P-glycoprotein drug transporters in the parasitic nematodes
Toxocara canis and Parascaris

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Iowa State University

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**P-glycoprotein drug transporters in the parasitic nematodes *Toxocara canis* and *Parascaris***

by

**Jeba Rose Jennifer Jesudoss Chelladurai**

A dissertation submitted to the graduate faculty

in partial fulfillment of the requirements for the degree of

**DOCTOR OF PHILOSOPHY**

Major: Veterinary Pathology (Veterinary Parasitology)

**Program of Study Committee:**
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Tomislav Jelesijevic

The student author, whose presentation of the scholarship herein was approved by the program of study committee, is solely responsible for the content of this dissertation. The Graduate College will ensure this dissertation is globally accessible and will not permit alterations after a degree is conferred.

Iowa State University
Ames, Iowa

2019

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<tr>
<th>Abbreviation</th>
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<tr>
<td>P-gp</td>
<td>P-glycoprotein</td>
</tr>
<tr>
<td>ABCB1</td>
<td>ATP binding cassette family B1</td>
</tr>
<tr>
<td>MDR1</td>
<td>Multi drug resistance gene 1</td>
</tr>
<tr>
<td>MLs</td>
<td>Macrocyclic Lactones</td>
</tr>
<tr>
<td>TMD</td>
<td>Transmembrane Domain</td>
</tr>
<tr>
<td>NBD</td>
<td>Nucleotide Binding Domain</td>
</tr>
<tr>
<td>ATP</td>
<td>Adenosine tri-phosphate</td>
</tr>
<tr>
<td>GluClR</td>
<td>Glutamate gated chloride channel receptor</td>
</tr>
</tbody>
</table>
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ABSTRACT

P-glycoproteins (P-gp) are ATP-binding cassette transporters capable of effluxing a wide range of structurally unrelated compounds from cells. Parasitic nematodes express P-gps that have the potential to contribute toward anthelmintic resistance via transport of macrocyclic lactone drugs. This thesis characterizes the expression and function of P-gps from ascarid nematodes. In canines, somatic larvae in bitches are the source of transplacentally transmitted *T. canis* to neonates. Somatic larvae are tolerant to the macrocyclic lactone (ML) class of anthelmintics. We hypothesized that P-gps may play a role in larval tolerance to MLs. This dissertation sought to identify P-gp expression in *T. canis* stages and to characterize the unique pharmacology and localization of Pgp-11, which has been implicated in resistance in other nematodes.

13 P-gps were identified in the *T. canis* genome, of which 10 were found to be expressed in adults and infective larvae. P-gps expression could not be induced in infective larvae treated with MLs *in vitro*, but Pgp-10 was upregulated in somatic larvae treated with moxidectin *in vivo*. P-gp mediated efflux in infective larvae could be mitigated by some P-gp inhibitors. *T. canis* Pgp-11 was heterologously expressed in a canine cell line expressing no endogenous P-gp and could not be inhibited by verapamil, cyclosporine A and reserpine. Pgp-11 was expressed in adult worm intestines but was absent in reproductive tissues.

P-gps have been implicated in ML resistance in the equine nematode *Parascaris spp.* Pgp-11 and Pgp-16 have been previously described from *Parascaris*, but the exact localization of their expression was unknown. This dissertation sought to semi-quantitatively determine the localization of Pgp-11 and Pgp-16 mRNA in adult worm tissues using a novel chromogenic *in situ* hybridization assay.
CHAPTER 1. GENERAL INTRODUCTION

1. Use of macrocyclic lactones against nematodes of veterinary importance

Nematodes are common parasites of domestic animals and chemotherapeutic interventions are the mainstay in veterinary medicine to mitigate their effects on animal health.

Resistance to anthelmintics is an emerging problem across all the drug classes licensed for veterinary use in many domestic species (Gasbarre et al., 2009). It is difficult to quantify the loss that is caused due to anthelmintic resistance given the variability of a number of factors, which include (a) parasite population characteristics such as refugia, predominance of dose-limiting species, (b) host factors such as age, nutrition, immune status, breed-dependent inherent resistance, (c) production factors such as stocking density and degree of environmental contamination. It has been suggested that an increase in anthelmintic resistance in nematodes is due to the indiscriminate overuse of anthelmintics, route of administration, use of slow-release formulations, failure to establish a refugia population of nematodes susceptible to anthelmintics and failure to treat during the quarantine period (Sutherland and Leathwick, 2011). Resistance often involves multigenic loci and mechanisms of resistance in nematodes to different drug classes have been studied (Whittaker et al., 2017). Several studies use the non-parasitic nematode Caenorhabditis elegans, which has proved to be a reasonable but not an absolute model to study anthelmintic resistance (Geary and Thompson, 2001).

Macrocyclic lactones (MLs) constitute a class of endectocide drugs that are effective against many nematodes and arthropods. MLs approved for veterinary use include ivermectin, selamectin, doramectin, eprinomectin, moxidectin and milbemycin oxime. They act on glutamate gated chloride channels (GluCl) in nematodes, insects, crustaceans, and molluscs. The phenotypic effects of ML action in nematodes observed in vitro include worm death and
impaired motility in adults, motility cessation and death in microfilaria and L3s, and potentiation of the immune system (Tompkins et al., 2010; Vatta et al., 2014). The phenotypic effects observed in vivo include alteration of embryonic morphology, degenerative changes to nematode enterocytes, microfilarial death in utero, abnormalities in the morphology and motility of adult worms, and adult worm death (Bazzocchi et al., 2008; McCall, 2005; McCall et al., 2008; Tompkins et al., 2010).

Resistance to MLs have risen in many important nematodes including *Dirofilaria immitis* of dogs (Bourguinat et al., 2011a), *Haemonchus contortus* of sheep (Xu et al., 1998), *Parascaris spp.* of equines (Boersema et al., 2002), *Cooperia spp.* of cattle (Fiel et al., 2001), *Onchocerca volvulus* in humans (Churcher et al., 2009) among others. Two main paradigms of resistance mechanisms have been extensively evaluated to understand the reduction of susceptibility or increased resistance to the MLs. These are (a) changes in the receptor target of the MLs – the glutamate gated chloride channels, and (b) efflux proteins that decrease effective drug concentration at the receptor site of action.

Glutamate gated chloride channels of nematodes are pentameric structures made of two or three different subunits and has been reviewed in (Lynagh and Lynch, 2012; Wolstenholme and Rogers, 2005). Sensitivity to ivermectin in nanomolar concentrations is conferred by a glycine residue (Gly-281, also known as M3-Gly) in the membrane bound helix M3 of the alpha subunit of GluClR (Hibbs and Gouaux, 2011; Lynagh and Lynch, 2010, 2012). Sensitivity to moxidectin was not due to the same interactions (Prichard et al., 2012). Changes in GluClR that were previously thought to confer ivermectin resistance on nematodes included: (a) an amino acid change (L256F) in the GluCl alpha3 subunit (Njue et al., 2004), (b) an amino acid change in the M3-Gly residue in the GluClR alpha3B subunit (Lynagh and Lynch, 2010), (c) changes in
the frequency of alleles encoding the alpha subunits (Blackhall et al., 1998b) and (d) changes in the expression levels of ligand gated chloride channel subunits glc-3, glc-5 (Williamson et al., 2011). However, these changes were not able to definitively provide an explanation for resistance in the field isolates.

The second most important mechanism of resistance to the macrocyclic lactones are the permeability glycoproteins (P-gp), also known as ATP Binding Cassette Family B1 (ABCB1). They belong to the large family of ATP Binding Cassette transporters and are capable of effluxing xenobiotic compounds (Lespine et al., 2012; Prichard and Roulet, 2007). Changes in P-glycoprotein that are thought to confer resistance to MLs include (a) SNPs in P-gp genes (Bourguinat et al., 2011b; Sangster et al., 1999; Xu et al., 1998) and (b) increased expression of P-gps in resistant field isolates (Dicker et al., 2011b; Janssen et al., 2013a; Tydén et al., 2014), in vitro (Raza et al., 2016b) and in experimental in vivo studies (Lloberas et al., 2013).

2. A brief note on Ascarids, genome status and P-glycoproteins

Ascarids are a group of large parasitic nematodes that belong to the Superfamily Ascaridoidea. Ascarids found in domestic hosts include Toxocara canis that infects canids, Toxocara cati that infects felids, Toxascaris leonina that infects canids and felids, Toxocara vitulorum that infects bovids, Parascaris univalens and Parascaris equorum that infect equids, and Ascaris suum and Ascaris lumbricoides that infect pigs and humans. Adult ascarids are generally susceptible to label doses of FDA approved macrocyclic lactones, benzimidazoles, imidathiazoles, tetrahydropyrimidines and cyclic depsipeptides, given at the appropriate time to the appropriate host species. Multiple reports of macrocyclic lactone resistance in adult Parascaris spp. has been documented across the world (Boersema et al., 2002; Craig et al., 2007; Slocombe et al., 2007). A single report of reduced susceptibility to albendazole in Ascaris spp. in humans also exists (Krücken et al., 2017). While resistance in adult Toxocara canis has not been
reported, somatic larvae which causes visceral larval migrans in humans and non-human hosts show an apparent tolerance to label doses of approved macrocyclic lactones (Fok and Kassai, 1998). Arrested somatic larvae are an important feature of the lifecycle of T. canis, T. cati and T. vitulorum, and are a reservoir of infection to progeny by transplacental or transmammary routes in the definitive hosts. Mechanisms of tolerance to the MLs in T. canis somatic larvae may be similar to mechanisms of resistance in other nematodes.

The genes encoding P-glycoproteins and their function in ML efflux have been studied in Parascaris spp. (Janssen et al., 2013a). But very little is known about P-glycoproteins in Toxocara canis and Ascaris spp. The availability of draft genomes of these organisms will facilitate the study of P-gp genes. One draft genome each of Ascaris lumbricoides, Parascaris equorum and P. univalens have been assembled (Consortium, 2019; Wang et al., 2017). Two draft genomes each of Ascaris suum and Toxocara canis have also been made available (Consortium, 2019; Jex et al., 2011; Wang et al., 2012b; Zhu et al., 2015). While draft genomes that are available as fragments and/or scaffolds are valuable resources for research, they do not provide a chromosome level resolution of the genome. Additionally, since ab initio gene prediction models are often used for gene prediction and annotation, single genes are often fragmented and genome quality may later be improved by empirical transcription data such as ESTs, RNA-seq and ChIP-seq (Wang et al., 2017).

3. Thesis organization

This thesis investigates P-glycoproteins in the ascarid nematodes Toxocara canis and Parascaris spp. Chapter 2 reviews the ATP binding cassette superfamily in general followed by a detailed summary of the methods of study of P-glycoproteins in nematodes. Chapter 3 examines the P-gp repertoire of T. canis expressed in adults and larval stages. Chapter 4 describes the unique pharmacology of T. canis Pgp-11. Chapter 5 shows the localization of two
P-gps in *Parascaris* adults. The final chapter summarizes the knowledge gap filled by this work and identifies future work that must be pursued in this field.

4. References


CHAPTER 2. METHODS IN THE STUDY OF P-GLYCOPROTEINS IN NEMATODE PARASITES OF VETERINARY IMPORTANCE

Jeba R. J. Jesudoss Chelladurai, Matthew T. Brewer

A review paper to be submitted

Abstract

Macrocyclic lactones (ML) are commonly used endectocides in veterinary medicine. But, resistance to the MLs is an emerging threat to their use. P-glycoproteins and other multidrug resistance transporters belonging to the ATP binding cassette (ABC) superfamily have been associated with ML resistance in many nematodes. Little is known about the repertoire and function of ABC superfamily members in nematodes. This review summarizes the available knowledge of ABC subfamilies studied in nematodes, followed by an overview of the methods used in the study and pharmacological characterization of the efflux proteins of the ABCB1 family in nematodes. The review also discusses the role of P-glycoproteins in nematodes and animals of veterinary importance.

1. ATP binding cassette (ABC) superfamily

Members of the ATP binding cassette superfamily are membrane proteins, characterized by an ATPase domain that hydrolyses ATP and an integral membrane domain that lies in the membrane. They are involved in multiple cellular functions such as the import of nutrients in prokaryotes, export of molecules in both eukaryotes and prokaryotes, in DNA repair, in translation and gene regulation processes (Dassa and Bouige, 2001).

1.1. Classification of the ABC superfamily

Proteins in the ABC superfamily can be broadly classified into 3 classes based on molecular structure: (a) Class 1 in which ATPase and membrane domains are fused and present as one long polypeptide, (b) Class 2 in which there are no membrane domains but the ATPase
domain is tandemly repeated and (c) Class 3 in which the ATPase and membrane domains may
or may not be on different polypeptides (Davidson et al., 2008). Eukaryotes including mammals
and nematode parasites possess several ABC proteins listed in Table 2-1. The functions of these
proteins have been studied in humans but are incompletely understood in nematodes. Their
presence in model non-pathogenic nematodes and a few pathogenic zoonotic nematodes have
been predicted by orthology of genes annotated with KEGG Orthology (KO) identifiers.

1.2. Roles of subfamilies of ABC transporters

Relatively little is known about ABC proteins other than ABCB1 transporters in parasitic
nematodes. But they have been reasonably researched in humans and C. elegans allowing for
broad inferences of putative functional roles of gene orthologues in parasitic nematodes. In the
following descriptions, a special emphasis has been laid on the ascarid nematode of dogs,
Toxocara canis, in order to provide context for the study of ABCB1 genes of the nematode
discussed in Chapters 3 and 4. A phylogenetic tree has been constructed to illustrate relationships
of ABC proteins of Toxocara canis and C. elegans (Figure 2-1).

ABCA proteins are large proteins generally involved in lipid transport and theorized to be
involved in drug sequestration in human carcinomas (Albrecht and Viturro, 2007). ABCA
orthologues exist in the model nematode Caenorhabditis elegans, C. briggsae and C. remanei
(Sheps et al., 2004). These include the genes Abt-1, Abt-2, Abt-3, Abt-4, Abt-5 and Ced-7 (Zhao
et al., 2007). Ced-7 is known to engulf cell corpses during programmed cell death in
embryogenesis in C. elegans (Wu and Horvitz, 1998). Among pathogenic nematodes, Toxocara
canis encodes a ced-7, whose function has not been experimentally studied.

ABCB proteins in nematodes and animals have been extensively studied in the context of
anthelmintic resistance and drug detoxification. These include the full transporter genes known
as pgp or mdr and half transporter genes known as hafs (Zhao et al., 2007). Cel-Pgp-3 has been
shown to protect *C. elegans* against naturally produced toxins such as colchicine and chloroquine (Broeks et al., 1995). Phylogenetic analysis of vertebrate and nematode P-gp orthologues reveal a significant divergence of nematode P-gps from mammalian and avian P-gps. In parasitic nematodes, several homologs of *C. elegans* P-gps exist. However, a few novel P-gps with no known *C. elegans* homologs have also been described. Nematode P-gps sequences share 35–64% identity with mammalian P-gps (Kerboeuf and Guégnard, 2011; Sangster et al., 1999). Other ABCB genes in nematodes include ABCB6/*hmt-1* which is involved in cadmium and copper detoxification in *C. elegans* (Schwartz et al., 2010). There is a knowledge gap in the identification and characterization of ABCB transporters in *T. canis*.

ABCC protein encoding genes in *Caenorhabditis* spp. includes at least 8 “MDR-related protein” genes that are known as *Mrp1* to *Mrp8* (Zhao et al., 2007). *Mrp-1* in *C. elegans* regulates the diapause state known as dauer (Yabe et al., 2005) and may be involved in resistance to heavy metals (Broeks et al., 1996). *Cel-mrp-5* is involved in heme and vitamin B12 transport during embryogenesis (Korolnek et al., 2014; Na et al., 2018). *Cel-Mrp-7* inhibits toxicity associated with methyl mercury (VanDuyn and Nass, 2014). The function of *mrp* genes in other nematodes is yet unknown.

ABCD protein encoding genes in *C. elegans* include several “Peroxisomal Membrane Protein” genes that are known as *pmp-1* to *pmp-5* (Zhao et al., 2007). *Cel-pmp-1*, *Cel-pmp-2* and *Cel-pmp-4* are involved in transport of very long chain fatty acids to peroxisomes, and are implicated in nematode development (Morita and Imanaka, 2012). Orthologs of *pmp* genes are encoded in the pathogenic nematode, *T. canis*, but their functions have not been experimentally studied.
The single ABCE protein encoding gene in *C. elegans* is known as *abce-1* (Yan et al., 2012a; Zhao et al., 2007). *Cel-abce-1* is involved in transcriptional and translational control, in protein transport between the nucleus and cytoplasm, and appears to be involved in molting (Zhao et al., 2004). SNPs in a ABCE1 gene were found to be IVM resistance associated (Luo et al., 2017). In the pathogenic nematode *T. canis*, an ortholog of *abce-1* is encoded in the genome, but its function is unknown.

ABCF protein encoding genes in *C. elegans* include at least 4 members named *abcf-1* to *abcf-4* (Zhao et al., 2007). *Abcf-3* in *C. elegans* appears to be involved in apoptosis regulation (Hirose and Horvitz, 2014). In the pathogenic nematode *T. canis*, orthologs of ABCF genes are encoded in the genome, but their functions have not been empirically tested.

ABCG/BCRP protein is represented by the *wht-6* gene in *C. elegans*, which is involved in the mitochondrial uptake of the Vitamin B12 (cobalamin) (McDonald et al., 2017). Orthologs of ABCG are encoded in the genome of *T. canis* but their functions have not been identified.

ABCH protein is encoded by a single gene in the *C. elegans* genome. There is no ortholog in the *T. canis* genome.

A number of other ATP binding cassette domain containing proteins have been assigned to the ABC superfamily that do not have significant similarity or identity with the classical ABC transporters described above. These include the structural maintenance of chromosomes (SMC) proteins that are involved in chromatin organization, DNA damage sensor proteins such as rfc-2, double stranded break (DSB) repair proteins and DNA mismatch repair protein (MutS) involved in mismatch repair (Dassa, 2011; Stergiou and Hengartner, 2004). These also include proteins involved in (a) cytokinesis such as kinesin-like protein, spindle apparatus protein lin-5, (b) molting such as mlt-10 related, (c) developmental cellular migration, (d) neurotransmitter release
such as nsf-1 etc. (Ali and Siddiqui, 2000; Barclay et al., 2012; Meli et al., 2010; Srinivasan et al., 2003; Stringham et al., 2002). There are orthologs of ABC ATPase genes in the *Toxocara canis* genome.

2. ABCB1 transporter family

2.1. P-glycoproteins

Members of the ABCB1 family are also known as permeability glycoprotein (P-gp) and multiple drug resistance 1 (mdr1) protein, and the terms are used interchangeably. Orthologous genes are named *mdr1* in humans and mice, and *pgp* with a number designation in nematodes. Rarely, *abcb1* is also used to refer to the protein. The first known member of this family was described as a 170kDa cell surface glycoprotein in Chinese hamster ovary cell line that was resistant to colchicine (Juliano and Ling, 1976). Since then, multiple studies have shown that P-glycoproteins have detoxification roles in mammals and are involved in multiple drug resistance in several neoplasia.

2.2. Structure of P-glycoprotein

P-glycoproteins are translated into a single polypeptide from mRNA transcripts. The crystal structure of P-glycoprotein has been elucidated for human *mdr1* (Kim and Chen, 2018), mouse *mdr1a* (Aller et al., 2009), and *C. elegans pgp-1* (Jin et al., 2012) using crystallography techniques, and were found to be structurally similar. P-gps have a pseudo two-fold symmetry with two transmembrane (TM) domains and two nucleotide binding domains (NBD), made of helices, bridges and beta strands. The two halves of the protein are connected by a flexible linker allowing flexibility in the conformation of the protein during the transport cycle (Ward et al., 2013). Each NBD is composed of Walker A, Walker B and C motifs, with the latter being the distinguishing feature that differentiates ABCB1 transporters from other proteins that bind ATP (Silva et al., 2015). The secondary structure of *C. elegans* Pgp-1 is illustrated in Figure 2-2 and
the transmembrane view of the ribbon model of the crystal structure in the inward facing conformation obtained by X-ray diffraction at 3.4 Å in Figure 2-3. Crystal structures of P-gps from pathogenic nematodes of veterinary or human importance are not available yet.

