Veterinary Parasitology 267 (2019) 75–83

Contents lists available at ScienceDirect

Veterinary Parasitology

journal homepage: www.elsevier.com/locate/vetpar

Research paper

Detection and quantification of *Parascaris* P-glycoprotein drug transporter expression with a novel mRNA hybridization technique

Jeba Jesudoss Chelladurai, Matthew T. Brewer*

Department of Veterinary Pathology, Iowa State University College of Veterinary Medicine, Ames, IA, USA

ARTICLE INFO

Keywords:
Parascaris
P-glycoprotein
Drug efflux
mRNA localization

ABSTRACT

Macrocyclic lactone-resistant *Parascaris* have been reported throughout the world. In part, the drug resistant phenotype is hypothesized to be associated with ATP-binding cassette transporters known as P-glycoproteins. In many systems, P-glycoproteins efflux drugs out of cells thereby precluding drug binding to target receptors. *Parascaris* may evade macrocyclic lactone-mediated death by effluxing drugs away from target receptors in the nervous system. Alternatively, P-glycoprotein expression in the gut or body wall could prevent penetration of drugs into the body of the parasite altogether. In the present study, we evaluate expression of *Peq-pgp-11* and *Peq-pgp-16* using a novel multiple nucleic acid hybridization method. This method allowed for visualization of individual mRNA transcripts within fixed tissue sections of *Parascaris* adults. Our investigation revealed expression of *Peq-pgp-11* and *Peq-pgp-16* in the intestine, body wall, nerves, lateral cords, and reproductive tissues of male and female parasites. These results suggest that Pgp could efflux drugs locally at the level of parasite neuronal tissue as well as at sites of entry for drugs such as the hypodermis and intestine. The multiple nucleic acid hybridization method could be useful for providing tissue context for gene expression in a variety of nematode parasites.

1. Introduction

Parascaris spp. is a cosmopolitan ascarid nematode that inhabits the small intestine of equids. These large nematodes reach up to 30 cm in length and are known to cause malnutrition and intestinal impaction when present in large numbers. Unfortunately, this parasite has developed resistance to macrocyclic lactone (ML) anthelmintics including ivermectin and moxidectin (Boersema et al., 2002). The first report of drug resistance in the United States was published in 2007 (Craig et al., 2007). Resistance is now considered to be relatively widespread in Europe and North America (Peregrine et al., 2014). Drug resistance has also been reported in *Parascaris* in South America (Molento et al., 2008), Oceania (Beasley et al., 2015; Bishop et al., 2014) and Asia (Shah et al., 2016). These findings have prompted the implementation of measures designed to delay the development of resistance in susceptible populations (Nielsen, 2016).

The ML group of anthelmintics includes the avermectins and milbemycins. These agents selectively act on glutamate-gated chloride channels of invertebrates causing a slow and permanent state of hyperpolarization due to excessive chloride ion influx leading to paralysis (Wolstenholme, 2012). The effects are concentration-dependent, but species-specific differences in potency due to biochemical and pharmacological differences have been demonstrated (Geary and Moreno, 2012). Resistance to the ML anthelmintics has often been attributed to mutations and decreased expression of glutamate-gated chloride channels (Whittaker et al., 2017). However, an understanding of other mechanisms of resistance is beginning to emerge, and includes metabolism by cytochrome P450 (Riga et al., 2014), and efflux due to overexpression by ABCB1 transporter family (James and Davey, 2009; Xu et al., 1998).

For several parasites of veterinary importance, there is a growing body of evidence that resistance to ML is, at least in part, mediated by ABC transporters (P-glycoproteins) (Whittaker et al., 2017). P-glycoproteins (Pgps) are members of the ATP-binding cassette transporter family that are well known to modulate drug resistance in bacteria and neoplastic tissues (Davidson and Chen, 2004; Licht et al., 1994). In helminths, Pgps are thought to contribute to drug resistance by effluxing anthelmintics away from their molecular target. Pgps associated with ML resistance have been characterized in several nematode parasites including the trichostrongyle *Haemonchus contortus* (Godoy et al., 2015a, 2016; Godoy et al., 2015b; Xu et al., 1998), cyathostomins (Drogemuller et al., 2004; Kaschny et al., 2015), and the filarid *Dirofilaria immitis* (Bourguinat et al., 2016; Mani et al., 2016).

Recent evidence suggests that increased levels of Pgp expression are

* Corresponding author at: Department of Veterinary Pathology, 2758 Vet Med, 1800 Christensen Dr., Ames, IA, 50011, USA.
E-mail address: brewermt@iastate.edu (M.T. Brewer).

https://doi.org/10.1016/j.vetpar.2019.02.002
Received 22 October 2018; Received in revised form 28 January 2019; Accepted 1 February 2019

highly correlated with reduced susceptibility of *Parascaris* to MLs (Janssen et al., 2013). Two Pgp genes, *Peq-Pgp-11* and *Peq-pgp-16*, have been identified. Of these two Pgp genes, it appears that *Peq-pgp-11* is more strongly associated with resistance (Janssen et al., 2013). Decreased susceptibility to MLs has been demonstrated with transgenically expressed *Peq-pgp-11* in *C. elegans* (Janssen et al., 2015). It is thought that the ML resistant phenotype occurs when expression of *Peq-pgp-11* is high, however, the factors regulating this expression are not well characterized (Nielsen et al., 2014).

Pgp-11 orthologs that alter susceptibility to MLs have been shown to occur in other parasitic nematodes including *D. immitis* (Mani et al., 2016), *Cooperia oncophora* (De Graef et al., 2013), *H. contortus* (Raza et al., 2016) and non-parasitic *Caenorhabditis elegans* (Bygarski et al., 2014). *Pgp-16* orthologs occur in *H. contortus* (Godoy et al., 2015a) and in *C. oncophora* (Demeler et al., 2013; Tydén et al., 2014) with expression levels increased after ML exposure in the latter. In contrast *Pgp-16* orthologs were not found in *C. elegans* (Issouf et al., 2014).

In *Parascaris,* tissue-specific expression of Pgp has been investigated by dissecting worm tissues and amplifying expressed genes by PCR (Janssen et al., 2013). However, this preparation method precludes making clean preparations of tortuous linear organs such as the testes (Janssen et al., 2013), and makes studying localization in nerve cords, and lateral cords impossible due to their small size. To overcome these obstacles, expression of Pgps can be studied *in situ* in sections of worm tissue that allow the preservation of anatomy and tissue architecture.

