was provided *ad libitum*. Animal procedures and management protocols were approved by the Ethics Committee (Animal Welfare Policy, act 12/2013) of the Faculty of Veterinary Medicine, Universidad Nacional del Centro de la Provincia de Buenos Aires (UNCPBA), Tandil, Argentina (http://www.vet.unicen.edu.ar).

2.2. P-glycoprotein *in vivo* expression study

Free parasite Corriedale lambs (30.8 ± 4.9 kg, n=4) were artificially infected with the IVM-highly resistant *H. contortus* isolate (7.000 L_3/animal) recovered from the above mentioned studies. Forty five days after infection, samples of adult *H. contortus* were collected from lambs sacrificed at 0 (untreated controls), 6, 12 and 24 h post-treatment with IVM (2 mg/kg, n=1/sampling point). Parasites (5 pools of approximately 80 female *H. contortus*/lamb) were recovered from the abomasum of each animal, gently washed in saline solution at 4°C and immediately frozen in vials placed in liquid nitrogen to study the nematode P-gp expression. All samples were stored at -70 ºC for future use.

2.3. RNA extraction and cDNA synthesis

Total RNA (tRNA) from female *H.contortus* collected from experimental animals sacrificed 0 (untreated), 6, 12 and 24 h post IVM treatment, was isolated using Trizol Reagent (Invitrogen, Carlsbad, CA, USA) according to the purchaser's protocol with an additional DNAse digestion step. Total RNA integrity, purity and concentration were determined accordingly Mate et al. (2015). The cDNA synthesis was carried out with 2 µg of tRNA using the High Capacity cDNA Reverse Transcription Kit (Life Technologies, Foster City, CA, USA) following the manufacturer's instructions.

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2.4. Reference and target genes

Actin (ACT) and *Hco-pgp2* primer pairs were designed by using Primer Express software (Applied Biosystems, Foster City, CA, USA). The *Hco-pgp-1*, *Hco-pgp-3*, *Hco-pgp-9.1*, *Hco-pgp-11* and *Hco-pgp-12* sequences were taken and tested against the P-gp sequences reported in Williamson and Wolstenholme (2012), and synthesized by Invitrogen (Carlsbad, CA, USA) (see Table 2).

Following the study of P-gp genetic expression by qPCR, several complete and partial P-gp sequences have been reported (Godoy et al., 2015; Issouf et al., 2014) and the *H. contortus* genome has been fully sequenced (Ardelli and Prichard, 2013). Even if the *H. contortus* genes have been predicted, the specific P-gp status gene names have not been reported yet. The primers sequences used in the qPCR approach were reanalyzed taking into account this information and the transcriptomic sequences obtained in the present work.

2.5. Quantitative real-time PCR (qPCR)

The relative expression levels of *Hco-pgp1*, *Hco-pgp2*, *Hco-pgp3*, *Hco-pgp9.1*, *Hco-pgp11* and *Hco-pgp12* amplicons (Williamson and Wolstenholme, 2012) were carried out using an ABI Prism 7500 Real Time PCR System (Applied Biosystems SA). The reaction mixtures were prepared using PCR power SYBR Green Master Mix 2X (Applied Biosystems SA) following manufacturer´s instructions, including 2 μL of each primer set (300 nM), 2 μL of cDNA diluted 1:100 and 4 μL of water to obtain a final volume of 20 μL. The qPCR was performed using the following thermal profile: initial hold at 95 °C for 10 min and then 40 cycles of 95 °C for 15 s followed by 1 min at 60 °C. Gene-specific amplification was confirmed by a melting-curve analysis.

Before experimental samples were quantified, calibration curves were built making dilutions of a cDNA pool at five-fold intervals in order to evaluate the qPCR efficiency for each primer pair. Calibration curves displayed slope values between -3.6 and -3.25 and r^2 higher than 0.985.

2.6. Library preparation and high-throughput RNA sequencing

RNA-seq analysis were performed to confirm the transcription levels of P-gp genes in two parasite pools (80 female *H. contortus* adults) recovered from each control and treated animal at

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12 and 24 h post-IVM administration. The RNA sequencing libraries were constructed from 1 µg of total RNA using TruSeq RNA Sample Prep (Illumina). Each cDNA library was tagged with a specific Illumina adapter sequence and the six 100-bp paired-end libraries were subsequently pooled and loaded on two Illumina HiSeq 4000 sequencing lane. The libraries were sequenced at the Vincent J. Coates Genomics Sequencing Laboratory (http://qb3.berkeley.edu/gsl/Home.html).

