INVESTIGATIONS INTO THE BIOLOGY AND GENETICS OF *CYLICOCYCLUS NASSATUS* AS RELATING TO THE MECHANISM OF ACTION AND SELECTION FOR RESISTANCE TO AVERMECTIN-MILBEMYCIN ANTHELMINTICS

by

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(Under the Direction of RAY KAPLAN)

ABSTRACT

The objectives of this dissertation were to study the feasibility of using Drenchrite® bioassay for the diagnosis of anthelmintic resistance in horse parasitic nematodes, determine the effects of moxidectin (an anthelmintic drug) selection on the population genetic diversity of *Cylicocyclus nassatus*, a common cyathostomin species and also to clone, sequence and characterize the orthologs of nematode glutamate-gated chloride channel (*GluCl*) genes from *C. nassatus*.

Cyathostomin are the principal parasitic pathogen and most important intestinal nematodes of horses. Resistance against two of the three available classes of anthelmintics has been reported in cyathostomins. Although no clinical case report of resistance against avermectin-milbemycin (AM) class exists at present, it is highly probable that cyathostomin will develop resistance to this group of drugs in near future. First objective: Studies on validation of a larval development assay (LDA) for detection of anthelmintic resistance in horses in the current situation of anthelmintic resistance determined that a LDA cannot be recommended for use in the field in the present scenario of anthelmintic resistance in horses. Second objective: Selection using a subtherapeutic dose of moxidectin for a period of 29 months (21 doses) leads to a

statistical decrease in population genetic diversity within *C. nassatus* as determined by Amplified Fragment Length Polymorphism (AFLP) for two of the three primer combinations tested. A high level of inherent genetic polymorphism in *C. nassatus* individuals was discovered in this study. Third objective: A homology based PCR approach was used to clone and sequence full-length cDNAs for two genes from *C. nassatus* which are orthologous to invertebrate glutamate-gated chloride channel genes. Phylogenetic analysis determined the close relationship of these genes to *GluCl* genes in other species. Radioligand studies using *GluCl* subunits expressed in mammalian cells demonstrated the presence of a high affinity ivermectin-binding site on the *C. nassatus GluCl*-α subunit but not on the *GluCl*-β subunit. Together these findings provide insights into some potential genetic aspects of AM resistance in cyathostomin nematodes and may serve as a basis for future studies aimed towards understanding the process of development of AM resistance in cyathostomins, as well as for the development of molecular diagnostic tools for detecting resistance.

INDEX WORDS: *Cylicocyclus nassatus*, Ivermectin, Moxidectin, Avermectin, Milbemycin, Resistance, Selection, *in vitro* larval development Assay, Drenchrite, Fecal Egg Count Reduction Test, cDNA library, Rapid amplification of cDNA ends, RT-PCR, glutamate-gated chloride channel, population genetic diversity, SSCP, AFLP, PCR, Cloning, *in vitro* expression, radioligand binding, COS-7 cells, ^{3H}ivermectin.

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DEDICATION

To my mother

Ms. Shanta Tandon

She is indeed the perfect exemplar of all nobleness.

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TABLE OF CONTENTS

Page

LIST OF TABLES

Page

LIST OF FIGURES

Page

CHAPTER 1

INTRODUCTION AND SIGNIFICANCE

Cyathostomins (small strongyles) are among the most important intestinal nematodes in horses (Lyons et al., 1999). Although individual horses may harbor tens of thousands of cyathostomins without developing detectable illness, an infection may result in weight loss, poor hair coat and intermittent diarrhea (Murphy and Love, 1997). Cyathostomins are sometimes responsible for producing a life-threatening syndrome usually seen in young horses, known as larval cyathostominosis. There is a lifelong susceptibility in horses to cyathostomins and they can cause clinical disease in horses of any age throughout the year (Love et al., 1999). Acquired immunity against cyathostomins is usually slow to develop and is often incomplete (Klei and Chapman, 1999).

More than 50 species of these parasitic nematodes have been described (Lichtenfels et al., 2002) but about 10 of them, including *Cylicocyclus nassatus,* comprise the major proportion of cyathostomins infecting horses worldwide (Reinemeyer 1986; Lyons et al. 1999).

The drugs used to control these parasites fall under three major groups: benzimidazoles (oxibendazole and fenbendazole), tetrahydropyrimidines (pyrantel) and avermectin-milbemycins (AM) (ivermectin and moxidectin). Resistance to benzimidazoles (BZ) was reported as early as 1965 (Drudge and Lyons, 1965) and since then BZ-resistant cyathostomins have been detected in many countries (Lyons et al., 1999). Resistance to pyrantel in cyathostomins has become a

common occurrence in recent years (Kaplan, 2002), leaving the AM group of drugs as the only effective group of drugs on many farms. Avermectin-milbemycin resistance has been reported in trichostrongylid nematodes (*H. contortus, C. oncophora, T. colubriformis, and T. circumcincta*) (Gopal et al., 1999; Wooster et al., 2001; Fiel et al., 2001; Sutherland et al., 2002; 2003, Chandrawathani et al., 2003). Although no reports of resistance to ivermectin or moxidectin in the cyathostomins have been published to date, a significant reduction in genetic diversity was reported on moxidectin selection in cyathostomin populations (Tandon et al., 2005). There is a likelihood of development of resistance against ivermectin in cyathostomins at some time in future (Kaplan, 2002).

Several methods have been described and used to detect anthelmintic resistance with differing sensitivity and reliability. Fecal egg count reduction test (FECRT) remains the practical gold standard for detecting resistance in horses in the field (Kaplan, 2002), although it is costly in terms of the amount of labor required to collect repetitive fecal samples and to perform fecal egg counts. Also only one drug can be tested at one time in FECRT. Therefore, it is essential to research alternate methods for diagnosis of anthelmintic resistance.

The level of genetic diversity present in cyathostomin populations and the genetic effects of drug selection on genetic structure have not been studied to date. These studies are important in order to provide insights into the dynamics of selection for resistance and also to gain a better understanding of anthelmintic selection mechanisms. Also, there are no reports characterizing the genes with probable involvement in the development of AM resistance in cyathostomins. Glutamate-gated chloride channels (*GluCl*s) are the putative target sites for the action of the AM group of drugs (Arena et al, 1995). These ion channels are reported to be present only in invertebrates (Cully et al., 1996). Although other proteins like aminobutyric acid (GABA)

receptors and p-glycoprotein (Burkhart, 2000) have been implicated in the mechanism of action of AM drugs, a correlation with AM resistance has only been established for *GluCl*s. Involvement of *GluCl*s in IVM resistance has been reported in studies on *C. elegans, H. contortus and C. oncophora* (Dent et al., 2000; Blackhall et al., 1998; Njue and Prichard, 2004). The fact that no AM resistance has yet been reported in cyathostomins even after more than two decades of continuous use of these drugs poses important questions on the differences in biology of cyathostomins compared to other strongylid nematodes as well as the factors contributing to the development of AM resistance in cyathostomins. In this dissertation, we have used a forward genetic approach by starting a drug (moxidectin) selection protocol to generate the AM resistant phenotype in cyathostomins and a reverse genetic approach by exploring at the probable target genes involved in the development of resistance against AM drugs in cyathostomins.

The specific objectives of this dissertation were to:

1. **Evaluate the Drenchrite® bioassay for the diagnosis of anthelmintic resistance under the current situation of anthelmintic resistance in horse nematodes.**
2. **Study the genetic diversity of cyathostomin populations and the effects of AM selection on this diversity and also search for probable genetic markers for AM resistance in *Cylicocyclus nassatus*.**
3. **Clone and sequence the orthologs of the invertebrate glutamate-gated chloride channel (*GluCl*) genes from *Cylicocyclus nassatus* and characterize their pharmacological properties by recombinant gene expression in mammalian cells and radioligand binding studies.**

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CHAPTER 2

LITERATURE REVIEW

Biology and History of Cyathostomins

Strongyles are the intestinal nematodes of equids (Lyons et al., 1999). Taxonomically they are grouped under the family Strongylidae (order Strongylida) (Lichtenfels et al., 1998) and are commonly designated as large or small strongyles based on their gross morphology. In the past, small strongyles have been referred to as cyathostomes in scientific literature, but recently the new term "cyathostomin" has been recommended as being scientifically more precise (Lichtenfels et al., 2002). A total of 52 species of cyathostomins have been recorded in horses, donkeys and zebras worldwide (Lichtenfels et al., 2002; Matthee et al., 2002). Some of the most common species are *Cyathostomum catinatum, Cylicocyclus nassatus, Cylicostephanus longibursatus, Cyathostomum coronatum, Cylicostephanus goldi, Cylicostephanus calicatus, Cylicostephanus minutus, Cylicocyclus leptostomus, Cylicocyclus insigne, Poteriostomum imparidentatum, Cyathostomum labiatum, Cylicocyclus ultrajectinus and Cyathostomum pateratum* (Reinemeyer, 1984, Collobert-Laugier et al., 2002).

