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ORIGINAL RESEARCH

P-glycoprotein gene expression analysis of ivermectin resistance in sheep naturally infected with *Haemonchus contortus*

Análisis de expresión del gen de la P-glicoproteína en la resistencia a la ivermectina en ovinos naturalmente infectadas con *Haemonchus contortus*

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ABSTRACT

The upregulation of P-glycoprotein genes in *Haemonchus contortus* suspected of ivermectin resistance (IVM-*Res*) was studied in sheep farms. A faecal egg count reduction test (FECRT) was used to evaluate IVM-*Res* on four farms (from A to D). Faecal samples positive to gastrointestinal nematodes (GINs) were used to identify infective larval stages (L_3) pre- and post-IVM treatment by Polymerase Chain Reaction (PCR) test. Ten P-glycoprotein (pgp) genes were analysed to estimate the over expression level in *H. contortus* L_3. Genes were quantified using the Retro Transcription–real time PCR technique. FECRT indicated low IVM efficacy on the four farms from zero to 33% of eggs per gram and *Haemonchus*, *Cooperia* and

Trichostrongylus were the GINs identified after IVM treatment. *H. contortus* isolates displayed different *pgp* gene upregulation levels ($p < 0.05$): *pgp4* from 19.03 to 25.96-fold on four farms; *3* and *16 pgp* from 32.19 to 134.21-fold on three farms; *1*, *2* and *12 pgp* from 7.14 to 56.43-fold on two farms; *10* and *11 pgp* from 6.82 to 32.86-fold on one farm. In conclusion, the examination of FECRT showed low IVM efficacy against GINs. Additionally, *pgp3*, *4* and *16* were the most frequent genes involved in sheep farms with IVM-*Res* problems in relation to IVM resistant *H. contortus*.

Keywords: *Haemonchus,* ivermectin, P-glycoprotein, resistance, sheep

RESUMEN

La sobre-regulación de los genes P-glicoproteínas en *Haemonchus contortus* con sospecha de resistencia a ivermectina (*Res*-IVM) fue estudiada en granjas de ovinos. La prueba de reducción del conteo de huevos (FECRT) fue usada para evaluar *Res*-IVM en cuatro granjas (de A a D). Muestras positivas de heces a nematodos gastrointestinales (NGIs) fueron usadas para identificar larvas infectantes (L_3) pre- y post- tratamiento con IVM por la prueba de reacción en cadena de polimeraza (PCR). Diez genes de P-glicoproteínas (*pgp*) fueron analizados para estimar la sobre expresión en *H. contortus* L_3. Los genes fueron cuantificados por la técnica de Retro Transcripción – tiempo real PCR. La FECRT indicó baja eficacia de IVM para las cuatro granjas de cero a 33% de huevos por gramo y *Haemonchus, Cooperia* y *Trichostrongylus* fueron los NGIs identificados post-tratamiento con IVM. Los aislados de *H. contortus* mostraron diferente nivel de sobre-regulación de los genes *pgp* ($p < 0.05$): *pgp4* de 19.03 a 25.96-veces en cuatro granjas; *pgp3* y *16* de 32.19 a 134.21-veces en tres granjas; *pgp1, 2* y *12* de 7.14 a 56.43-veces en dos granjas y *pgp10* y *11* de 6.82 a 32.86-veces en una granja. En conclusión, la examinación de la FECRT mostró baja eficacia a IVM contra NGIs. Además, *pgp3, 4* y *16* fueron los genes involucrados con mayor frecuencia en las granjas ovinas con problemas de *Res* a IVM.

Palabras clave: *Haemonchus,* ivermectina, P-glicoproteína, ovinos, resistencia

1. INTRODUCTION

Gastrointestinal nematodes (GINs) are a significant problem in small ruminant production (Craig, 2018). GIN damage is caused through larval invasion and their feeding habits in the gastrointestinal tract. Different inflammatory cells are activated to regulate nematode infection and repair tissue damage (Li *et al*., 2007; Craig, 2018; Maza-Lopez *et al*., 2020; Cruz-Tamayo *et al*., 2021). However, susceptible hosts may have acute clinical signs, or they may present persistent parasitic problems that affect their long-term health (Besier *et al*., 2016; Zajac & Garza, 2020). The use of anthelmintics with broad-spectrum drug mechanisms of action can reduce nematode infections, but a problem with the proper use anthelmintic drugs has contributed to reduce their efficacy (Khöler, 2001; Kaplan *et al*., 2020; Martin *et al*., 2021).