We examined Pgp expression using an *in situ* multiple nucleic acid RNA hybridization method that allowed visualization of individual mRNA transcripts through a novel signal amplification technique (Wang et al., 2012). This method utilizes a double Z probe design based on a target mRNA with the base of the Z having a complementary binding sequence. The 18- to 25- nucleotide base and the 14- nucleotide tail of the Z are connected by a spacer. Binding of two adjacent Z probes allows the formation of a 28 nucleotide binding site made of two tails of the Z probes. A preamplifier binds to the site, and is turn bound by an amplifier, which is bound by an alkaline phosphatase labeled signal amplifying probe. A chromogenic substrate is added that allows visualization. The chromogenic signal, indicating an individual mRNA transcript, can be viewed as a punctate dot by light microscopy. The aim of this present study was to determine the tissue distribution of *Peq-pgp-11* and *Peq-pgp-16* as determined by multiple nucleic acid RNA hybridization.

2. Materials and methods

2.1. Parasites

Adult male and female *Parascaris* were obtained opportunistically from foals necropsied at the College of Veterinary Medicine, Iowa State University. All procedures were conducted in accordance with applicable institutional animal care and use committee protocols and guidelines.

Adult worms were fixed in 10% neutral buffered formalin (NBF) (Fisher Scientific, NJ) for 22 h. Ten percent NBF was injected into the worms to fix internal organs, and the whole worms were placed in 10% NBF. Four segments from each of three body regions (anterior, middle, posterior) were excised from a male and female worm and embedded in paraffin blocks. Five μm tissue sections were prepared on microscope slides by standard histological procedures in the Department of Veterinary Pathology at Iowa State University.

2.2. Probe targets

Probes for chromogenic multiple nucleic acid *in situ* mRNA hybridization were obtained from Advanced Cell Diagnostics (Hayward, CA). Multiple nucleic acid probes targeting the nucleotides 687–2489 of *Peq-pgp-11* (GenBank Accession number JX308230.1) and nucleotides 2592–3541 of *Peq-pgp-16* (GenBank Accession number JX308231.1) were used. A positive control probe targeted the nucleotides 5–1305 of *Peq-beta-tubulin* (GenBank Accession number JN034256.1), a component of the eukaryotic cytoskeleton. This probe could also weakly bind isotype 2 of *P. equorum* β-tubulin (GenBank Accession number KC713798.1). A negative control probe was designed to target the nucleotides 414–862 of the dapB sequence of *Bacillus subtilis* (GenBank Accession number EF191515), encoding the bacterial enzyme dihydrodipicolinate reductase. Proprietary programs were used to assure target probe specificity. Probes were stored at 4 °C and used according to the manufacturer's protocol.

Chromogenic *in situ* mRNA hybridization was performed using RNAscope 2.5 HD Assay - Red reagents (Advanced Cell Diagnostics, Hayward, CA) on 5 μm thick sections of the adult worms, and mounted according to the manufacturer's protocol. Fast Red was used as substrate for alkaline phosphatase in the chromogenic reaction.

2.3. Analysis

Photomicrographs were obtained on Olympus BX40 and BX60 microscopes with a Olympus DP70 camera using CellSens software (Olympus, Waltham, MA). The intestine, body wall, lateral cords with excretory canals, dorsal, and ventral nerve cords were examined. The ovaries, uteri, and testes were examined when present. Signal was measured from n = 5 images from each of 3 tissue sections obtained from each body region (anterior, middle, posterior). Hybridization signal was resolved as punctate, stained spots located within parasite tissues. Areas of positive signal were measured using the ISH 2.2/RNAscope module of HALO image analysis software (Indica Labs, Advanced Cell Diagnostics, Hayward, CA). Ratio of probe hybridization area to total tissue area was calculated and used for data analysis. Differences in expression levels were compared among tissues as well as within individual tissues across anterior, middle and posterior of the worms by one-way ANOVA with Tukey-Kramer HSD (Honestly Significant Difference) post-hoc test on SAS JMP Pro 12.

3. Results

3.1. Multiple nucleic acid hybridization is sensitive and specific for Parascaris pgp

To characterize the tissue-specific expression of *Peq-pgp-11* and *Peq-pgp-16*, adult worm tissues were analyzed with a multiple nucleic acid hybridization technique. Multiple nucleic acid hybridization allows for specific signal amplification, with low background, resulting in individual mRNA transcripts appearing as distinct spots in tissue sections. Probes binding *Bacillus subtilis* DapB was used as the negative control for the assay. No positive signal indicative of DapB mRNA was found in any of the organs examined (Fig. S1). *Parascaris* β-tubulin was used as the positive control for the assay. Positive signal indicative of β-tubulin mRNA was found to be highly expressed in all the organs examined (Fig. S1).

3.2. Qualitative analysis of Peq-pgp-11 and Peq-Pgp-16 mRNA expressed in Parascaris

Positive signal indicative of *Peq-pgp-11* mRNA and *Peq-pgp-16* mRNA visualized as red-pink dots were found in a variety of parasite tissues. Histologic features and location of mRNA hybridization are presented in Fig. 1(male) and Fig. 2(female) and described below.

3.2.1. Body wall

The ascarid body wall is typically composed of three distinct layers - a cuticle, hypodermis and a single layer of coelomyarian muscle cells (Watson, 1965). Red-pink dots indicative of *Peq-pgp-11* and *Peq-pgp-16* mRNA were visualized in the hypodermis and in the muscle cells, but