In typical natural infections, a small number of species including *Cylicocyclus nassatus* comprise the majority of cyathostomin worm burden. Reinemeyer et al., (1984) necropsied 55 adult horses over a 15-month period, counted and identified cyathostomin species recovered from the cecum and colon. They reported that the 5 most prevalent species of cyathostomins comprising 84% of the total lumen small strongyle population were *Cyathostomum catinatum,*

Cylicocyclus nassatus, Cylicostephanus longibursatus, Cyathostomum coronatum and Cylicostephanus goldi. The species that were most prevalent also occurred in the highest average numbers. A quantitative post-mortem study of 150 horses in Victoria, Australia determined that 93% per cent of all horses harbored adult cyathostome worms. The 3 most prevalent species were *Cylicostephamus longiburstatus* (76%); *Cyathostomum catinatum* (68%) and *Cylicocyclus nassatus* (54%). (Bucknell et al., 1995). In a necropsy study conducted in 9 states of Brazil, the most prevalent species were *C. longibursatus* (100%), *C. nassatus* (97.22%) and *C. catinatum* (94.44%) (Silva et al., 1999). Retrieval and identification of cyathostomins expelled after deworming in Sweden showed that six species including *C. nassatus*, *C. catinatum*, *C. longibursatus*, *C. leptostomus*, *C. minutus* and *C. calicatus* comprise 91% of the total burden of cyathostomins (Osterman Lind et al., 2003).

The identification of cyathostomin species is generally done based on microscopic morphological features of worms that are distinguishable only in the adult stage (Fig. 2.2). It is often impossible to identify larval and egg stages of cyathostomins morphologically down to species level (Reinemeyer, 1986). Recently a molecular tool (PCR-ELISA) has been reported for identification of several cyathostomin species. It utilizes species specific oligoprobes designed from intergenic spacer region sequences of ribosomal DNA. The intergenic spacer region is amplified from sample worms by PCR using conserved primers and the probes are tested for hybridization to this amplicon (Hodgkinson et al., 2003, 2005).

Cyathostomins have a direct life cycle; adult females lay eggs in the host cecum and colon that are passed in the feces of the host into the environment. There, an embryo develops within the egg to the first stage larva. This larva hatches out in the environment and develops consecutively to the second and third stage larva, which is the infective stage. Infective larvae

are ingested by the host while grazing. These larvae penetrate the mucosa and submucosa of cecum and colon of the host and encyst in the intestinal wall for variable lengths of time (Love et al., 1999). The larvae then emerge from the intestinal wall into the lumen, mature into female and male worms, mate, and the cycle is completed when the eggs produced in large numbers by female worms are shed in the feces of the host. Completion of this lifecycle can occur in as few as 5 weeks, but larvae may remain encysted in the intestinal mucosa for many months or even years. The magnitude of larval and adult populations within the host displays seasonal variations with the peak numbers occurring in early spring and autumn in the United States (Reinemeyer, 1986). Horses are typically infected with many thousands of cyathostomins and it is not uncommon for a single horse to harbor more than 100,000 worms (Chapman et al., 2003). Cyathostomins have not been successfully cultured *in vitro* after the third larval stage and there are no published reports of attempted culture retrievable in PubMed. *In vitro* growth upto adult stage would provide an opportunity for selective breeding of worms and therefore would be important for the experiments studying genetic aspects of drug resistance or selection. Maintaining laboratory lines of cyathostomins would help tremendously in the drug selection studies by providing genetically similar populations (isolates) of worms which are hard to obtain in *in vivo* conditions due to random mating.

Cyathostomins are presently considered the principal parasitic pathogen of horses (Lyons et al., 1999). Although severe infections are reported occasionally in adult horses, infection may result in weight loss, poor hair coat**,** and intermittent diarrhea (Murphy and Love, 1997). Animals infected with cyathostomins may develop hypoalbuminaemia and/or neutrophilia but there are no clinico-pathological features specific for the disease (Love et al., 1999). A more severe form of disease syndrome is caused by cyathostomins, termed as larval cyathostominosis. This is the

acute form of the disease caused by simultaneous emergence of the larvae from the encysted stage in the intestinal wall and may result in death by verminous enteritis (Mirck, 1977). From the clinical and pathological evidence of the effects of cyathostomin infection, it is evident that worms induce a protein-losing enteropathy (Love et al., 1999).

Control and Treatment of Cyathostomins

The control and treatment of cyathostomins in horses is largely based on drug treatment. The drugs used to treat helminth parasites are termed as anthelmintics. For horses, three chemical classes of anthelmintics are available in the market: benzimidazoles (BZ) (fenbendazole, oxfendazole and oxibendazole), tetrahydropirimidines (pyrantel) and avermectin-milbemycins (AM) (ivermectin and moxidectin). All the three classes of anthelmintics are available over the counter in the United States and are relatively inexpensive.

In the 1960s, the haphazard approach to deworming was replaced by an epidemiological approach with recommendations for treatment of horses every 6 to 8 weeks (Drudge and Lyons, 1966). This dosing interval was introduced primarily to control the highly pathogenic large strongyles with the newly available benzimidazole anthelmintics. The great success of this system in reducing the parasitic burden in horses made this practice a routine procedure on horse farms worldwide. The effects of this system on the selection for resistance were largely neglected.

After the introduction of ivermectin in 1980s a dramatic reduction in the prevalence and intensity of *Strongylus vulgaris* occurred, which is the most pathogenic of the three large strongyle species (Herd, 1990). This was believed to be due to high efficacy of ivermectin

against migrating larval stages of *S. vulgaris,* which are responsible for the serious pathological effects. The pathogenic potential of cyathostomins is now being realized and they have become the focus of attention in last two decades.

Molecular mechanism of anthelmintic action

There have been several studies to elucidate the molecular mechanisms of action of anthelmintics belonging to different chemical classes.

Benzimidazoles: The benzimidazole group of anthelmintics including thiabendazole, mebendazole, oxfendazole and fenbendazole, are broad-spectrum anthelmintics that are synthetically produced. Thiabendazole was the first anthelmintic of the benzimidazole class introduced in 1961. Benzimidazoles act against gastrointestinal nematodes and also against trematodes at higher concentrations. The mode of action of the benzimidazoles started to be understood when it was realized that mebendazole given to *Ascaris* produces damage to the intestinal cells of the parasite. It was reported that there was a loss of cytoplasmic tubules of both the intestinal cells and teguments of cestodes and nematodes (Van den Bossche & De Nollin, 1973; van den Bossche et al., 1982) and this was associated with a loss of transport of secretory vesicles and the failure of the intestinal cells to take up glucose. Thus following application of mebendazole, the parasite starved. It was later realized that mebendazole bound to cytoplasmic proteins that had a molecular weight of 50 kDa and 100 kDa, which are monomers and dimers of tubulin. It was also realized that the benzimidazoles anthelmintics competed for the binding site on β-tubulin with colchicine, a substance known to block cell division in the metaphase (Sangster et al., 1985; Lacey & Gill, 1994).

Normally dimers of β-tubulin together with α-tubulin polymerize to form microtubule structures. Binding of benzimidazoles to β-tubulin subunits prevents the self-association of β-tubulin onto the growing microtubules (Lacey, 1988). Microtubules are important in the cellular transport and coordination and their loss disrupts cellular integrity. It was reported that benzimidazoles have higher affinity for nematode tubulin compared to mammalian tubulin which explains their selective toxicity (Friedman and Platzer, 1980).

Tetrahydropyrimidines: Pyrantel, morantel, and oxantel are the commercial members of the tetrahydropyrimidine class and are synthetically produced. Pyrantel is prepared for use as a tartrate, embonate, or pamoate salt. The pamoate salt of pyrantel is poorly soluble in water; this offers the advantage of reduced absorption from the gut and allows the drug to reach and be effective against parasites in the lower end of the large intestine, which makes it useful in horses. Anthelmintics of this class act by binding to the nicotinic acetylcholine receptors (nAChR). Studies using electrophysiological techniques have shown that the surface of somatic muscle cells of nematodes possess nAChRs (Martin et al., 1996). Binding of these compounds to the recognition site of this excitatory receptor produces depolarization and spastic paralysis of the nematode muscle that can result in parasite expulsion (Martin et al., 1997). nAChR receptors are typically heteropentameric, transmembrane glycoproteins. Two members of the pentamer are α-subunits which contain acetylcholine (ACh) binding site. Rest three subunits are non-α heterogenous subunits and are referred to as strucutural subunits. Together with α-subunits they help form the ion channel, which on binding agonists such as pyrantel or ACh, opens and allows the passage of cations which leads to depolarization of the cell. The orthologs of *C. elegans* unc-38 (Fleming et al., 1997) encoding nAChR-α-subunit have been cloned from *T. colubriformis*, *O.*

circumcincta and *H. contortus* (Hoekstra et al., 1997; Wiley et al., 1996) but not from any cyathostomin species.

Avermectin-Milbemycin: Based on the chemical structure, anthelmintics of avermectin-milbemycin class are also called as macrocyclic lactones. These drugs are effective in controlling a broad spectrum of arthropod pests as well as nematodes and hence are called as endectocides. AM drugs are also able to kill the free living nematode *C. elegans* at therapeutic concentrations making it a useful model system in which to examine mechanisms of ivermectin toxicity and resistance.