2.3. Mechanism of drug efflux

P-gp molecules have a substrate binding pocket that involves most of the transmembrane helices (Ward et al., 2013). In the inward facing conformation, the most likely active state, two portals allow molecules access to the substrate binding pocket. These allow access from the cytoplasm and inner leaflet of the cell membrane but preclude access from the extracellular space or the outer leaflet. There are two leading models to explain efflux action – the pump or vacuum cleaner model and the flippase model.

In the vacuum cleaner model, P-gp substrate molecules partition and aggregate near the lipid headgroups of the inner layer of the cell membrane bilayer where they enter the substrate binding pocket of P-gp and are effluxed into the extracellular space. In this model, some drugs that enter the bilipid membrane may be effluxed out before gaining entry into the cytosol. Evidence for this model is provided by transport studies with fluorescent molecules such as Hoechst 33342 (Shapiro and Ling, 1997; Sharom, 2014).

In the flippase model, P-gp substrate molecules aggregate near phospholipid acyl cores of the inner leaflet and by interaction with the drug binding pocket are translocated to the outer leaflet of the membrane from where they passively diffuse into extracellular space. Evidence for this model is provided by the efflux of phospholipid and glycosphingolipids (Eckford and Sharom, 2005; Romsicki and Sharom, 2001; Sharom, 2014).

The two models are not mutually exclusive and experimental studies to accurately ascertain the mechanism is constrained by available technologies (Sharom, 2014). Conformational change from the inward facing confirmation to the outward facing conformation
occurs when a substrate is bound to the substrate binding pocket. ATP binding causes
dimerization of the NBD. ATP hydrolysis causes disruption of the NBD dimers, causing a
reversal back to the inward facing conformation. ATP binding is seen only in the inward binding
state, but not in the outward state (Aller et al., 2009).

2.4. Substrate binding and prediction

There is a marked “substrate promiscuity” by which structurally diverse compounds can
be effluxed by P-gps. There is a large amount of flexibility in the transmembrane domains
manifested by helical kinking and/or unwinding of the helices allowing for the binding of diverse
substrates (Wen et al., 2013). Many of the compounds effluxed are hydrophobic and amphipathic
with variable molecular weights, chemical sidechains, linkages and topologies. In computer-
based molecular dynamics studies, distinct binding sites are not observed but rather a “binding
belt” of amino acids that interact with drugs (Jagodinsky and Akgun, 2015). Thus, the versatility
displayed by the amino acid residues in the drug binding pocket of the protein in addition to
diversity in the substrates are the biggest challenge to substrate binding prediction by in silico
methods. Despite the challenges, docking studies have shown the P-gps from pathogenic
nematodes such as Haemonchus contortus can be modelled using in silico algorithms on the
experimentally obtained crystal structure of C. elegans Pgp-1 (David et al., 2018). These can be
improved as higher resolution crystal structures of nematode P-gps are made available in the
future.

2.5. Substrates and inhibitors of P-gps

P-gps efflux a range of chemically diverse compounds, that may be hydrophobic or
amphipathic. Several fluorescent substrates of P-gps such as Rhodamine 123, Hoechst 33342,
Calcein-AM are known (Lebedeva et al., 2011) and are frequently used in research studies. P-gp
substrates approved as drugs for human and animal use have been reviewed and include
antibacterials like erythromycin, antifungals like ketoconazole, cardiac drugs such as diltiazem, opioids such as loperamide among others (Mealey and Fidel, 2015). Anthelmintics such as macrocyclic lactones and monepantel are effluxed by P-gps and compete with rhodamine 123 for efflux (Raza et al., 2016a). Eprinomectin, abamectin, doramectin, selamectin were capable of causing an accumulation of Rhodamine 123 by competing for P-gp binding. Moxidectin, a milbemycin, showed lower potency than the others (Lespine et al., 2007).

P-gp inhibitors are used frequently used to cause a reversal of resistant to susceptible phenotype in human neoplasia (Nanayakkara et al., 2018). These act on the protein in one of three different ways, viz. (a) blocking transport of another compound by direct interaction and competitive or non-competitive antagonism at the active site; (b) inhibiting ATP hydrolysis, or (c) altering drug interactions with the cellular membrane (Stouch and Gudmundsson, 2002).

There are three generations of P-gp inhibitors: (a) first generation inhibitors had low P-gp specificity and were agonists of other receptors. These include verapamil, cyclosporine A, caffeine, (b) second generation inhibitors had higher specificity and fewer side effects. These include valspodar, dexverapamil and dextiguldipine, (c) third generation inhibitors have high specificity and efficacy. These include zosuquidar, elacridar, tariquidar, and (d) fourth generation inhibitors having the highest efficacy. These include peptidomimetics and dual activity ligands like flavonoids, quercetin (Palmeira et al., 2012).

ATPase activity is also altered by the P-gp modulators, with the drugs being classified into three classes: (a) Class I drugs like verapamil inhibit ATPase activity at high concentrations, but enhance activity at low concentrations, (b) Class II drugs like valinomycin have no inhibitory action but act in a dose dependent manner to enhance ATPase function, (c) Class III drugs like Cyclosporine A inhibit basal and stimulated ATPase activity (Ambudkar et al., 1999).
Several of these inhibitors have been used to inhibit P-glycoproteins in nematodes in vitro and in vivo. However, nematode specific P-gp inhibitors have not been discovered and are the holy grail of nematode research in order to overcome anthelmintic resistance.

2.6. Role of ABCB1 efflux proteins in veterinary species

In clinical veterinary medicine, there is evidence that P-gps in host animals interact with multiple classes of drugs and alter pharmacokinetics and bioavailability in different tissues (Virkel et al., 2019). They are briefly reviewed here.

In dogs, basal levels of tissue expression of the sole P-gp protein - mdr1 and MDR-related proteins (mrp-1 and -2) have been studied (Conrad et al., 2001). P-gp in dogs is expressed in enterocytes, endothelia of brain capillaries, canalicular cells of the liver, proximal tubular cells of the kidneys, testes and placenta (Dowling, 2006). The most important clinical application of studying the canine MDR1 gene has been the identification of breed related distribution and elimination of oncotherapeutic drugs, macrocyclic lactones and several other drugs (Mealey, 2013). A 4-basepair deletion resulting in a mutated Can-mdr-1 gene conferring increased ivermectin sensitivity in collies and herding breeds has been well characterized (Mealey et al., 2001).

In adult cats, tissue distribution of P-gps at basal levels have been studied by immunohistochemistry and found to similar to dogs (Van Der Heyden et al., 2009). Distribution and function in neonatal or geriatric cats is unknown (Merola and Eubig, 2012). A premature stop codon in the feline mdr-1 gene conferring a phenotype similar to the collie when exposed to P-gp substrates has also been identified (Mealey and Burke, 2015).

In bovines, MDR1 is expressed in the rumen wall (Haslam and Simmons, 2014). It is also expressed in mammary epithelial cells, and may be upregulated by mdr1 substrates (Yagdiran et al., 2016).
In swine, \textit{abcb1} gene function and pharmacology have been recently studied. Co-administration of verapamil was found to increase the intestinal absorption of enrofloxacin (Guo et al., 2016). There was also a decrease in \textit{abcb1} gene expression when fenbendazole or flunixin meglumine were administered to certain breeds of commercial swine (Howard et al., 2015).

In horses, the P-gp gene is expressed in the intestines, liver and kidney. It is about 100 amino acids longer than P-gps of dogs, humans, rhesus macaques, sheep, mice and rats (Tydén et al., 2009).

2.7. Role of ABCB1 efflux proteins in nematodes

P-glycoproteins in the nematodes \textit{C. elegans} and \textit{Haemonchus contortus} were first discovered by Sangster et al. (Sangster, 1994). The role of nematode P-glycoproteins in (a) the physiological maintenance of homeostasis and (b) their role in anthelmintic resistance have long been debated (Sangster, 1994).

\textit{C. elegans} has been used as a model to study P-gp pharmacology. \textit{C. elegans} is a rhabditid non-parasitic nematode and phylogenetically is a Clade IV nematode (Blaxter et al., 1998). The suitability of \textit{C. elegans} as a model for parasitic nematodes of distant clades such as the Clade III ascarids (\textit{Ascaris, Parascaris, Toxocara}) and spirurids (\textit{Dirofilaria, Brugia, Onchocerca}) and Clade I (\textit{Trichuris}) has been questioned (Beech et al., 2011). However, this might not be true for P-gps as an \textit{in silico} analysis revealed a close relationship between various P-gps genes from \textit{C. elegans}, \textit{C. brenneri}, \textit{C. briggsae}, \textit{C. remanei} and the parasitic nematodes \textit{Ascaris spp.}, \textit{Brugia spp.}, \textit{Cooperia spp.}, \textit{Haemonchus spp.}, \textit{Loa spp.}, \textit{Onchocerca spp.}, \textit{Parascaris spp.}, \textit{Strongyloides spp.}, \textit{Trichuris spp.} and \textit{Wuchereria sp.} (Figueiredo et al., 2018).

A physiological role for P-gps in nematodes can be argued for, on the evidence of basal levels of P-gp mRNA expression even in the absence of drugs (Figueiredo et al., 2018; Raza et al., 2016a). Additionally, there is evidence that p-glycoproteins significantly contribute to
molecular defenses against natural toxins in *C. elegans* (Lindblom and Dodd, 2006). However, physiological roles of p-gps in parasitic nematodes have not been empirically demonstrated yet.

P-glycoproteins have been strongly associated with macrocyclic lactone resistance in several parasitic nematodes (Janssen et al., 2015; Xu et al., 1998). In *C. elegans*, there are 14 functional P-gps and 1 pseudogene. Deletion/loss of function strains in which any one of the 14 functional P-gps had been altered showed a greater susceptibility to ivermectin, with pgp-11 and pgp-14 being particularly important (Janssen et al., 2013b).

In the past, P-gps were also associated with benzimidazole resistance (Beugnet et al., 1997; Lespine et al., 2012) but subsequent research has shown that benzimidazoles do not significantly modulate P-gp activity (Kerboeuf and Guégnard, 2011; Kwa et al., 1998).

Several molecular techniques and model systems have been used to study nematode P-glycoproteins. These are reviewed in detail below. These can be broadly classified into (a) nematode P-gp gene identification studies by *in silico* mining of genomes, (b) gene expression, polymorphism and induction studies (c) pharmacological studies in heterologous systems (d) pharmacological studies in whole organisms (e) localization studies and (f) studies to alter parasite p-gp pharmacology *in vivo*.

### 3. Methods used in the study of nematode P-gps

#### 3.1. Gene identification

In general, nematodes possess more than one ABCB1/P-glycoprotein gene when compared to mammals of veterinary interest which typically possess one gene with one or more isoforms. The model nematode *C. elegans* has 14 functional genes and 1 pseudogene (Janssen et al., 2013b). Isoforms are known to exist in some nematode P-gp genes, such as *pgp-9* of *Haemonchus contortus* which has three isoforms - *Hco-pgp-9.1, Hco-pgp-9.2* and *Hco-pgp-9.3*.
P-gp genes that have been described in nematodes of veterinary and human importance are listed in Table 2-2.

Several of these genes were described in the context of known clinical anthelmintic resistance while others were identified by genomic analysis. Table 2-2 is not a comprehensive list as more genes are expected to be described as genome qualities of whole genomes improve and as annotations are validated by cloning approaches, RNA-seq, gene silencing studies and proteomics for each nematode genome separately (Palevich et al., 2018). Alternatively, accurate chromosomal mapping and annotation of genes may result in a reduction in the number of total genes. In a recent study, transcripts analyzed in the past as $Hco$-pgp-1 and $Hco$-pgp-11 were revealed to be products of a single gene, as were the transcript of $Hco$-pgp-12 and $Hco$-pgp-14, which matched $Hco$-pgp-13 (Maté et al., 2018).

Accurate gene naming is important for systematic comparison as inventories of genes that do not follow gene naming conventions leads to confusion (Ardelli et al., 2010). However, accurate identification of nematode genes are compounded by the fact that (a) only draft genomes exist for several parasitic nematodes (Consortium, 2019) and (b) P-gp mRNAs have multiple transcription start sites (Ince and Scotto, 1995) and different transcripts may map differently to genomic sequences leading to inaccuracies in the annotation of draft genomes.

3.2. Gene expression, polymorphism and induction studies

P-glycoprotein genes have become increasingly associated with clinical resistance of nematodes to macrocyclic lactones in companion and food animals. The extremely complex nature of the transcriptional regulation mechanisms that govern P-gp mRNA expression have been studied in eukaryotes (Ince and Scotto, 1995; Scotto and Egan, 1998; Shtil and Azare, 2005) but not in nematodes. P-gps are constitutively expressed in all life stages of Cooperia oncophora (De Graef et al., 2013). It is not known if this is true of all parasitic nematodes.
In the model nematode *C. elegans*, exposure to ever increasing doses of ivermectin (IVM) was demonstrated to increase P-gp mRNA expression and induce resistance (James and Davey, 2009). Selection for tolerance by exposure to non-toxic doses of MLs in *C. elegans* caused an induced upregulation of certain P-gp mRNA (Pgp-1, Pgp-6, Pgp-10 and Pgp-14 in IVM selected strains and Pgp-1, Pgp-6, Pgp-8 and Pgp-14 in MOX selected strains) (Ménez et al., 2016). Similar research had previously led to the conclusion that certain individual P-gp genes may not play a critical role in conferring IVM resistance (Yan et al., 2012b). P-gp upregulation appears to occur in short time scales without sustained changes even over 2.5 hours in individual genes in *C. elegans* in MOX-selected and IVM-resistant strains (Bygarski et al., 2014). P-gp upregulation in parasitic nematodes may not necessarily be accurately reflected by studies in *C. elegans*, since P-gp homologs of certain genes such as *pgp-6* and *pgp-8* have not been found in parasitic nematodes so far (Table 2-2).

Several methods have been used to quantify expression changes and understand allelic variations in different stages of the nematodes. These are summarized in Table 2-3. The most frequently used assay is quantitative PCR (qPCR) to estimate mRNA transcription levels of specific P-gps. Basal levels of P-gp transcription, upregulation or downregulation of transcription when clinically susceptible or resistant nematode stages are exposed to drugs *in vivo or in vitro* can be quickly and conveniently calculated using qPCR. Since substrate specificity changes can occur even in the presence of silent mutations in mdr1 genes, SNP analysis after sanger sequencing has also been used as a method to correlate genetic mutations with clinical anthelmintic resistance (Kimchi-Sarfaty et al., 2007). RNA-seq analysis is a promising new -omics platform tool that has been used to quantify transcription levels of P-gp genes (Kenealy, 2019; Maté et al., 2018). The drawbacks that would have to be addressed in its
use are: a possible transcript length bias in detecting differentially expressed genes (Oshlack and Wakefield, 2009), over-detection of highly expressed transcripts (Young et al., 2010), and biochemical selection bias in cDNA library preparation (Roberts et al., 2011). The emergence of these assays in the 2000s based on improved molecular technologies have largely supplanted older radioactive isotope-based assays used in the 1990s such as southern blotting (hybridization of DNA with DNA probes) to detect restriction fragment length polymorphism and northern blotting (hybridization of RNA with DNA or RNA probes) to detect RNA transcription.

There is considerable variation in the conclusions reached in these studies that can be attributed to (a) differences in genetic make-up of isolates studied, (b) differences in mechanisms of resistance and (c) differences in assay sensitivity. As assay technologies improve, future studies are needed to understand how changes in gene expression correlate with clinically detectable resistance/tolerance.

### 3.3. Nematode P-gps in heterologous systems

Heterologous expression systems are useful for expression of intact and mutant variants of a protein in isolation. Mammalian P-gps have not been successfully expressed in prokaryotic systems but have been expressed in yeast, baculovirus/insect cell systems and eukaryotic cell lines (Bartley et al., 2009; Evans et al., 1995; Mealey et al., 2017; Tang et al., 2002). Nematode P-gp genes have been expressed in cell lines, yeast or *C. elegans* to study efflux activity and pharmacological interactions, with the most frequently used being the epithelial pig kidney cell line LLC-PK1. An ideal cell line must allow protein translation from mRNA transcribed from a transfected plasmid, followed by post-translational modifications and trafficking to the correct protein compartment. In the study of P-gps, endogenous P-gp levels in the native cell lines must be accounted for on a cell line to cell line basis. LLC-PK1 is a good model because it has very low endogenous P-gp expression (Goh et al., 2002). This allows true estimation of transfected
Pgp, when compared to other cell lines like Madin-Darby canine kidney (MDCK) in which the higher endogenous activity leads to underestimation of activity of the transfected P-gp (Kuteykin-Teplyakov et al., 2010).

Protein expression in heterologous models is often confirmed using Western blotting using the anti-mammalian-Pgp antibody UIC2. However, the relevance of this is questionable as (a) UIC2 binds to a conformational epitope in human P-gp (but not mouse Pgp) (Vahedi et al., 2018), and (b) the absence of the same conformational epitope in nematode P-gps. Some researchers have used antibodies raised against the specific nematode P-gp under study (Godoy et al., 2015b). In these studies, glycosylation differences must be accounted for, as inter-species glycosylation differences exist. Rat P-gp has an apparent molecular weight of 140 kDa due to lower levels of glycosylation than human P-gp which has a higher apparent molecular weight of 170kDa due to higher glycosylation (Dong et al., 1998). The effect of these glycosylation differences may result in differences in pharmacological responses in the heterologous system.

Pharmacology of transporters are determined by biochemical assays after heterologous expression of the protein. Given the increasing association between anthelmintic resistance and P-gp expression, there has been a growing interest in the pharmacological interaction of anthelmintic molecules with nematode P-glycoproteins. The most frequently used assays in nematode P-gp study are competitive efflux of a fluorescent substrate such as Rhodamine 123, Hoechst 33342, Calcein-AM in the presence of a drug such as a macrocyclic lactone. Ivermectin, eprinomectin, abamectin, doramectin, selamectin and moxidectin, interact with mammalian P-gps by competitively inhibiting efflux of a fluorescent P-gp substrate (Lespine et al., 2007). Other anthelmintics such as closantel, triclabendazole and rafoxanide also competitively inhibit Rhodamine 123 efflux by mammalian P-gp (Dupuy et al., 2010). However, since nematode P-
gps share only 35 - 64% identity with mammalian P-gps, efflux capabilities of the former cannot be directly extrapolated from data of the latter. Additionally, different nematode P-gps may have different affinities and responses to drugs, which can only be determined by empirical testing. Other assays including measurements of ATPase activity have also occasionally been used. Drug interaction studies in heterologous systems are summarized in Table 2-4.

3.4. Whole organism pharmacology

One or more free-living stages of nematodes are often used to assess the pharmacological response of P-gps with the assumption that free-living stages are a surrogate for parasitic stages. These assays involve the incubation of eggs or larvae with different drugs followed by flow cytometry, development or motility assay (Demeler et al., 2012; Demeler et al., 2010; Kerboeuf et al., 1996). In vitro tests possess the advantage that receptors and proteins are expressed in their homologous state, and specific or class-wide ML resistance can be detected (Kotze et al., 2014). They suffer from the disadvantage that free living stages are not generally accessible to drugs and tissue specific responses may not be the same in different life stages unless empirically proven. In some groups of nematodes such as the ascarids, no free-living larval stages exist. Therefore, assays have to be optimized with biological relevance in mind. Relevant whole organism studies of P-gps have been summarized in Table 2-5.