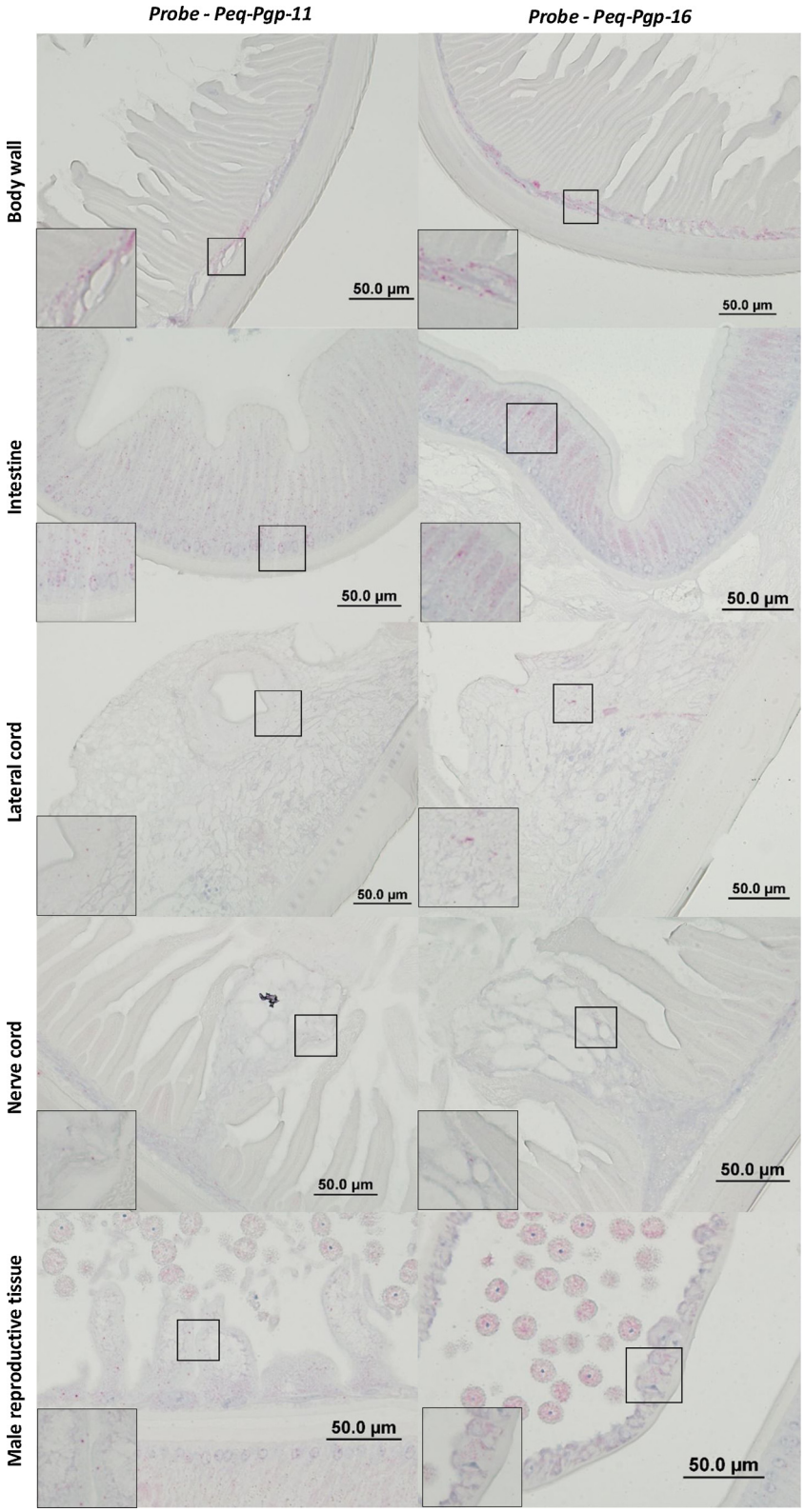

Fig. 1. Representative images of adult male *Parascaris*. Positive signal resulting from probe hybridization appeared as red punctate dots. Black boxes indicate the location of high magnification insets. A description of staining in specific tissues is given in Section 3.2. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article).

not in the cuticle in sections of male and female worms.

3.2.2. Intestine

Intestines of ascarids consist of a single layer of epithelial cells with an apical microvilli/ bacillary layer and a layer of dense cytoplasm on the luminal side called a plasma cap. The basal part has a basal lamella and an outer mesenteric membrane. Brown granular inclusions were present in sections of the intestines, typical of the ascarid gut (Kessel

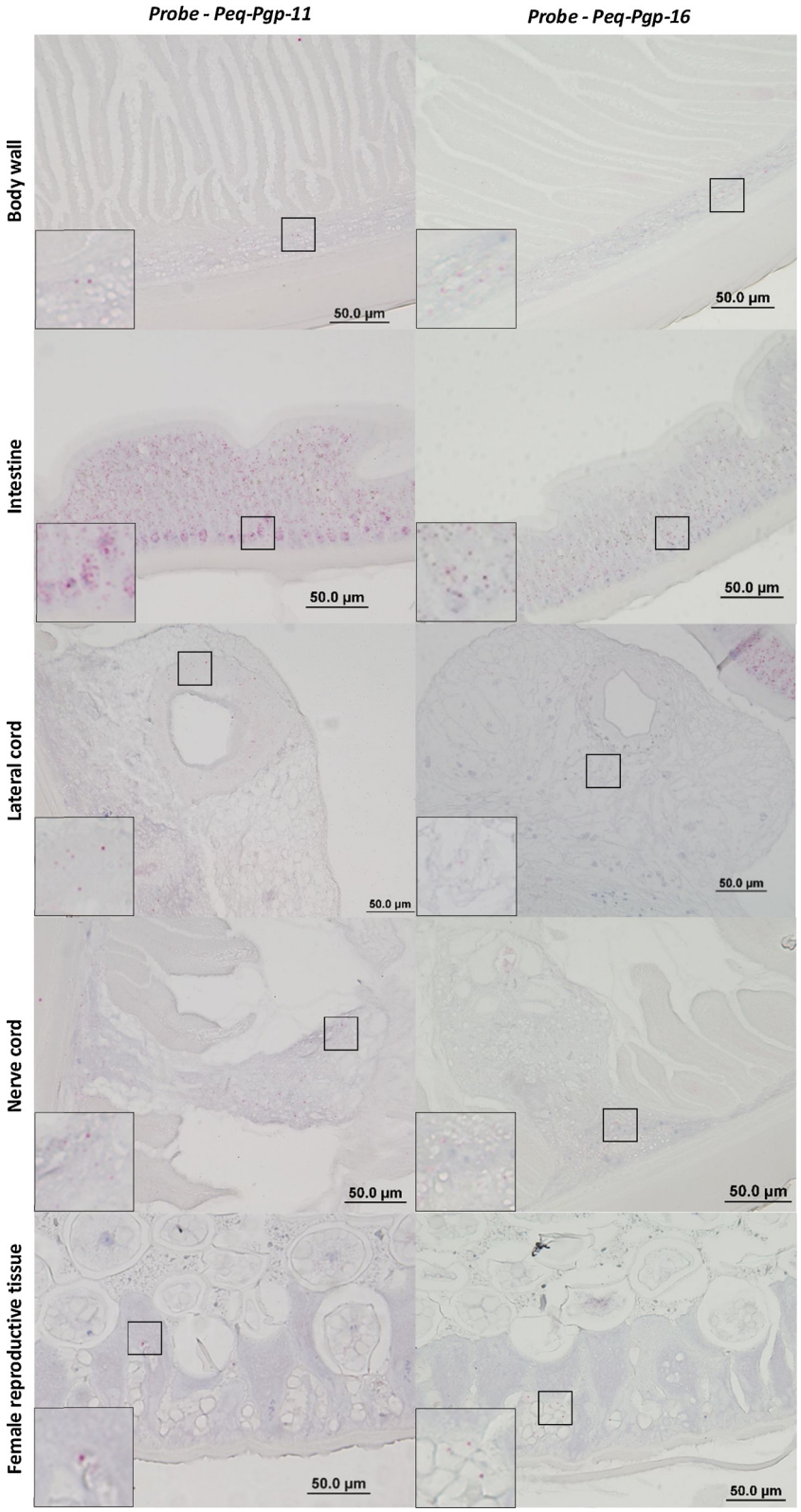

Fig. 2. Representative images of adult female *Parascaris*. Positive signal resulting from probe hybridization appeared as red punctate dots. Black boxes indicate the location of high magnification insets. A description of staining in specific tissues is given in Section 3.2. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article).

et al., 1961). These features are of note so as not to confuse them with nucleic acid hybridization signal. *Peq-pgp-11* and *Peq-pgp-16* mRNA was visualized in moderate numbers throughout the cell including around the nuclear envelope, but was absent at the microvilli, plasma cap, basal lamella and mesenteric membrane in both male and female worms.