The avermectins are produced as a mixture of eight different components from natural fermentation by the soil dwelling bacteria *Streptomyces avermitilis* (Campbell et al., 1983). These natural components are denoted A1a, A_{1b}, A_{2a}, A_{2b}, B_{1a}, B_{1b}, B_{2a} and B_{2b}. The A-components have a methoxy group at the C-5 position (Fig. 2.1), whereas the B-components have a hydroxyl group. Out of these eight components only A_{2a}, B_{1a} and B_{2a} are produced in quantity during fermentation, making them desirable candidates for drug development. B_1 homologs possess the highest potency and breadth of spectrum against nematodes. Ivermectin (22, 23-dihydro-avermectin B_1) was the first avermectin to be commercialized (Chabala et al., 1980; Egerton et al., 1980) and its release for use in animals came in 1981. It is synthesized by selective hydrogenation of the cis 22, 23-double bond of avermectin B_1. Milbemycins are produced by a variety of *Streptomyces spp.* isolated throughout the world (Carter et al., 1988) and can also be subdivided into A- and B-components based on hydroxy or methoxy groupings at the C-5 position. Ivermectin and moxidectin are similar in structure except for the presence of a dissacharide at C-13 in case of ivermectin, presence of a methoxine moiety at carbon-23 in moxidectin and different side chains in two compounds at carbon-25 (Fig. 2.1).

Avermectin-milbemycins produce a flaccid paralysis of the somatic worm musculature and inhibit feeding of the nematode by blocking pharyngeal pumping (Paiement et al., 1999). Nematode feeding has been shown to be more sensitive to the effects of the AM drugs than the effects on motility in vitro (Geary et al., 1993). However, Sheriff et al., (2005) found that ivermectin had no effect on feeding by *H. contortus* in vivo. In this study, nematodes recovered from sheep treated with ivermectin 4 h prior to the [^{3}H]inulin administration showed equivalent feeding levels (over a 1 hour period) to those recovered from sheep not treated with ivermectin. In addition, there was no difference in the radioactivity in nematodes of an ivermectin-susceptible and an ivermectin-resistant isolate recovered from individual sheep with concurrent infections after a dose with ivermectin.

The identity of the target protein of these drugs has been controversial (Arena et al., 1995). Early studies on the mechanism of action of AM drugs showed that they are antagonists of the GABA (gamma-aminobutyric acid) receptor and act by blocking hyperpolarization of nematode somatic muscle membranes (Holden-Dye and Walker, 1990). However, considerably higher concentrations of ivermectin are required for GABA-antagonist effect than for the effects on the glutamate-gated chloride channels (*GluCl*) (Cull-Candy and Usherwood, 1973). Subsequently, it was shown that injection of *C. elegans* mRNA into *Xenopus laevis* oocytes results in expression of an avermectin-sensitive chloride current (Arena et al., 1991). Construction of a *C. elegans* cDNA library and screening by expression in *Xenopus laevis* oocytes resulted in identification of a pool of 5000 cDNAs, which was sensitive to glutamate as well as ivermectin-phosphate. Subfractionation of these cDNAs yielded two cDNA clones *pGluCl-α* and *pGluCl-β* that expressed glutamate and ivermectin-phosphate current, respectively (Cully et al., 1994). The nematocidal activity of avermectin analogues was found to correlate

with their activity to activate and potentiate glutamate-sensitive current in oocytes injected with mRNA (Arena et al., 1995). Based on these studies GluCls were deemed as the target of avermectin in nematodes. GluCls were later found to be expressed in certain muscle cells and neurons in the pharynx of *C. elegans* (Dent et al., 1997; Laughton et. al., 1997). *GluCl*s have been found only in invertebrates (Cully et al., 1996), which explains why they are selectively toxic to invertebrate parasites and not to the vertebrate host. Only *GluCl-α* type subunits have been found to interact with ivermectin in *C. elegans. GluCl-α* subunits in *C. elegans* are encoded by a family of genes including *glc-1, avr-14, avr-15, glc-3* and *glc-4* (reviewed by Yates et al., 2003). It was reported that sensitivity to ivermectin in *C. elegans* is interplay of at least three *GluCl-α* genes (avr-14, avr-15 and glc-1) (Dent et al., 1997).

A study on the effects of ivermectin and moxidectin on pharyngeal pumping in *H. contortus* has shown that both drugs may share a common mechanism of action but that there may be subtle differences in the response to the target site between these compounds (Paiement et al., 1999). There is no information available describing the mode of action of AM drugs on cyathostomes, but it is believed that the drugs act in a similar fashion in all nematodes (Sangster and Gill, 1999).

Anthelmintic resistance

Anthelmintic resistance is defined as the ability of worms in a population to survive drug treatments that are generally effective against the same species and stage of infection at the same dose rate (Sangster and Gill, 1999). This shift in susceptibility is caused by changes in the gene frequencies of relevant genes and results from drug selection (Anderson et al., 1998).

Cyathostomins have been found to be resistant to benzimidazoles and pyrantel (Kaplan, 2002, 2004) leaving the AM group of drugs as the only effective group of drugs on many farms.

Benzimidazoles: Resistance against benzimidazole anthelmintics (thiabendazole) was reported after only a few years of use (Drudge and Lyons, 1965) and resistance to these drugs is the most prevalent and widespread of the three major classes of anthelmintics (Lyons et al., 1999). Recent studies in several regions of the world have reported a prevalence of resistance to benzimidazole (Lyons and Tolliver, 2003; Cirac et al., 2004). In *H. contortus*, two types of β-tubulin have been recognized: isotype 1 and isotype 2 and there is reduction in the number of isotype 1 alleles for β -tubulin during the appearance of benzimidazole resistance (Roos et al., 1995). A point mutation, detected in the BZ resistant fungi and *C. elegans*, was also found in resistant isolates of *H. contortus* (Roos et al., 1995). This mutation (Phe200Tyr) was found to be extremely important as it was able to confer a resistant phenotype to susceptible *C. elegans* when the β-tubulin gene from BZ-resistant *H. contortus* was transfected into this free living nematode (Kwa et al., 1995). This mutation has also been linked to BZ-resistance in *H. contortus* and *T. circumcincta* in association studies (Elard et al., 1996; Kwa et al., 1994). A PCR-based technique was later developed to identify resistant individuals based on this mutation (Elard et al., 1999). Cyathostomins possess both isotype-I and isotype-II β-tubulin genes (Clark et al., 2005) and the BZ-resistant populations have either the Phe200Tyr or a Phe167Tyr mutation (Kaplan, 2002). Cyathostomins resistant to a broad range of BZ anthelmintics remain sensitive to oxibendazole for a limited time. No clear associations between differential sensitivity to fenbendazole or oxibendazole and β-tubulin sequence or isotype expression have been found (Kaplan, 2002). It is possible that slight differences in the pharmacokinetics of these two drugs are responsible for this effect.

Pyrantel: Use of pyrantel in horses dates back to 1970's, yet reports of pyrantel-resistant cyathostomins have only recently become common. The resistance against pyrantel has been difficult to measure because even at their release in the 1970s, the efficiency of these compounds against the cyathostomins was in the range of 91-100%. The discriminating level of 90% efficacy is inappropriate as a cutoff for resistance in this case. First published report of pyrantel resistance in cyathostomins (Chapman et al., 1996) described worm count reduction of 25-83%. Subsequently, Lyons et al., (1996) reported a fecal egg count reduction of 72% for pyrantel. A recent study in the southern United States reported a prevalence of pyrantel resistance of more than 40% (Kaplan, 2004).

Pyrantel resistant isolates of pig parasite *Oesophagostomum dentatum have* been recovered and characterized (Roepstorff et al., 1987). These isolates were about two fold resistant than the sensitive worms. A patch-clamp study on these isolates of *O. dentatum* membranes reported alteration of the subtype profile and mean open time of the nicotinic acetylcholine receptors compared to the susceptible isolate (Robertson et al., 2000). Studies on mammalian nicotinic acetylcholine receptors demonstrate that the phosphorylation state of the receptor plays an important role in modulating channel opening (Hoffman et al., 1994). In nematodes, consensus sites for phosphorylation on nicotinic acetylcholine receptor subunits have been found in *C. elegans* (Fleming et al., 1997) and *Trichostongylus colubriformis* (Wiley et al., 1996). Electrophysiological work on *Ascaris* muscle has shown that the response to levamisole (a drug with similar mechanism of action as pyrantel) and acetylcholine can be reduced by tamoxifen, a protein kinase C antagonist (Trim et al., 1998). These results suggest that compounds that promote receptor phosphorylation would be expected to increase the response to

these anthelmintics. Such compounds when used in association with these anthelmintics could render previously resistant parasite isolates susceptible to chemotherapy.