Larval development assay (LDA) is based on the inhibition of the biological development and hatching of larvae from a single cell/morula that is present in a freshly laid egg in the presence of drugs. Some drugs such as the benzimidazoles have known ovicidal properties while the ovicidal effects of others such as the ivermectin depends on the route of administration in in vivo studies (Tyrrell et al., 2002). Several lines of evidence led to the conclusion that LDA suffers from having poor correlation between clinical phenotype and in vitro assay output, being the best for benzimidazoles such as thiabendazole, which has known ovicidal property, and the
worst for levamisole and ivermectin (Tandon and Kaplan, 2004). This was further demonstrated in a comparison of two variants of the LDA in which the assays were able to detect benzimidazole and levamisole resistance but not ivermectin resistance (Várady et al., 2009). But, a more recent study has demonstrated its use in distinguishing susceptible and resistant isolates of nematodes (Dolinská et al., 2013). Some researchers have used the LDA with known P-gp inhibitors and Mls to test if P-gp inhibition leads to developmental arrest/death. The LDA has only been used to study strongyle nematodes.

The Larval motility inhibition assay/test (LMIA/LMIT) is based on the behavioral propensity of third larval stages of strongyles to actively migrate up grass blades to await ingestion by a host. The *in vitro* assay setup involves a filter mesh through which larvae are allowed to migrate in the presence or absence of drugs (Rothwell and Sangster, 1993). The assay can be modified to detect paralysis after hatching in the presence of avermectins (Gill et al., 1995).

The larval feeding inhibition assay is based on the inhibition of bacterivoral behavior of first stage strongyle larvae in the presence of drugs (Alvarez-Sánchez et al., 2005). This assay cannot be used in nematode groups such as ascarids, oxyurids and spirurids which do not have free-living L1s.

Motility as measured by number of movements of the posterior end of the adult worm per unit time, as measured by an operator has been used in studies of filarial nematodes (Tompkins et al., 2010). Given the great variation in sizes of nematode life stages, motility measurements cannot be easily compared between groups of nematodes. Further research is needed to optimize motility measures for each drug class and each parasitic nematode.
Phenotypic assays such as pharyngeal pumping and velocity have been used as outputs in the model organism *C. elegans* to demonstrate the role of P-gps in cross resistance of IVM and MOX (Bygarski et al., 2014). These have not been used in parasitic nematodes so far.

### 3.5. Localization studies

Tissue specific transporter activity is important for understanding pharmacokinetic disposition of drugs. In nematodes, P-gp expression in different tissues is relevant in understanding compartmentalization of anthelmintics. Antibodies raised against mammalian P-gps are frequently used as reagents to understand nematode P-gp localization. Nucleic acid-based assays including chromogenic *in situ* hybridization RNAscope have been recently used. These are summarized in Table 2-6.

### 3.6. In vivo assays

ML resistance in parasitic nematodes is due to adult populations that survive anthelmintic treatments. P-gp activity in these parasites can be inhibited by administering P-gp inhibitors to host animals. Pharmacokinetic studies have shown that plasma concentrations of the ivermectin and moxidectin increase when P-gp inhibitors such as loperamide, quercetine, verapamil, closantel, ketoconazole,itraconazole, albendazole and triclabendazole are co-administered (Alvarez et al., 2008; Alvinerie et al., 2008; Ballent et al., 2007; Cromie et al., 2006; Dupuy et al., 2003; Lifschitz et al., 2009; Lifschitz et al., 2002; Molento et al., 2004). Several *in vivo* studies that have examined the effect of co-administration of drugs on parasites are summarized in Table 2-7.

### 4. Conclusions

Macrocyclic lactone resistance in nematodes of veterinary importance is an emerging issue which has morphed into a massive economic burden on producers in recent years. In companion animals, emerging loss of efficacy of MLs in controlling *Dirofilaria immitis*
has resulted in poor life quality. Research efforts have identified a role for P-glycoproteins in ML resistance and tolerance. However, several questions remain about the mechanism by which P-gps confer resistance.

Given the variation in expression levels obtained by different research groups in different nematodes, empirical studies are essential in parasites in which P-gps have not been studied so far. Methods used in the study of nematode P-gps have to be developed and optimized for each nematode with biologically relevant life stages prioritized over easily obtainable free-living stages. Regulatory mechanisms that govern P-gp expression such as transcription factors (Ménez et al., 2019) must be identified, along with interactions between glutamate-gated chloride channels, other ion channels and P-gps. Candidate gene based approaches in the study of macrocyclic lactone resistance are being slowly supplanted by whole genome scale approaches when chromosomal scale genome assemblies are available (Doyle and Cotton, 2019), but these have yet to be used in P-gp studies.

Pharmacological profiles of different nematode P-gps have to be individually assessed to identify those with unique profiles that can be exploited to reverse drug resistance. Further, there is a need for P-gp inhibitors that will specifically inhibit nematode P-gps.

Finally, selection protocols may dictate the development of resistance and more than one mechanism may exist for resistance development (Gill et al., 1998). Clinically relevant anthelmintic selection and use protocols, tailored for use in different vegetation and climate belts of the world must be developed to lower the selection pressure on parasites.

5. References


Figure 2-1. UPGMA phylogenetic tree of ABC superfamily proteins annotated in the genomes of *Toxocara canis* and *C. elegans*.
Figure 2-2. Secondary structure of *C. elegans* Pgp-1 after (Jin et al., 2012), created using RCSB PDB (PDB: 4F4C) with DSSP, RESID and PSI-MOD tools (Garavelli, 2004; Kabsch and Sander, 1983; Montecchi-Palazzi et al., 2008). The secondary structure has an estimated 54% helical and 9% beta sheet structure.
Figure 2-3. Transmembrane view of the X-ray diffraction crystal structure of C. elegans Pgp-1 at 3.4Å in the inward facing confirmation created using NGL viewer PDB ID: 4F4C after (Jin et al., 2012).
Table 2-1. ABC transporter genes in humans and nematodes, derived from the KEGG pathways (Kanehisa et al., 2017).

<table>
<thead>
<tr>
<th>Family</th>
<th>Sub family</th>
<th>HGNC name</th>
<th>Function in humans</th>
<th>Present in nematodes</th>
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<td><strong>Class 1 systems</strong></td>
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<td><strong>DPL</strong></td>
<td></td>
<td></td>
<td>Drug, peptides and lipid</td>
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<td>P-gp</td>
<td>ABCB1</td>
<td></td>
<td>Efflux pump xenobiotic compounds with broad substrate specificity, multiple drug resistance</td>
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<tr>
<td>TAP</td>
<td>ABCB2</td>
<td></td>
<td>Transport of degraded cytosolic peptides</td>
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<tr>
<td>TAP</td>
<td>ABCB3</td>
<td></td>
<td>Peptide transport</td>
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<td>HMI/HMT</td>
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<td>MDR/TAP</td>
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<td>MDR/TAP</td>
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<td>Organic and inorganic molecule transport from mitochondria</td>
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<td>MDR/TAP</td>
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<td>ABCC1</td>
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<td></td>
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<td>ABCC2</td>
<td>Drug resistance in mammalian cells</td>
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<td>ABCC3</td>
<td>Organic anion transport in biliary and intestinal excretion</td>
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<td>Function in humans</td>
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<td>ABCC10</td>
<td>Cellular detoxification, lipophilic anion extrusion</td>
<td><em>T. spiralis, N. americanus</em></td>
</tr>
<tr>
<td></td>
<td></td>
<td>ABCC11</td>
<td>Transport of lipophilic anions, glucose, bile salts, organic acids</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>ABCC12</td>
<td>Transmembrane transport</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>ABCC13</td>
<td>Pseudogene in humans</td>
<td></td>
</tr>
<tr>
<td>FAE</td>
<td></td>
<td>ABCD</td>
<td>very long chain Fatty Acid Export</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>ABCD1</td>
<td>Fatty acid transport in peroxisomes</td>
<td><em>L. loa</em></td>
</tr>
<tr>
<td></td>
<td></td>
<td>ABCD2</td>
<td>Fatty acid transport in peroxisomes</td>
<td><em>C. elegans, C. briggsae, N. americanus</em></td>
</tr>
<tr>
<td></td>
<td></td>
<td>ABCD3</td>
<td>Fatty acid transport in peroxisomes</td>
<td><em>C. elegans, C. briggsae, N. americanus</em></td>
</tr>
<tr>
<td></td>
<td></td>
<td>ABCD4</td>
<td>Fatty acid transport in peroxisomes</td>
<td><em>C. elegans, C. briggsae, B. malayi, L. loa, T. spiralis, N. americanus</em></td>
</tr>
<tr>
<td>EPD</td>
<td>WHITE</td>
<td>ABCG or BCRP</td>
<td>Eye pigment precursors and drugs</td>
<td><em>C. elegans</em></td>
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</tbody>
</table>

**Class 2 systems**

<table>
<thead>
<tr>
<th>Family</th>
<th>Sub family</th>
<th>HGNC name</th>
<th>Function in humans</th>
<th>Present in nematodes</th>
</tr>
</thead>
<tbody>
<tr>
<td>RLI</td>
<td></td>
<td>ABCE1</td>
<td>RNase L inhibitor in mammals</td>
<td></td>
</tr>
<tr>
<td>ART</td>
<td>REG</td>
<td>ABCF</td>
<td>Translation regulation</td>
<td></td>
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</tbody>
</table>

**Class 3 systems**

<table>
<thead>
<tr>
<th>Family</th>
<th>Sub family</th>
<th>HGNC name</th>
<th>Function in humans</th>
<th>Present in nematodes</th>
</tr>
</thead>
<tbody>
<tr>
<td>DRA</td>
<td>ABCA; previously ABC1</td>
<td>ABCA</td>
<td>Exclusive to multicellular eukaryotes; Lipid trafficking</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>ABCA1</td>
<td>Cholesterol efflux regulatory protein/cholesterol metabolism</td>
<td><em>C. elegans, C. briggsae, B. malayi, L. loa, N. americanus</em></td>
</tr>
<tr>
<td>Family</td>
<td>Sub family</td>
<td>HGNC name</td>
<td>Function in humans</td>
<td>Present in nematodes</td>
</tr>
<tr>
<td>--------</td>
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<td>---------------------------------------------------------</td>
<td>---------------------------------------</td>
</tr>
<tr>
<td>DRA</td>
<td>ABCA</td>
<td>ABCA2</td>
<td>Macrophage lipid metabolism and neural development</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>ABCA3</td>
<td>Resistance to xenobiotics and engulfment during programmed cell death</td>
<td><em>C. elegans, C. briggsae, L. loa, N. americanus, T. spiralis</em></td>
</tr>
<tr>
<td></td>
<td></td>
<td>ABCA4</td>
<td>Retina photoreceptor specific ABC transporter that transports N-retinylidene-PE</td>
<td><em>T. spiralis</em></td>
</tr>
<tr>
<td></td>
<td></td>
<td>ABCA5</td>
<td>Possibly lysosomal trafficking; Substrate and function unknown</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>ABCA6</td>
<td>Macrophage lipid homeostasis</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>ABCA7</td>
<td>Immune cell lipid homeostasis</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>ABCA8</td>
<td>Formation and maintenance of myelin; possibly drug transport</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>ABCA9</td>
<td>Monocyte differentiation</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>ABCA10</td>
<td>Lipid transport</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>ABCA11P</td>
<td>Pseudogene in humans</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>ABCA12</td>
<td>Lipid transport in skin</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>ABCA13</td>
<td>Lipid transport</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>ABCA17P</td>
<td>Pseudogene in humans</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>ABCA14 to ABCA17</td>
<td>Present in rodents only</td>
<td></td>
</tr>
</tbody>
</table>

*HGNC= Human Gene Nomenclature Committee*
Table 2-2. P-glycoprotein encoding genes in pathogenic nematodes of veterinary importance

<table>
<thead>
<tr>
<th>Species</th>
<th>Protein encoded</th>
<th>Gene</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Clade V nematodes</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td><em>Haemonchus contortus</em></td>
<td>Pgp-2 /Pgp-A</td>
<td><em>Hco-Pgp</em>-2</td>
<td>(Xu et al., 1998)</td>
</tr>
<tr>
<td></td>
<td>Pgp-1</td>
<td><em>Hco-Pgp</em>-1</td>
<td>(Le Jambre et al., 1999)</td>
</tr>
<tr>
<td></td>
<td>Pgp 3</td>
<td><em>Hco-Pgp</em>-3</td>
<td>(Issouf et al., 2014; Raza et al., 2016b; Williamson and Wolstenholme, 2012)</td>
</tr>
<tr>
<td></td>
<td>Pgp-4</td>
<td><em>Hco-Pgp</em>-4</td>
<td>(Williamson and Wolstenholme, 2012)</td>
</tr>
<tr>
<td></td>
<td>Pgp-9.1</td>
<td><em>Hco-Pgp</em>-9.1</td>
<td>(Issouf et al., 2014; Raza et al., 2016b; Williamson and Wolstenholme, 2012)</td>
</tr>
<tr>
<td></td>
<td>Pgp-9.2</td>
<td><em>Hco-Pgp</em>-9.2</td>
<td>(Issouf et al., 2014; Raza et al., 2016b)</td>
</tr>
<tr>
<td></td>
<td>Pgp-9.3</td>
<td><em>Hco-Pgp</em>-9.3</td>
<td>(Issouf et al., 2014; Raza et al., 2016b)</td>
</tr>
<tr>
<td></td>
<td>Pgp-10</td>
<td><em>Hco-Pgp</em>-10</td>
<td>(Issouf et al., 2014; Raza et al., 2016b; Williamson and Wolstenholme, 2012)</td>
</tr>
<tr>
<td></td>
<td>Pgp-11</td>
<td><em>Hco-Pgp</em>-11</td>
<td>(Issouf et al., 2014; Raza et al., 2016b; Williamson and Wolstenholme, 2012)</td>
</tr>
<tr>
<td></td>
<td>Pgp-12</td>
<td><em>Hco-Pgp</em>-12</td>
<td>(Raza et al., 2016b)</td>
</tr>
<tr>
<td></td>
<td>Pgp-13</td>
<td><em>Hco-Pgp</em>-13</td>
<td>(David et al., 2018)</td>
</tr>
<tr>
<td></td>
<td>Pgp-14</td>
<td><em>Hco-Pgp</em>-14</td>
<td>(Issouf et al., 2014; Raza et al., 2016b; Williamson and Wolstenholme, 2012)</td>
</tr>
<tr>
<td></td>
<td>Pgp-16</td>
<td><em>Hco-Pgp</em>-16</td>
<td>(Issouf et al., 2014; Raza et al., 2016b)</td>
</tr>
<tr>
<td></td>
<td>Pgp-17</td>
<td><em>Hco-Pgp</em>-17</td>
<td>(Laing et al., 2016; Maté et al., 2018)</td>
</tr>
<tr>
<td><em>Oesophagostomum dentatum</em></td>
<td>2 P-gps</td>
<td></td>
<td>(Laing et al., 2016)</td>
</tr>
<tr>
<td><em>Necator americanus</em></td>
<td>Pgp10 tandem duplication</td>
<td></td>
<td>(Laing et al., 2016)</td>
</tr>
<tr>
<td></td>
<td>Novel Pgp not present in <em>H. contortus</em> or <em>C. elegans</em></td>
<td>(Laing et al., 2016)</td>
<td></td>
</tr>
<tr>
<td><strong>Teladorsagia circumcincta</strong></td>
<td>Pgp-2</td>
<td><em>Tci-Pgp</em>-2</td>
<td>(Dicker et al., 2011b)</td>
</tr>
<tr>
<td></td>
<td>Pgp-3 or 4</td>
<td><em>Tci-Pgp</em>-3</td>
<td>(Dicker et al., 2011b)</td>
</tr>
<tr>
<td></td>
<td>Pgp-9</td>
<td><em>Tci-Pgp</em>-9</td>
<td>(Dicker et al., 2011b; Turnbull et al., 2018)</td>
</tr>
<tr>
<td></td>
<td>Putative Pgp10</td>
<td><em>Tci-Pgp</em>-10</td>
<td>(Dicker et al., 2011b)</td>
</tr>
<tr>
<td>Species</td>
<td>Protein encoded</td>
<td>Gene</td>
<td>Reference</td>
</tr>
<tr>
<td>------------------------------</td>
<td>-----------------</td>
<td>------------</td>
<td>------------------------------------------------</td>
</tr>
<tr>
<td><em>Cooperia oncophora</em></td>
<td>Pgp-1</td>
<td><em>Con-Pgp-1</em></td>
<td>(De Graef et al., 2013)</td>
</tr>
<tr>
<td></td>
<td>Pgp-2</td>
<td><em>Con-Pgp-2</em></td>
<td>(Demeler et al., 2013)</td>
</tr>
<tr>
<td></td>
<td>Pgp-3</td>
<td><em>Con-Pgp-3</em></td>
<td>(Demeler et al., 2013)</td>
</tr>
<tr>
<td></td>
<td>Pgp-9</td>
<td><em>Con-Pgp-9</em></td>
<td>(Areskog et al., 2013; Tydén et al., 2014)</td>
</tr>
<tr>
<td></td>
<td>Pgp-11</td>
<td><em>Con-Pgp-11</em></td>
<td>(De Graef et al., 2013; Tydén et al., 2014)</td>
</tr>
<tr>
<td></td>
<td>Pgp-12</td>
<td><em>Con-Pgp-12</em></td>
<td>(Demeler et al., 2013; Tydén et al., 2014)</td>
</tr>
<tr>
<td></td>
<td>Pgp-16</td>
<td><em>Con-Pgp-16</em></td>
<td>(Demeler et al., 2013; Tydén et al., 2014)</td>
</tr>
<tr>
<td><em>Cylicocyclus elongatus</em></td>
<td>Pgp-9</td>
<td><em>Ceg-Pgp-9</em></td>
<td>(Kaschny et al., 2015; Peachey et al., 2017)</td>
</tr>
<tr>
<td>Other cyathostome spp.</td>
<td></td>
<td></td>
<td>(Drogemuller et al., 2004)</td>
</tr>
<tr>
<td><strong>Clade III nematodes</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td><em>Parascaris spp.</em></td>
<td>Pgp-11</td>
<td><em>Peq-Pgp-11</em></td>
<td>(Janssen et al., 2013a; Janssen et al., 2015;</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>Jesudoss Chelladurai and Brewer, 2019)</td>
</tr>
<tr>
<td></td>
<td>Pgp-16</td>
<td><em>Peq-Pgp-16</em></td>
<td>(Janssen et al., 2013a; Jesudoss Chelladurai</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>and Brewer, 2019)</td>
</tr>
<tr>
<td><em>Dirofilaria immitis</em></td>
<td>Pgp-3</td>
<td><em>Dim-Pgp-3</em></td>
<td>(Bourguinat et al., 2016)</td>
</tr>
<tr>
<td></td>
<td>Pgp-10</td>
<td><em>Dim-Pgp-10</em></td>
<td>(Bourguinat et al., 2016)</td>
</tr>
<tr>
<td></td>
<td>Pgp-11</td>
<td><em>Dim-Pgp-11</em></td>
<td>(Bourguinat et al., 2016; Mani et al., 2016)</td>
</tr>
</tbody>
</table>
Table 2-3. Summary of gene expression and polymorphism studies in parasitic nematodes of veterinary importance.