Numerous studies have reported nematode resistance to benzimidazole (BZ), imidazothiazole (IMZ) and macrocyclic lactone (ML), and a relatively new anthelmintic, namely monepantel (Kotze & Prichard, 2016; Ploeger & Everts, 2018). In Mexico, the efficacy of BZ, ivermectin (IVM, ML family) and levamisole (IMZ family) has also been reported (Campos *et al*., 1990; Montalvo *et al*., 2006; Becerra-Nava *et al*., 2014). Due to the importance of conserving the efficacy of anthelmintic drugs, diverse studies concerning the mechanisms of action associated with resistance have been performed (Whitaker et al., 2017; Khöler, 2001; Baltrušis *et al*., 2020). For instance, BZ resistance in parasitic nematodes is related to a single nucleotide change in the *β-tubulin* gene localised at codons 167/200 [phenylalanine (T*T*C) to tyrosine (T*A*C)] and 198 [glutamate (G*A*A) to alanine (G*C*A)], as reported previously (Kwa *et al*., 1994; Ghisi *et al*., 2007). In addition, a change at position 200 in *β-tubulin* has frequently been observed in GINs, which is considered an important mutation as it is related to the phenotype BZ-resistant trait in grazing sheep infected with GINs (Silvester & Humbert, 2000; Baltrušis *et al*., 2020, Bartley et al., 2021). In contrast, resistance to ML drugs, specifically IVM, is related to various regulations and changes in genes involved in the transportation of xenobiotics, for instance, increasing the expression of the efflux transporter P-glycoprotein genes (*pgp1, 2, 3, 4, 9, 10, 11, 12, 14* and *16*) and enhancing the metabolism of biotransformation enzymes such as cytochrome oxidase (P-450, CYP14) and glutathione S-transferases (GST-4 and -10), as well as inducing nucleotide changes in the regulator of ligand-gated ion channels (e.g. *GluClc*, *Gly*, *GABA* genes) (Ardelli, 2013; Kellerová *et al*., 2019; Reyes-Guerrero *et al*., 2020; Martin *et al*., 2021). In addition, nematodes such as *Haemonchus*, *Cooperia*, *Trichostrongylus*, *Oesophagostomum* and *Teladorsagia/Ostertagia* have shown high prevalence in temperate and tropical regions (> 80%) and anthelmintic treatment is required (Villa-Mancera & Reynoso-Palomar,2019; Scott *et al*., 2018, 2019; Zajac & Garza, 2020). An appropriate management of anthelmintic drugs in high-risk regions and the study of genes involved in GIN resistance in infected hosts could contribute to a reduction in nematode infections. However, more studies are required considering the importance of preserving the efficacy of anthelmintic drugs, thus, the aim of the present study was to evaluate the relative expression and upregulation of ten functional P-glycoprotein genes probably involved in IVM resistance in four GIN populations on monitored and under anthelmintic treatment sheep farms in Mexico, where *H. contortus* was the main parasite nematode.

2. MATERIALS AND METHODS

2.1. Localisation

Four sheep farms in Puebla State, Mexico, were selected based on the number of naturally infected sheep with GINs and a high suspicion of anthelmintic resistance to IVM. Farms were located in Zacatlán municipality, with a temperate climate – sub humid with a mean annual temperature around 23 °C, located at latitude 18º 50’ 11” N and elevation of 500 m above sea level.

2.2. Animals and farms

Seven hundred and fifty-eight hair sheep, aged six months to two years, naturally infected with GINs, were selected based on two criteria: (1) a minimum number of eggs per gram (EPG) of 200; and (2) the selection of naturally infected sheep treated with anthelmintics 45 days prior to the beginning of this study to avoid residual anthelmintic effects (Coles *et al*., 1992, 2006). Additionally, farmers treated sheep with anthelmintics (BZ or IVM) from 21 days to three months based on the animals' corporal condition, and they did not have a record of which animals were treated (MSci Sara Olazarán-Jenkins, personal communications)