Avermectin-Milbemycin: AM resistance is highly prevalent in trichostrongylid nematodes (*H. contortus, T. colubriformis, T. circumcincta and Cooperia oncophora)* (Gopal et al. 1999; Wooster et al., 2001; Fiel et al., 2001; Sutherland et al. 2002; 2003, Chandrawathani et al., 2003). Although no reports of resistance to ivermectin or moxidectin in cyathostomins have been reported to date, a statistically significant decrease in the genetic diversity of moxidectin selected population compared to the ivermectin naïve population has been reported (Tandon et al., 2005). It is believed that an important factor for the lack of resistance to ivermectin in cyathostomins may be due to the lack of efficiency of this drug against encysted mucosal larval stages. A huge number of larvae encyst in the mucosa and therefore provide a large refugia (proportion of a parasite population that is not exposed to a particular drug, thereby escaping selection for resistance) (Sangster and Gill, 1999; Kaplan, 2004). Means of inheritance of the resistance trait can also influence the rate of development of AM resistance. It was shown by computer modeling that resistance evolves fastest when it is inherited as dominant trait, more slowly when co-dominant, and slowest when it is recessive (Barnes et al., 1995). In *H. contortus*, ivermectin resistance has been shown to be inherited as a completely dominant trait (LeJambre et al., 1995). Levamisole resistance is inherited as an autosomal recessive trait in *H. contortus*, while in *T. colubriformis* levamisole resistance is inherited as a recessive sex-linked trait (Dobson et al., 1996). Inheritance of resistance to levamisole, benzimidazole and ivermectin in *H. contortus* and *T. colubriformis*, varies between drugs and parasites; incomplete dominant, complete dominant, incomplete recessive, sex linked recessive, and autosomal recessive inheritance have all been reported (Dobson et al., 1996). The pattern of inheritance of a trait can only be determined after

that trait is expressed phenotypically. Because there are no reports of resistance against AM drugs in cyathostomins at present, there is no way to establish what the pattern of inheritance will be. If inheritance of the ivermectin resistance trait in cyathostomes is recessive, then this would also greatly decrease the rate of the selection process toward resistance (Kaplan, 2002).

Involvement of more than one gene in the development of resistance can also influence the rate of development of resistance. Dent et al (2000) demonstrated that simultaneous mutation of three genes, avr-14, avr-15, and glc-1, encoding glutamate-gated chloride channel (*GluCl*) α-type subunits in *Caenorhabditis elegans* conferred high-level resistance to ivermectin. Interestingly, mutating any two channel genes conferred modest or no resistance. Based on these results it was proposed that ivermectin sensitivity in *C. elegans* is mediated by genes affecting parallel genetic pathways defined by the family of *GluCl* genes. Several other genes (unc-7, unc-9, and the Dyf) were also found to modulate ivermectin sensitivity in *C. elegans*. These studies show a multigenic nature of AM resistance in *C. elegans*. Changes in allele frequencies of the putative *H. contortus GluCl*-gene were linked with resistance to ivermectin and moxidectin (Blackhall et al., 1998) though a similar analysis of the *H. contortus* -subunit gene showed no significant differences in allele frequencies between the unselected and drug-selected strains. These results are meaningful in the context that *GluCl*- subunit binds to ivermectin whereas subunit is the binding site for glutamate and does not bind ivermectin (Cully et al., 1994). Also, selection at a p-glycoprotein gene was documented in *H. contortus* in moxidectin and ivermectin resistant strains (Blackhall et al., 1998). Significant differences were also shown in the allele frequencies of a GABA-receptor gene, HG1 in *H. contortus* between the parent unselected strain and the resistant ivermectin or ivermectin+moxidectin-selected strains (Blackhall et al., 2003). These results hint towards the multigenic nature of AM resistance in parasitic nematodes.

Although it is possible that the nature of resistance in cyathostomins is different from *C. elegans* and *H. contortus* a multigenic nature may provide explanations for its slow onset. *In H. contortus* AM resistance is found to be multigenic as well as dominant trait. This can be explained by possibility of linkage of genes conferring AM resistance in *H. contortus*. This genetic linkage may be absent in case of cyathostomins and the genes responsible for conferring AM resistance in these parasites may be located on different chromosome or at distant locations on the same chromosome.

Diagnosis of anthelmintic resistance: larval development assays

The controlled test where worms are recovered, enumerated and identified following necropsy at a certain period after treatment (Clark and Turton, 1973; Drudge and Lyons, 1977) is considered the gold standard for testing anthelmintic efficacy. However, it is only practical in research settings and cannot be used in on-farm studies because the animals used in these studies need to be euthanized. Fecal egg count reduction test (FECRT) remains the most common and practical method for detecting resistance in the field (Kaplan, 2002). FECRT involves collection of fecal samples before and after treatment and enumeration of eggs per gram of feces sampled. Several other *in vitro* methods have been described and used to detect anthelmintic resistance, with differing sensitivity and reliability in horses such as Egg Hatch Assay (Ihler and Bjorn, 1996, Craven et al., 1999), Larval Development Assay (Ihler and Bjorn, 1996), and DrenchRite® Larval Development Assay (Young et al., 1999 and Pook et al., 2002).

Fecal egg count reduction test is costly in terms of the amount of labor required to collect repetitive fecal samples and to perform fecal egg counts. Also it is less sensitive, it can only

detect resistance when the proportion of resistant worms in the population is at least 25% (Martin et al., 1989). EHA is only useful for benzimidazoles (BZ) and has an absolute requirement for undeveloped eggs. Larval paralysis, migration and motility tests are only effective for drugs that affect muscular activity and these tests utilize complicated instruments. Use of molecular techniques to detect resistance is not a viable option at present because knowledge of the molecular mechanisms of anthelmintic resistance is limited and mainly confined to the detection of a particular mutation conferring benzimidazole resistance using a PCR based approach (Elard et al., 1999). Biochemical tests are not available for most of the drugs and are generally not feasible because they require a large quantity of worm material. Larval development tests may provide a cost-effective and reliable alternative to other anthelmintic resistance detection methods for several reasons: there is no requirement for repetitive sampling, simultaneous testing of several drugs is possible, there are minimal restrictions on the mechanism of action for the drug, it is not essential to have undeveloped eggs and there is no requirement for sophisticated instruments.

The DrenchRite® LDA (Microbial Screening Technologies, Kemps Creek, NSW, Australia) was developed by Commonwealth Scientific Industrial Research Organization (CSIRO) in Australia. This is a larval development assay where the parasite eggs extracted from host feces are incubated for a week in the presence of increasing concentrations of agar-impregnated drugs in a 96 well plate. Inhibition of larval development is measured by counting the larval stages surviving at various drug concentrations. This test was initially developed and validated for use with ovine parasites and has been effectively used in sheep and goats (Lacey, 1990 and Gill et al., 1995). Another study showed that results from DrenchRite® assay were consistent with FECRT in goats (Terrill et al., 2001). Detection of anthelmintic resistance in

cyathostomin parasites of horses using LDA has been investigated but without conclusive results (Ihler and Bjorn, 1996; Craven et al., 1999; Young et al., 1999 and Pook et al., 2002).

Genetic diversity in nematode parasites and effects on anthelmintic selection

Large effective population sizes, high gene flow among populations and rapid rates of nucleotide sequence evolution in parasitic nematodes lead to high within-population diversities (Blouin et al., 1995). This provides a suitable genetic background for the selection of resistance because of the increased probability of presence of an allele, which may confer some selective advantage under drug pressure. Genetic analysis of a number of genes in *H. contortus* has indicated high genetic diversity (Prichard, 2001). This high diversity seems to be a general feature of parasitic nematode populations (Braisher et al., 2004). Several methods for studying genetic diversity in nematodes have been utilized by researchers, such as sequencing of mitochondrial DNA (mtDNA)(Dame et al., 1993; Blouin et al., 1999; Nieberding et al., 2005; Braisher et al., 2004; van der Veer and de Vries, 2004; Leignel and Humbert, 2001), RAPD markers (Nadler et al., 1995; Muller-Graf et al., 1999; Jobet et al., 2000; Pradeep Kumar et al., 2002), microsatellites (Otsen et al., 2000, Higazi et al., 2001), restriction fragment length polymorphism (RFLP) (Cameron et al., 1988) and isoenzyme electrophoresis (Nadler et al., 1995; Muller-Graf et al., 1999; Gasnier and Cabaret., 1998). Variation in DNA sequences of a particular gene has also been used widely. Ruiz et al., (2004) reported significant variation in nucleotide diversity within and between North American (NA) and Spanish (SP) strains of *H. contortus* by looking at cysteine protease genes. Anderson and Jaenike, (1997) studied polymorphisms in the intron regions in nuclear genes and in the ribosomal DNA spacer regions of *Ascaris sp.* and found that 65% of nuclear genetic variation was within populations. Host

(human or pig) explained 18% of this variation, while geographical variation within host-associated populations explained 17% of the variation. Nematode mtDNA undergoes a high rate of mutation, which can be up to ten times higher in *H. contortus* than in vertebrates (Blouin et al., 1995). Comparing five populations of *O. ostertagi*, Dame et al., (1993) reported that greater than 98% of the total mitochondrial DNA sequence diversity is present within a single population. mtDNA diversity gives an indication of the overall diversity in a particular species. A major contributing factor to the rate at which resistance develops is the initial frequency of resistance alleles in the target population (Anderson et al., 1998). The hypothesis here is that development of resistance against anthelmintic drugs is due to selection of existing alleles rather than new mutations. The more diverse or heterozygous the population is, the greater the chances that a resistance allele will be present. Studies in four species of trichostrongylid nematodes showed that 96-99% of the nucleotide diversity is found within populations (Blouin et al., 1992). Application of the drug would kill the drug- sensitive individuals and most individuals that survive would possess an allele conferring resistance. Reproduction of these resistant worms would lead to a buildup of resistant populations over a number of generations. The ability to predict the dynamics of drug resistance evolution depends on measurement of allele frequencies and not simply the ability to detect such alleles. Therefore DNA sequences of individual parasites should be examined while using genetic tools to dissect the changes associated with the development of resistance (Anderson et al., 1998).