2.6.1. Identification of H. contortus P-gp genes

Over the past years, protein sequence from several *H.contortus* P-gp gene have been reported (Issouf et al., 2014; Williamson and Wolstenholme, 2012). These sequences of *H. contortus* together with *Caenorabditis elegans* (*C. elegans*) P-gps protein genes were download from NCBI database (https://www.ncbi.nlm.nih.gov/). The above sequences were used for tbastn (https://blast.ncbi.nlm.nih.gov/Blast.cgi) search against genome assembly sequences of *H. contortus* at NCBI.

A database search on PRJEB506 genome assembly of *H. contortus* using the tBLASTx algorithm (National Center for Biotechnology Information) was performed to identify the closest matching P-gp sequence. The P-gp genes identified in the PRJEB506 *H. contortus* genome are described in Table 1.

2.7. Statistical analysis

Relative expression of each *Hco-pgp* to ACT gene was calculated by the comparative delta-Cq method (Livak and Schmittgen, 2001), incorporating the calculated amplification efficiency for each primer pair. Unpaired Student's T-test and Welch correction if it was necessary were used to compare the *Hco-pgp* expression results obtained in control and IVM-treated groups at different sampling times. A value of $P<0.05$ was considered statistically significant.

Tophat2-DESeq2 (Kim et al., 2013; Love et al., 2014) and Kallisto–Sleuth (Bray et al., 2016; Pimentel et al., 2017) analyses were used for the comparison of transcriptome paired-end libraries. All reads were trimmed with Trimmomatics (Bolger et al., 2014) with a Phred quality threshold of 30. Reads were then mapped either to genome assembly of *H. contortus* (project ID

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PRJEB506) with Tophat2 or with Kallisto. Differentially-expressed genes analysis was performed with Dseq2 and Sleuth Bioconductor packages using the Tophat2 and Kallisto output, respectively.

3. Results

3.1. Laboratory-selected isolate

No adverse events were observed in the animals involved in the current trials after IVM treatments at high dose. The individual faecal egg counts as well as the overall faecal egg counts (arithmetic mean ± SD) obtained on days -1 and 14 after treatment are shown in Table 1, including the results of the FECRT with its lower and upper confidence intervals 95% at the two consecutive efficacy trials carried out in sheep. After the first treatment with IVM (2 mg/kg, dose x10) the result of the FECRT was 72.2%, indicating the presence of *H. contortus* resistant to IVM. In addition, in the second efficacy trial, in which parasite free lambs were infected with L_3 obtained from the *H. contortus* survivors, a decrease in the FECRT value was observed (32.6%).

3.2. Expression levels of P-gp by qPCR analysis

Calibration parameters for qPCR analysis were properly validated. Data are summarized in Supplementary Table 1.

In this work, the gene expression of the PCR products amplified with the putative *Hco-pgp-1*, *Hco-pgp-2, Hco-pgp-3*, *Hco-pgp-9.1* and *Hco-pgp-11* in adult specimens of *H. contortus* was quantified. *Hco-pgp-12* was not detected under the current qPCR conditions. The relative gene expression profiles of these transporters in adult parasites exposed to IVM *in vivo*, expressed as fold change compared to control samples, are shown in Figure 2. Whereas the transcription levels of *Hco-pgp-3* and *Hco-pgp-9* declined progressively throughout the sampling time, the *Hco-pgp-2* gene expression was increased 1.81 (6 h), 2.08 (12 h) and 2.49 (24 h) -fold compared to its expression in control parasites. One notable aspect was the large range of expression levels detected for this transporter, which resulted in no statistically significant differences among groups (P>0.05). The expression profiles of *Hco-pgp-1* and *Hco-pgp-11* determined by qPCR were almost identical, showing not substantial changes in a time-depending manner.