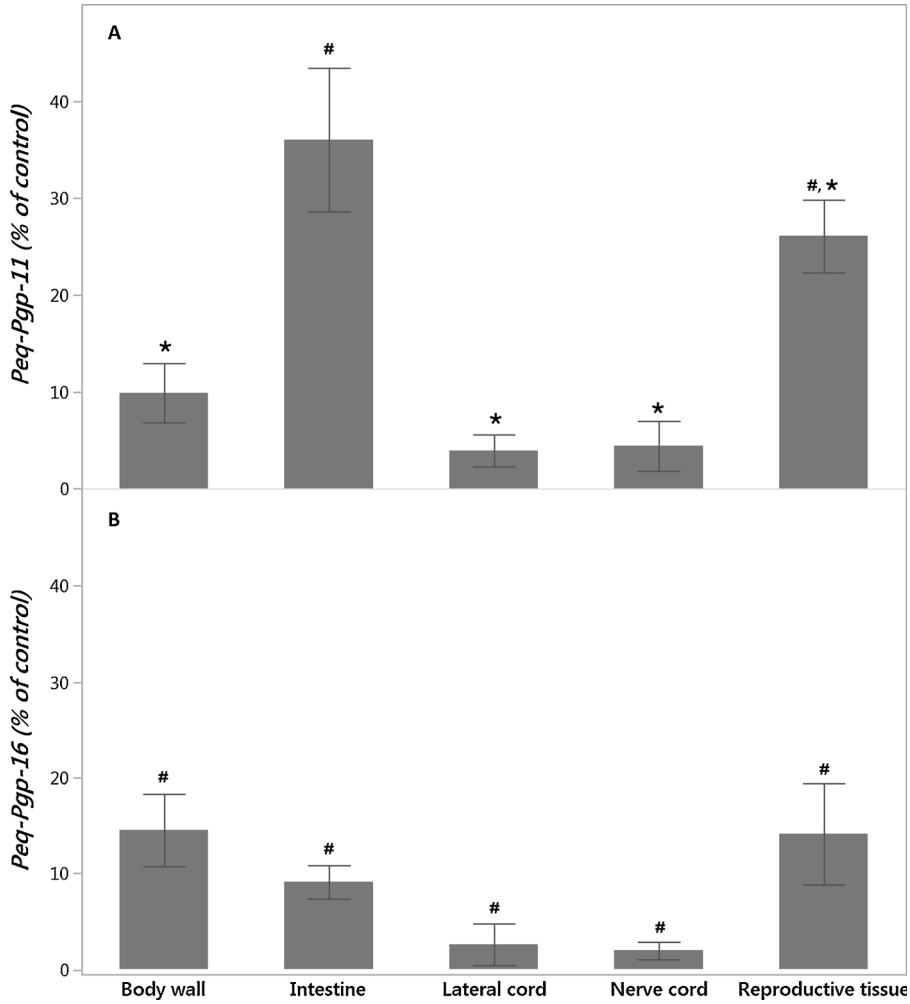

Fig. 3. Expression of (A) *Peq-pgp-11* and (B) *Peq-pgp-16* in adult male *Parascaris*. Values represent percent area of the tissue with positive staining relative to beta-tubulin ± S.E.M. Columns with different symbols are significantly different ($p < 0.05$).

3.2.3. Lateral cords

Ascarids have a H-shaped excretory system that is embedded in the lateral lines, and extend from the nerve ring to about the middle of the body as a continuous canal with a lumen, after which the canal appears to degenerate. The lateral line tissue also contributes to this drainage through the many intercellular spaces that it contains (Dankwarth, 1971). *Peq-pgp-11* mRNA and *Peq-pgp-16* mRNA were noted in the central excretory canal and in the tissue of the lateral lines in both male and female worms in very low numbers.

3.2.4. Nerve cords

The dorsal and ventral nerve cords, which are the major components of the ascarid nervous system, originate at the nerve ring and run along the length of the body (del Castillo et al., 1989). *Peq-pgp-11* mRNA and *Peq-pgp-16* mRNA were visualized in the dorsal and ventral nerve cords in very low numbers in male and female worms.

3.2.5. Male reproductive tissue

The male ascarid reproductive tissue consists of a single convoluted, tubular testis, seminal vesicle and a terminal vas deferens (Foor, 1976). *Peq-pgp-11* mRNA and *Peq-pgp-16* mRNA were seen in all parts of the male reproductive tissue in low numbers.

3.2.6. Female reproductive tissue

The female reproductive tissue consists of convoluted tubular ovaries lined by a single layer of simple cuboidal epithelium, that eventually become two tubular uteri proceeding anteriorly, and which terminate in a single uterus with simple cuboidal to columnar epithelium with the presence of some free intercellular space, depending on their location in the body (Lýsek and Ondrus, 1992). *Peq-pgp-11* mRNA and *Peq-pgp-16* mRNA were visualized in low numbers in the ovaries and uterus

Our results demonstrate that *Peq-pgp-11* and *Peq-pgp-16* mRNA expression could be qualitatively visualized using multiple nucleic acid hybridization *in situ* in the enterocytes of the intestines, hypodermis and coelomyarian muscles of the body wall, epithelia of the ovaries and uteri in the female, cells of the reproductive tract in the male, excretory canal, lateral line, and nerve cords. These findings are generally in agreement with Pgp expression data assessed by qPCR (Janssen et al., 2013).

3.3. Quantitative analysis of Peq-pgp-11 and Peq-Pgp-16 mRNA expressed in Parascaris

In order to make comparisons of signal among tissues, positive signal was quantified as dots per μm^2 for each probe. Expression levels of *Peq-pgp-11* mRNA and *Peq-pgp-16* mRNA relative to beta tubulin mRNA (positive control probe) were analyzed (Figs. 3 and 4). Expression levels were also analyzed in each organ in sections from the anterior, middle and posterior of the worms (Figs. 5 and 6).