2.3. Faecal egg count reduction test (FECRT) study

The FECRT was conducted following the method described by the World Association for the Advancement of Veterinary Parasitology (Coles *et al*., 1992, 2006). Farmers from four different sheep units from the state of Puebla, Mexico, were invited to participate and agreed to collaborate. Infected sheep were randomly allocated into two groups between 11–14 animals, designated as control and IVM groups. The control group was not treated, and the treated groups received 200 $\mu g\ kg^{-1}$ of IVM (Merck, Laboratory) per kg of body weight subcutaneously on day zero. The EPG per animal and group was determined using the McMaster technique before the beginning of the experiment on day zero and between 11–14 days of the IVM treatments. The McMaster technique was performed in duplicate using 2 g of faeces soaked in a 50-mL plastic tube with 28 mL of salt solution (density 1.2 g/mL). In addition, faecal cultures were assessed before and after treatment per group to identify infective larvae (L_3) of GIN species by molecular identification. Cultures were covered with foil and incubated at 28 °C for seven days. The infective larvae (L_3) of the GIN isolates were recovered from faecal cultures using the migration technique (12 h) (Cedillo-Borda *et al*., 2020).

2.4. Infective larval stages of GINs

Larval stages of *H. contortus* susceptible to IVM were collected from the National Centre of Disciplinary Research in Animal Health and Innocuity of the National Institute of Research in Forestry, Agriculture and Livestock (CENID-SAI, INIFAP) and used as a reference for molecular techniques (Reyes-Guerrero *et al*., 2020). Faecal samples from grazing sheep were collected to quantify the EPG before and after anthelmintic treatment. In addition, the coproculture technique of positive faecal samples to GINs was used to obtain L_3 stages, which were collected by the migration technique (Cedillo-Borda *et al*., 2020). The L_3 were washed with PBS at pH 7.4 and treated with 40% sucrose and 0.187% sodium hypochlorite (Cloralex, Sta. Catarina, Nuevo León, Mexico) to remove detritus and the second larval moult from L_3. Biological material was recovered and transferred into tubes with PBS, pH 7.4 and antibiotic-antimycotic 100X (Invitrogen, Carlsbad CA, USA) for two hours (Contreras-Ochoa *et al*., 2019). Clean larvae were kept at −20 °C to perform the molecular technique.

2.5. Anthelmintic resistance and molecular studies

2.5.1. Genotyping of GIN species

The multiplex polymerase chain reaction (PCR) was used to identify the main nematode species collected from naturally infected sheep in this study. After parasitological

procedures, infective larval stages were identified from two faecal samples at 0 and 14 days from two experimental groups. The sequences used for PCR were specific and obtained from internal and external transcribed spacers of the ribosomal DNA regions to identify the genera *Haemonchus*, *Teladorsagia*, *Oesophagostomum*, *Trichostrongylus* and *Cooperia* (Zarlenga *et al*., 2001). Genomic DNA (gDNA) was purified from ~ 2,000 GIN L_3 per farm using the commercial DNeasy Blood and Tissue kit (Qiagen, Hilden, Germany), following the procedure cited by Cedillo-Borda *et al*. (2020). The gDNA concentration was calculated in a NanoPhotometer NP80 (IMPLEN, Germany), and integrity was visualised via 1.5% agarose gel electrophoresis and ethidium bromide. The PCR reaction was performed with GoTaq® Green Master Mix (Promega, Madison, USA) following the manufacturer's instructions. The PCR assays were performed in a C1000 Touch Thermocycler (Bio-Rad Technologies, Hercules, CA, USA) as described previously by Cedillo-Borda *et al*. (2020). Each genotype fragment for nematode identification was visualised via 3% agarose gel and ethidium bromide.

2.5.2. Resistance to IVM using P-glycoprotein genes on *H. contortus* by RT-qPCR

2.5.2.1. RNA extraction and retro transcription (RT) techniques

Total RNA (RNA_T) was collected from a pool of GINs L_3 including *H. contortus*. All infective larvae were treated with TRIzol (Invitrogen, Carlsbad CA, USA), following the manufacturer's instructions. Previously, larvae were disrupted at 400 rpm for 40 s using 1-mm zirconium beads twice in a BeadBug Microtube D1030 (Benchmark Scientific, South Plainfield, USA). The RNA_T concentration was calculated by spectrophotometry using a NanoPhotometer NP80 (IMPLEN, USA); RNA_T integrity was visualised by ethidium bromide stained via 2.5% agarose gel electrophoresis. Subsequently, RT was performed for cDNA synthesis at 300 ng of RNA_T concentration, using the commercial product, ImProm-II Reverse Transcription System® (Promega, Madison, USA), following the manufacturer's instructions and the procedure of Cedillo-Borda *et a*l. (2020).