<table>
<thead>
<tr>
<th>Nematode</th>
<th>Assay and experimental set-up</th>
<th>Significant result</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>Haemonchus contortus</em></td>
<td>Restriction fragment length polymorphism of <em>Hco-Pgp</em>-2 in adult male worms from IVM or MOX selected and unselected strains</td>
<td>Polymorphism in P-gp genes as a result of allelic differences in genes may be associated with IVM and MOX resistance.</td>
<td>(Blackhall et al., 1998a)</td>
</tr>
<tr>
<td></td>
<td>(a) Northern blotting of RNA from eggs from IVM selected and unselected strains. (b) Southern blotting of DNA on adults of both strains</td>
<td>Increased expression of P-gp mRNA in IVM-selected strain. Qualitative differences in DNA restriction patterns between IVM selected strain and unselected strain.</td>
<td>(Xu et al., 1998)</td>
</tr>
<tr>
<td></td>
<td>(a) Cloning and sequencing of internucleotide binding domains. (b) Northern blotting of RNA from eggs, immature adult and adult males from IVM susceptible strain. (c) Southern blotting of DNA from adult worms of IVM resistant strain</td>
<td>Heterogeneity in IBDs of Hco-Pgps found. P-gp mRNA is developmentally regulated and differentially expressed in life stages. There is evidence for P-gp involvement in IVM resistance.</td>
<td>(Sangster et al., 1999)</td>
</tr>
<tr>
<td></td>
<td>PCR followed by RFLP of <em>Hco-Pgp</em>-2 in cambendazole selected and unselected strains</td>
<td>Difference in allelic frequency between cambendazole selected and unselected strains</td>
<td>(Blackhall et al., 2008)</td>
</tr>
<tr>
<td></td>
<td>PCR followed by RFLP of <em>Hco-Pgp</em>-9 in IVM selected and unselected worms</td>
<td>Allelic frequency increase in ML selected worms compared to unselected</td>
<td>(Le Jambre et al., 1999)</td>
</tr>
<tr>
<td></td>
<td>Southern blotting to analyze restriction fragment length polymorphism between susceptible and resistant strains</td>
<td>First study to show that P-gps did not show any selection in BZ or LEV resistance</td>
<td>(Kwa et al., 1998)</td>
</tr>
</tbody>
</table>
Table 2-3 continued

<table>
<thead>
<tr>
<th>Nematode</th>
<th>Assay and experimental set-up</th>
<th>Significant result</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>Haemonchus contortus</em></td>
<td>qPCR to analyze P-gp transcription patterns in an isolate resistant to benzimidazole, levamisole and avermectins</td>
<td><em>Hco-Pgp</em>-2 and -9 levels were significantly upregulated; <em>Hco-Pgp</em>-1 level was downregulated in the resistant strain compared to susceptible lab isolate. No changes in expression levels of <em>Hco-pgp</em>-3, -4, -10, -11, -12 or -14 were noted.</td>
<td>(Williamson et al., 2011)</td>
</tr>
<tr>
<td></td>
<td>qPCR to analyze P-gp transcription patterns in isolate experimentally selected for IVM resistance over 3 generations</td>
<td>In L3 of the selected strain, no significant changes in expression level were found in <em>Hco-Pgp</em>-1, -2, -3, -4, -9, -10, -11, -12 and -14.</td>
<td>(Williamson and Wolstenholme, 2012)</td>
</tr>
<tr>
<td></td>
<td>qPCR to analyze P-gp transcription pattern of <em>Hco-Pgp</em>-2 in resistant isolate treated in vivo with MLs</td>
<td>Only ivermectin, not abamectin or moxidectin caused upregulation of P-gp expression in adults</td>
<td>(Lloberas et al., 2013)</td>
</tr>
<tr>
<td></td>
<td>qPCR to analyze P-gp transcription patterns in levamisole resistant strains</td>
<td>No consistent changes in P-gp gene expression seen</td>
<td>(Sarai et al., 2013)</td>
</tr>
<tr>
<td></td>
<td>qPCR to analyze P-gp transcription patterns in <em>H. contortus</em> larvae exposed to eosinophil granules</td>
<td><em>Hco-Pgp</em>-3 and -16 were upregulated when larvae were exposed to eosinophil granules</td>
<td>(Issouf et al., 2014)</td>
</tr>
<tr>
<td></td>
<td>qPCR to analyze P-gp transcription patterns in IVM resistant adult worms from lambs 14 days post treatment</td>
<td>IVM dosed at 10x recommended dose did not modify P-gp homolog expression</td>
<td>(Alvarez et al., 2015)</td>
</tr>
<tr>
<td>Nematode</td>
<td>Assay and experimental set-up</td>
<td>Significant result</td>
<td>Reference</td>
</tr>
<tr>
<td>-------------------</td>
<td>------------------------------------------------------------------------------------------------</td>
<td>-------------------------------------------------------------------------------------------------------------</td>
<td>--------------------</td>
</tr>
<tr>
<td><em>Haemonchus</em></td>
<td>qPCR to analyze P-gp transcription patterns in susceptible worms exposed to monepantel</td>
<td>Transcription of <em>Hco-pgp</em>-11, -12 and -14 genes were upregulated, with a sustained increase in <em>Hco-pgp</em>-11 after drug removal</td>
<td>(Raza et al., 2016a)</td>
</tr>
<tr>
<td><em>contortus</em></td>
<td>qPCR to analyze P-gp transcription patterns in L3s hatched from eggs derived from sheep feces</td>
<td>L3s from resistant isolate expressed significantly higher levels of <em>Hco-Pgp</em>-1, -2, -3, -9.1, -9.2, -9.3, -10, -11, -12, -14 and -16 compared to susceptible isolate</td>
<td>(Raza et al., 2016b)</td>
</tr>
<tr>
<td></td>
<td>qPCR to analyze P-gp transcription patterns in L3s exposed <em>in vitro</em> to drugs for 3 – 6 hrs.</td>
<td>No changes in the susceptible isolate following exposure to IVM for 3 hrs. or 6 hrs. <em>Hco-pgp</em>-2, -9.1, -11, -1, -10 upregulated in resistant isolate after IVM or LEV exposure for 3 hrs. but not 6 hrs. LEV exposure also upregulated <em>Hco-pgp</em>11 at 6hrs.</td>
<td>(Raza et al., 2016b)</td>
</tr>
<tr>
<td></td>
<td>RNA-seq and qPCR to analyze P-gp gene transcription in adult males and females after <em>in vivo</em> exposure to 10x the therapeutic dose</td>
<td><em>Hco-pgp</em>-3 and -9 were downregulated and <em>Hco-Pgp</em>-2 was upregulated over 24 hrs after <em>in vivo</em> IVM exposure. <em>Hco-pgp</em>-1 and -11 levels did not change over time</td>
<td>(Maté et al., 2018)</td>
</tr>
</tbody>
</table>
Table 2-3 continued

<table>
<thead>
<tr>
<th>Nematode</th>
<th>Assay and experimental set-up</th>
<th>Significant result</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>Haemonchus contortus</em></td>
<td>(a) qPCR to analyze basal P-gp transcription patterns in adult male and female worms. (b) qPCR to analyze transcription response to low doses of IVM <em>in vitro</em> in adult males and females.</td>
<td><em>Hco</em>-Pgp-2 was the highest basal transcription level in females while <em>Hco</em>-Pgp-3 and -9.1 had the highest basal transcription levels in males. Significant upregulation of <em>Hco</em>-pgp-9.2 in IVM exposed males and of <em>Hco</em>-pgp-10 and -11 in IVM exposed females</td>
<td>(Kellerová et al., 2019)</td>
</tr>
<tr>
<td><em>Cooperia oncophora</em></td>
<td>SNP analysis of <em>Con</em>-Pgp-2 and <em>Con</em>-Pgp-3 in various field isolates</td>
<td>32 SNPs found among 6 isolates that caused increase/ decrease/ complete change in amino acid variability in <em>Con</em>-Pgp-2, while <em>Con</em>-Pgp-3 had low to no variability and was highly conserved</td>
<td>(Demeler et al., 2013)</td>
</tr>
<tr>
<td></td>
<td>Reverse transcription-qPCR to analyze transcription response to selection with IVM <em>in vivo</em> 10 days post treatment in IVM selected and unselected populations. Amplified fragment length polymorphism (AFLP) to detect gene diversity variations between pre and post treatment populations.</td>
<td><em>Con</em>-Pgp-9 expression was higher in female worms than male worms. No differences in expression pattern between IVM selected and unselected groups were observed. No differences in gene diversity between pre and post treatment populations were observed.</td>
<td>(Areskog et al., 2013)</td>
</tr>
<tr>
<td>Nematode</td>
<td>Assay and experimental set-up</td>
<td>Significant result</td>
<td>Reference</td>
</tr>
<tr>
<td>--------------------------</td>
<td>-----------------------------------------------------------------------------------------------</td>
<td>---------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------</td>
<td>--------------------------------------------------------------------------</td>
</tr>
<tr>
<td><strong>Nematode</strong></td>
<td></td>
<td><strong>Significant result</strong></td>
<td><strong>Reference</strong></td>
</tr>
<tr>
<td></td>
<td>qPCR to analyze basal P-gp expression after <em>in vivo</em> and <em>in vitro</em> ML exposure of IVM resistant and susceptible isolates</td>
<td>Basal levels of P-gp expression in eggs, L3 and adult was not significantly different between resistant and susceptible worms. Significant upregulation of <em>Con-pgp-11</em> was found in IVM-resistant adults exposed to IVM. <em>Con-pgp-2</em> and <em>-11</em> were upregulated IVM-resistant adults exposed to MOX. <em>Con-pgp-12</em> and <em>Con-pgp-16</em> were upregulated in IVM-exposed susceptible and IVM-resistant L3s.</td>
<td>(De Graef et al., 2013)</td>
</tr>
<tr>
<td></td>
<td>qPCR to analyze transcription in adult worms of a field selected isolate exposed to MLs <em>in vivo</em></td>
<td><em>Con-pgp-9</em>, -11, -12 and -16 basal expression levels were higher in male and female worms in field-selected isolate compared to susceptible isolate. <em>Con-Pgp-16</em> was upregulated after treatment with ivermectin in field and lab selected isolates. No changes were induced by doramectin.</td>
<td>(Tydén et al., 2014)</td>
</tr>
<tr>
<td><strong>Teladorsagia</strong></td>
<td>qPCR to analyze transcription of <em>Tci-pgp-9</em> in susceptible and triple resistant (FBZ, LEV, IVM) field isolate</td>
<td>Triple resistant field isolate had higher levels of <em>Tci-pgp-9</em> than the susceptible isolate at all life stages (eggs, L1, exsheathed L3, L4, adults)</td>
<td>(Dicker et al., 2011b)</td>
</tr>
</tbody>
</table>
Table 2-3 continued

<table>
<thead>
<tr>
<th>Nematode</th>
<th>Assay and experimental set-up</th>
<th>Significant result</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>Teladorsagia circumcincta</em></td>
<td>SNP analysis of partial <em>Tci-pgp-9</em> in susceptible and triple resistant (FBZ, LEV, IVM) field isolate</td>
<td>Partial sequence analysis of the second internucleotide binding domain of <em>Tci-pgp-9</em> revealed several non-coding SNPs between the susceptible and triple resistant isolates.</td>
<td>(Dicker et al., 2011b)</td>
</tr>
<tr>
<td></td>
<td>Transcriptomic (EST) analysis following <em>in vitro</em> exposure of resistant adult worms to IVM</td>
<td>P-gp genes were poorly represented in the exposed and unexposed EST datasets.</td>
<td>(Dicker et al., 2011a)</td>
</tr>
<tr>
<td></td>
<td>Sequence and SNP analysis of complete <em>Tci-pgp-9</em> in IVM, LEV and BZ isolate</td>
<td>9 non-synonymous SNPs were found in the multidrug resistant isolate. High intra-worm allelic variability in IBD region was found. Selection for specific gene variants observed in resistant strain.</td>
<td>(Turnbull et al., 2018)</td>
</tr>
<tr>
<td><em>Mixed spp. of Cyathostomins</em></td>
<td>qPCR to quantify transcription of <em>Pgp-9</em> in mixed population L3s after exposure to IVM <em>in vitro</em></td>
<td>Higher levels of <em>Pgp-9</em> transcription was found in IVM resistant isolate after IVM exposure compared to the IVM sensitive isolate.</td>
<td>(Peachey et al., 2017)</td>
</tr>
<tr>
<td><em>Parascaris spp.</em></td>
<td>qPCR to quantify expression of <em>Peq-pgp-11</em> and -16 in eggs, pre-adults and adults of resistant and susceptible populations, SNP analysis of <em>Peq-Pgp-11</em> in resistant and susceptible populations</td>
<td>No significant differences P-gp expression were found in embryonated eggs derived from ML resistant and susceptible populations. <em>Peq-pgp-11</em> was overexpressed in preadults resistant to MLs. IVM incubation of adults did not affect <em>Peq-pgp-11</em> and -16 expression. Three SNPs were identified in <em>Peq-Pgp-11</em> and were associated with ML resistance.</td>
<td>(Janssen et al., 2013a)</td>
</tr>
</tbody>
</table>
Table 2-3 continued

<table>
<thead>
<tr>
<th>Nematode</th>
<th>Assay and experimental set-up</th>
<th>Significant result</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>Dirofilaria immitis</em></td>
<td>SNP analysis of <em>Dim-Pgp-11</em></td>
<td>Partial sequence analysis of <em>Dim-Pgp-11</em> revealed 2 SNPs (one in the coding region in the IBD and one in non-coding region) between putative IVM resistant and susceptible isolates. Diploptype analysis revealed significant high presence of GG-GG in resistant isolates.</td>
<td>(Bourguinat et al., 2011b)</td>
</tr>
<tr>
<td></td>
<td>qPCR to quantify expression of <em>Dim-Pgp-10, Dim-Pgp-11</em> in male and female adults treated in vitro with IVM, doxycycline or both</td>
<td>In IVM treated females, both genes were downregulated, but were upregulated when treated with DOX alone. Combination therapy caused upregulation of <em>Dim-Pgp-10</em>.</td>
<td>(Lucchetti et al., 2019)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>In IVM treated males, both genes were upregulated in IVM treated and combination therapy but not when treated with DOX alone.</td>
<td></td>
</tr>
</tbody>
</table>
Table 2-4. Drug interaction studies of nematode P-gps in heterologous systems.

<table>
<thead>
<tr>
<th>Model</th>
<th>P-gp studied</th>
<th>Assay used to study</th>
<th>Inhibitors used</th>
<th>Significant results</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>Pig epithelial kidney cell line - LLC-PK1</td>
<td>Hco-Pgp-2</td>
<td>Rhodamine 123 and Calcein-AM accumulation in a ligand competition assay</td>
<td>IVM, ABA, MOX, Valspodar</td>
<td>IVM and abamectin but not MOX had inhibitory effect on R123 efflux similar to VSP. All three MLs had an inhibitory effect on Calcein-AM efflux</td>
<td>(Godoy et al., 2015b)</td>
</tr>
<tr>
<td></td>
<td>Hco-Pgp-9.1</td>
<td>Rhodamine 123 and Calcein-AM accumulation in a ligand competition assay</td>
<td>IVM, ABA, MOX, VSP</td>
<td>IVM and abamectin but not MOX had inhibitory effect on R123 efflux, similar to VSP</td>
<td>(Godoy et al., 2016)</td>
</tr>
<tr>
<td></td>
<td>Hco-Pgp-16</td>
<td>Rhodamine 123 and Calcein-AM accumulation in a ligand competition assay</td>
<td>IVM, ABA, MOX, VSP</td>
<td>IVM and abamectin and high concentrations of moxidectin had inhibitory effect on R123 efflux, similar to VSP</td>
<td>(Godoy et al., 2015a)</td>
</tr>
<tr>
<td></td>
<td>Dim-Pgp-11</td>
<td>Rhodamine 123 and Hoechst 33342 and Calcein-AM accumulation in a ligand competition assay</td>
<td>IVM, SEL, MOX, MBO, VSP</td>
<td>IVM and selamectin but not MOX had inhibitory effect on R123 and H33342 efflux</td>
<td>(Mani et al., 2016)</td>
</tr>
<tr>
<td></td>
<td>Pichia pastoris Hco-Pgp-13</td>
<td>Vanadate sensitive ATPase activity</td>
<td>IVM, Actinomycin D</td>
<td>Inhibition by IVM and stimulation of ATPase activity by ACD in a concentration depended biphasic manner</td>
<td>(David et al., 2018)</td>
</tr>
<tr>
<td>Model</td>
<td>P-gp studied</td>
<td>Assay used to study</td>
<td>Inhibitors used</td>
<td>Significant results</td>
<td>Reference</td>
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<tr>
<td><em>Saccharomyces cerevisiae</em> lacking seven endogenous transporters</td>
<td><strong>Pgp-9</strong> from <em>Cylicocyclus elongatus</em></td>
<td>Direct Growth assay</td>
<td>Actinomycin D, Daunorubicin, Valinomycin, Ketaconazole, Thiabendazole</td>
<td><em>Ceg-Pgp-9</em> decreased yeast susceptibility to Ket. EC50 was higher for actinomycin, daunorubicin and valinomycin. No differences were found for TBZ</td>
<td>(Kaschny et al., 2015)</td>
</tr>
<tr>
<td><em>Pgp-9</em> from <em>Cylicocyclus elongatus</em></td>
<td>Indirect growth assay; competitive killing assay in the presence of Ketaconazole</td>
<td>IVM, EPM, MOX, SEL, DORA</td>
<td>Fungicidal effect of Ket was significantly increased when EPM, IVM and MOX, but not SEL or DORA are added</td>
<td></td>
<td></td>
</tr>
<tr>
<td><em>Ceg-Pgp-9</em></td>
<td>Antibody binding in the presence of drugs</td>
<td>IVM, MOX, SEL, Daunorubicin</td>
<td>IVM, MOX, daunorubicin increased binding of UIC2 to yeast cells. Selamectin had no effect.</td>
<td></td>
<td></td>
</tr>
<tr>
<td><em>C. elegans</em> deficient in Pgp11</td>
<td><strong>Pgp-11</strong> <em>Parascaris spp.</em></td>
<td>Thrashing assay</td>
<td>IVM</td>
<td>EC50 for IVM in transfected strain of <em>C. elegans</em> was higher than control strain</td>
<td>(Janssen et al., 2015)</td>
</tr>
</tbody>
</table>
Table 2-5. *In vitro* P-gp-drug interaction assays with whole organisms.