Glutamate- gated chloride channels

The invertebrate glutamate-gated chloride channels (*GluCl*) are the putative receptor molecules and a target for the avermectin-milbemycin group of drugs in *C. elegans.* (Arena et

al., 1995). *GluCl*s have been found to be associated with ivermectin resistance in the soil dwelling nematode *C. elegans* (Dent et al., 2000) as well as the parasitic nematode *C. oncophora* (Njue and Prichard., 2004). These ion channels are expressed in the pharyngeal and extrapharyngeal neurons in *C. elegans* (Dent et al., 2000) and are widely distributed in the nervous system of *H. contortus* (Portillo et al., 2003). In *C. elegans* a small family of six *GluCl* genes encoding eight possible subunits has been reported (Dent et al., 1997, 2000; Laughton et al., 1997; Vassilatis et al., 1997). The gene structure and phylogenetic analyses for *C. elegans GluCl* genes has shown that these genes belong to a discrete ligand-gated ion channel family orthologous to the vertebrate glycine channels (Vassilatis et al., 1997). It is proposed that the properties of orthologous *GluCl* subunits are conserved across species, although the repertoire and relative importance of those subunits may vary (Cheeseman, 2001). In *H. contortus*, two alternatively spliced *GluCl* subunit cDNAs (*HcGluCla* and *HcGluClb*) were cloned (Forrester et al., 1999) that exhibit different sized ligand binding domains. The longer sequence was expressed and found to bind IVM with high affinity ($K_d \approx 10^{-10}$ M). Other *H. contortus GluCl* subunit cDNAs that have been sequenced are *Hc-GBR2A*, *Hc-GBR2B* (alternatively spliced form of *Hc-GBR2A*) (Jagannathan et al. 1999) and *HG4* (Delany et al., 1998). HcGBR2 is expressed on the nerve ring, the ventral and dorsal nerve cords, the anterior portion of the dorsal sub-lateral cord and motor-neuron commissures in *H. contortus* (Jagannathan et al., 1999), whereas *HG4* is expressed in motor neuron commissures in the anterior portion of *H. contortus* from the nerve ring to just anterior of the vulva (Delany et al., 1998). Cloning of a *Drosophila melanogaster* glutamate-gated chloride channel, *DrosGluCl-α* is reported that shares 48% amino acid and 60% nucleotide identity with the *C. elegans GluCl* channels (Cully et. al., 1996). Recently, two cDNAs encoding *D. immitis GluCl* subunits were cloned and found to be orthologs of the

alternatively spliced *GluCl*α3A and α3B subunits (encoded by the avr-14 gene) previously identified in *C. elegans* and in *H. contortus* (Yates and Wolstenholme, 2004). Two full-length *GluCl* cDNAs, encoding *GluClα3* and *GluClβ* subunits, were cloned from ivermectin-susceptible (IVS) and resistant (IVR) *Cooperia oncophora* adult worms. When expressed in *Xenopus* oocytes, IVS and IVR *GluClα3* subunits both formed ivermectin and moxidectin sensitive glutamate-gated channels. Mutations in the IVR *GluClα3* subunit caused a significant 2.5-fold reductions in sensitivity to ivermectin and moxidectin whereas mutations in the IVR *GluClβ* subunit abolished responsiveness to glutamate (Njue et al., 2004). These results suggest presence of binding site for AM drugs on *GluCl-α* subunit and also show the involvement of *GluCl-α* subunits in AM resistance development. Based on these results and also the studies done in *C. elegans, GluCl α type* subunits appear to play important role in the development of AM resistance in nematodes. The β type *GluCls* appear to be the binding site of glutamate which is the natural ligand for *GluCls* and probably these subunits have lesser or no involvement in AM drugs binding and development of resistance.

Glutamate binding properties of unselected and ivermectin-selected adult and larvae of *H. contortus* were studied by preparing the crude membranes from these stages (Paiement et al., 1999). Resistant adults showed a 2 fold increase in B_{max} and the larvae a 2 fold decrease in Kd for glutamate binding suggesting that resistant parasites overcome the effects of ivermectin by enhancing glutamate binding at this binding site, either through an increase in binding affinity, in the case of the larvae, or by making more receptor sites available for glutamate binding in adults. Rohrer et al. prepared membranes from ivermectin-sensitive and -resistant *H. contortus* L_3 larvae and examined for the presence of [^{3}H]ivermectin binding sites. Both tissue preparations displayed high affinity drug binding sites (Kd = 0.13 nM). Receptor density was the same in both

the sensitive and resistant nematodes suggesting that target site modification was not involved in the development of drug resistance in this particular strain of *H. contortus*. These studies suggest that mechanisms other than the decrease in binding affinity of *GluCls* for AM drugs may be involved in the development of AM resistance in nematodes.

Discussion

In the model nematode *C. elegans*, resistance to AM drugs is thought to involve alterations in the target sites, as well as a reduction in the drug concentration at the site of action. The delay in development of AM resistance in cyathostomins is intriguing. The fact that cyathostomins have developed widespread resistance against benzimidazoles and pyrantel but AM drugs still remain highly effective is also intriguing. Further complicating this issue is the presence of AM resistant trichostrongyle nematodes in ruminants. Trichostrongyles belong to the same taxonomical order as cyathostomins. This raises questions about the different ways the biology of cyathostomin influences the development of resistance against a particular chemical class of drug. Although research on development of AM resistance in *C. elegans, H. contortus, C. oncophora* and *D. immitis* provides us with a model to guide us in this investigation, it is possible that the factors involved in the development of AM resistance are completely different in cyathostomins.

The complexity of AM resistance development in cyathostomins can be addressed by the traditional forward and reverse genetic approaches. We started a drug (moxidectin) selection protocol in an effort to generate the AM resistant phenotype in cyathostomins and then investigated the genetic alterations posed by this selection. This is a forward genetic approach and we expected to find specific genes or loci that can be linked to the development of AM

resistance in cyathostomins. We also used a reverse genetic approach where we utilized the information gained from AM resistance studies on other nematode species. Glutamate-gated chloride channels (*GluCls*) are the putative target sites of AM action in *C. elegans* (Cully et al., 1994) and have also been associated with AM resistance in nematodes (Dent et al., 2000, Njue and Prichard, 2004). We investigated the presence of genes encoding *GluCls* in *C. nassatus,* one of the most common cyathostomin species. Properties of the individual genes were investigated by studying the characteristics of their translated protein sequences and their ivermectin binding affinities on *in vitro* expression in mammalian cells. Phylogenetic analysis of *C. nassatus GluCl* proteins was done to find out their closely related orthologs in other species and to shed light on their evolutionarily conserved function and properties. Although mutations in *GluCls* in *C. elegans* were shown to confer 4000 fold resistance to ivermectin *in vitro* (Dent et al., 2000) it is possible that the mechanism of AM resistance in Cyathostomins does not involve *GluCls.* Other genes that are associated with ivermectin resistance in *C. elegans* and parasitic nematodes are *GABA-gated chloride channels* (Blackhall et al., 2003), *p-glycoprotein* (Xu et al., 1998), *dyf* genes and *unc* genes (Dent et al., 2000). In order to investigate the involvement of genes other than *GluCls* in the development of resistance in cyathostomins, we followed a genome based approach and screened the moxidectin-selected and AM-naïve population of worms for the presence of particular molecular markers that could be linked to AM resistance.

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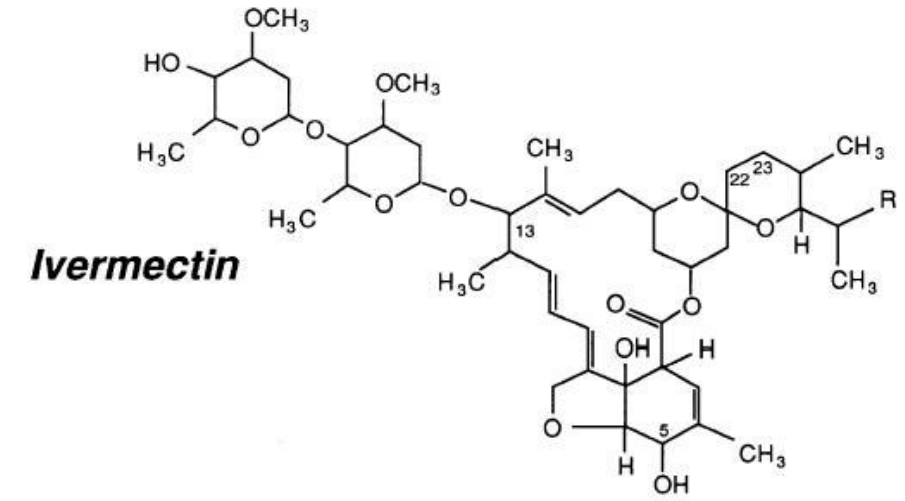

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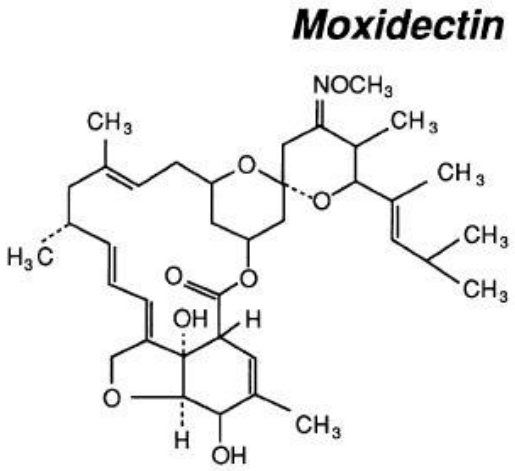

Fig. 2.1 The chemical structures of ivermectin and moxidectin (Martin et al., 1997).