2.5.2.2. RT-real-time PCR (qPCR) assays

Nucleotide sequences were synthesised from partial encoding domain sequences localised in the union of nucleotides from *pgp* genes specific for *H. contortus* (*Hco-pgp).* The RT-qPCR assays were performed in triplicate per gene for each farm, including the *H. contortus* susceptible isolate as reference (Reyes-Guerrero *et al*., 2020). The qPCR reaction for each *Hco-pgp* and housekeeping (HK, *β-tubulin* and *GAPDH*) gene was carried out at a final volume of 20 µL in 0.2-mL microtubes. The reaction was prepared using GoTaq® RT-qPCR Master Mix (Promega, Madison USA), 20 µM oligonucleotides and 300 ng of cDNA. The PCR conditions were as follows: initial denaturation at 95 °C for 5 min, followed by 40 cycles of denaturation at 95 °C for 10 s, annealing at 63 °C for 15 s and extension at 72 °C for 20 s. The procedure ended with a step to determine the dissociation temperature increasing from 65 to 95 °C (Cedillo-Borda *et al*. 2020). The RT-qPCR was carried out in at Rotor-Gene 6000 thermocycler (Corbet Research, Hilden, Germany) to amplify PCR products at 470 and 510 nm. Oligonucleotide sequences of *Hco-pgp* and HK genes used in the present study were obtained from Cedillo-Borda *et al*. (2020).

2.5.2.3. Relative expression of *Hco-pgp* genes

For relative expression, the comparative threshold cycle (C_T) method was used (Pfaffl, 2001). The gene expression analysis was based on the number of cycles at which the amplification plot crossed the threshold. Raw data from *H. contortus* isolates collected from each farm and the reference were analysed to obtain the fold change and the p-value. The C_T value for *β-tubulin* and *GAPDH* genes was subtracted from the C_T value for each target expression gene to normalise the mRNA content and provide a relative expression value for each *pgp* gene. This value was defined as ΔC_T. To assess the effects of IVM, the means of ΔC_T from *H. contortus* isolates from each farm were subtracted from the mean ΔC_T control group, identified as *H. contortus* susceptible to IVM. These values were defined as $\Delta\Delta C_T$. The relative fold increases or decreases were then calculated as $2^{\Delta\Delta CT}$. The fold change was analysed using the Student's t-test ($p = 0.05$), using the ΔΔCT for each replicate (n = 3) of each target gene, HK genes and negative control group via the Qiagen GeneGlobe platform (https://www.qiagen.com).

3. RESULTS

3.1. Faecal egg count reduction test (FECRT) and nematode identification

According to the FECRT analyses, four farms in the municipality of Zacatlán showed low percentages of IVM nematocidal efficacy (< 50%), and some farms displayed zero reduction of EPG after anthelmintic treatments (Table 1). However, after anthelmintic treatments, *Haemonchus* was the main identified nematode resistant to IVM on all farms, followed by *Cooperia* and *Trichostrongylus* species identified on three sheep farms. In contrast, no resistance to the IVM anthelmintic was observed in *Teladorsagia* and *Oesophagostomum* nematode species.

3.2. Expression level of *pgp* genes for *H. contortus* L_3

Comparative analysis of *pgp* genes to estimate the relative expression levels between isolates of *H. contortus* resistant and susceptible to IVM drugs displayed significant upregulation ($p < 0.05$) on different sheep farms per gene (Table 2) (Figs. 1–2). Gene *pgp4* was identified on four farms, *pgp3* and *16* on three farms, associated with IVM-*Res*, followed by *pgp1*, *2* and *12* on two farms and *pgp10* and *11* genes on one farm. The expression of genes*9* and *14* was not significantly ($p > 0.05$) associated with IVM-*Res*. It should be mentioned that the *pgp4* gene displayed an important relation with the four farms with a severe IVM-*Res* percentage observed through FECRT, followed by the *pgp3* and *16* genes identified on three sheep farms with a low reduction of EPG and one farm showing IVM-*Res*.

Table 1. Faecal egg count reduction test recorded in control and ivermectin treated groups of sheep infected with gastrointestinal nematodes from four farms in Puebla, Mexico.