<table>
<thead>
<tr>
<th>Organism</th>
<th>Stage used</th>
<th>Assay</th>
<th>Results/comments</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>Haemonchus contortus</em></td>
<td>Eggs</td>
<td>Rhodamine 123 accumulation in a ligand competition assay using flow cytometry</td>
<td>Higher levels of accumulation of R123 when verapamil was added to IVM resistant strain eggs</td>
<td>(Kerboeuf et al., 1999)</td>
</tr>
<tr>
<td>Eggs</td>
<td>Rhodamine 123 accumulation in a ligand competition assay</td>
<td>MLs except ivermectin stimulated P-gps</td>
<td>(Kerboeuf and Guégnard, 2011)</td>
<td></td>
</tr>
<tr>
<td>Eggs</td>
<td>Rhodamine 123 accumulation after exposure to eosinophil granules</td>
<td>Eosinophil granules caused a dose dependent increase in R123 accumulation</td>
<td>(Issouf et al., 2014)</td>
<td></td>
</tr>
<tr>
<td>Eggs and L3s</td>
<td>Larval migration inhibition assay with IVM and P-gp inhibitors. Larval development assay</td>
<td>LMA: Third generation P-gp inhibitors increased larval sensitivity to ivermectin. Synergism seen between tariquidar and IVM in the resistant strain in the LMA. Synergism was also seen between zosuquidar and IVM, and between verapamil and IVM in larval development assays.</td>
<td>(Raza et al., 2015)</td>
<td></td>
</tr>
<tr>
<td>L3s</td>
<td>Rhodamine 123 efflux spectrophotometrically measured in supernatant following ligand competition assay</td>
<td><em>In vitro</em> exposure to high doses of monepantel caused increased efflux of Rhodamine 123</td>
<td>(Raza et al., 2016a)</td>
<td></td>
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Table 2-5 continued

<table>
<thead>
<tr>
<th>Organism</th>
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<th>Results/comments</th>
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</tr>
</thead>
<tbody>
<tr>
<td><em>Haemonchus contortus</em></td>
<td>L3s</td>
<td>Larval migration inhibition assay with IVM or LEV dilutions after pre-exposure to monepantel</td>
<td>Dose response curves obtained. In vitro pre-exposure to high doses of monepantel increased larval migration at high concentrations of IVM but not LEV</td>
<td>(Raza et al., 2016a)</td>
</tr>
<tr>
<td></td>
<td>L3s</td>
<td>Rhodamine 123 efflux spectrophotometrically measured in supernatant following ligand competition assay. Larval migration inhibition assay in the presence of different concentrations of drugs with in vitro pre-exposure to IVM or LEV</td>
<td>In vitro exposure to IVM and LEV increased efflux of Rhodamine 123. Dose response curves obtained. In vitro pre-exposure to high concentrations of IVM increased larval motility through a filter mesh system.</td>
<td>(Raza et al., 2016b)</td>
</tr>
<tr>
<td>Eggs and L3s</td>
<td>Larval development assay with IVM and P-gp inhibitor. Larval migration inhibition assay in the presence of different concentrations of IVM and P-gp inhibitor</td>
<td>Dose response curves obtained. In LDA, crizotinib decreased IVM IC50 in both susceptible and resistant strains. In LMIA, crizotinib caused a 2.6 fold decrease in IVM IC50 in the resistant but not susceptible strain.</td>
<td>(Raza et al., 2016c)</td>
<td></td>
</tr>
<tr>
<td>Eggs</td>
<td>Larval development assay with ML resistant strain against IVM, MOX, EPR</td>
<td>Dose response curves obtained with MOX having 29 fold and 280 fold lower EC50 than IVM and EPR</td>
<td>(Ménez et al., 2016)</td>
<td></td>
</tr>
<tr>
<td>Organism</td>
<td>Stage used</td>
<td>Assay</td>
<td>Results/comments</td>
<td>Reference</td>
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<tr>
<td>Adults</td>
<td>HPLC analysis of flubendazole in worms incubated ± verapamil</td>
<td>Flubendazole accumulation was not affected by Ver in any strains</td>
<td>(Bártíková et al., 2012)</td>
<td></td>
</tr>
<tr>
<td><em>Haemonchus placei</em></td>
<td>L3s</td>
<td>Larval migration inhibition assay in the presence of different concentrations of IVM with fixed concentrations of P-gp modulators</td>
<td>Increased IVM efficacy with a reduction in EC50 was seen with cyclosporine A, dexamethasone, verapamil, vinblastine, ceftriaxone, quercetin, trifluperazine</td>
<td>(Heckler et al., 2014)</td>
</tr>
<tr>
<td><em>T. circumcinta</em> and <em>H. contortus</em></td>
<td>L1s</td>
<td>Larval feeding inhibition by IVM in the presence and absence of P-gp interfering agents with FITC-E. coli</td>
<td>Resistant worms required more IVM than sensitive worms to arrest feeding behavior. P-gp inhibitors decreased larval feeding.</td>
<td>(Bartley et al., 2009)</td>
</tr>
<tr>
<td><em>C. oncophora</em></td>
<td>Eggs (Eggs and larvae counted in output)</td>
<td>Larval development assay with IVM±VPL</td>
<td>Dose response curves obtained. Resistance worms had higher EC50 than susceptible worms in the absence of VPL. In the presence of VPL, resistance worms had lower EC50s than susceptible strain.</td>
<td>(Demeler et al., 2013)</td>
</tr>
<tr>
<td>Sheathed</td>
<td>Larval migration inhibition assay (LMIA) with IVM ± VPL</td>
<td>Dose response curves obtained. IVM resistant isolates had higher EC50 in the presence of IVM only. VPL addition lowered the EC50 below that of the susceptible isolate.</td>
<td>(Demeler et al., 2013)</td>
<td></td>
</tr>
</tbody>
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Table 2-5 continued

<table>
<thead>
<tr>
<th>Organism</th>
<th>Stage used</th>
<th>Assay</th>
<th>Results/comments</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>C. oncophora</em> and <em>Ostertagia ostertagi</em></td>
<td>Eggs and L3s</td>
<td>Larval development assay Larval migration inhibition</td>
<td>Dose response curves obtained. Verapamil and piperonyl butoxide caused a decrease in EC50 of ivermectin In LMIA, verapamil increases susceptibility of ivermectin</td>
<td>(AlGusbi et al., 2014)</td>
</tr>
<tr>
<td>Mixed population of Cyathostomin</td>
<td>Eggs and L3s</td>
<td>Larval development assay Larval migration inhibition test</td>
<td>Dose response curves obtained. P-gp inhibitors - Ketaconzole and Pluronic 85 caused a higher reduction in EC50 in susceptible population than in the resistant population in LDA. Ketaconazole and ivermectin-aglycone caused a decrease in EC50 in the resistant population only in LMIT</td>
<td>(Peachey et al., 2017)</td>
</tr>
<tr>
<td><em>Heligmosomoides bakeri</em></td>
<td>Eggs and L3</td>
<td>Rhodamine 123 accumulation measured as % of R123 positive eggs following a ligand competition assay</td>
<td>Verapamil co-incubated with phytochemicals had an additive lethal effect on eggs and larvae</td>
<td>(Doligalska et al., 2011)</td>
</tr>
<tr>
<td>Organism</td>
<td>Stage used</td>
<td>Assay</td>
<td>Results/comments</td>
<td>Reference</td>
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</tr>
<tr>
<td><em>Brugia malayi</em></td>
<td>Male and female adults</td>
<td>Motility measured as no. of movements per minute in adults with IVM ± verapamil, cyclosporine A, vinblastine, daunorubicin</td>
<td>Motility was significantly inhibited in males and females when P-gp inhibitors were co-administered with ivermectin. Motility of microfilariae were lower when co-incubated with IVM + verapamil, quinidine, vincristine, vinblastine, colchicine, actinomycin d, daunorubicin, doxorubicin, etoposide, rhodamine and forskolin over a period of 7 days</td>
<td>(Tompkins et al., 2011)</td>
</tr>
<tr>
<td></td>
<td>Male and female adults</td>
<td>Motility measured as no. of movements per minute in adults with MOX ± Verapamil or daunorubicin</td>
<td>Motility was significantly inhibited in females only but not males when inhibitors were co-incubated. Motility of microfilariae were lower when co-incubated with MOX + verapamil, vinblastine, colchicine, etoposide, rhodamine, and forskolin.</td>
<td>(Stitt et al., 2011)</td>
</tr>
</tbody>
</table>
Table 2-6. Localization studies in nematodes of veterinary importance.

<table>
<thead>
<tr>
<th>Parasite</th>
<th>Life stage</th>
<th>Assay and reagent</th>
<th>Results/comments</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>Haemonchus contortus</em></td>
<td></td>
<td><em>In situ</em> hybridization using a labelled cDNA probe</td>
<td>P-gps were mainly expressed in the intestine and lateral cords. no differences between ML-selected and susceptible strains</td>
<td>(Smith and Prichard, 2002)</td>
</tr>
<tr>
<td>Eggs</td>
<td></td>
<td>Flow cytometry with mammalian P-gp antibodies UIC2 and C219</td>
<td>P-gps detected on eggshells</td>
<td>(Kerboeuf et al., 2003)</td>
</tr>
<tr>
<td>Eggs, L1s, L2s, L3s, adults</td>
<td></td>
<td>Indirect immunofluorescence with mammalian P-gp antibody UIC2</td>
<td>P-gps expressed in external layers of the eggshell, sheath of L3 larvae, cuticles of larval stages, intestinal cells of male worms, intestinal cells of L1 and L2 larvae</td>
<td>(Riou et al., 2005)</td>
</tr>
<tr>
<td>Eggs</td>
<td></td>
<td>Flow cytometry with mammalian P-gp antibody UIC2</td>
<td>P-gps colocalized with raft-like cholesterol-enriched microdomains on the egg shells</td>
<td>(Riou et al., 2010)</td>
</tr>
<tr>
<td><em>Cooperia oncophora</em></td>
<td>Eggs</td>
<td>Flow cytometry with mammalian P-gp antibody UIC2</td>
<td>Higher binding seen with resistant eggs than with susceptible eggs</td>
<td>(Demeler et al., 2013)</td>
</tr>
<tr>
<td><em>Parascaris spp.</em></td>
<td>Adults</td>
<td>Dissection of worm and qPCR for P-gp expression</td>
<td><em>Peq-pgp-11</em> expression was highest in the intestines in both males and females. <em>Peq-pgp-16</em> expression was highest in the body wall of males.</td>
<td>(Janssen et al., 2013a)</td>
</tr>
</tbody>
</table>
Table 2-7. *In vivo* studies of effect on P-gps when MLs are co-administrated with P-gp inhibitors.

<table>
<thead>
<tr>
<th>Parasite</th>
<th>Host animal used</th>
<th>P-gp inhibitor used</th>
<th>Results</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>Haemonchus contortus</em></td>
<td>Jird</td>
<td>Verapamil with IVM or MOX</td>
<td>Efficacy of IVM determined by worm count at necropsy increased from 80% (without Ver) to 93% (with Ver) and MOX from 70% (without Ver) to 96% (with Ver)</td>
<td>(Xu et al., 1998)</td>
</tr>
<tr>
<td></td>
<td>Sheep</td>
<td>Loperamide with IVM</td>
<td>FECRT improved from 78.6% with IVM alone to 96% with IVM+LPM. Worm counts improved from 0% efficacy with IVM alone to 72.5% with IVM+LPM</td>
<td>(Lifschitz et al., 2010a)</td>
</tr>
<tr>
<td></td>
<td>Sheep</td>
<td>IVM + Ketaconazole</td>
<td>Worm burden in sheep given IVM with P-gp inhibitor decreased by 16% and 51%</td>
<td>(Bartley et al., 2012)</td>
</tr>
<tr>
<td></td>
<td>Sheep</td>
<td>IVM + Pluronic 85</td>
<td></td>
<td></td>
</tr>
<tr>
<td><em>Trichostrongylus axei</em></td>
<td>Sheep</td>
<td>Loperamide with IVM</td>
<td>Worm counts improved from 98.6% efficacy with IVM alone to 99.6% with IVM+LPM</td>
<td>(Lifschitz et al., 2010a)</td>
</tr>
<tr>
<td><em>Trichostrongylus colubriformis</em></td>
<td>Sheep</td>
<td>Loperamide with IVM</td>
<td>Worm counts improved from 77.9% efficacy with IVM alone to 96.3% with IVM+LPM</td>
<td>(Lifschitz et al., 2010a)</td>
</tr>
</tbody>
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Table 2-7 continued

<table>
<thead>
<tr>
<th>Parasite</th>
<th>Host animal used</th>
<th>P-gp inhibitor used</th>
<th>Results</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>Nematodirus spp.</em></td>
<td>Sheep</td>
<td>Loperamide with IVM</td>
<td>Worm counts improved from 85.5% efficacy with IVM alone to 93% with IVM+LPM</td>
<td>(Lifschitz et al., 2010a)</td>
</tr>
<tr>
<td>Mixed infection with resistant</td>
<td>Cattle</td>
<td>Loperamide with IVM</td>
<td>FECRT improved from 23.5% with IVM alone to 50% with IVM+LPM. FECRT improved from 69% with MOX alone to 87.1% with MOX+LPM.</td>
<td>(Lifschitz et al., 2010b)</td>
</tr>
<tr>
<td><em>Ostertagia</em>, <em>Trichostrongylus</em>, <em>Cooperia</em>, <em>Haemonchus</em></td>
<td></td>
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</tbody>
</table>
CHAPTER 3. EFFECTS OF IN VITRO AND IN VIVO EXPOSURE TO MACROCYCLIC LACTONES ON THE EXPRESSION PATTERNS OF P-GLYCOPEPTIDES IN TOXOCARA CANIS LARVAE

Jeba R J Jesudoss Chelladurai, Alan Robertson, Matt T Brewer

Modified from a manuscript to be submitted

Abstract

Toxocara canis has a complex lifecycle that includes somatic larval stages in the tissues of adult dogs, paratenic hosts and humans. These larvae are tolerant to therapeutic doses of macrocyclic lactones. We investigated hatched T. canis L3s and observed that ivermectin did not halt motility of larvae. However, a combination of ivermectin and the P-glycoprotein inhibitor verapamil decreased the area and wavelength during swimming motility of larvae. Whole organism assays revealed total P-glycoprotein efflux activity could be mitigated by drugs known to inhibit these proteins in mammals, but in an unusual rank order of potency. Analysis of the T. canis draft genome resulted in the identification of 13 annotated P-gp genes which enabled naming of genes and isoforms. Quantitative PCR was used to measure P-glycoprotein mRNA expression in adult worms, hatched larvae, and somatic larvae derived from experimentally infected mice. 10 of the predicted genes were expressed in adults and hatched larvae, and 6 were expressed in visceral larvae. There were no differences in expression patterns between adults and hatched larvae. Tca-Pgp-10 was significantly upregulated in somatic larvae from mice treated with moxidectin. Further studies are needed to understand the role of the different ATP binding cassette transporters leading to macrocyclic lactone tolerance in T. canis.

1. Introduction

Toxocara canis is a cosmopolitan zoonotic nematode of dogs. T. canis larvae are transplacentally transferred from bitches to neonatal puppies and after a complex hepato-pulmonary-tracheal migration develop to adult worms in the small intestines. In older puppies
and adult dogs, larvae that hatch following the ingestion of infective eggs migrate to the skeletal muscles, kidneys, liver and heart and persist for years as somatic larvae (Schnieder et al., 2011). Somatic larvae in the tissues of bitches are reactivated during pregnancy and are a reservoir of infection for up to three litters following a single infection (Soulsby, 1983). Prenatal transmission of reactivated larvae has been stopped in infections using a few experimental drug regimens (Burke and Roberson, 1983; Krämer et al., 2006; Payne and Ridley, 1999). However, somatic non-reactivated larvae are not killed by macrocyclic lactones in dogs. Somatic larvae in paratenic hosts such as mice are not amenable to death by ivermectin (Carrillo and Barriga, 1987; Fok and Kassai, 1998). Larval migration to somatic musculature or brain allowed survivability and protection against drugs (Abo-Shehada and Herbert, 1984). These studies point to an unknown mechanism of tolerance to the macrocyclic lactones exhibited by somatic T. canis larvae to the macrocyclic lactones.

Ivermectin (IVM) is multimodal in its mechanism and in the effects that it produces on nematodes. Ivermectin acts on glutamate-gated chloride channels, causing hyperpolarization of muscles resulting in pharyngeal muscle paralysis, ES pore-associated muscle paralysis, and inhibition of larval motility (Geary et al., 1993; Gill et al., 1991; Moreno et al., 2010). It potentiates the adherence of mononuclear cells and activated neutrophils to nematode cuticle (Vatta et al., 2014). IVM and other macrocyclic lactones are also substrates of P-glycoproteins and multidrug resistance proteins (MRPs) that efflux xenobiotics from cells (Lespine et al., 2006; Lespine et al., 2007).

P-glycoproteins are encoded by genes from the ATP-binding cassette (ABC) class B1 family. Typically, one or two isoforms of the ABCB1 gene are expressed in vertebrates. In contrast, nematodes commonly express multiple ABCB1 genes. Gene identification, assessment
of expression and function have been carried out for a few nematode P-gps (Godoy et al., 2015b). The role of P-glycoproteins in anthelmintic resistance in ruminant gastrointestinal nematodes and filarid nematodes have been widely studied (Bourguinat et al., 2016; Lespine et al., 2012). Several in vitro studies have demonstrated that P-gp substrates and blockers can compete for efflux at the binding site and potentiate the others’ effects (Kaschny et al., 2015; Raza et al., 2016c). Initial preliminary observations of Toxocara canis larval motility in the presence of P-gp substrate – ivermectin, and P-gp competitive inhibitor – verapamil led to the investigation of P-gps. However, the identity, expression and function of genes encoding P-glycoproteins in T. canis are unknown.

Based on our observations on larval motility in the face of ML treatment, we hypothesized that P-glycoproteins may play a role in larval tolerance to MLs, since somatic larvae of T. canis are present at sites where macrocyclic lactones are bioavailable. Additionally, we hypothesized that known inhibitors may affect function of P-gp in T. canis. To those ends, the aim of this study was to identify P-gp genes in T. canis by bioinformatically mining the draft genome and to evaluate constitutive P-gp gene expression in adults and larvae. We also sought to detect differences in P-gp expression in larvae exposed to macrocyclic lactones in vitro and in vivo.

2. Methods

2.1 Ethics statement

All experiments were conducted in accordance with the recommendations of the NIH Guide for the care and use of laboratory animals. The studies were approved by the Iowa State University Institutional Animal Care and Use Committee.
2.2 Parasites

*Toxocara canis* were obtained from dogs that expelled adult worms after treatment with anthelmintics. Worms were washed in tap water and eggs were isolated from the uteri of female adult worms by careful dissection. Eggs were washed and incubated in 1x phosphate buffered saline (PBS) at room temperature for at least 2 weeks to allow development of larvae to the third larval (L3) stage. L3 larvae were isolated by a chemical hatching protocol (Ponce-Macotela et al., 2011).

2.3 *In vitro* motility assays

Stock solutions of 10mM ivermectin and verapamil were prepared in 100% DMSO and diluted in 1x PBS to obtain working dilutions of drugs with 0.1% final DMSO concentration. Hatched *T. canis* L3 larvae were individually transferred to 24 well plates in RPMI1640. The drugs were added to the wells and the final volume adjusted to 400μL using RPMI. Videos of larval motility were recorded using the WormLab software (MBF Bioscience, Williston, VT) using the default settings. All larvae were tracked for 2 minutes and videos analysed using the default settings. Motility parameters including wavelength, amplitude, linear speed and peristaltic speed, linear and peristaltic track length and mean area occupied by the larvae were obtained as outputs. One-way ANOVA with Tukey’s multiple comparison test was used to compare each motility parameter of the larvae using GraphPad Prism version 8 (San Diego, CA).

2.4 *In vitro* larval dye efflux assay

The larval efflux assay was modified from Raza et al. (2016b). Hatched larvae were washed in 1x Dulbecco’s PBS and exposed to 10 μM ivermectin or P-gp inhibitors (cyclosporine A, loperamide, reserpine, verapamil or tariquidar) for 1 hr at 37°C with horizontal shaking at 200
rpm. The larvae were washed in 1x D-PBS and then incubated in the fluorescent P-gp substrate 15μM Hoechst 33342, a fluorescent P-gp substrate for 10 min at 37°C. Images obtained by fluorescence microscopy were analyzed using the fluorescence area module in Halo (Indica Labs, Advanced Cell Diagnostics, Hayward, CA). Percentage of Hoechst staining in larvae was calculated using the formula: (fluorescently stained area/ total area of larva) x 100. Percentage of fluorescence was compared to larvae that were not exposed to drugs. One way ANOVA with Tukey’s multiple comparison test was used to analyze percentage of staining in GraphPad Prism version 8 (San Diego, CA).

2.5 Genomic survey and Phylogenetic analysis

The 317Mb draft genome of Toxocara canis (Zhu et al., 2015) on NCBI (Toxocara canis isolate PN_DK_2014, whole genome shotgun sequencing project, GenBank Accession JPKZ00000000) was surveyed and annotated P-gp genes obtained. These were annotated as either pgp-1 or pgp-3. Nucleotide sequences of P-gp genes of Toxocara canis retrieved from GenBank were used to design custom primers to amplify partial sequences from cDNA. Protein sequences translated from nucleotide sequences of P-gp genes described in other nematodes such as Haemonchus contortus, Cooperia oncophora, Teladorsagia circumcincta, Cylicocyclus spp., Parascaris spp., Dirofilaria immitis, and C. elegans were obtained from GenBank and aligned with the MAFFT algorithm (Katoh and Standley, 2013). Substitution model was selected using the SMS tool with Bayesian Information Criteria (Lefort et al., 2017). The model with lowest BIC value was LG+F+I+G with 4 parameter gamma distribution (Le and Gascuel, 2008). Maximum likelihood phylogenetic analyses performed using PhyML3.0 (Guindon et al., 2010). The tree was visualized using Mega X (Kumar et al., 2018).
2.6 qPCR in adults *Toxocara canis*

Expression levels of P-gp genes was determined using qPCR in adult male and female worms separately and results pooled. qPCR primers were designed to amplify the genes (Table 3-S1) and specificity was confirmed using BLAST *in silico*. RNA extracted from pools of adult male and female worms was extracted with Trizol reagent (Ambion by Life Technologies, Carlsbad, CA) followed by purification using the Direct-zol RNA Miniprep kit (Zymo Research) according to the manufacturer's instructions. cDNA was synthesized from 50 ng of total RNA in a 20µL volume using the iScript cDNA synthesis kit (Bio-rad), using random oligonucleotides. qPCR reactions were individually optimized using diluted cDNA synthesized from adult *Toxocara canis* worms. Specificity was determined using melt curve analysis and sequencing. 18S was used as a reference gene (Durant et al., 2012). qPCR was carried out in a volume of 20 µL with 2 µL of diluted cDNA, 1x of SSoAdvanced Universal SYBR Green Master Mix (Bio-rad) and 0.2 - 0.5 µM of diluted primers. PCR efficiency was determined for each primer pair using LinRegPCR (Ramakers et al., 2003) and change in gene expression was calculated using the efficiency corrected ΔCt method based on single samples (Pfaffl, 2004). One way ANOVA with Tukey’s multiple comparison test was used to analyze fold changes in expression using GraphPad Prism version 8 (San Diego, CA).