3.3. RNA-seq analysis

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To validate the results of the qPCR analysis, both cDNA P-gp sequences from *H. contortus* reported in the literature (Issouf et al., 2014; Williamson and Wolstenholme, 2012) and protein sequences from *C. elegans* were used in order to locate the genes corresponding to the PBR506 *H.contortus* genome. The best match between each P-gp and the transcript sequences of *H.contortus* is displayed in Table 1. This analysis showed that the sequence HM635772 reported by Williamson and Wolstenholme (2012) as P-gp1 and used in this work for the quantification of *Hco-pgp-1* by qPCR, matches the exons 26-27 from gene HCOI00233200, corresponding to P-gp17(David et al., 2018; Laing et al., 2013). Unfortunately, the sequence HM635770 used for the design of the *Hco-pgp-11* primers includes exons 5 to 7 of the same gene. Consequently, the gene expression results of *Hco-pgp-1* and *Hco-pgp-11* reflect the expression of a single underlying gene. This explains why the expression profiles of *Hco-pgp-1* and *Hco-pgp-11* determined by qPCR were almost identical (Figure 3). Similarly, the sequences HM635773 and HM635767 reported by Williamson and Wolstenholme (2012) as P-gp12 and P-gp14, respectively, both match the complete P-gp13 mRNA sequence KX844966 published in GenBank (David et al., 2018) and corresponding to gene HCOI01115500 on the *H. contortus* reference genome (Laing et al., 2013).

RNA-seq analysis were performed in two parasites pools (80 female/pool) recovered from each control and treated animal at 12 and 24 h post IVM administration. For six pool samples, a total of 140 million reads were obtained and used for mapping to the *H. contortus* genome. According to the mapping results, approximately 90% were mapped successfully to the reference. The ranking of transcription level, expressed as transcript per million (tpm) of P-gp genes from the higher to the lower was P-gp-11, P-gp2, P-gp3, P-gp1 and finally P-gp9.1 (Table 3).

Only the P-gp transcripts that were quantified by qPCR are reported here. The results of RNAseq differential expression approach correlate with qPCR analysis. While P-gp2 gene expression was increased in parasites after 12h of IVM exposure, P-gp3 and P-gp9 transcripts decreased significantly at both 12 and 24 h post IVM exposure. By contrast, P-gp1 and P-gp11 expression showed not significant changes in a time-dependent manner (Table 3, Figure 3).

4. Discussion

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The potential changes in the genetic expression of different P-gps occurring in a highly IVM-resistant isolate of *H. contortus in vivo* exposed to IVM were assessed in the current experimental work. The highly resistant isolate was obtained after treatment of lambs infected with a pre-selected isolate with 10 X the IVM therapeutic dose (Treatment II). The anthelmintic efficacy (assessed by FECRT) was only 32.6% when IVM was administered at ten times the therapeutic dose. Furthermore, no statistical differences in EPG counts were observed between day -1 and day 14 post-treatment, which demonstrate the complete therapeutic failure of IVM against this isolate at a dose as high as 2 mg/kg. The simple "selection scheme" reported here, resulted a suitable method for isolation of a highly resistant population of *H. contortus*. The rationale behind this experimental approach was that potential genetic changes underlying resistance may be easier to be shown in highly resistant individuals. To assess these potential genetic changes, two different experimental approaches, qPCR and RNA-seq analysis, were applied.

P-glycoprotein, responsible for some cases of multidrug resistance reported in cancer cells (Gottesman and Pastan, 1993), may also contribute in different ways to ML resistance in *H. contortus*. Resistance occurs when a susceptible population decrease its response to a drug treatment and is complete when the maximum dose of this drug has no anthelmintic effect (Coles, 2006). The individuals that survive drug treatment pass on their genes to the next generation, increasing the frequency of survivors over many generations. Understanding the development of drug resistance in parasitic helminths is crucial to prolong the lifespan of currently used anthelmintic drugs and to develop markers to monitor drug resistance. It was also be beneficial in the design of new chemotherapeutic agents to overcome or prevent resistance and in the identification of new drug targets (Bartram et al., 2012). There is considerable evidence that nematodes may regulate the expression levels of P-gp and other ABC transporters through transcriptional or post-transcriptional mechanisms to counteract the toxic effects of anthelmintic drugs (James and Davey, 2009; Lloberas et al., 2013; Raza et al., 2016a). However, nematode drug transporter genes do not show a consistent pattern of regulation following exposure to anthelmintics. In this regard, some studies on parasitic nematodes have reported an increase in

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the transcriptional level of transporter genes following *in vivo* (Lloberas et al., 2013; Xu et al., 1998b) and *in vitro* exposure to anthelmintic drugs (Raza et al., 2016a, 2016b; Williamson et al., 2011; Xu et al., 1998a). However, other studies have reported no transporter transcriptional changes in nematodes collected from animals treated with IVM (Alvarez et al., 2015; Areskog et al., 2013).