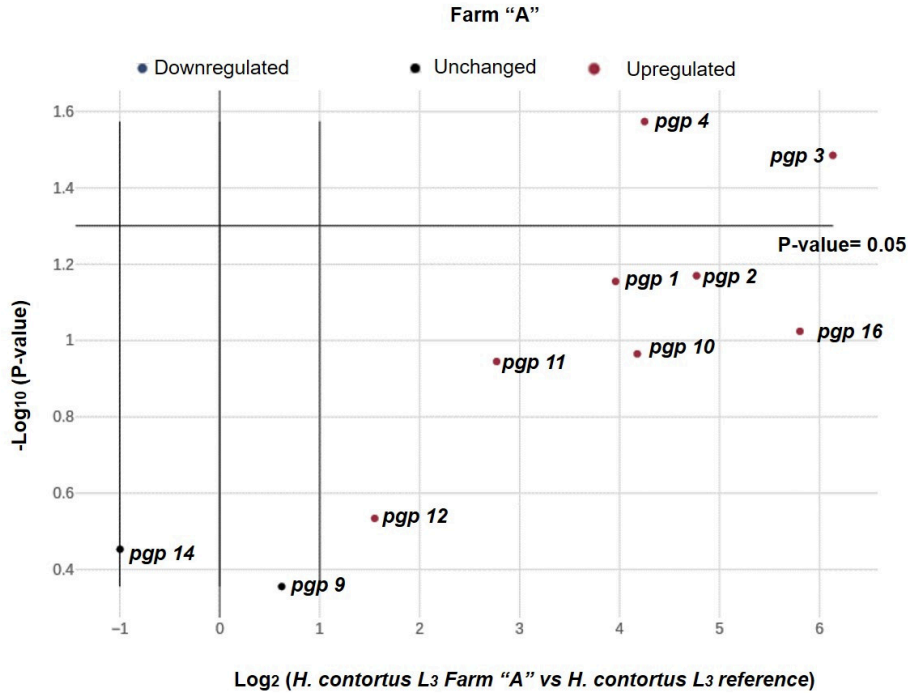
Ivermectin treatments (Macrocyclic Lactone)					
Farms	Control	Treated	% EPG reduction	I.C. 95 % Upper-Lower limits	Phenotype
	n = 11 - 14				
	EPG				
A	236.36 ± 352.20	559.09 ± 1111.49	0.00	51.00 – 0.00	*Res*
B	336.36 ± 571.44	254.54 ± 395.89	24.00	82.00 – 0.00	*Res*
C	3500.00 ± 6366.79	4000.00 ± 4949.34	0.00	72.00 – 0.00	*Res*
D	2642.86 ± 2767.31	1761.53 ± 2536.00	33.00	76.00 – 0.00	*Res*

n = number of sheep per group; ***Res*** = resistant; **C.I. 95%** = 95% confidence interval; **EPG** = number of eggs per gram

Table 2. Relative expression level of ten P-glycoprotein genes of infective larval stages of *Haemonchus contortus* isolates from four sheep farms suspicious to ivermectin.

Relative expression level (fold-change)					
Hco-pgp genes	Farms				*p-gp* genes upregulated level per farm
	A	B	C	D	*p < 0.05*
1	**15.56**	**53.51***	**39.08***	**36.97**	2
2	**27.28**	**56.43***	**28.21***	**42.47**	2
3	**70.20***	**124.64***	**42.47***	**92.09**	3
4	**19.03***	**47.12***	**32.63***	**25.96***	4
9	1.54	**4.31**	1.90	**2.32**	0
10	**18.08**	**4967.60**	**87.53***	**15.94**	1
11	**6.82**	**13.88**	**11.20**	**32.86***	1
12	**2.93**	**8.03***	**8.47**	**7.14***	2
14	0.50	0.82	*0.25*	*0.48*	0
16	**55.84**	**134.21***	**53.88***	**32.19***	3

Hco-pgp = *Haemonchus contortus* P-glycoprotein genes; **bold number** = upregulation of the expression level; **italic number** = downregulation of the expression level; * = *p < 0.05*