2.7 qPCR following *in vitro* drug exposure

Groups of 500 hatched L3 larvae were washed in 1x Dulbecco’s PBS and exposed to 10 µM Ivermectin (MP Biomedical, Solon, OH) or no drugs (control) in RPMI 1640 (Gibco, Grand Island, NY) at 37°C with horizontal shaking at 200 rpm for 24 hours. Drug exposure assays were carried out separately with three different isolates of *Toxocara canis* eggs. Immediately after drug exposure, larvae were washed in RPMI1640. Sterile 0.1mm, 0.5mm and 2mm Zymo
bashing beads (Zymo research, Irvine, CA) were used to homogenize larvae in Trizol reagent and total RNA was extracted following manufacturer’s protocol. RNA concentration and purity were measured using a Nanodrop spectrophotometer and stored at -80°C. cDNA was synthesized from 50 ng of total RNA in a 20μL volume using the iScript cDNA synthesis kit (Bio-rad), using random oligonucleotides and stored at -20°C till use. To determine differences between drug exposed and unexposed larvae, qPCR was carried out in a volume of 20 μL with 2 μL of undiluted cDNA, 1x of SSoAdvanced Universal SYBR Green Master Mix and 0.2 - 0.5 μM of diluted primers. PCR efficiency was determined for each primer pair using LinRegPCR (Ramakers et al., 2003). Fold changes in gene expression were calculated using the efficiency corrected ΔCt method based on single samples (Pfaffl, 2004). One way ANOVA with Tukey’s multiple comparison test was used to analyze fold changes in expression using GraphPad Prism version 8 (San Diego, CA).

2.8 qPCR following in vivo drug exposure in mice

Male and female C3H/HEJ mice were obtained from Jackson Labs and housed in ventilated rack cage system with standard enrichment. After acclimatization, mice were gavaged with 5000 larvated *Toxocara canis* eggs. Mice were injected subcutaneously with ivermectin (200 μg/kg), moxidectin (500 μg/kg), or sham (saline) on day 7 PI and euthanized on day 10 PI. Liver, lungs, brain were collected from each mouse and frozen at -80°C for RNA extraction. qPCR following cDNA synthesis was performed using the methods outlined above for *T. canis* adult and larvae. One way ANOVA with Tukey’s multiple comparison test was used to analyze fold changes in expression using GraphPad Prism version 8 (San Diego, CA).
3. Results

3.1 In vitro motility assay

*T. canis* larvae exhibit sinusoidal thrashing motility in liquid media without progressive or forward movement. The WormLab software tracked swimming larvae using anterior, middle and posterior markers. Larvae treated with a combination of ivermectin and verapamil had a lower mean area than larvae in any other treatment group (p<0.05, Figure 3-1). Larvae treated with the combination also had lower wavelength than verapamil alone(p<0.05). Speed of larvae was reduced following treatment with the combination, although this change was not statistically different (P=0.0942).

3.2 In vitro efflux of P-gp is inhibited

Total P-gp activity and inhibition was measured by a Hoechst 33342 efflux assay. Fluorescent H33342 is a substrate of P-gps which efflux it from the plasma membrane. It does not fluoresce when not associated with membranes (Shapiro et al., 1997), allowing fluorescence microscopy of larvae without fluorescent background signal on a slide. Larvae were co-incubated with and without P-gp inhibitors, photographed, and positive H33342 staining was quantitated using computer software (Figure 3-2). Untreated larvae had constitutive P-gp efflux activity, indicated by absence of H33342 staining. In contrast, larvae treated with P-gp inhibitors had low P-gp activity leading to retention of H33342. Interestingly, reserpine, verapamil, and tariquidar caused a significant decrease in P-gp activity (p<0.05, Figure 3-3). On the other hand, P-gp inhibition by ivermectin, cyclosporine A, and loperamide were modest and not statistically different (Figure 3-3).

3.3 Thirteen P-gp genes identified in *T. canis* genome

The draft genome of *Toxocara canis* on NCBI GenBank nucleotide database was initially used for primer design. Thirteen P-gp protein sequences with length > 450 amino acids annotated
in the *T. canis* genome were identified in the analysis. Conceptually translated amino acid sequences from P-gp genes annotated in the *T. canis* genome, annotated in genomes of other nematodes obtained from GenBank, and cloned sequences obtained in this study were used in the Maximum Likelihood phylogenetic analyses (Figure 3-4). Names for the 13 P-gp genes are given in Table 3-1. Based on the phylogenetic analysis, several of these were determined to be isoforms. Isoforms have been observed in other nematode P-gps including *Haemonchus contortus* Pgp-9 (Godoy et al., 2016). In our analysis, three annotated isoforms of Pgp-11, two isoforms each of Pgp-9, Pgp-13 and Pgp-16 were found. One P-gp (KHN86334) has an ambiguous position with low statistical support in the tree and was named (*Tca-Pgp-16.3/3.2*). PCR investigation resulted in six partial and one full length Pgp cloned from mRNA. Thus, these seven annotated P-gp genes are not pseudogenes.

### 3.4 Ten P-gp genes are expressed in adults

Constitutive expression levels of P-gp genes were determined using qPCR in adult *T. canis* nematodes and infective larvae hatched from eggs. Ct values from 10 P-gp genes and isoforms were compared between adults and larvae (Figure 3-5). Variability in expression levels were seen between biological replicates with no significant differences between the genes. Specific primer sets could not be designed/optimized for *Tca-Pgp-3, Tca-Pgp-11.3* and *Tca-Pgp-13.2* because of very high sequence cross-identity and the presence of multiple peaks in melt curve analyses of products, reinforcing our finding that isoforms of the genes exist as predicted in the phylogenetic analysis.

### 3.5 *In vitro* effects of IVM on P-gp expression in larvae

Expression profile of 10 P-gp genes and isoforms in *T. canis* larvae was determined before and after treatment with 10 μM ivermectin for 24 hrs (Figure 3-6). Variability in expression levels were seen between biological replicates with no differences between the genes.
3.6 In vivo effects of MLs on P-gp expression in somatic larvae

Expression profile of P-gp genes was determined in somatic *T. canis* larvae derived from infected mice. P-gp expression was detected in larvae from mice treated with ivermectin or moxidectin (Figure 3-7). There were no differences among genes in the ivermectin-treated group. *Tca-Pgp-10* was significantly upregulated in larvae from moxidectin-treated mice. *Tca-Pgp-10* was also significantly upregulated compared to *Tca-Pgp-16.1*. Several P-gp genes and isoforms could not be amplified from somatic larvae due to poor RNA quality, RNA degradation and overabundance of host RNA.

4. Discussion

Bitches harboring somatic *T. canis* larvae are reservoirs for infection of puppies which occurs primarily by a transplacental route. Arrested somatic larvae evade drug-mediated killing until they are reactivated, and the reason for this drug-resistant phenotype has not been elucidated. Our hypothesis is that nematode P-glycoproteins contribute to evasion of drug-mediated killing of somatic larvae. In this study we described the repertoire of P-gp genes, phenotypic effects of P-gp inhibition, and induction of P-gp mRNA expression. In addition, experiments with hatched larvae revealed larval expulsion of the P-gp substrate H33342 and this activity could be blocked by some but not all P-gp inhibitors tested.

Motility has been used to determine the activity of anthelmintics against different stages of nematodes (Blanchard et al., 2018; Kotze et al., 2004; Storey et al., 2014). Interestingly, we observed that ivermectin treatment did not alter motility of *T. canis* larvae (Figure 3-1). Combination of ivermectin and the P-gp inhibitor verapamil resulted only in modest changes in measures of motility. Our results are largely in agreement with previous studies of nematodes in which exposure to physiological levels of macrocyclic lactones fails to
inhibit larval motility, thus supporting the hypothesis that ivermectin exerts an effect beyond paralysis (Vatta et al., 2014).

An important finding of this study was that *T. canis* larvae constitutively expel H33342, indicating the presence of functional P-gps. The P-gp inhibitors tariquidar, verapamil, and reserpine caused significant inhibition of H33342 efflux. Interestingly, numerous other known inhibitors failed to inhibit P-gp activity in *T. canis* larvae. Although our assays represent the net effect of inhibition on total larval P-gp activity, we demonstrated that *T. canis* P-gp cannot be inhibited by all P-gp inhibitors unlike mammalian P-gps. This supports further investigation of these putative drug targets.

Analysis of the *T. canis* draft genome led to an estimate of 530 genes that were likely to be transporters (out of the 18,596 protein coding genes), of which 10.8% were predicted to be ABC transporters by computer algorithms (Zhu et al., 2015). Of all the genes annotated as P-gp genes in the genome of *T. canis*, our phylogenetic analysis suggests that only 13 genes likely exist, of which several are isoforms. >1000 base pair fragments of seven of these genes could be cloned from mRNA, suggesting that they are functional genes. Gene naming conventions based on *C. elegans* genes as suggested by Beech et al. (2010) were used except where cases of ambiguity existed such as *Tca-Pgp-16.3/3.2*. Uncertainties in the assigning of genes can be resolved as genome assemblies of organisms improve and as paralogs are studied in closely related nematodes.

We demonstrated that at least 10 of the 13 named genes/isoforms are expressed in *T. canis* adults and larvae. While comparisons of adults and hatched infective larvae did not yield significant differences, constitutive expression of P-gps are likely to have functional significance in both stages. In larvae exposed to ivermectin *in vitro* for 24 hours, significant changes in P-gp
gene expression did not occur. These results have to be cautiously interpreted as biological compensation may occur by which a single P-gp gene may be upregulated to compensate for the downregulation of another as has been shown in *H. contortus* (Maté et al., 2018). Additionally, larval gene regulatory changes may occur earlier than 24 hours. Further research is essential to map fine scale temporal gene expression changes that result from drug exposure, with an assay that provides greater molecular resolution such as RNA-seq.

Finally, P-gp gene expression was determined in *T. canis* somatic larvae derived from liver granulomas of experimentally infected mice. Mice are natural paratenic hosts for *T. canis* and are a tractable model for study of somatic larval migrans of humans. Ivermectin at various doses and routes is unable to eliminate larvae in mice (Fok and Kassai, 1998). Several genes in larvae from ivermectin and moxidectin treated animals had differential expression, with only one gene – *Tca-Pgp-10* having a significant increase. Gene expression in larvae derived from other organs in the paratenic host is essential to determine if larvae in relatively immuno-privileged sites exhibit the similar gene changes.

This study revealed significant findings regarding P-gps of the zoonotic nematode *Toxocara canis*. First, we curated and named putative P-gp genes present in the genome which was supported by phylogenetic analysis and molecular cloning. We then demonstrated phenotypic evidence that *T. canis* larvae exhibit physiologic responses to P-gp inhibition with a unique pharmacological profile. In addition, we detected the expression of numerous P-gp genes in adult worms, larvae hatched *in vitro*, and somatic larvae recovered from mice. While a few P-gps were induced by macrocyclic lactone treatment, many were constitutively expressed in both larvae and adults. In conclusion, *T. canis* expresses a large repertoire of P-gps, some of which
appear unresponsive to mammalian P-gp inhibitors. Taken together, our findings support further study of this protein family in the search for new nematode-specific drug targets.

5. References


Figure 3-1. WormLab output obtained with *Toxocara canis* larvae exposed to DMSO control, 10 μM ivermectin, 10 μM verapamil or combination of 10 μM ivermectin with 10 μM verapamil. Various motility parameters are shown: (A) track length in μm (forward + reverse), (B) peristaltic track length in μm (forward – reverse), (C) speed in μm/s (track length/time), (D) peristaltic speed in μm/sec (peristaltic track length/time), (E) mean area occupied by the larvae, (F) Wavelength in μm, (G) mean amplitude in μm and (H) maximum amplitude in μm. Statistically significant differences are marked by * (One-way ANOVA, p<0.05)
Figure 3-2. Representative bright-field overlays and fluorescence images of *T. canis* larvae stained with Hoechst 33342 in the absence of drugs (A,B) and presence of inhibitors - ivermectin (C,D), cyclosporine A (E, F), loperamide (G, H), reserpine (I, J), verapamil (K, L) and tariquidar (M,N) (400x). The brightfield image was used to annotate the outline of the larvae which was overlaid on the fluorescent image. The area stained was quantitated using image analysis software (Figure 3-3).
Figure 3-3. Area of H33342 staining of *T. canis* larvae exposed to P-gp inhibitors. Experiments were performed in triplicate with larvae hatched from different batches of eggs (n ≥ 5 larvae per replicate). Mean ± SE are shown, asterisks denote statistical differences (p<0.05).
Figure 3-4. Maximum Likelihood phylogenetic tree of conceptually translated P-gp genes. Pgp genes predicted in the genome (o) and cloned (●) are highlighted along with assigned names for *T. canis* P-gp genes provided in red.
Figure 3-5. Mean + SE of expression levels of P-gp genes in adult *T. canis* worms. Three pools of adult worms with two technical replicates each were compared to three biological replicate populations of hatched larvae from different pools of eggs with two technical each. Fold change was calculated using the efficiency corrected ΔCt method using *T. canis* 18S as the reference gene.
Figure 3-6. Mean + SE of induction of P-gp expression following exposure to ivermectin. Three biological replicate populations of hatched larvae from different pools of eggs with two technical replicates were used to obtain Ct values. Each biological replicate had ivermectin treated and untreated groups of larvae derived from the same population of eggs. Fold change was obtained using the efficiency corrected ΔCt method using *T. canis* 18S as the reference gene.
Figure 3-7. Mean + SE of expression of P-gp genes in somatic *T. canis* larvae derived from mice treated with (A) ivermectin or (B) moxidectin. Six biological replicate populations of somatic larvae with two technical replicates each were used to obtain Ct values. Fold change was calculated using the efficiency corrected ΔCt method using *T. canis* 18S as the reference gene.
Table 3-1. Nomenclature for P-gp protein sequences in *Toxocara canis*

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The following is the supplementary data to this article:

Table 3-S 1. qPCR primers used in this study

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CHAPTER 4. IDENTIFICATION AND FUNCTIONAL CHARACTERIZATION OF P-GLYCOPROTEIN 11.1 OF *TOXOCARA CANIS*

Jeba Jesudoss Chelladurai, Tomislav Jelesijevic, Matthew T. Brewer

Modified from a manuscript to be submitted

Abstract

The interaction of macrocyclic lactones with P-glycoproteins has been studied in nematodes with reduced susceptibility to the drug class. *Toxocara canis* somatic larvae exhibit a remarkable tolerance to systemically bioavailable macrocyclic lactones and resist death, except when they are hormonally reactivated during the third trimester of pregnancy in dogs. We hypothesized that the tolerance to MLs could be due to P-glycoprotein mediated efflux of drugs from their sites of action. In the present study, a full length *Toxocara canis* P-gp was studied by cloning, and by phylogenetic analysis was named *Tca-Pgp-11.1*. The cloned P-gp was expressed and its pharmacological substrate profile was studied by fluorescent substrate efflux in transfected cells using flow cytometry in the presence and absence of known P-gp substrates and inhibitors. Tissue distribution of two isoforms of *Tca-Pgp-11* mRNA in adult male and female worms was studied using chromogenic *in situ* hybridization and expression was found to be the highest in the intestine and absent in the reproductive tissues. We have previously shown that somatic larvae express isoforms of *Tca-Pgp-11*. Taken together, these results indicate that *Tca-Pgp-11.1* transports macrocyclic lactones, which could contribute to somatic larval tolerance to MLs.

1. Introduction

*Toxocara canis* is a cosmopolitan ascarid nematode of dogs. Dogs acquire infection by one of four routes - ingestion of eggs with infective L3 larvae, ingestion of paratenic hosts harboring infective L3, transplacentally transferred larvae or lactogenically derived larvae from
infected dams. Upon ingestion of infective eggs, the larvae are released in the stomach and undergo extensive migration in the body. In dogs that are > 8 weeks of age, the larvae are retained in skeletal muscles, kidneys, liver, heart, lungs and diaphragm as arrested somatic larvae (Schnieder et al., 2011). Transplacentally and lactogenically transmitted *T. canis* larvae are important in neonates and worms grow to adulthood in the small intestines.

The efficacy of the macrocyclic lactones in the treatment of infections with adult *T. canis* is well-established and there have been no reports of resistance. However, somatic larvae are not completely eliminated by macrocyclic lactones. We hypothesized that P-glycoproteins may be involved in the transport of MLs out of target cells in somatic larvae, as ML resistance in several nematodes has been associated with P-gp mediated efflux of drugs from target sites.

P-glycoproteins are a family of transporters located on the cell membrane that have a characteristic ATP-binding cassette and are involved in the ATP-mediated efflux of a wide range of xenobiotics. Nematode P-gps have several paralogs that have been studied in the context of anthelmintic resistance (Figueiredo et al., 2018). Specifically, Pgp-11 appears to be important in parasitic nematodes of veterinary importance and its role has been studied in *Parascaris* (Janssen et al., 2013a; Janssen et al., 2015), *Dirofilaria immitis* (Mani et al., 2016), *Haemonchus contortus* (Issouf et al., 2014), *Cooperia oncophora* (De Graef et al., 2013) and others.

The pharmacological interactions of drugs and *Toxocara canis* P-glycoproteins have not been characterized. The draft genome of *T. canis* has been surveyed to identify ABC transporters and the expression levels of P-gps have been studied in adults, hatched third stage larvae and somatic larvae recovered from mice (Chapter 3 of this thesis). In this study, we cloned and bioinformatically analyzed a P-gp gene from *Toxocara canis*. We characterized the unique
pharmacology of \textit{Tca-Pgp-11.1} in stably transfected MDCK II cMDR1-KO cells and localized gene expression in adult worm tissue by chromogenic \textit{in situ} hybridization.

\textbf{2. Materials and methods}

\textbf{2.1. Ethics statement}

All experiments were conducted in accordance with the recommendations of the NIH Guide for the care and use of laboratory animals. The studies were approved by the Iowa State University Institutional Animal Care and Use Committee.

\textbf{2.2. Molecular and phylogenetic characterization}

Adult \textit{Toxocara canis} worms were obtained from feces of puppies recently treated with anthelmintics. To extract RNA for cloning, adult worms were homogenized with Trizol reagent (Life Technologies) followed by purification using the Direct-zol RNA Miniprep kit (Zymo) according to the manufacturer’s instructions. RNA was eluted into 50\(\mu\)L of nuclease free water, quantified using a Nanodrop1000 spectrophotometer, and stored at \(-80^\circ\)C. RNA quality was assessed using the 260/280 ratio and by electrophoresis in a denaturing bleach agarose gel (Aranda et al., 2012). cDNA was synthesized using 0.5\(\mu\)g of total RNA, 200U of SuperScript IV Reverse Transcriptase enzyme (Invitrogen) and 2.5\(\mu\)M Oligo \textit{d(T)}\textsubscript{20} primers, using the manufacturer’s recommended protocol.