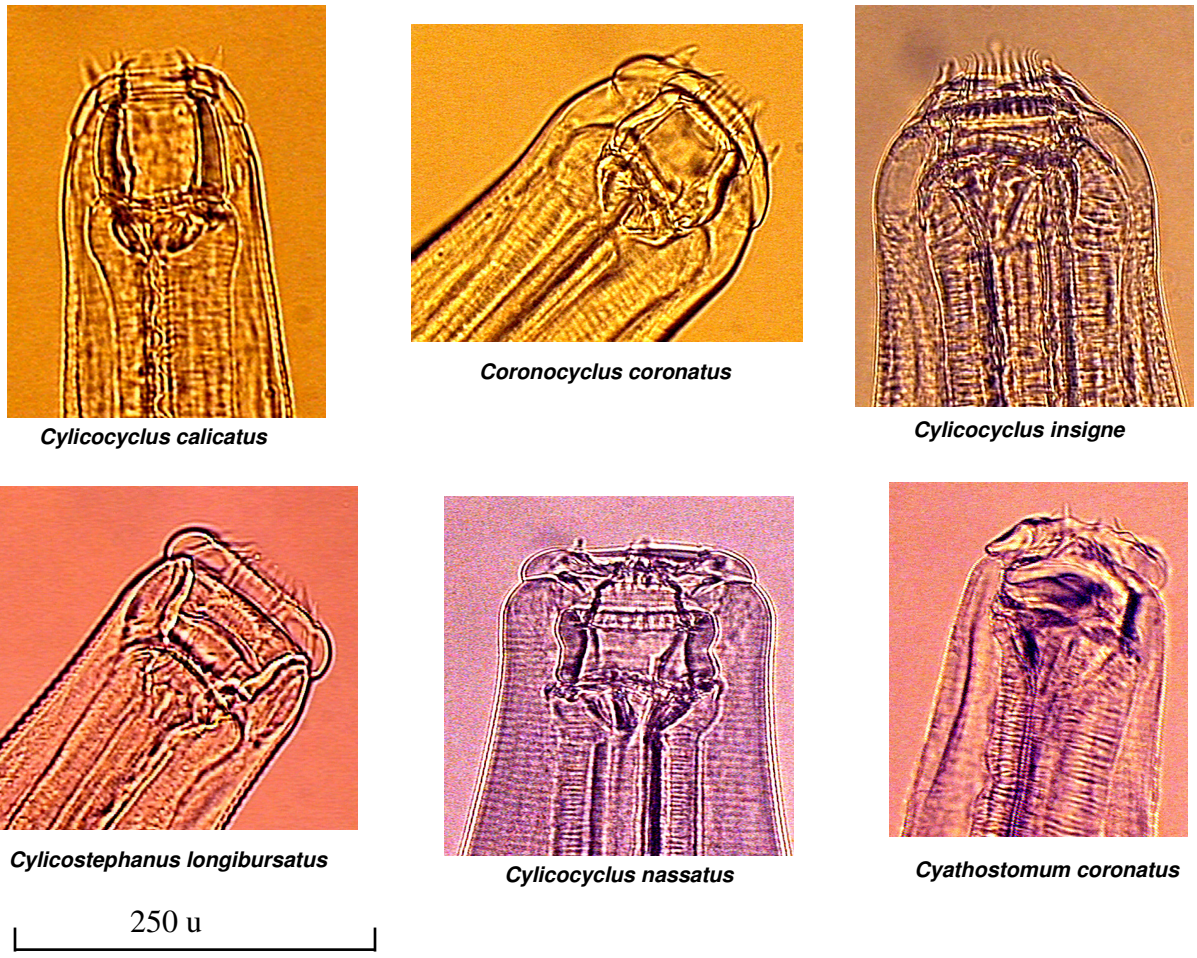

Fig. 2.2 Some of the common species of cyathostomins as identified by their mouth parts. Worms were cleared in Lactophenol overnight and photographed at 400X magnification.

CHAPTER 3

EVALUATION OF A LARVAL DEVELOPMENT ASSAY (DRENCHRITE®) FOR THE DETECTION OF ANTHELMINTIC RESISTANCE IN CYATHOSTOMIN NEMATODES OF HORSES*

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* Tandon, R. and Kaplan, R. M. 2004. Veterinary Parasitology. 121 (1-2). Reprinted here with the permission of publisher.

Abstract

A larval development assay (LDA, DrenchRite®) was evaluated to determine the effectiveness of this method in detecting anthelmintic resistance in cyathostomin nematodes of horses. A total of 15 horse farms from Georgia and South Carolina (USA) and Population S ponies from the University of Kentucky (USA) were included in this study. Nematode eggs were extracted from pooled fecal samples and placed into the wells of a DrenchRite® plate for testing against thiabendazole (TBZ), levamisole (LEV) and 2 ivermectin (IVM) analogs (IVM-1, IVM-2). After a 7-day incubation larvae in each well were counted and data were analyzed by logistic regression. Resistance status of each farm for different drugs was determined in a separate study using a fecal egg count reduction test. LDA were performed on the 15 farms once, however, the Population S cyathostomins were assayed on 3 separate occasions to estimate the consistency of results between assays. Mean TBZ LC_{50} for oxibendazole resistant, suspected resistant and sensitive farms were 0.2015, 0.1625, and 0.1355 μM, respectively. For LEV, mean LC_{50} for PYR resistant, suspected resistant and sensitive farms were 1.590, 1.8018 and 1.4219 μM, respectively. All 15 farms had worms susceptible to IVM; mean LC_{50} for IVM-1 and for IVM-2 were 7.5727 and 87.9718 nM, respectively. A linear mixed model was fitted to the data to determine the relationship between LC_{50} and LC_{95} and resistance status for each farm. No meaningful relations were found. Consistency of assays varied between drugs, being best for TBZ and worst for LEV and IVM-1. All farms in this study had benzimidazole-resistant nematodes; therefore usefulness of DrenchRite® for discriminating susceptibility versus resistance to this drug class could not be accurately assessed. Moreover, since all farms tested were sensitive to IVM and resistance to this drug class has not yet been reported in cyathostomins, it is not possible to assess accurately the usefulness of DrenchRite® LDA for

detecting IVM resistance at this time. Assay results for LEV suggest that LEV in a LDA does not yield data that is useful in estimating PYR efficacy in vivo. Based on results for PYR/LEV, the current high prevalence of benzimidazole resistance, no known cases of IVM resistance, and the sometimes extreme variation in results seen in many of the assays, DrenchRite® LDA cannot be considered a useful tool for the diagnosis of resistance in *cyathostomins* of horses at present.

1. Introduction

Small strongyles or cyathostomins are the most important intestinal parasites of horses worldwide (Lyons et al., 1999). Cyathostomins typically cause subclinical disease that is characterized by decreased level of performance, decreased rates of growth, weight loss, colic, rough hair coat, and debilitation (Uhlinger, 1991). These parasites may also cause a severe clinical disease known as larval cyathostominosis, which is characterized by weight loss and chronic diarrhea (Love et al., 1999). The primary lesion underlying the pathogenic effect of cyathostomins is an inflammatory enteropathy due to larval penetration into and larval emergence from the large intestinal mucosa (Love et al., 1999).

"Drug-resistance" is defined as the ability of worms in a population to survive drug treatments that are generally effective against the same species and stage of infection at the same dose rate (Sangster, 1999) and is caused by a change in the gene frequency of that population resulting from drug selection (Blackhall et al., 1998). In recent years, resistance to anthelmintics has emerged as a serious problem for the control of many species of important livestock nematodes, and is highly prevalent in nematodes of sheep, goats (Bjorn et al., 1991; Uhlinger et al., 1992; Waller, 1997; Van Wyk et al., 1999 and Mortensen et al., 2003) and horses (Kaplan, 2002). Cyathostomins have developed resistance to every major anthelmintic group except

avermectins/milbemycins (AM) complicating parasite control strategies (Kaplan, 2002). Although no resistance has been reported against AM in cyathostomins; sheep, goat and cattle parasites have already developed resistance to AM (Le Jambre, 1993; Vermunt et al., 1995; Coles et al., 1998; Gopal et al., 1999 and Sargison et al., 2001) and extensive use of these drugs in horses may lead to resistance in the near future (Sangster, 1999). It is essential to detect anthelmintic resistance early in the course of its development so that appropriate control strategies can be designed and implemented to prevent the further development and spread of resistant worms. It is also necessary for minimizing the impact of suboptimal parasite control on equine health and other costs associated with the use of ineffective drugs.