In male parasites, overall expression of *Peq-pgp-11* was significantly higher in the intestine and reproductive tissue (which were not

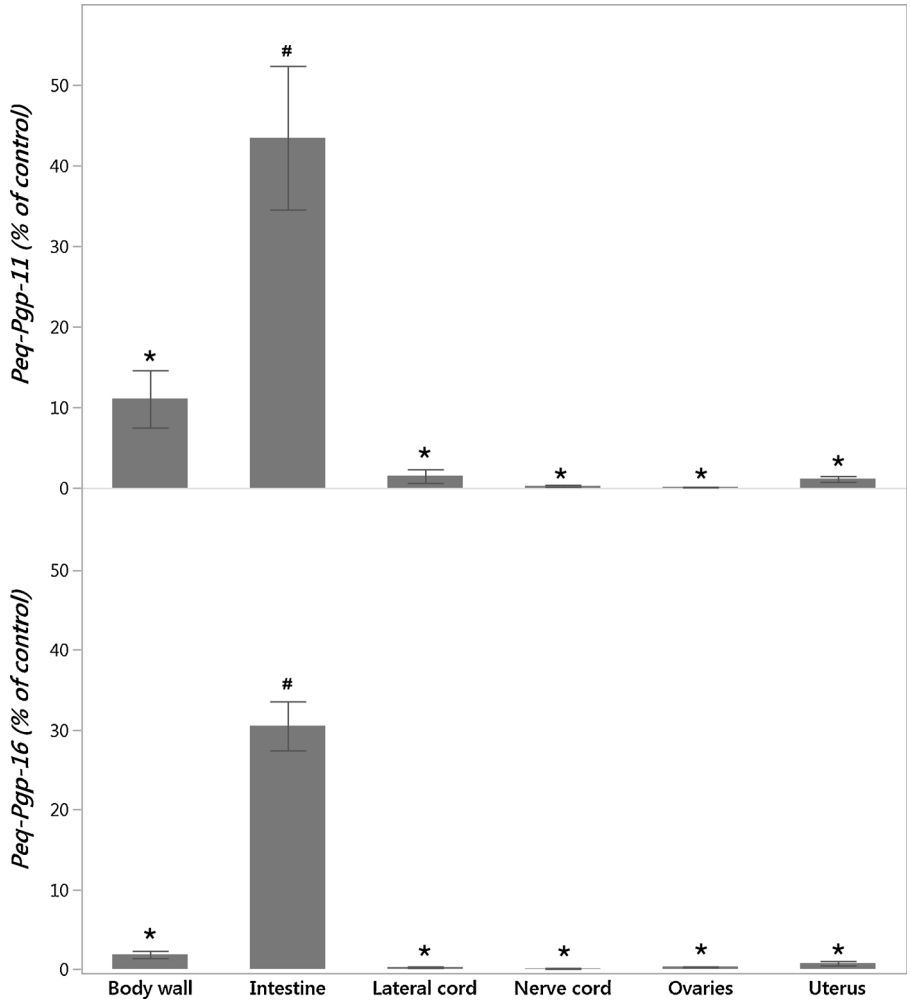

Fig. 4. Expression of (A) *Peq-pgp-11* and (B) *Peq-pgp-16* in adult female *Parascaris*. Values represent percent area of the tissue with positive staining relative to beta-tubulin ± S.E.M. Columns with different symbols are significantly different ($p < 0.05$).

significantly different from each other) than in the body wall, nerve cords and lateral cords (Fig. 3A). In contrast, expression of *Peq-pgp-16* mRNA was not significantly different among the tissues studied (Fig. 3B). However, significant differences in the relative expression levels of *Peq-pgp-11* and *Peq-pgp-16* mRNA were observed in body wall, intestine, nerve cord and reproductive tissue when comparing the anterior, middle and posterior regions of the parasite. (Fig. 5).

In female parasites, overall expression of *Peq-pgp-11* was significantly higher in the intestine when compared with the body wall, ovaries, uterus, lateral and nerve cords (Fig. 4A). Similarly, expression of *Peq-pgp-16* was significantly higher in the intestine when compared to all other tissues studied (Fig. 4B). Significant differences of relative expression of *Peq-pgp-11* and *Peq-pgp-16* were observed in the body wall, intestine, nerve cords and uterus when comparing the anterior, middle and posterior regions of the parasite (Fig. 6).

Thus, *Peq-pgp-11* and *Peq-pgp-16* mRNA expression could be quantitatively analyzed using multiple nucleic acid hybridization in the tissues of male and female worms, and this was in agreement with the previously published report for *Peq-pgp-11* (Janssen et al., 2013). Comparisons between expression levels in the same organs in different sections of the worms were also possible with this technique.

4. Discussion

P-glycoproteins have been associated with macrocyclic lactone resistance in nematode parasites, including *Parascaris*. This is the first study to examine *in situ* P-glycoprotein mRNA localization in an ascarid or equine nematode parasite. More broadly, our results suggest the multiple nucleic acid hybridization technique can be used as a quantitative measure of mRNA transcripts in parasitic nematodes.

Previously, localization studies have been conducted using antibody-based detection of Pgp protein in tissues of Clade V nematodes. In transgenic *C. elegans*, *Cel-Pgp-3* and *Cel-Pgp-1* proteins localized to the apical membranes of the excretory and intestinal cells and the anterior pharynx (Broeks et al., 1995). In *Haemonchus contortus*, antibodies developed by immunization with synthesized peptides detected *Hco-Pgp-2* in the pharynx, lateral nerve cords, deirids and mid-intestine (Godoy et al., 2015b). A monoclonal antibody targeting human Pgp bound to the cuticle, eggs, and intestinal cells of *H. contortus* (Riou et al., 2005). Fewer studies have assessed the localization of Pgp mRNA transcripts *in situ*. In *H. contortus*, *in situ* hybridization revealed that mRNA of Pgp-A (*Hco-Pgp-2)* was present in the worm gut, anterior to the pharyngeal intestinal junction, lateral cords, vas deferens and spicules, with no significant differences being observed in males or females (Smith and Prichard, 2002).

In the present study, multiple nucleic acid hybridization revealed significant levels of *Peq-pgp-11* transcripts in the intestines of both sexes and in the reproductive tissue of the male and significant levels of *Peq-pgp-16* mRNA transcripts in the intestines of male and female *Parascaris*. This technique has a significant advantage over worm dissection and qPCR, as tubular organs such as the testes that take significant skill to isolate without contamination. In addition, small organs such as nerve