To clone a full length \textit{Toxocara canis} P-gp gene, the predicted nucleotide sequence was derived from the \textit{T. canis} draft genome assembly Scaffold1303 on NCBI GenBank - Accession number JPKZ01003065.1, and specific primer pairs were designed using the Primer3 software (version 2.2.3) on the PrimerQuest Tool platform (Integrated DNA technologies, Coralville, IA). Primers were synthesized at the Iowa State University DNA Facility. \textit{Tca-Pgp-11.1} was cloned as two separate fragments initially using the conserved nematode spliced leader primer SL3 and
custom designed primers (Table 4-S 1). Gibson assembly was used to fuse and clone the full-length sequence into the pCR-XL-TOPO vector (Life Technologies) for sequencing. PCR reactions consisted of 1x OneTaq HotStart DNA polymerase master mix (New England BioLabs), 1µM of each primer and 2µL of cDNA. A touch down PCR cycle of initial denaturation at 94°C for 2 min, followed by 20 cycles of cyclic denaturation at 94°C 15 sec, annealing at 65°C for 15 sec with a drop of 1°C per cycle, cyclic extension at 68°C for 6 min, and again followed by 20 cycles of cyclic denaturation at 94°C 15 sec, annealing at 45°C for 15 sec, cyclic extension at 68°C for 6 min and final extension at 68°C for 10 min was used. PCR products were visualized on an agarose gel, purified using Wizard SV Gel and PCR Clean-Up system (Promega), cloned into pCR-XL-TOPO, and transformed into OneShot TOP10 Chemically competent E. coli (Life Technologies). Transformants were analysed by PCR and sequenced using a primer walking strategy on an Applied Biosystems 3730xl DNA analyzer at the Iowa State University DNA Facility. The amplicon was double digested using HindIII and BamHI (Thermo Scientific) after reamplification using primers with restriction sites and His tag, gel purified and directionally sub-cloned into pcDNA3.1+ Mammalian Expression Vector (Invitrogen). Transformants were analysed by restriction digestion and sequencing by primer walking.

2.3. Bioinformatic analyses

Sequences were assembled using GeneStudio Professional Edition version 2.2.0.0, and conceptually translated to the corresponding peptide sequence using EMBOSS Transeq (Rice et al., 2000). The BLAST suite was used to align and confirm sequence identities of nucleotide and translated peptide sequences by comparison to draft Toxocara canis genomes WGS: JPKZ01, LYYD01 and UYWY01 (NCBI Genome database assemblies GCA_000803305.1,
GCA_001680135.1 and GCA_900622545.1 respectively) (Johnson et al., 2008). The 1283 amino acid long conceptually translated protein sequence was analyzed with ScanProsite (de Castro et al., 2006) and InterPro for residue annotation (Mitchell et al., 2019). Protein secondary structure was predicted using Porter 5.0 (Torrisi et al., 2019) with secondary structure (SS3, SS8) output as defined by DSSP (Kabsch and Sander, 1983) with prediction confidence intervals (0 to 9). Protein tertiary structure was predicted using ModWeb using the combined sequence-profile and PSI-BLAST fold assignment methods (Pieper et al., 2014). Multiple sequence alignments of nucleotide and translated protein sequences were visualized with Multalin version 5.4.1 (Corpet, 1988).

Translated protein sequence of other nematode P-gp sequences were obtained from NCBI GenBank protein database, aligned using MAFFT (Katoh and Standley, 2013). Substitution model was selected using the SMS tool with Bayesian Information Criteria (Lefort et al., 2017). The model with lowest BIC value was LG+F+I+G with 4 parameter gamma distribution (Le and Gascuel, 2008) and maximum likelihood phylogenetic analyses performed using PhyML3.0 (Guindon et al., 2010). The tree was visualized using Mega X (Kumar et al., 2018).

2.4. Heterologous expression in MDCK cells

*Tca-Pgp-11.1* was heterologously expressed in MDCK cells lacking expression of endogenous P-gp due to CRISPR/Cas9-mediated deletion of *MDR1* (Karlgren et al., 2017). Cells were maintained in Eagle’s minimum essential medium with 10% heat inactivated fetal bovine serum (Atlanta biologicals) at 37°C with 5% CO2. Chemical transfection was carried out using Lipofectamine 2000 reagent, according to the manufacturer’s protocol. 5µg of the plasmid pcDNA3.1+ containing the *Tca-Pgp-11.1* gene was transfected into the cells at 90% confluence.
in 24 well plates. Transfected cells were selected using 400 µg/mL of Geneticin/G418 sulfate, and after 2 weeks of selection, stably transfected colonies were transferred to cell culture flasks.

The expression profile of Tca-Pgp11 was quantitated using RT-qPCR. Cells were removed from cell culture flasks using 0.25% Trypsin-EDTA and used for RNA extraction using Trizol reagent. cDNA was synthesized using iScript Select cDNA synthesis kit (Bio-rad). The synthesized cDNA was used as template for qPCR using the primers listed in Table 4-S 1. Tca-Pgp-11.1 and CanMDR1 expression levels were measured in all cell lines. CanGAPDH was used as the reference gene. qPCR was carried out in a QuantStudio 3 Real-time PCR system (Applied Biosystems) using the Sso Advanced Universal SYBR Green Supermix (Bio-rad). qPCR efficiencies for each reaction were calculated by linear regression analysis of amplification curves using LinregPCR (Ramakers et al., 2003). Relative fold differences between cells were calculated using the efficiency corrected ΔCt method based on individual samples (Pfaffl, 2004).

Cells were harvested at 100% confluency and lysed using sterile beads (0.1mm, 0.5mm and 2mm Zymo bashing beads). Total protein concentrations were determined using the Coomassie Plus (Bradford) Assay. 30 µg of total protein per well was resolved in a discontinuous denaturing SDS-polyacrylamide gel with 4% stacking and 8% resolving gel. Proteins were transferred to a PVDF membrane, blocked with Superblock blocking buffer in PBS (Thermo Scientific) and incubated with primary antibody (1:250 dilution). Antibodies were raised in rabbits targeting three 14 amino acid peptides (Table 4-1) predicted to be encoded by the cloned Tca-Pgp11.1 (GenScript). Binding was detected by a 50:50 mixture of 1:5000 anti-rabbit IgG and IgM (Jackson Labs) and Super signal picoluminescence (Thermo Fisher). The same samples were blotted on a nitrocellulose membrane for a dot blot assay. Primary and
secondary antibodies were used at the same dilutions as the western blotting experiment with NBT/BCIP substrate (Thermo Fisher)

2.5. Tca-Pgp-11.1 mediated efflux assays

*Tca-Pgp-11.1*-mediated efflux of known P-gp substrates was measured using flow cytometry. WT MDCK cells expressing canine endogenous MDR1 (Pgp) were used as positive control and are hereafter referred to as WT cells. Non-transfected MDCK KO cells were used as negative control and are hereafter referred to as KO cells. Fluorescent substrates included Rhodamine 123 (R123), EffluxIDGreen (Enzo Life Sciences) and Calcein-AM (Invitrogen). EffluxID Green was tested with specific inhibitors for MDR1/P-gp (Verapamil), MRP1/2 (MK-571) and BCRP (Novobiocin) respectively. Calcein-AM was used to test the efflux of known mammalian P-gp inhibitors - Verapamil (Acros Organics), Cyclosporine A, Tariquidar, Reserpine (SelleckChem), Ivermectin (MP Biomedicals), Milbemycin oxime (US Pharmacopia). Cells were grown to 90-100% confluency, trypsinized, triturated to create a single cell suspension, and washed with 1x D-PBS. Cells were incubated at 37°C with dilutions of drugs in 1x D-PBS for 30 min, followed by with Calcein-AM (1μM) at 37°C for 15 min, washed twice with D-PBS, and incubated at 37°C for 60 mins prior to flow cytometry. Fluorescence was determined by in a BD Accuri C6 flow cytometer with laser excitation of 488nm and emission filter 530/30 (FITC channel), acquiring a minimum of 30,000 events per sample. Pharmacological profile was recorded and normalized median fluorescence intensity (nMFI) was calculated as a ratio of MFI of cells exposed to inhibitor drugs + dye and MFI of cells exposed to no drugs or dye as described in (Chan et al., 2013). Additionally, ratio of mean fluorescence intensities of transfected cells treated with drugs and transfected cells that were not treated with inhibitors as described in (Mealey et al., 2017).
2.6. Chromogenic in situ mRNA hybridization

Multiple nucleic acid hybridization was carried out to determine tissue specific expression of isoforms of Tca-Pgp-11. Adult male and female worms were formalin fixed for 22 hours and embedded in paraffin for chromogenic hybridization studies.

Probes targeting the nucleotides 421 - 1364 of Tca-Pgp-11.1 were obtained from Advanced Cell Diagnostics. The probes were also able to bind to putative Tca-Pgp-11.2 mRNA which has high identity (>90%) with Tca-Pgp-11.1. Positive control probes targeting nucleotides 4 - 1004 of JPKZ01000754.1:68516-75076 of the T. canis β-tubulin component encoded by the ttb4 gene and negative control probes targeting the nucleotides 414 - 862 of the dapB gene of Bacillus subtilis (GenBank Accession number EF191515) were obtained. Probes were stored at 4°C until use. RNAscope 2.5 HD Assay-Red reagents (Advanced Cell Diagnostics) were used with Fast Red substrate. Chromogenic in situ mRNA hybridization was carried out on 5μm thick sections according to the protocol optimized for ascarid nematodes (Jesudoss Chelladurai and Brewer, 2019). Positive mRNA signal after amplification was observed as red-pink dots in nematode tissue.

2.7. Statistical analysis

Two-way ANOVA with Tukey’s multiple comparison tests was performed for the analysis of flow cytometry data. All statistical analyses were performed using GraphPad Prism version 8.

3. Results

3.1. Bioinformatic analysis of Tca-Pgp-11.1

The full length cloned Tca-Pgp-11.1 gene is 3,849 base pairs in length, and the conceptually translated protein is 1283 amino acids in length. In BLAST analyses, the cloned sequence showed 98.88%, 91.88% and 53.84% identity with T.canis draft genome-predicted P-
gp sequences KHN73709, KHN87227 and KHN89031. The cloned sequence demonstrated 72% identity to Pgp11 from Parascaris equorum (72%), Brugia malayi (55%) and Dirofilaria immitis (55%), consistent with similar comparisons in other nematodes (David et al., 2018).

Structural bioinformatic analyses of translated protein sequence showed tandem arrangement of ABC transporter type-1 fused domain and ATP-binding domain in tandem in each half of the protein which is typical of P-glycoproteins (Figure 4-1). The ATP transporter family signature motif (LSGGQ) as well as the conserved Walker A, Walker B, Q loop/lid, D loop and H loop/switch motifs were also present. Predicted secondary structures with the SS3 (3 state secondary structure) and SS8 (8 state secondary structure) are also depicted. Multiple sequence alignments of predicted nucleotide and protein sequences from the T.canis draft genome (GenBank Accession number JPKZ01003065 and KHN73709) and Parascaris Peq-Pgp-11 (GenBank Accession numbers JX308320 and AGL08022) and are shown in Figures 4-S1 and S2.

Maximum Likelihood phylogenetic analysis of P-gp sequences revealed that Pgp-11 sequences from C. elegans and other parasitic nematodes formed a distinct clade (Figure 4-2). Three predicted T. canis Pgps were observed in the Pgp11 clade – KHN73709, KHN87227 and KHN89031. It is likely that the three are isoforms of Pgp11.

3.2. Expression of Tca-Pgp-11.1 in CRISPR-edited MDCK cells

The cloned sequence of Tca-Pgp-11.1 was ligated into the pcDNA3.1+ vector and stably transfected into MDCK cells CRISPR-edited to remove endogenous canine MDR1 expression. Expression of Tca-Pgp-11.1 mRNA was found to be on average 4000 times higher in transfected cells than in the KO control cells (Figure 4-3). Translation of the protein was demonstrated by immunoblotting (Figure 4-4). Western blotting revealed several bands, suggesting variations in glycosylation of translated protein, including one of the expected (140kDa) size.
3.3. Functional transport activity by *Tca-Pgp-11.1*

Functional activity of *Tca-Pgp-11.1* was characterized by flow cytometry using the P-gp substrates EffluxID Green (Figure 4-5), Calcein-AM, and R123 (Figure 4-6). EffluxID Green is a non-specific dye that can be effluxed by mammalian MDR1, MRP and BCRP (Lebedeva et al., 2011). Functional assays revealed efflux activity by *Tc-Pgp-11.1*-transfected cells that was similar to WT, but significantly higher than KO cells (Figure 4-5). Similarly, R123 efflux was apparent in both wild type and *Tc-Pgp-11.1*-transfected cells as compare to KO (Figure 4-6). Interestingly, Calcein AM efflux was evident in *Tca-Pgp-11.1*-transfected cells, but not WT or KO cells (Figure 4-6). Additionally, MK-571 (50 µM) caused marked losses in cell viability (9.4% - 16% viable) in WT or KO cells compared to nematode P-gp (35% viable) (Data not shown).

3.4. Unique pharmacology of *Tca-Pgp-11.1*

*Tca-Pgp-11.1* activity was assessed with Calcein-AM in the presence of six drugs – two macrocyclic lactones and four known mammalian P-gp inhibitors. Mean fluorescence intensities ratios calculated at various concentrations of the six drugs are presented in Figure 4-9 and Table 4-S2. A mean fluorescence intensity ratio of <1 represents low levels of efflux and conversely, a ratio >1 represents higher Calcein-AM retention. An overlay of histograms of different concentrations of the six drugs in *Tca-Pgp-11.1* transfected are presented in Figure 4-S3.

P-gp activity of WT cells expressing mammalian MDR1 was sensitive to verapamil, cyclosporine A, reserpine, and tariquidar in a dose-dependent manner. Interestingly, *Tca-Pgp-11.1* was insensitive to these inhibitors, exhibiting efflux activity that was similar to KO cells in the presence of these drugs (p<0.05, Figure 4-8).

P-gp activity was increased in *Tca-Pgp-11.1* and WT compared to KO cells, while *Tca-Pgp-11.1* and WT efflux activity were equivalent for all doses of ivermectin and milbemycin
oxime studied (p<0.05, Figure 4-8). High dose ivermectin (100 µM) and milbemycin oxime
(≥ 50 µM) caused ≥ 50% reduction in viability compared to cells exposed to lower
concentrations of drugs in all three cell lines (Data not shown).

3.5. Tissue specific expression of Tca-Pgp-11

*In situ* multiple nucleic acid RNA hybridization was used to visualize mRNA transcripts
in tissues of adult male and female worms (Figure 4-10). No signal was detected in any
nematode tissue for the negative control (*Bacillus subtilis* DapB) (Figure 4-S4). Positive control
for the assay was a β-tubulin component encoded by the *tib4* gene in *T. canis*, which was
visualized throughout the parasite (Figure 4-S5). Ascarid anatomy for interpreting *in situ*
hybridization has been outlined in a previous study (Jesudoss Chelladurai and Brewer, 2019).
Red-pink dots indicative of *Tca-Pgp-11* mRNA were observed in the hypodermis, but not in the
cuticle of both male and female worms. Increased positive signal was also observed in the single
layer of columnar cells of the intestine in both sexes. A large number of vacuoles were also
observed in the intestine in both sexes. Red-pink dots indicative of *Tca-Pgp-11* mRNA was
observed in low numbers in the lateral cords and nerve cords. No hybridization signal was
visualized in either the male or female reproductive tracts at any transverse section measured.

4. Discussion

*Toxocara canis* somatic larvae in dog tissue are not susceptible to macrocyclic lactone
anthelmintics. Macroyclic lactone efflux has been associated with P-glycoproteins in other
parasitic nematodes but much is unknown about the role that P-gp orthologs play in mediating a
drug-resistant phenotype (Kaschny et al., 2015). The present study described *Toxocara canis*
Pgp-11.1, which is an ortholog of Pgp-11 in the related Clade III nematodes *Parascaris spp.*, *Dirofilaria immitis* and *Brugia malayi* (Bourguinat et al., 2011a; Janssen et al., 2013a; Stitt et al.,
2011) and in the unrelated Clade V nematodes *Haemonchus contortus, Cooperia oncophora* and
Caenorhabditis elegans (De Graef et al., 2013; Kellerová et al., 2019; Ménez et al., 2016). In these nematodes, resistance has been associated with SNPs in the DNA sequence and/or mRNA expression changes in Pgp-11. In order to study the role of Pgp-11 in Toxocara canis, we cloned and heterologously expressed \textit{Tca-Pgp-11.1} and assessed the interaction of MLs and known P-gp inhibitors by flow cytometry. We present bioinformatic and phylogenetic analyses of the cloned gene, the localization of mRNA expression, and evidence that \textit{Tca-Pgp-11.1} has a unique pharmacological inhibition profile.

The cloned sequence was confirmed to be an ortholog of Pgp-11 by phylogenetic analysis and was designated \textit{Tca-Pgp-11.1} in accordance with gene naming conventions (Beech et al., 2010). Multiple sequence alignments revealed that the cloned sequence had very close identity to Pgp-11 of the ascarid nematode \textit{Parascaris} and slightly lower identity to Pgp-11 of \textit{C. elegans}. Phylogenetic analysis revealed that the cloned gene was found in the same clade in the ML tree as \textit{Parascaris} Pgp-11 and \textit{C. elegans} Pgp-11. Orthologs from the spirurid nematode \textit{Dirofilaria immitis}, \textit{Onchocerca volvulus}, \textit{Brugia malayi} and \textit{Acanthocheilonema vitae} formed a distinct sub-clade that did not contain any ascarid Pgp-11 sequences. Two other protein sequences encoded in the \textit{T. canis} draft genome were found in the Pgp-11 clade. It is currently unknown if these are paralogs or isoforms of Pgp-11. As genome assembly qualities improve, questions about gene ontology can be resolved by the use of advanced new techniques such as RNA-seq.

Bioinformatic assessment of the \textit{Tca-Pgp-11.1} gene revealed that the mRNA transcript in adult \textit{T. canis} possessed a trans-spliced nematode leader SL3, which was not found at the 5’ end of P-gp genes in all three draft genomes. While the spliced leader sequence has been used to facilitate cloning of full length P-glycoprotein genes (Bourguinat et al., 2016; David et al., 2018),
its role in gene regulation remains elusive 23 years after the question was first posed (Blaxter and Liu, 1996).

*Tca-Pgp-11.1* is a classical P-gp with two each of transporter and ATP-binding domains. The tertiary structure deduced from the crystal structure of *C. elegans* Pgp-1 (Jin et al., 2012) revealed that *Tca-Pgp-11.1* has two transmembrane domains with 6 transmembrane helices where drugs may bind. This modelled structure can be used to screen compounds by *in silico* methods prior to testing in *in vitro* models (David et al., 2018) in order to expedite screening of large libraries of compounds that might be transported by *Tca-Pgp-11.1*.

*Tca-Pgp-11.1* was expressed without codon optimization changes, in order to accurately assess the function of the nematode gene. In this study, KO cells, altered by CRISPR-Cas9 technology, were used for nematode P-gp expression. This cell line has been a tractable model in our hands to study nematode P-gps as it expresses no endogenous MDR1 (P-gp) similar to LLC-PK1 pig kidney cells (which has low endogenous MDR1 levels), with the added advantage of having increased growth rates. Transfected cells both transcribed and translated *Tca-Pgp-11.1* which was detectable by qPCR as well as polyclonal antibodies targeting exposed regions of the protein. Thus, we developed a novel method for studying nematode P-glycoproteins in the absence of interference by mammalian MDR1. This system will be highly valuable for studying the pharmacology of nematode P-gp.

In the functional efflux assays, three fluorescent dyes were tested to determine the best candidate for use in the MDCK KO model since individual dyes have different transport rates in various cell lines under different experimental conditions (Lebedeva et al., 2011). The observation that *Tca-Pgp-11.1* mediated efflux of all three substrates reinforced the suitability of the model. Calcein-AM was chosen for further drug assessments because of light stability and
ease of handling. Calcein-AM is a non-fluorescent Pgp substrate that is hydrolyzed intracellularly to fluorescent Calcein molecules, which cannot be effluxed (Liminga et al., 1994). Efflux data suggests that *Tca-Pgp-11.1* is capable of transporting both avermectin and milbemycin class drug candidates. Additionally, *Tca-Pgp-11.1* mRNA is expressed in hatched and somatic larvae (Chapter 3 of this thesis). Taken together, these points provide evidence that *Tca-Pgp-11.1* plays some role in the perceived tolerance of somatic larvae to macrocyclic lactones. Further research to explore the regulation of Pgp-11 is essential to aid in understanding how modulating/down-regulating the action/expression of P-gps can help overcome tolerance in clinical situations.