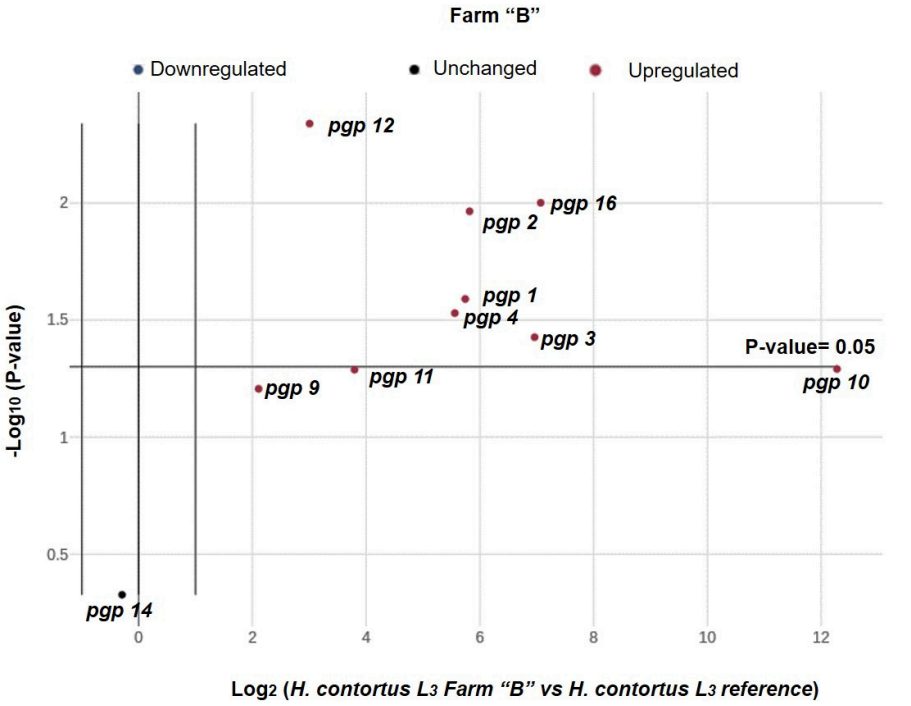


Fig. 1. Relative quantification of mRNA transcripts from *pgp* genes in *H. contortus* L_3 from farms A and B. RT-qPCR assays were performed in triplicate from three independent experiments. The expression level of *pgp* showed variability. Differences were considered statistically significant when $p < 0.05$.