Williamson and Wolstenholme (2012) were able to quantify P-gp13 expression (reported in the manuscript as P-gp12 and P-gp14) by qPCR in the L3 stage of *H. contortus*. Recently, David et al. (David et al., 2018) reported the P-gp13 protein localization in several organs of the digestive, neuronal, excretory and epithelial systems in larvae and adult *H. contortus* cryosections by immunohistochemistry. By contrast, we were not able to quantify P-gp13 expression neither by qPCR nor RNA-seq analysis in female specimens of the *H. contortus* isolate here studied. Although one possible reason could be a technical limitation of both approaches (qPCR and RNA-seq analysis) for detecting P-gp13 mRNA expression, an alternative explanation could be that P-gp13 is not expressed in female *H. contortus* parasite. Further studies using both sexes are needed to clarify this situation.

Under the current experimental conditions, either by qPCR or RNA-seq analysis, the transcription profile of both P-gp-3 and P-gp-9.1 tends to slightly decrease *in vivo* exposed adult parasites. Issouf *et al.* (Issouf et al., 2014) reported up regulation of P-gp3 in *H. contortus* exposed to sheep eosinophil granules, suggesting a role of this P-gp in detoxification of host immune cell products. In addition, the expression of P-gp3 and P-gp9.1 in intestinal excretory cells of the nematode *C. elegans* suggests they may play a role in the protection of worms against xenobiotic compounds (Ardelli and Prichard, 2013). By contrast, P-gp*2* gene expression tends to increase in adult parasites following *in vivo* exposure to 10X the IVM therapeutic dose compared to those recovered from untreated animals. P-gp2 is expressed in the nematode digestive and adjacent nervous system, protecting them from the effects of anthelmintic drugs (Godoy et al., 2015). In fact, P-gp2 was the first nematode P-gp related to IVM resistance (Xu et al., 1998a). In agreement

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with our results, Lloberas et al. (2013) reported that the IVM treatment significantly increased the P-gp2 expression in resistant *H. contortus* collected from treated lambs. Furthermore, similar to results reported by Williamson and Wolstenholme (2012), P-gp mRNA levels vary considerably among different intragroup samples in the *H. contortus* isolate analysed in the current work, especially for P-gp2. In fact, it is likely that this variability precluded us to find, either by qPCR or RNA-seq analysis, significant changes in the expression of P-gp2 following *in vivo* parasite exposure to IVM. A variety of studies have reported extremely high levels of genetic diversity within *H. contortus* populations, with the population size being a major determinant of this high genetic diversity (Gilleard and Redman, 2016; Laing et al., 2013; Otsen et al., 2001; Redman et al., 2008; Schwarz et al., 2013; Troell et al., 2006). This feature enables *H. contortus* populations to quickly adapt to climate changes, hosts and drug treatments (Emery et al., 2016), the latter of which can lead to anthelmintic drug resistance even after a short time of drug use (Gilleard and Beech, 2007; Prichard, 2001). In this sense, a variety of studies suggest that laboratory strains of *H. contortus* retain high levels of genetic diversity, even if they are subjected to drug selection (Gilleard and Redman, 2016). In fact, a shallow inspection of the RNA sequences obtained from the *H. contortus* IVM-highly resistant isolate studied here showed a high level of polymorphism. It has been shown that the transcriptomic profile of *H. contortus*, changes throughout its life cycle and between adult sexes (Issouf et al., 2014; Schwarz et al., 2013). In this work, pools of female parasites were used for reasons of technical simplicity. Females can contain eggs that could interfere with P-gp expression analysis, maybe contributing to the large variation in the ARNs expression among different samples from the same parasite isolate.

One of the most cited mechanisms in the development of IVM resistance in nematodes is the overexpression of ABC transporters. However, the increased expression levels that were previously observed (Ardelli and Prichard, 2004; Dicker et al., 2011; Lloberas et al., 2013; Raza et al., 2016b, 2016a; Williamson et al., 2011) were modest and *per se* could not be sufficient to explain the high level of resistance to ML now reported in *H. contortus* populations. In agreement, there are some evidence suggesting that resistance to ML may develop by multiple mechanisms.