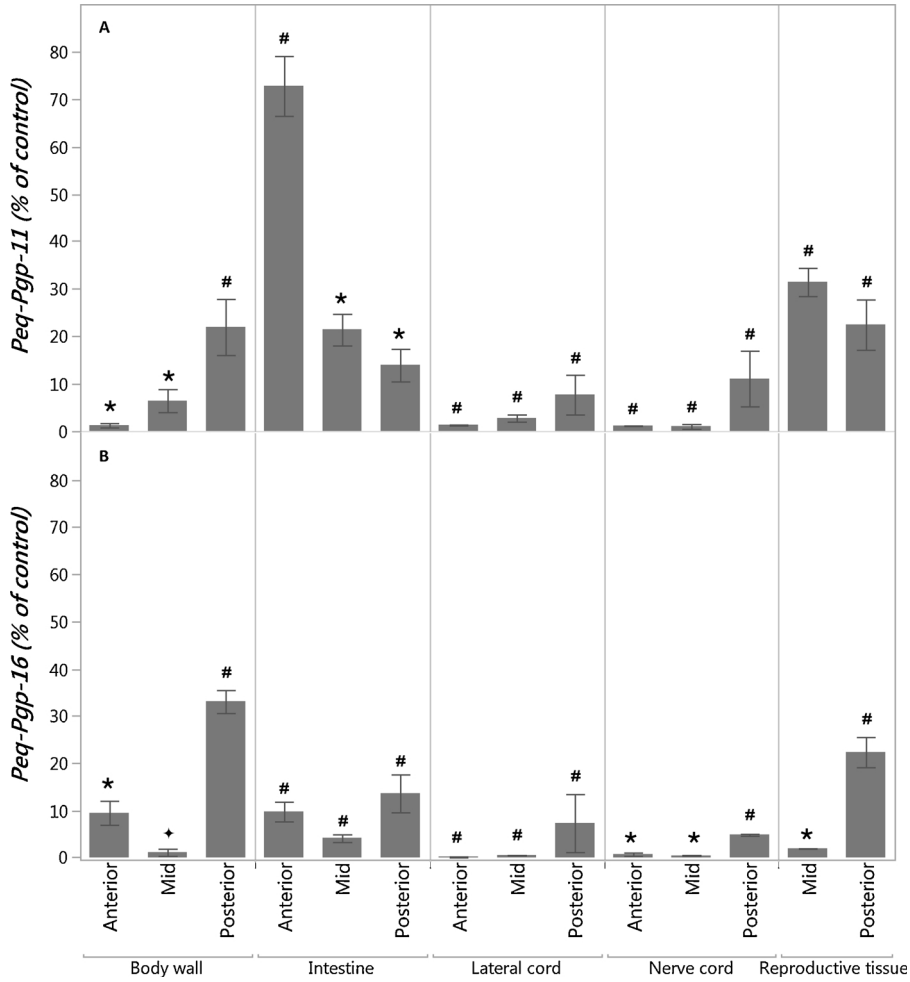

Fig. 5. Expression of (A) *Peq-pgp-11* and (B) *Peq-pgp-16* in different body regions of adult male *Parascaris*. Values represent percent area of the tissue with positive staining relative to beta-tubulin ± S.E.M. Columns with different symbols are significantly different from other regions of a specific tissue ($p < 0.05$).

cords can be studied in the context of the surrounding tissues.

P-glycoproteins are thought to efflux MLs out of cells and prevent drug binding to nematode glutamate-gated chloride channels. It is unclear if Pgp-mediated drug efflux acts at a local level, near nematode nervous tissues expressing ion channels. Another possibility is that Pgp eliminates anthelmintics at the level of the nematode body wall or intestine, which may initially encounter the drug. Alternatively, Pgp efflux could trap anthelmintics in specific tissue compartments thereby sequestering them away from target receptors. In order to develop a model for understanding Pgp-mediated ML resistance, it is important to determine the location and expression patterns of Pgps.

Detection of Pgp transcripts in some cell types suggests that Pgps could prevent entry of ML into the body of the nematode. Pgp mRNA was visualized in the hypodermis and in the coelomyarian musculature. Since transcuticular diffusion of anthelmintics has been demonstrated in ascarids (Alvarez et al., 2001; Martin et al., 1992), anthelmintic efflux could be relevant at the level of the body wall. High levels of Pgp were also observed within intestinal cells. In mammals, the intestinal epithelium is the first line of defense and effluxes many foreign substances. There is a possibility that ingested anthelmintics are effluxed at the level of the intestine, thereby preventing their entry into the body of the nematode.

Pgp transcripts were also visualized, albeit in lower numbers, in the lateral cords through which the excretory canal passes (Martin et al., 1992; Sanglas et al., 2009). It has been postulated that since nematodes lack a liver or kidneys, drug and xenobiotic clearance occurs through the excretory canal by P-glycoproteins on the surface of cell membranes (Broeks et al., 1995). In *Ascaris*, vacuolated cells of the lateral cords absorb ML from the peri-enteric fluid (Martin et al., 1992). Thus, the presence of Pgps in the lateral cords may enable drug efflux into the excretory canal causing a reduction in effective concentration of the drug at other active sites in the parasite.

In mammals, nervous system Pgps exclude xenobiotics at the level of the blood brain barrier (de Boer et al., 2003). In the present study, *Pgp* mRNA was visualized in low numbers in the dorsal and ventral nerve cords. One binding site for MLs is in the outer monolayer of the plasma membrane of muscle cells and nerve-cords (Martin et al., 1992), therefore, efflux of anthelmintics could also be relevant at the level of the nerves bearing ligand-gated ion channels. Thus, although the mammalian body components are different structurally, the function of Pgp-mediated neuroprotection could be similar.

Pgp mRNA was visualized in the reproductive tissues of both the male and female worms. Increased uterine expression of *Peq-pgp-11* and *Peq-pgp-16* has also been detected by PCR for *Parascaris* (Janssen et al., 2013). Uterine muscles are sites of action of for MLs and the presence of Pgps in the uterus and uterine muscles has been proposed (Godoy et al., 2015a; Prichard, 2001) However, the potential function of Pgps in the male reproductive organs such as the testis, vas deferens, and seminal vesicles are largely unknown.

Overall, the multiple nucleic acid hybridization method is useful for detecting specific transcripts within tissues of nematodes. The present study suggests that *Peq-pgp-11* and *Peq-pgp-16* mRNAs are expressed in many tissues of *Parascaris*. Our results indicate that Pgps could protect nematodes from anthelmintics locally, at the level of the neuron, but