Several methods have been described and used to detect anthelmintic resistance with differing sensitivity and reliability in sheep: fecal egg count reduction test (FECRT) (Whitlock et al., 1980), egg hatch paralysis assay (Bjorn et al., 1991), egg hatch assay (EHA) (Dobson et al., 1986), larval development assay (LDA) (Taylor, 1990), larval paralysis assay and larval micromotility assay (Varady and Corba, 1999). In horses FECRT (Whitlock et al., 1980), EHA (Ihler and Bjorn, 1996, Craven et al., 1999), LDA (Ihler and Bjorn, 1996), and DrenchRite® LDA (Young et al., 1999 and Pook et al., 2002) have been used. Although the controlled test (Clark and Turton, 1973 and Drudge and Lyons, 1977) is considered the gold standard for testing anthelmintic efficacy it is rarely used and is only practical in research settings because the animals used in these studies need to be euthanazed. Lacking a better method, FECRT remains the practical gold standard for detecting resistance in the field (Kaplan, 2002), but misinterpretation of results is possible (Grimshaw et al., 1996). Also, FECRT is costly in terms of the amount of labor required to collect repetitive fecal samples and to perform fecal egg counts. EHA is only useful for benzimidazoles (BZ) and has an absolute requirement of

undeveloped eggs. Larval paralysis, migration and motility tests are only effective for drugs that affect muscular activity and these tests utilize complicated instruments. Use of molecular techniques to detect resistance is not a viable option at present because knowledge of the molecular mechanisms of anthelmintic resistance is limited and mainly confined to the BZ. Biochemical tests are not available for most of the drugs and generally are not feasible because they require a large amount of worm material.

Larval development tests may provide a cost-effective and reliable alternative to other anthelmintic resistance detection methods for several reasons: there is no requirement for repetitive sampling, simultaneous testing of several drugs is possible, there are minimal restrictions on the mechanism of action for the drug, it is not essential to have undeveloped eggs, and there is no requirement for sophisticated instruments.

The DrenchRite® LDA (Microbial Screening Technologies, Kemps Creek, NSW, Australia) was developed by Commonwealth Scientific Industrial Research Organization (CSIRO) in Australia. This assay uses a 96-well microtiter plate containing drug-impregnated agar to detect anthelmintic resistance to four different anthelmintic groupings: BZ, levamisole (LEV), a BZ/LEV combination and avermectins. This test was initially developed and validated for use with ovine parasites and has been effectively used in sheep and goats (Lacey et al., 1990 and Gill et al., 1995). Another study showed that results from DrenchRite® assay were consistent with FECRT in goats (Terrill et al., 2001). Detection of anthelmintic resistance in cyathostomin parasites of horses using LDA has been investigated but without conclusive results (Ihler and Bjorn, 1996; Craven et al., 1999; Young et al., 1999 and Pook et al., 2002). In this study, usefulness of LDA in detecting resistance in equine cyathostomin nematodes has been assessed by correlating the results of LDA with the results of a parallel in vivo study using FECRT and

also by testing the repeatability of the assay by retesting the same population of cyathostomins on separate occasions.

2. Materials and methods

2.1. Sample collection

Fecal samples from six horses not treated with anthelmintic for at least 8 weeks were collected on 15 horse farms in Georgia and South Carolina (USA). Each of these farms also participated in a study investigating the prevalence of anthelmintic resistance to fenbendazole (FBZ), oxibendazole (OBZ), pyrantel (PYR) pamoate, and ivermectin (IVM); however, not all farms were tested with all drugs. Resistance status on each farm was classified based on results of FECRT using the following criteria: resistant (R) if FECR was less than 80%, suspected resistant (SR) if FECR was between 80 and 90%, and sensitive (S) if FECR was greater than or equal to 90%. Fecal samples were also obtained from the Population S pony herd maintained at the University of Kentucky (Lyons et al., 1996). This group of ponies has been maintained as a closed herd for 29 years and has never been exposed to avermectins of any form, though cyathostomins infecting this herd are known to be resistant to both PYR and BZ (Lyons et al., 1996 and Lyons et al., 2001). In this study, cyathostomins from Population S ponies served as an IVM-sensitive control and also were used to determine the consistency of results across assays performed on the same nematode population.

2.2. DrenchRite® assay

Assays were performed according to manufacturer's directions (Horizon Technology, 1996) with minor modifications. In brief, during the process of egg isolation DrenchRite® plates were warmed to room temperature, examined for moisture content, and 10 or 20 μl of distilled water

(depending upon the extent of dryness) was added to wells as required. Feces from six horses with the highest FEC for a given farm were made into a slurry with water, filtered through #40 (425 μm) and #80 (180 μm) sieves and then again through 85 μm and 30 μm nylon filters. The retained fecal material containing the eggs was rinsed from the 30 μM filter with distilled water, and reduced to a volume of 10–15 ml. Sucrose gradients were prepared by diluting a saturated sucrose stock solution (4.06 M, specific GRAVITY = 1.275) (J.T. Baker, Phillipsburg, NJ, USA) by 1:4 (A), 1:6 (B) and 1:8 (C) and then layering 10 ml of C, 10 ml of B and 15 ml of A in a 50 ml centrifuge tube (Marquardt, 1961). The egg slurry was then added on top of the sugar gradients and the tube was centrifuged at 3500 rpm for 7 min at 4 °C using slow acceleration and deceleration speed. After centrifugation, the layer of eggs present between A and B layers of the gradient was retrieved using a Pasteur pipette. Eggs were rinsed with distilled water to remove all sucrose, and volume was adjusted to give a final concentration of 50 to 70 eggs per 20 μl. Ninety microliters of fungizone (amphotericin B, supplied with kit) was added per ml of egg suspension and 20 μl of egg suspension was added to each well. Assay plates were sealed with parafilm to prevent excess drying during incubation and were placed in a humidifying incubator at 25 °C. After 24 h control wells were examined to ensure that more than 80% of eggs were hatched and 20 μl of nutritive media (supplied with kit and diluted by 50% with distilled water) was added to each well. Plates were checked daily to insure that a thin layer of moisture covered the agar of each well and 10 μl distilled water was added to any wells that looked dry. The assay was ended on day 7 by adding 20 μl of 50% lugols iodine to all wells. Ten grams of feces from the initial slurry were also used for larval culture (Steffan et al., 1989) to determine the percentage of cyathostomin larvae present in the sample.

To improve the accuracy for measuring larval development, after addition of the Lugols iodine, contents of each well were transferred to a clean 96-flat well plate. Transferring of larvae was accomplished by adding 50 µl of water to all wells followed by thorough up and down rinsing using a multi-channel pipette to dislodge the larvae. Larvae were then transferred to the corresponding well on the clean plate and the original plate was observed under a dissecting microscope to ensure that all the larvae were successfully transferred. If more than three larvae were left in any well, 50 µl of additional water was added and the washing process was repeated to retrieve all possible larvae. All assay plates were stored at 4 °C until counting was completed. For counting, one drop (approx. 15 µl) of 10% sodium thiosulfate solution was added to each well to destain. Larvae in each well were then counted using an inverted head microscope at 200× to 400× and stages of development (L_1/L_2, L_3) of all the larvae in each well along with the presence of unhatched eggs were enumerated. The DrenchRite® plate contains two rows of wells each containing thiabendazole (TBZ), LEV, and mebendazole/LEV combination, and 1 row each of two different ivermectin analogs. Eleven concentrations of TBZ, IVM-1 and IVM-2 and eight concentrations of LEV are tested in the DrenchRite® assay: for TBZ (µM): 0.010, 0.020, 0.040, 0.078, 0.156, 0.313, 0.625, 1.250, 2.500, 5.000 and 10.000., for IVM-1 (nM): 0.500, 0.970, 1.900, 3.900, 7.800, 15.600, 31.250, 62.500, 125.000, 250.000 and 500.000, for IVM-2 (nM): 0.970, 1.900, 3.900, 7.800, 15.600, 31.250, 62.500, 125.000, 250.000, 500.000 and 1000.000 and for LEV (µM): 0.195, 0.390, 0.780, 1.560, 3.125, 6.250, 12.560 and four replicates of 25.000.

2.3. Data analysis

The data for individual anthelmintics were analyzed by logistic regression models to determine LC_{50} (Waller et al., 1985) (LOGIT program for TBZ and LEV; SAS/STAT® software Version 8, Cary, NC, USA for IVM-1 and IVM-2) and LC_{95} (SAS/STAT® software Version 8, Cary, NC,

USA for all drugs tested), which are defined as the anthelmintic concentration where development to L_3 stage is inhibited in 50% and 95% of the larvae, respectively. The LDA does not evaluate PYR, which was used as the anthelmintic in one group of horses in the parallel FECRT study. However LEV, which is used in the DrenchRite® plate, has a mode of action similar to that of PYR (Martin, 1997). Based on FECRT all the farms had resistance to FBZ, therefore resistance status to OBZ was used in the analysis for assignment of resistant status of farm for TBZ. A linear mixed model was fitted to determine the relationship between LC_{50}/LC_{95} and farm drug resistance status (R, SR, S) based on FECRT. F-test was used to determine the significance of this relationship between LC_{50}/LC_{95} and farm drug resistance status (SAS/STAT® software Version 8, Cary, NC, USA). Calculations of FECRT and LC_{50}/LC_{95} are based on the assumption that all of the strongyle eggs found in egg counts and used in assays were of cyathostomins, which is supported by previous studies (Herd et al., 1981; Drudge et al., 1988; Uhlinger, 1990; Borgsteede et al., 1993 and Lyons and Tolliver, 2003) and examination of larvae recovered in cultures (data not shown).