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ML bind nematode glutamate-gated chloride channels (GluCls), leading to paralysis of the worm's muscles (Cully et al., 1994; McCavera et al., 2009). A potential resistance mechanism could consist on allele frequency changes in genes encoding the GluCl channel subunits and/or g-aminobutyric acid (GABA) receptor (Blackhall et al., 2003, 1998). It has been shown that substitutions of single amino acids of GluCl channels from *Cooperia oncophora* and GABA receptors from *H. contortus* could modulate drug (Feng et al., 2002; Njue et al., 2004). Alternatively, IVM treatment may select for some alleles of P-gp that possess a higher binding affinity for IVM, without changing the expression profile of this transporter (Molento and Prichard, 2001).

5. Conclusion

In summary, changes in the transcription level of some P-gp genes in an isolate of adult *H. contortus* resistant to IVM are here reported. However, the biological significance of the observed changes could not be sufficient to explain the high level of IVM resistance displayed by the isolate under assessment. Further studies focusing in *e.g.* examination of different *H. contortus* isolates, P-gp protein expression and activity, and gene polymorphism analysis, are needed in order to elucidate the mechanisms responsible for IVM resistance in *H. contortus* populations.

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Tables

Table 1. Nematode egg per gram counts (EPG, individual and arithmetic mean ± SD) and reduction percentages of faecal egg counts (FECR) with its lower and upper confidence intervals 95%, after the oral administration of ivermectin (IVM, 2 mg/kg) to artificially infected lambs.

Efficacy trials	Animals	EPG Counts		FECR[1] (CI)
		Day -1	Day 14	
First Efficacy Trial	1	10600	840	
	2	3260	2840	
	3	2940	460	
	4	10840	3280	
	5	5360	2240	
	6	5300	800	
	Mean ± SD	**6383 ± 3506**	**1743 ± 1196**	**72.7%** (43-87)
Second Efficacy Trial	1	3880	3500	
	2	7680	1020	
	3	3300	2840	
	4	1840	2680	
	5	8160	5580	
	6	2800	3020	
	Mean ± SD	**4610 ± 2654**	**3107 ± 1475**	**32.6%** (0-64)

[1]FECR estimated according to McKenna (McKenna et al., 1990). CI: lower and upper confidence intervals.

Table 2. Primers and GenBank accession numbers/ Reference for sequences used for quantitative PCR.

Putative gene name	Primer Sequence (5´→3´)	GenBank Accession N°/Reference
H. contortus		
ACT	F: GCTCCCAGCACGATGAAAA	DQ080917
	R: CACCAATCCAGACAGAGTATTTGC	(Kotze and Bagnall, 2006)
Hco-pgp-1	F: GACTTTCAGCTACCCATCACG	HM635772
	R:GTCCGGTTCGTAGAATCTCTC	(Williamson and Wolstenholme, 2012)
Hco-pgp-2	F: CGGCAGCAGATCTCATGGT	AF003908
	R: TCGGTTAGACGAGCTGTGAGATT	(Xu et al., 1998a)
Hco-pgp-3	F: TGAACCTAAGCGTCCGACATG	HM635768
	R: TAGTGATTCCACACAAAGCATCG	(Williamson and Wolstenholme, 2012)

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Hco-pgp-9.1	F: CATCCGGTACGGACGAGAGA	HM635771
	R: TGATTCGTCGAGAGCGCTAGTG	(Williamson and Wolstenholme, 2012)
Hco-pgp-11	F: ACGATCACCACGAAGCTGAACG	HM635770
	R: CCCAAATGCACACCAGAGTG	(Williamson and Wolstenholme, 2012)
Hco-pgp-12	F: CCAAGGCTATTTTCGGGAACG	HM635773
	R: CGTCCAGCAGCAATATCTTGG	(Williamson and Wolstenholme, 2012)

ACTB: actin; bp: base pair; F: Forward; R: Reverse; P-gp: P glycoprotein.

Table 3. Sleuth output of differential expression analysis of *H. contortus* P-gp genes.