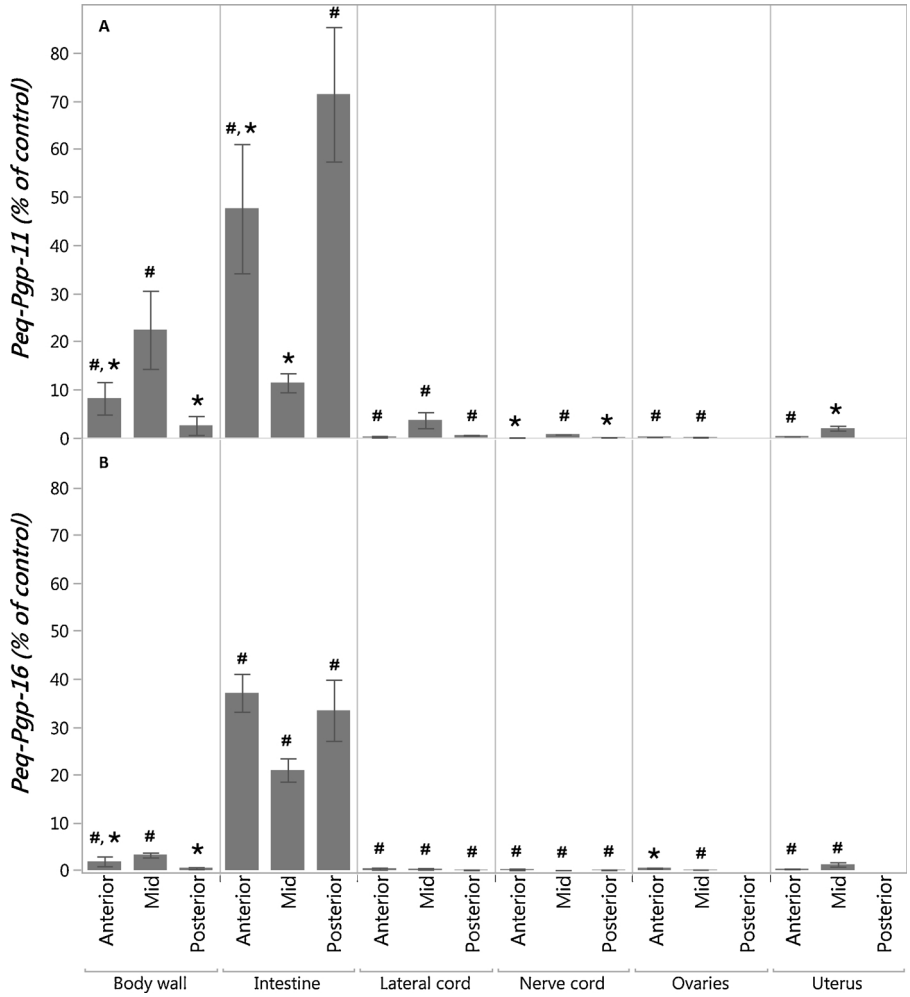

Fig. 6. Expression of (A) *Peq-pgp-11* and (B) *Peq-pgp-16* in different body regions of adult female *Parascaris*. Values represent percent area of the tissue with positive staining relative to beta-tubulin ± S.E.M. Columns with different symbols are significantly different from other regions of a specific tissue ($p < 0.05$).

also at sites of entry such as the hypodermis and intestine. The parasites studied in this report were not known to be drug-resistant, so it appears these Pgps are expressed constitutively in a variety of tissues. Therefore, ML-induced hyperexpression or post-translational changes in Pgps require additional investigation. The multiple nucleic acid hybridization approach can be used in combination with protein-detection techniques to enable future studies comparing drug-resistant and -susceptible nematodes.

Conflict of interest

None.

Acknowledgement

This research was supported by start-up funds from the Iowa State University College of Veterinary Medicine provided to MTB.

Appendix A. Supplementary data

Supplementary material related to this article can be found, in the online version, at doi:https://doi.org/10.1016/j.vetpar.2019.02.002.

References

Alvarez, L.I., Mottier, M.L., Sánchez, S.F., Lanusse, C.E., 2001. Ex vivo diffusion of albendazole and its sulfoxide metabolite into ascaris suum and Fasciola hepatica. Parasitol. Res. 87, 929–934.

Beasley, A., Coleman, G., Kotze, A.C., 2015. Suspected ivermectin resistance in a south-east Queensland Parascaris equorum population. Aust. Vet. J. 93, 305–307.

Bishop, R.M., Scott, I., Gee, E.K., Rogers, C.W., Pomroy, W.E., Mayhew, I.G., 2014. Sub-optimal efficacy of ivermectin against Parascaris equorum in foals on three Thoroughbred stud farms in the Manawatu region of New Zealand. N. Z. Vet. J. 62, 91–95.

Boersema, J.H., Eysker, M., Nas, J.W., 2002. Apparent resistance of Parascaris equorum to macrocylic lactones. Vet. Rec. 150, 279–281.

Bourguinat, C., Che, H., Mani, T., Keller, K., Prichard, R.K., 2016. ABC-B transporter genes in Dirofilaria immitis. Int. J. Parasitol. Drugs Drug Resist. 6, 116–124.

Broeks, A., Janssen, H.W., Calafat, J., Plasterk, R.H., 1995. A P-glycoprotein protects Caenorhabditis elegans against natural toxins. EMBO J. 14, 1858–1866.

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.

Craig, T.M., Diamond, P.L., Ferwerda, N.S., Thompson, J.A., 2007. Evidence of ivermectin resistance by Parascaris equorum on a Texas horse farm. J. Equine Vet. Sci. 27, 67–71.

Dankwarth, L., 1971. [Ultrastructure and function of the excretory organ of Ascaris lumbricoides L. (Nematoda)]. Z. Zellforsch. Mikrosk. Anat. 113, 581–608.

Davidson, A.L., Chen, J., 2004. ATP-binding cassette transporters in bacteria. Annu. Rev. Biochem. 73, 241–268.

De Graef, J., Demeler, J., Skuce, P., Mitreva, M., Von Samson-Himmelstjerna, G., Vercruysse, J., Claerebout, E., Geldhof, P., 2013. Gene expression analysis of ABC transporters in a resistant Cooperia oncophora isolate following in vivo and in vitro exposure to macrocyclic lactones. Parasitology 140, 499–508.

del Castillo, J., Rivera, A., Solórzano, S., Serrato, J., 1989. Some aspects of the neuromuscular system of Ascaris. Q. J. Exp. Physiol. 74, 1071–1087.

Demeler, J., Krücken, J., AlGusbi, S., Ramünke, S., De Graef, J., Kerboeuf, D., Geldhof, P., Pomroy, W.E., von Samson-Himmelstjerna, G., 2013. Potential contribution of P-glycoproteins to macrocyclic lactone resistance in the cattle parasitic nematode Cooperia oncophora. Mol. Biochem. Parasitol. 188, 10–19.