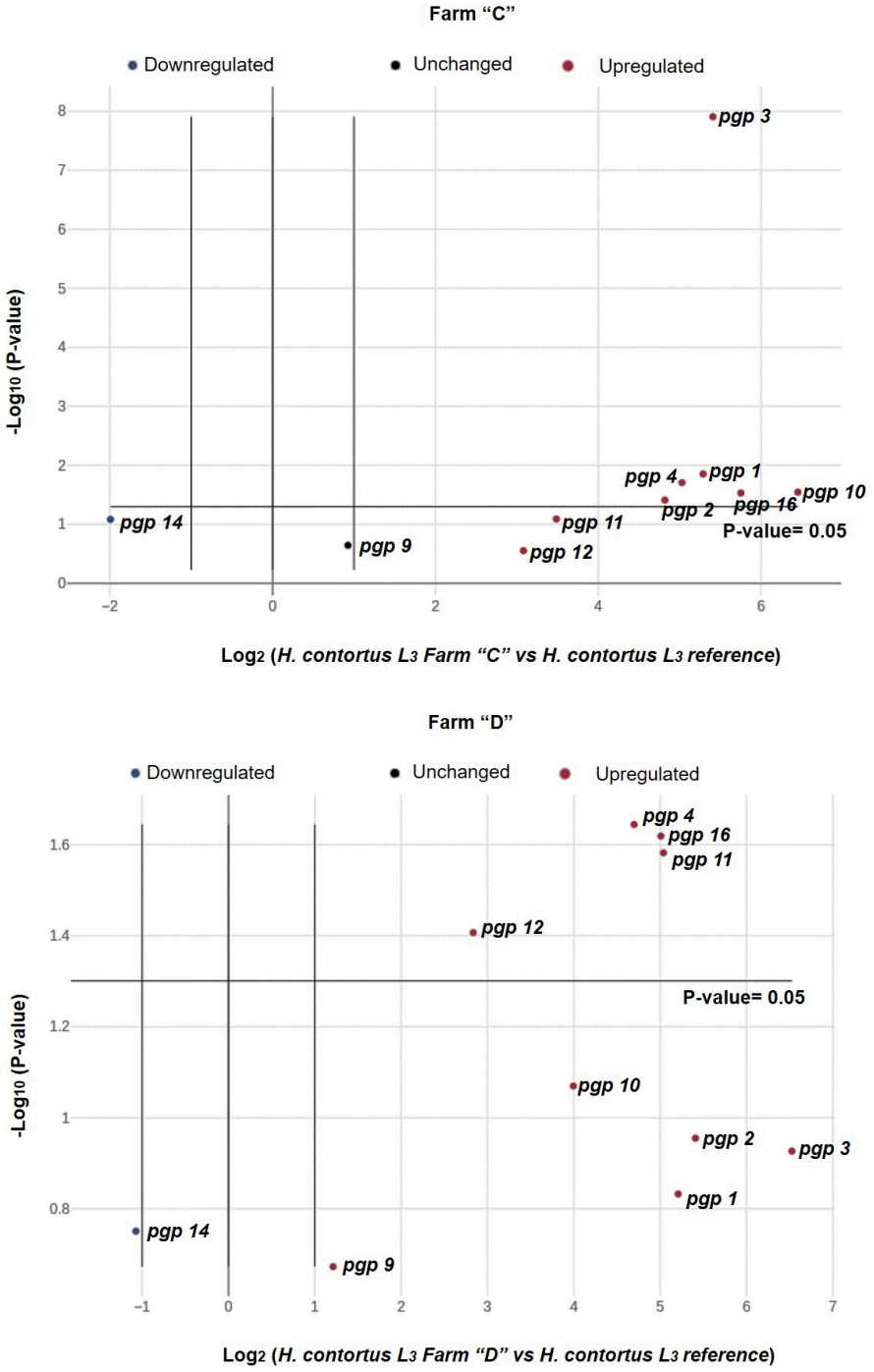


Fig. 2. Relative quantification of mRNA transcripts from *pgp* genes in *H. contortus* L_3 from farms C and D. RT-qPCR assays was performed in triplicate from three independent experiments. The expression level of *pgp* showed variability. Differences were considered statistically significant when $p < 0.05$.

4. DISCUSSION

The widespread issue of anthelmintic resistance is a concern in the livestock industry due to the infection problem of GINs for grazing ruminants (Villa-Mancera & Reynoso-Palomares, 2019; Charlier *et al*., 2020; Zajac & Garza, 2020). Although diverse commercial anthelmintic families are currently on the market against parasites, farmers frequently use IVM or BZ because of their broad efficacy, for instance, IVM against nematodes and ectoparasites (e.g. *Amblyoma*, *Demodex*, etc.), and BZ against trematodes (e.g. *Fasciola*), cestodes (e.g. *Taenia*), nematodes (e.g. *Haemochus*) and protozoa (e.g. *Eimeria*, *Giardia*, *Trypaonosama*) (Atez-Alagoz, 2016; Velázquez-López et al., 2016; Martin *et al*., 2021). However, this advantage may also represent a problem due to the anthelmintic problem observed in most GINs in ruminants. In the present study, our results confirmed the low efficacy of IVM to reduce GINs by means of the FECRT (EPG reduction range from 0 to 33%) on four sheep farms localised in temperate climates; where the frequent use of anthelmintic treatments is necessary (Besier *et al.* 2016; Villa-Mancera & Reynoso-Palomar, 2019). These data indicate the importance of still monitoring the IVM-*Res* in GINs that affect small and large ruminants not only to conserve the efficacy of IVM, but also because IVM has been indicated as a P-glycoprotein substrate and an interaction at this level should be considered as a possible molecular marker. The upregulation is one of the main molecular changes associated with IVM-Res (Martin *et al*., 2021). The IVM upregulation of *pgp3*, *4* and *16*, as main genes in GIN populations with a resistance phenotype to IVM, suggests genetic functions to induce resistance in the *H. contortus* isolates localised in Zacatlán, Puebla, Mexico. Of these three *pgp* genes, the *pgp16* has been associated with IVM resistance in other *H. contortus* isolates (on larval or adult stages) and *Cooperia oncophora* (a cattle GIN) resistant to IVM, and also the equine nematode *Parascaris* sp. (Godoy *et al*., 2015; Raza *et al*., 2016; Tydén *et al*., 2014; Martin *et al.,* 2021). Although, differences in the expression level of *pgp16* between larvae and adults of *H. contortus* were observed with higher levels in adult stages than larvae; larval stages may be important to support a kind of diagnosis for farmers in Mexico (Tydén *et al*., 2014, Reyes-Guerrero *et al*., 2020). For instance, the *pgp*16 had the highest level of expression among the *pgp* under study on all sheep farms with IVM problems, suggesting its importance in these *H. contortus* populations. On the other hand, the active function of *pgp3* and *4* genes in *H. contortus* was observed in a previous study after exposure of larval stages to different concentrations of levamisole anthelmintic, showing resistance to this drug (Sarai *et al*., 2013). These data are interesting since P-glycoprotein is a multidrug transporter from a wide range of compounds (e.g. levamisole and IVM), which is advantageous, but it can also be a disadvantage and may interfere with the delivery of drugs to target tissues resulting in multidrug resistance. Therefore, in the present study, we were looking for specific *pgp* genes with major possibilities of being upregulated in *H. contortus* L_3 resistant to IVM as one of the main anthelmintic drugs used by farmers, because we know that more studies are necessary to understand these functions in Mexican populations. Thus, other *pgp* genes (*1*, *2*, *10*, *11* and *12)* identified on farms A and B were previously mentioned in different GIN species and isolates such as *H. contortus*, *C. oncophora* and *Parascaris* spp. with reduced efficacy of IVM similar to this study (Areskog *et al*., 2013; Janssen *et al*., 2013; Godoy *et al*., 2015, 2016; Jesudoss Chelladurai & Brewer, 2019; Gerhard *et al*., 2020). To improve IVM efficacy as well as