P-gp	Target_id	0h1	0h2	12h1	12h2	24h1	24h2	tpm	q-value
P-gp-1	HCOI00146500	402	384	298	319	406	394	6.59	0.146
P-gp-2	HCOI00025600	1273	1145	1605	1469	1400	1507	15.06	0.117
P-gp-3	HCOI00117000	1167	901	418	471	846	827	14.39	0.015
P-gp-9.1	HCOI01671700	511	421	224	260	223	253	6.24	0.024
P-gp-11	HCOI00233200	1268	671	1218	748	1199	1220	15.64	0.876

Columns: 1 P-gp name, 2 Target id: P-gp genes identified in the PRJEB506 *H. contortus* genome, 3-8 Normalized reads counts in adult *Haemonchus contortus* parasites collected from lamb orally treated with IVM at 2 mg/Kg, at 12 and 24 h post-treatment in comparison with parasites (control, 0 h) collected from untreated lambs, 9 tpm: Transcript per million mean expression across all samples, 10 qval: false discovery rate (FDR) adjusted p-value.

Figure captions

Figure 1. Schematic representation of the selection of the *Haemonchus contortus* ivermectin-highly resistant isolate used in the current trial. IVM: ivermectin; FECRT: faecal egg count reduction test; EPG: nematode egg per gram of faeces counts.

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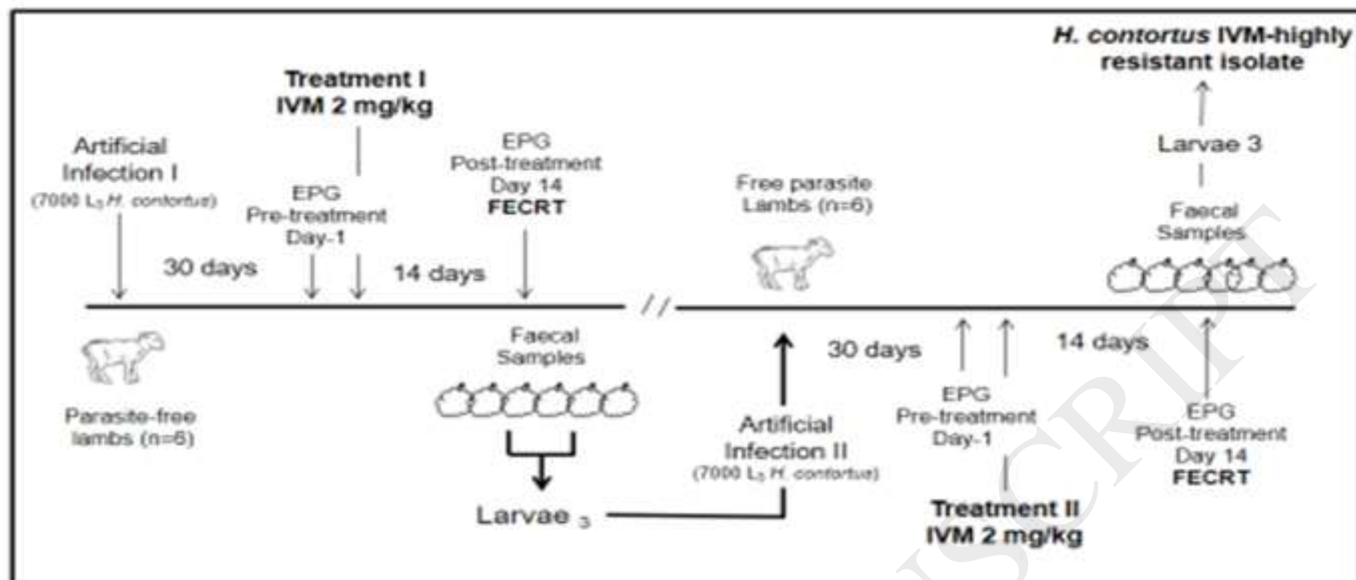

Figure 2. Relative expression of P-glycoprotein mRNA in adult *Haemonchus contortus* parasites collected from lambs orally treated with IVM at 2 mg/Kg, at 6, 12 and 24 h post-treatment in comparison with parasites collected from untreated lambs. Data are expressed as –fold change relative to control which was normalized to one, and reported as the mean±SEM (n=5 pool/group, composed each one of 80 adult female *H. contortus*).