Drogemuller, M., Schnieder, T., von Samson-Himmelstjerna, G., 2004. Evidence of p-glycoprotein sequence diversity in cyathostomins. J. Parasitol. 90, 998–1003.
Foor, W.E., 1976. Structure and function of the glandular vas deferens in Ascaris suum (Nematoda). J. Parasitol. 62, 849–864.
Geary, T.G., Moreno, Y., 2012. Macrocyclic lactone anthelmintics: spectrum of activity and mechanism of action. Curr. Pharm. Biotechnol. 13, 866–872.
Godoy, P., Che, H., Beech, R.N., Prichard, R.K., 2015a. Characterization of Haemonchus contortus P-glycoprotein-16 and its interaction with the macrocyclic lactone anthelmintics. Mol. Biochem. Parasitol. 204, 11–15.
Godoy, P., Lian, J., Beech, R.N., Prichard, R.K., 2015b. 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., 2016. Characterisation of P-glycoprotein-9.1 in haemonchus contortus. Parasit. Vectors 9, 52.
Issouf, M., Guégnard, F., Koch, C., Le Vern, Y., Blanchard-Letort, A., 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, e87802.
James, C.E., Davey, M.W., 2009. Increased expression of ABC transport proteins is associated with ivermectin resistance in the model nematode Caenorhabditis elegans. Int. J. Parasitol. 39, 213–220.
Janssen, I.J., Krücken, J., Demeler, J., Basiaga, M., Kornaś, S., von Samson-Himmelstjerna, G., 2013. Genetic variants and increased expression of Parascaris equorum P-glycoprotein-11 in populations with decreased ivermectin susceptibility. PLoS One 8, e61635.
Janssen, I.J., Krücken, J., Demeler, J., von Samson-Himmelstjerna, G., 2015. Transgenically expressed Parascaris P-glycoprotein-11 can modulate ivermectin susceptibility in Caenorhabditis elegans. Int. J. Parasitol. Drugs Drug Resist. 5, 44–47.
Kaschny, M., Demeler, J., Janssen, I.J., Kuzmina, T.A., Besognet, B., Kanellos, T., Kerboeuf, D., von Samson-Himmelstjerna, G., Krücken, J., 2015. Macrocyclic lactones differ in interaction with recombinant P-glycoprotein 9 of the parasitic nematode Cylicocylus elongatus and ketoconazole in a yeast growth assay. PLoS Pathog. 11, e1004781.
Kessel, R.G., Prestage, J.J., Sekhon, S.S., Smalley, R.L., Beams, H.W., 1961. Cytological studies on the intestinal epithelial cells of Ascaris lumbricoides suum. Trans. Am. Microsc. Soc. 80, 103–118.
Licht, T., Pastan, I., Gottesman, M., Herrmann, F., 1994. P-glycoprotein-mediated multidrug resistance in normal and neoplastic hematopoietic cells. Ann. Hematol. 69, 159–171.
Lýsek, H., Ondrus, J., 1992. Morphology of the uterus of Ascaris lumbricoides in the region where fertilization and formation of egg-shell occur. Folia Parasitol (Praha) 39, 41–50.
Mani, T., Bourguinat, C., Keller, K., Ashraf, S., Blagburn, B., Prichard, R.K., 2016. Interaction of macrocyclic lactones with a Dirofilaria immitis P-glycoprotein. Int. J. Parasitol. 46, 631–640.
Martin, R.J., Kusel, J.R., Robertson, S.J., Minta, A., Haugland, R.P., 1992. Distribution of a fluorescent ivermectin probe, bodipy ivermectin, in tissues of the nematode parasite Ascaris suum. Parasitol. Res. 78, 341–348.
Molento, M.B., Antunes, J., Bentes, R.N., Coles, G.C., 2008. Anthelmintic resistant nematodes in Brazilian horses. Vet. Rec. 162, 384–385.
Nielsen, M.K., 2016. Evidence-based considerations for control of Parascaris spp. Infections in horses. Equine Vet. Educ. 28, 224–231.
Nielsen, M.K., Reinemeyer, C.R., Donecker, J.M., Leathwick, D.M., Marchiondo, A.A., Kaplan, R.M., 2014. Anthelmintic resistance in equine parasites—current evidence and knowledge gaps. Vet. Parasitol. 204, 55–63.
Peregrine, A.S., Molento, M.B., Kaplan, R.M., Nielsen, M.K., 2014. Anthelmintic resistance in important parasites of horses: does it really matter? Vet. Parasitol. 201, 1–8.
Prichard, R., 2001. Genetic variability following selection of Haemonchus contortus with anthelmintics. Trends Parasitol. 17, 445–453.
Raza, A., Kopp, S.R., Bagnall, N.H., Jabbar, A., Kotze, A.C., 2016. Effects of in vitro exposure to ivermectin and levamisole on the expression patterns of ABC transporters in Haemonchus contortus larvae. Int. J. Parasitol. Drugs Drug Resist. 6, 103–115.
Riga, M., Tsakireli, D., Ilias, A., Morou, E., Myridakis, A., Stephanou, E.G., Nauen, R., Dermauw, W., Van Leeuwen, T., Paine, M., Vontas, J., 2014. Abamectin is metabolized by CYP392A16, a cytochrome P450 associated with high levels of acaricide resistance in Tetranychus urticae. Insect Biochem. Mol. Biol. 46, 43–53.
Riou, M., Koch, C., Delaleu, B., Berthon, P., Kerboeuf, D., 2005. Immunolocalisation of an ABC transporter, P-glycoprotein, in the eggshells and cuticles of free-living and parasitic stages of Haemonchus contortus. Parasitol. Res. 96, 142–148.
Sanglas, L., Aviles, F.X., Huber, R., Gomis-Rüth, F.X., Arolas, J.L., 2009. Mammalian metallopeptidase inhibition at the defense barrier of Ascaris parasite. Proc. Natl. Acad. Sci. U. S. A. 106, 1743–1747.
Shah, S.Z.A., Khan, S., Compston, P., Upjohn, M., Jobling, R., 2016. Gastrointestinal parasite infestation and the efficacy of Fenbendazole and Ivermectin in working equids in selected areas of Pakistan. J. Equine Vet. Sci. 39 (Supplement), S103.
Smith, J.M., Prichard, R.K., 2002. Localization of p-glycoprotein mRNA in the tissues of Haemonchus contortus adult worms and its relative abundance in drug-selected and susceptible strains. J. Parasitol. 88, 612–620.
Tydén, E., Skarin, M., Höglund, J., 2014. Gene expression of ABC transporters in Cooperia oncophora after field and laboratory selection with macrocyclic lactones. Mol. Biochem. Parasitol. 198, 66–70.
Wang, F., Flanagan, J., Su, N., Wang, L.C., Bui, S., Nielson, A., Wu, X., Vo, H.T., Ma, X.J., Luo, Y., 2012. RNAscope: a novel in situ RNA analysis platform for formalin-fixed, paraffin-embedded tissues. J. Mol. Diagn. 14, 22–29.
Watson, B.D., 1965. The fine structure of the body-wall and the growth of the cuticle in the adult nematode Ascaris lumbricoides. J. Cell. Sci. 3, 83–91.
Whittaker, J.H., Carlson, S.A., Jones, D.E., Brewer, M.T., 2017. Molecular mechanisms for anthelmintic resistance in strongyle nematode parasites of veterinary importance. J. Vet. Pharmacol. Ther. 40, 105–115.
Wolstenholme, A.J., 2012. Glutamate-gated chloride channels. J. Biol. Chem. 287, 40232–40238.
Xu, M., Molento, M., 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.