the conservation of other anthelmintic drugs, genes involved in resistance are under interesting studies, using different biological models including nematode genome and transcriptome, etc., to identify those molecular mechanisms that could contribute to inhibit or reduce the resistance problem (Kotze & Pritchard, 2016; Jiang *et al*., 2013; Ménez *et al*., 2019). Reduced efficacy of IVM on *H. contortus*, *Cooperia* spp. and *Trichostrongylus* spp. was observed in this study, and in previous studies these species have been noted as the dominant species in temperate and tropical areas in small grazing ruminants (Alcalá-Canto *et al*., 2016; Craigh, 2018; Claerebout *et al*., 2020; Ramos *et al*., 2020). Although an understanding of IVM requires more information, considering the *pgp* genes and other genes (e.g. *GluCl*$^{-}$ and *GlyCl*$^{-}$ and cytochrome oxidase genes) related to IVM resistance in parasitic nematodes, it is important to still monitor those genes that appear relevant and contribute in the study of control (Kotze & Pritchard, 2016; Kellerová *et al*., 2019). Thus, this study provides information concerning genes involved in specific drug resistance that might support future research to increase the efflux of drugs in order to improve deworming programmes.

The examination of IVM resistance on GINs was confirmed in all experimental sheep farms through FECRT, the only diagnostic currently used in the field; however, it has low sensitivity (75%). Therefore, the study of IVM resistance using molecular tools contributes to identifying the nematode species involved after IVM treatment and to evaluate the level of upregulation that three *pgp* genes ($p < 0.05$) displayed on all sheep farms. Our results contribute to the research of *pgp* genes that could be important such as *pgp16* that were notified in an autochthonous *Haemonchus* resistant isolate from Chiapas, Mexico or from those GINs resistant to IVM related to *pgp3* and *4*, previously notified. Regarding IVM and *pgp* genes, more evidence for the present study is required due to the different *pgp* relative expression analysis. Recently, the studies of differential expression analyses between parasitic nematode isolates using RNAseq are trying to identify *pgp* and other genes implicated as candidates in anthelmintic resistance genes and in other important functions on parasitic nematodes.

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CONFLICT OF INTEREST

The authors declare that they have no conflicts of interest.

ETHICAL STATEMENT

Blood and faecal samples were collected from infected and non-infected sheep according to the Mexican Ethical Norms for Animal Health NOM-051-ZOO-1995 and NOM-062-ZOO-1995 (http://www.gob.mx/senasica) as well as the Federal Law of

Animal Health, DOF 07-06-2012 (https://www.diputados.gob.mx/LeyesBiblio/ref/lfsa.htm).

CREDIT AUTHOR STATEMENT

Lopez-Arellano ME & Olazaran-Jenkins S: conception, investigation, designed the present study, writing – original draft and funding acquisition. Bonilla-Suarez HA & Reyes-Guerrero DE: methodology, formal analysis, validation, and writing- original manuscript. Olmedo-Juárez A: statistical support and writing. Maza-Lopez J: methodology. Mendoza-de-Gives P: Validation, manuscript review and editing.

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