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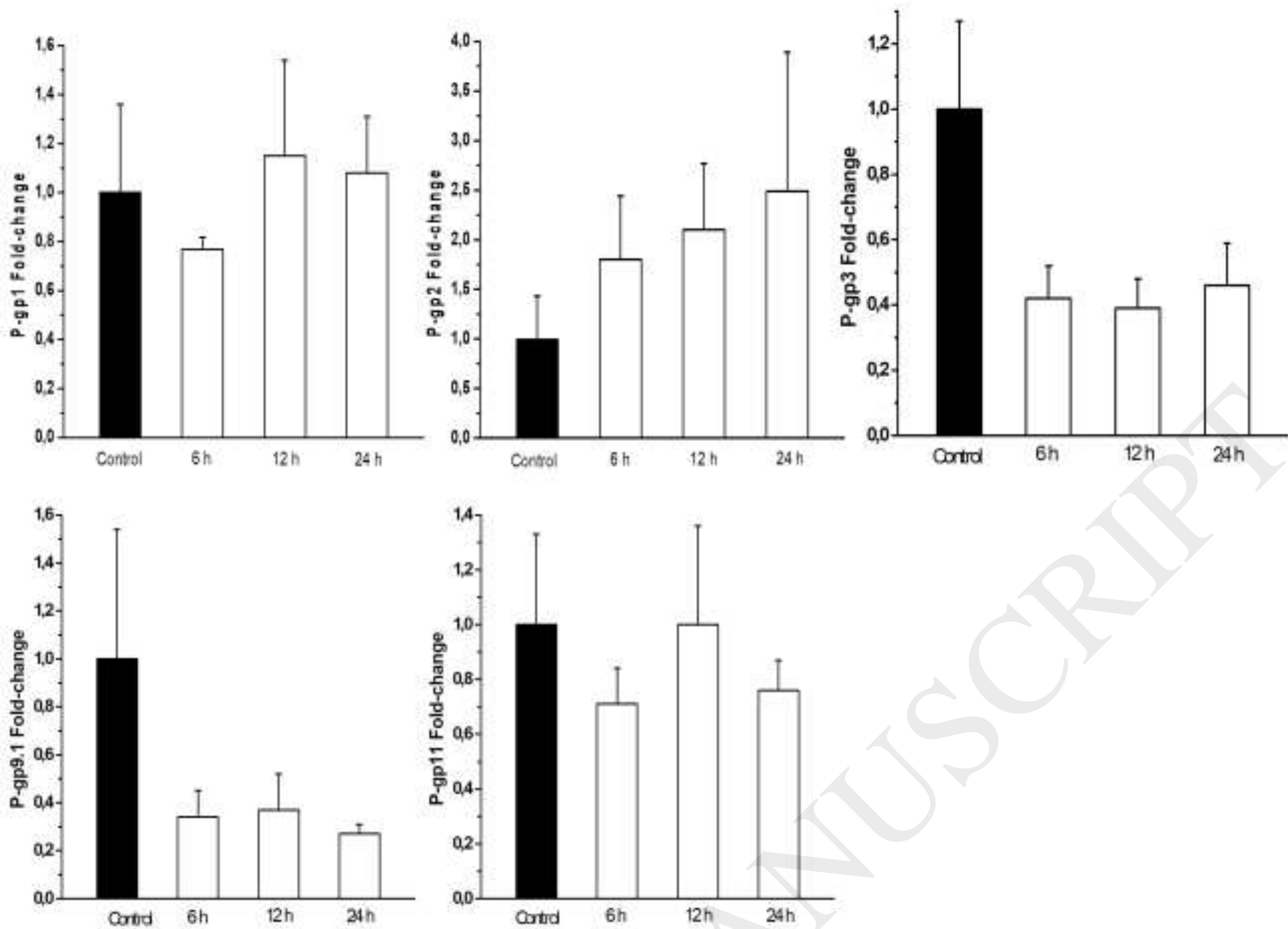

Figure 3. Boxplot showing differences in expression of P-glycoprotein mRNA measured by RNAseq in adult *Haemonchus contortus* parasites collected from lamb orally treated with IVM at 2 mg/Kg, at 12 and 24 h post-treatment in comparison with parasites collected from untreated lambs. Data are expressed as normalized reads count. The intrasampling variance was obtained by 100 bootstrap sample using the Sleuth R package.

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