Mammalian P-gp inhibition has been extensively studied because of the role that P-gps play in the MDR phenotype exhibited by many mammalian tumors (Briz et al., 2019; Zandvliet and Teske, 2015). Verapamil (also a calcium channel blocker) and Cyclosporine A (also an immunosuppressive agent) are first generation inhibitors that are themselves substrates of P-gps, and compete with other substrates for P-gp mediated efflux (Palmeira et al., 2012). Reserpine (an antihypertensive alkaloid) inhibits mammalian P-gp activity but also affects the catecholamine pathways (Abdelfatah and Efferth, 2015). Tariquidar is a third generation P-gp inhibitor that was specifically designed to allosterically inhibit mammalian P-gps but is not itself a substrate (Weidner et al., 2016).

Interestingly, our studies revealed that *Tca-Pgp-11.1* was insensitive to verapamil, cyclosporine A, reserpine, and tariquidar (Figure 4-8, Figure 4-S3). This is in disagreement with some studies using first generation inhibitors both *in vitro* and *in vivo* with the intention of nematode P-gp inhibition (AlGusbi et al., 2014; Stitt et al., 2011; Xu et al., 1998). We hypothesize that whole organism assays are measurements of net P-gp activity that is a result of
simultaneous expression of numerous P-gps. It is also possible that past studies have made observations resulting from these drugs interacting with other cellular targets. Additional research with other P-gp orthologs, paralogs from *T. canis* including allelic variations among individuals, as suggested by (Kaschny et al., 2015) is necessary to understand the fine scale molecular mechanism of action of these inhibitors.

Localization studies have been conducted in ascarid nematodes to study P-gps using the multiple nucleotide chromogenic *in situ* hybridization assay (Jesudoss Chelladurai and Brewer, 2019). The sites of mRNA expression of more than one isoform of *Tca-Pgp-11* was determined in this study as probes used were unable to distinguish between *Tca-Pgp-11.1* and *Tca-Pgp-11.2* due to their high level of identity. Differential expression of Pgp-11 mRNA was observed with high levels in the intestines and low levels in the body wall, nerve and lateral cords of adult *T. canis* were similar to patterns of *Peq-Pgp-11* expression observed in adult *Parascaris* (Jesudoss Chelladurai and Brewer, 2019). However, there was no *Tca-Pgp-11* mRNA expression in the reproductive tissues in either sex of *T. canis*, while *Peq-Pgp-11* was expressed in the reproductive tissues of both sexes. Further research is necessary to understand the localization patterns of P-gps paralogs in adult tissue and to determine the significance of differential P-gp gene expression in nematode reproductive tissues.

In this study, we identified an ATP-binding cassette transporter from the nematode parasite *T. canis*. Phylogenetic and bioinformatic analysis allowed us to designate this gene *Tca-Pgp-11.1*, which is an ortholog of Pgp-11 in related ascarids. Nucleic acid hybridization studies demonstrated expression of *Tca-Pgp-11* in adult parasites. Heterologous expression assays revealed that *Tca-Pgp-11.1* has P-gp activity, measurable by efflux of fluorescent P-gp substrates. Significantly, we used a gene-edited cell line enabling the study of *Tca-Pgp-11.1* in
isolation from mammalian P-gp which has the potential to confound such heterologous studies. Flow cytometric analysis of cells expressing the nematode protein revealed a novel pharmacological profile, being insensitive to many agents known to cause P-gp inhibition. Further studies are needed to investigate the repertoire of P-glycoprotein activity in nematodes and to identify parasite-specific inhibitors that could be used to potentiate anthelmintic activity.

5. References


Figures and tables

Figure 4-1. Bioinformatic analysis of conceptually translated protein sequence of Tca-Pgp-11.1. (A) ScanProsite predicted domain arrangements in conceptually translated protein sequence of Tca-Pgp-11.1. (B) ModWeb predicted tertiary structure modelled after C. elegans Pgp-1 crystal structure (PDB: 4f4c). (C) Porter 5.0 predicted secondary structure of conceptually translated protein sequence
of Tca-Pgp-11.1 with domain architecture annotations from ScanProsite and residue level annotation from InterPro. Line 1 denotes conceptually translated Tca-Pgp-11.1 protein sequence (SEQ). Line 2 denotes SS3, the 3-class secondary structure prediction where H = helix (alpha helix, 3-10 helix, pi-helix) classes, E = strand (extended strand, beta-bridge classes) and C = the rest (turn, bend etc.). Line 3 denotes 3 class Secondary structure prediction confidence: a number between 0 and 9, with 9 signifying maximal confidence. Line 4 denotes SS8, the 8-class secondary structure prediction where H = alpha helix, G = 3-10 helix, I = pi-helix, E = extended strand, B = beta-bridge, T = turn, S = bend and C = the rest. Line 5 denotes 8 class Secondary structure prediction confidence: a number between 0 and 9, with 9 signifying maximal confidence. Helices are colored red and coils are colored blue.
Figure 4-2. Maximum likelihood phylogenetic tree of cloned and predicted *T. canis* Pgp genes in comparison with those from other nematodes. Mouse, human and dog Pgp (MDR1) protein sequences were used as outgroup. The cloned *Tca-Pgp-11* is highlighted with a black bullet (●).
Figure 4-3. Expression of *Tca-Pgp-11.1* in transfected MDCK-ΔMDR cells. Relative fold change was calculated with the efficiency corrected $\Delta$Ct method based on single samples using transfected cells as samples, KO cells as calibrators, and canine GAPDH as the reference gene.

Figure 4-4. Western blot and dot blot using anti-Tca-Pgp-11 polyclonal antibodies raised in rabbits against (A) whole cell lysate of *Tca-Pgp-11.1* transfected cells, (B) whole cell lysate of
KO cells and (C) protein marker (PageRuler Plus, ThermoScientific). Each lane was loaded with 30 µg of total protein. Expected 140 KDa band is indicated by a green arrow.

Figure 4-5. Flow cytometry histograms of EffluxID dye transport activity in WT cells, Tca-Pgp-11 transfected, and KO cells with (A) EffluxID Green with no drugs (B) EffluxID Green + 20µM Verapamil, (C) EffluxID Green + 50µM MK-571, (D) EffluxID Green + 100µM Novobiocin. A
minimum of 10,000 gated cells are represented in each histogram, except where indicated in the text. Normalized mean fluorescence obtained with Calcein-AM (B) and Rhodamine 123 (D) as fluorophores. Significant differences in a two-way ANOVA are depicted (*) (p<0.05).

Figure 4-6. Flow cytometry histograms of Calcein-AM (A) and Rhodamine 123 (C) dye transport activity in WT cells, Tca-Pgp-11 transfected, and KO cells with no drugs. A minimum of 10,000 gated cells are represented in each histogram, except where indicated in the text. Normalized mean fluorescence obtained with Calcein-AM (B) and Rhodamine 123 (D) as fluorophores.
Figure 4-7. Flow cytometry histograms of Calcein-AM dye transport activity in WT cells, Tca-
Pgp-11 transfected, and KO cells. Flow plots of the three cell lines with dilutions of ivermectin 
(A-G), milbemycin oxime (I-O), verapamil, (P-V), cyclosporine A (W-AC), reserpine (AD-AJ) 
and tariquidar (AK-AQ). A minimum of 10,000 gated cells are represented in each histogram, 
except where indicated in the text.
Figure 4-8. Normalized mean fluorescence obtained with Calcein-AM in the presence of ivermectin, milbemycin oxime, verapamil, cyclosporine A, reserpine and tariquidar.
Figure 4-9. Mean fluorescence intensity ratios in *Tca-Ppg-11* transfected cells co-incubated with Calcein-AM and inhibitors.
Figure 4-10. Representative images of multiple nucleic acid in situ hybridization in adult female and male *Toxocara canis*. Positive signal resulting from hybridization of *Tca-Pgp-11* probes.
appeared as red punctate dots in all tissues except the reproductive tract. Black boxes indicate the location of high magnification insets. Black arrows point to red dots in locations where signal was low.

Table 4-1. Peptide sequences used as antigens for antibody production in rabbits.

<table>
<thead>
<tr>
<th>Antigen</th>
<th>Length</th>
<th>Antigenicity/Surface/ Hydrophilicity</th>
</tr>
</thead>
<tbody>
<tr>
<td>VDAASSKQMKGNAEC</td>
<td>14 aa</td>
<td>1.51/0.86/0.48</td>
</tr>
<tr>
<td>CGQKQRIAIARTIAR</td>
<td>14 aa</td>
<td>1.20/0.57/0.29</td>
</tr>
<tr>
<td>IKSEGSIQFSDVHFC</td>
<td>14 aa</td>
<td>0.99/0.64/-0.00</td>
</tr>
</tbody>
</table>
The following is supplementary data to this article:

Figure 4-S1. Multiple sequence alignments of nucleotide sequences of cloned Tca-Pgp-11.1, Parascaris gene Peq-Pgp-11 (GenBank Accession number JX308320) and predicted P-gp from draft genome of T. canis (GenBank Accession number JPKZ01003065).
Figure 4-S 2. Multiple sequence alignments of conceptually translated protein sequences of cloned *Tca*-Pgp-11.1, *Parascaris* Peq-Pgp-11 (GenBank Accession number AGL08022) and predicted P-gp from draft genome of *T. canis* (GenBank Accession number KHN73709).
Figure 4-S 3. Flow cytometric analysis of transport activity of ABC transporters exposed to (A) Ivermectin, (B) Milbemycin oxime, (C) Verapamil, (D) Cyclosporine A, (E) Reserpine and (F) Tariquidar.
Figure 4-S 4. Representative images of adult female and male *Toxocara canis*. No positive signal resulting from hybridization of *B. subtilis* DapB probes appeared in any nematode tissue studied in either sex.
Figure 4-S 5. Representative images of adult female and male *Toxocara canis*. Positive signal resulting from hybridization of *Toxocara canis* tubulin gene *ttb4* probes appeared as red punctate dots in all tissues studied in both sexes.
Table 4-S 1. Primers used to clone *Tca-Pgp-11.1* and in qPCR

<table>
<thead>
<tr>
<th>Primer name</th>
<th>Sequence</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>SL3</td>
<td>5’- GGT TTA ATT ACC CAA GTT TGA G -3’</td>
<td>(Gems et al., 1995)</td>
</tr>
<tr>
<td>Pgp11R</td>
<td>5’- GTA TGA GTT CGG CGT AT -3’</td>
<td>Custom designed</td>
</tr>
<tr>
<td>Pgp11 forward</td>
<td>5’- ACT GAA AAC CCC TTT TGG GGA AGC</td>
<td>Custom designed</td>
</tr>
<tr>
<td>Pgp11 HindIII</td>
<td>5’- TTG CCG CCA CCA TGG ATG ACA ATC GCA AGG ACT C -3’</td>
<td>Custom designed</td>
</tr>
<tr>
<td>Pgp11 reverse</td>
<td>5’- ACT GAA AAC CCC TTT TGG GGG GAT</td>
<td>Custom designed</td>
</tr>
<tr>
<td>Pgp11 BamH1</td>
<td>5’- CCT TAA TGG TGA TGG TGG TGG TGG CTG CGC AGA TCC TGT TTG CGT ATG AGT TCG GCG TAT -3’</td>
<td>Custom designed</td>
</tr>
<tr>
<td>qTcPgp11-2F</td>
<td>TGCAACGTCAGGAAACGAT</td>
<td>Custom designed</td>
</tr>
<tr>
<td>qTcPg11-2R</td>
<td>AATATCGCCCGCTCTTTGA</td>
<td>Custom designed</td>
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Table 4-S 2. Ratio of Mean fluorescence Intensities in *Tca-Pgp-11.1* transfected cells with Calcein-AM with the experimental drugs at various concentrations

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<thead>
<tr>
<th></th>
<th>Ivermectin</th>
<th>Milbemycin oxime</th>
<th>Verapamil</th>
<th>Cyclosporine A</th>
<th>Reserpine</th>
<th>Tariquidar</th>
</tr>
</thead>
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<tr>
<td>100 µM</td>
<td>0.65248</td>
<td>0.56614</td>
<td>1.10767</td>
<td>1.21652</td>
<td>0.64602</td>
<td>1.63992</td>
</tr>
<tr>
<td>50 µM</td>
<td>0.99269</td>
<td>0.84915</td>
<td>1.20057</td>
<td>1.20963</td>
<td>0.96822</td>
<td>2.23853</td>
</tr>
<tr>
<td>10 µM</td>
<td>1.21566</td>
<td>1.00715</td>
<td>1.06833</td>
<td>1.18814</td>
<td>1.06587</td>
<td>3.58703</td>
</tr>
<tr>
<td>5 µM</td>
<td>0.96961</td>
<td>0.85356</td>
<td></td>
<td></td>
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</tr>
<tr>
<td>1 µM</td>
<td>1.31677</td>
<td>1.22984</td>
<td>0.99923</td>
<td>0.95178</td>
<td>1.05937</td>
<td>1.92403</td>
</tr>
<tr>
<td>0.1 µM</td>
<td>0.49697</td>
<td>0.89788</td>
<td>0.98064</td>
<td>0.92345</td>
<td>0.99778</td>
<td>1.00113</td>
</tr>
<tr>
<td>0.01 µM</td>
<td></td>
<td></td>
<td>1.06221</td>
<td>0.97367</td>
<td>0.95830</td>
<td>1.34620</td>
</tr>
<tr>
<td>0 µM</td>
<td></td>
<td></td>
<td>1</td>
<td>1</td>
<td>1</td>
<td>1</td>
</tr>
</tbody>
</table>
CHAPTER 5. DETECTION AND QUANTIFICATION OF PARASCARIS P-GLYCOPROTEIN DRUG TRANSPORTER EXPRESSION WITH A NOVEL MRNA HYBRIDIZATION TECHNIQUE

Jeba R J Jesudoss Chelladurai, Matt T Brewer

Modified from a manuscript published in Veterinary Parasitology

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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 P-gp 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, P-gps are thought to contribute to drug resistance by effluxing anthelmintics away from their molecular target. P-gps 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 P-gp expression are highly correlated with reduced susceptibility of *Parascaris* to MLs (Janssen et al., 2013a). Two P-gp genes, *Peq-pgp-11* and *Peq-pgp-16*, have been identified. Of these two P-gp genes, it appears that *Peq-pgp-11* is more strongly associated with resistance (Janssen et al., 2013a). 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., 2016b) 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 P-gp has been investigated by dissecting worm tissues and amplifying expressed genes by PCR (Janssen et al., 2013a). However, this preparation method precludes making clean preparations of tortuous linear organs such as the testes (Janssen et al., 2013a), and makes studying localization in nerve cords, and lateral cords impossible due to their small size. To overcome these obstacles, expression of P-gps can be studied *in situ* in sections of worm tissue that allow the preservation of anatomy and tissue architecture.

We examined P-gp 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., 2012a). 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 an 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* P-gp

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 (Figure 5-S1). *Parascaris* \( \beta \)-tubulin was used as the positive control for the assay. Positive signal indicative of \( \beta \)-tubulin mRNA was found to be highly expressed in all the organs examined (Figure 5-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 Figure 5-1 (male) and Figure 5-2 (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 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 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.

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.

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 (Foer, 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 P-gp expression data assessed by qPCR (Janssen et al., 2013a).
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 μm² 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 (Figure 5-3, Figure 5-4). Expression levels were also analyzed in each organ in sections from the anterior, middle and posterior of the worms (Figure 5-5 and Figure 5-6).

In male parasites, overall expression of Peq-pgp-11 was significantly higher in the intestine and reproductive tissue (which were not significantly different from each other) than in the body wall, nerve cords and lateral cords (Figure 5-3A). In contrast, expression of Peq-pgp-16 mRNA was not significantly different among the tissues studied (Figure 5-3B). 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. (Figure 5-5).

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 (Figure 5-4A). Similarly, expression of Peq-pgp-16 was significantly higher in the intestine when compared to all other tissues studied (Figure 5-4B). 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 (Figure 5-6).

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 P-gp 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 P-gp bound to the cuticle, eggs, and intestinal cells of *H. contortus* (Riou et al., 2005). Fewer studies have assessed the localization of P-gp 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 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 P-gp eliminates anthelmintics at the level of the nematode body wall or intestine, which may initially encounter the drug. Alternatively, P-gp 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 P-gp transcripts in some cell types suggests that P-gps could prevent entry of ML into the body of the nematode. P-gp 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 P-gp 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.

P-gp 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 P-gps 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 P-gps exclude xenobiotics at the level of the blood brain barrier (de Boer et al., 2003). In the present study, P-gp 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.

P-gp 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., 2013a). Uterine muscles are sites of action of for MLs and the presence of P-gps in the uterus and uterine muscles has been proposed (Godoy et al., 2015a; Prichard, 2001). However, the potential function of P-gps 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 P-gps could protect nematodes from anthelmintics locally, at the level of the neuron, but 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 P-gps are expressed constitutively in a variety of tissues. Therefore, ML-induced hyperexpression or post-translational changes in P-gps 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.

Acknowledgement

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

5. References


Figure 5-1. 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.
Figure 5-2. 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.
Figure 5-3. 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).
Figure 5-4. 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).
Figure 5-5. 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).
Figure 5-6. 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).
Supplementary data

The following is supplementary data to this article:

Figure 5-S 1. Representative images of adult Parascaris. Positive signal resulting from positive and negative control probe hybridization appeared as red punctate dots.
CHAPTER 6. GENERAL CONCLUSIONS

In order to understand the function of P-glycoproteins in ascarid worms, a series of studies were conducted in *Toxocara canis* and *Parascaris*. The principle findings of these experiments were:

**P-gp genes are expressed in *T. canis***

Phylogenetic analysis and molecular cloning revealed 13 P-gp encoding genes present in *T. canis*, with several isoforms predicted. 10 of these were constitutively expressed in adults and infective larvae, while 6 were expressed in somatic larvae. *Tca-Pgp-10* expression was upregulated in somatic larvae derived from mice treated with moxidectin. Larval inhibition assays provided phenotypic evidence of functional P-gp activity with a unique pharmacological profile. Further work is required to study the function of these genes and discover nematode specific P-gp inhibitors.

**Tca-Pgp-11 has unique pharmacology and localization**

A full length was cloned and designated *Tca-Pgp-11*. *Tca-Pgp-11* was stably expressed in a cell line lacking endogenous P-gp expression, and the pharmacological profile was studied using flow cytometry. These experiments revealed that macrocyclic lactones are transported by *Tca-Pgp-11* and that this transporter has a inhibition profile that differs from mammalian P-gps. Notably, *Tca-Pgp-11* was insensitive to the P-gp inhibitors verapamil, cyclosporine A, reserpine and tariquidar. *Tca-Pgp-11* expression was restricted to intestines, body wall, nerve and lateral cords and was completely absent from the reproductive tissue in adult worms.

**Peq-pgp-11 and Peq-pgp-16 are expressed by several cell types in adult parasites**

Macrocyclic lactone resistance is a clinical problem in *Parascaris* and has been
associated with *Peq-pgp-11*. This research demonstrated that P-gp mRNA localization could be quantitatively measured using a multiple nucleic acid hybridization technique in formalin-fixed paraffin embedded nematode tissue sections. *Peq-pgp-11* and *Peq-pgp-16* transcripts were detected in several tissues in male and female worms indicating that P-gps could protect the worm from MLs locally in the intestines, neurons, reproductive tissue and hypodermis. Further work is necessary to understand if tissue localization of P-gp mRNA changes with ML-induced hyperexpression in resistant isolates of *Parascaris*. mRNA *in situ* hybridization in combination with immunohistochemistry would provide a snap-shot of mRNA and protein expression to understand drug resistance mechanisms better.