Delineating doses (DD) and percentage L_3 survival at these doses were calculated manually. For our study, delineating doses are defined as the highest drug concentration where greater than or equal to 5 or 1% of larvae developed to the L_3 stage. Percentage of L_3 surviving at a given DD was calculated as a percentage of the mean number of L_3 in control wells. Five %DD were used for TBZ and LEV and 1% DD were used for IVM. Five individual DrenchRite® assays were performed on Population S cyathostomins over a 2-year time period on three separate occasions. Results of these assays were tested for consistency using the PROBIT procedure (SAS/STAT® software Version 8, Cary, NC, USA), which calculates maximum likelihood estimates of regression parameters and the natural response rate for quantal response data. Ninety five percent

confidence intervals (CI) of the natural response rate estimate for each drug was calculated and consistency was determined by analyzing the overlap of the CI.

3. Results

3.1. Logistic analysis and interpretations based on LC_{50} and LC_{95}

Based on the criteria of effective development of L_3 in control wells, assays for all 15 farms, and for four of five Population S assays appeared to work well. However, on some farms, although assay results appeared reasonable for some drugs, other drugs yielded LC_{50} and LC_{95} values that were far greater than what would seem reasonable (Table 3.1 and Table 3.2). LC_{50} and LC_{95} values are shown for each farm in Table 3.1 and Table 3.2. All farms had BZ-resistant worms as determined by FECRT using FBZ. However, OBZ may retain efficacy against BZ-resistant worms (Drudge et al., 1979). This was observed in farm J (Table 3.1). LC_{50} of TBZ for this farm was 0.1355 μM. Mean LC_{50} for the OBZ R group (*n*=10) was 0.2015 μM (range 0.0415–0.3232 μM) and for OBZ SR group (*n*=3) was 0.1625 μM (range 0.0861–0.2221 μM). PYR was used in the FECRT study whereas LEV was used in the LDA. For this group, sensitive farms (*n*=5) had a mean LC_{50} of 1.4219 μM (range 0.8326–2.3110 μM), resistant farms (*n*=6) had a mean LC_{50} of 1.5900 μM (range 0.5164–4.4250 μM) and suspected resistant farms (*n*=3) had a mean LC_{50} of 1.8018 μM (range 0.6055–3.0910 μM). All the farms had worms sensitive to IVM; IVM-1 (*n*=15) had a mean LC_{50} of 7.5727 nM (range 0.1777–54.5985 nM) and IVM-2 (*n*=15) had a mean LC_{50} of 87.9719 nM (range 0.0286–834.6967 nM). Means of responses from the logistic analysis model from S, SR and R farms were plotted and compared between groups for OBZ and PYR and between IVM naïve and exposed populations for IVM-1 and IVM-2 (GraphPad Prism ver 3.00 for Windows, GraphPad Software, San Diego, CA, USA) (Fig. 3.1).

For OBZ, a small shift to the right was observed in the dose response curve of the resistant population as compared to the susceptible and suspected-resistant populations (Fig. 3.1 a). For PYR, there was a shift to the right for susceptible and suspected-resistant populations whereas this right shift would be expected for the resistant population (Fig. 3.1 b). IVM-1 and IVM-2 showed a distinct right side shift for IVM exposed populations compared to the IVM naïve population (Fig. 3.1 c and 3.1 d). A linear mixed model was fitted to the data to determine the relationship between LC_{50}/LC_{95} and resistance status. Results of the *F*-test for LC_{50} (Table 3.3) showed that interaction between drug and status is significant ($P<0.0001$), which indicate that the LC_{50} corresponding to a drug depends on resistance status. However, when individual drugs were examined the analysis yielded no meaningful information for the specific drugs tested. This is best illustrated with the results for LEV, which demonstrated a significant difference ($P<0.0001$) between R and S status although the model-adjusted mean LC_{50} for R and S farms were 0.7438, and 1.3269, respectively. This is opposite of what would be expected. Results for LC_{95} did not show a significant interaction between drug and status (Table 3.3).

3.2. Delineating doses (DD)

Although every farm demonstrated BZ resistance, DD for TBZ were not very high. No larvae were found surviving the highest concentration of TBZ (10.0 μM), the 5% DD was not more than 2.50 μM in any assay (Fig. 3.2 a) and 16 out of 20 assays (including BZ-resistant Population S) had a 5% DD less than or equal to 0.625 μM (Fig. 3.2 b). For LEV there was a tendency for resistant farms to have higher DD; 7 of the 11 assays from resistant populations had a DD of 12.5 or 25 μM whereas only one susceptible farm had a DD at this level (Fig. 3.3). Delineating doses (1%) for IVM-2 showed some noticeable differences between IVM-naïve and exposed populations (Fig. 3.4 c). With IVM-2, all of the Population S IVM-naïve assays had DD

less than or equal to 250 nM, whereas 9 out of 15 IVM-exposed farms had DD or 500 nM or higher. In contrast, DD for IVM-1 did not demonstrate any distinction between these populations and, in one assay performed on Population S 100% of larvae developed to the L_3 stage at the highest concentration tested in the plate (500 nM) (Fig. 3.4 b).

3.3. Analysis of consistency of assays

Data from five separate assays performed on Population S cyathostomins were analyzed for consistency. Larval development in the control wells of assay 2 was the poorest of the five assays, and results from this plate were inconsistent with the other assays for LEV and both IVM analogs. Additionally, this was the only assay that was not done in duplicate. These factors suggest that assay 2 was a "bad assay" and probably should not be considered in evaluation of between-assay consistency. For LEV, even if assay 2 is omitted, the CI of Assay 1 does not overlap the CI of the other three assays indicating poor repeatability between assays (Fig. 3.5 b). For IVM-2, if results from assay 2 are omitted, the remaining 4 assays all have overlapping CI indicating that results were consistent (Fig. 3.5 d). For IVM-1, LC_{50} and CI could not be calculated for assay 5 due to a high rate of development to the L_3 stage at all drug concentrations tested. This result suggests that consistency with IVM-1 is poor. For TBZ, CI of all five assays were found to overlap one another demonstrating consistency between all assays (Fig. 3.5 a).

4. Discussion

In this investigation we attempted to determine the effectiveness of the DrenchRite® LDA for detecting anthelmintic resistance in cyathostomin nematodes of horses. Data from a parallel study investigating the prevalence of resistance in cyathostomin nematodes using FECRT gave us the opportunity to categorize farms by drug susceptibility. However, results of the FECRT

indicated that all farms were BZ resistant and none were IVM resistant. Since no BZ susceptible or IVM resistant populations were available for comparison, our ability to fully interpret results was limited. However, we were able to collect data on Population S cyathostomins, which are completely naïve to IVM. This provided a sensitive control with which to compare the farm populations, which have been under many years of IVM selection. Additionally, because OBZ often retains efficacy in otherwise BZ resistant cyathostomin populations, we were able to use the FECRT data for OBZ to segregate farms into sensitive, suspected resistant, or resistant for this drug.

For TBZ, LC_{50} values obtained for the 15 farms in this study ranged from 0.0415 to 0.3232 μM with a mean of 0.1845 μM. These values are in similar range to the values reported previously (Table 3.4) and the mean LC_{50} is close to the post-treatment mean LD_{50} of 0.2024 μM reported for nine farms with resistance to OBZ (Pook et al., 2002). Since only one farm was susceptible to OBZ in the FECRT it was not possible to do a statistical comparison with R or SR farms, but the LC_{50} from this one S farm was less than 10 of 13 farms designated as either R or SR. Lack of sensitive controls for comparison made it impossible to calculate resistance ratios for TBZ. However, the relatively low LC_{50} and DD found in many OBZ-resistant populations and the high variability in results between farms suggest that it may be difficult to reliably segregate BZ-sensitive from BZ-resistant populations using this assay. Tests for between assay consistencies with TBZ demonstrated that the DrenchRite® LDA gave repeatable results for this drug. Overall, the assay data from TBZ suggests that the DrenchRite® LDA is probably a fairly good assay for detecting resistance in cyathostomins among a group of farms, but confidence in results for any particular farm would not be high. However, because all farms tested had BZ resistance it is difficult to reach any firm conclusion regarding the usefulness of LDA for BZ resistance

detection. For PYR/LEV, one must ask the question whether efficacy against PYR in vivo can be accurately measured using LEV in the LDA. Available evidence suggests that these two drugs share a very similar mechanism of action (Martin, 1997) and side resistance has been demonstrated in trichostrongylid nematodes of sheep between LEV and morantel, which is the methyl ester of PYR (Sangster et al., 1979). Based on these similarities, it should be possible to use the DrenchRite® assay plates as manufactured with LEV to determine efficacy for PYR. In our study, approximately equal numbers of farms that were sensitive or resistant to PYR were tested, which allowed us to perform statistical contrasts of results. Although contrasts for LEV between LC_{50} and resistance status yielded a significant relationship (P=0.0001), model-adjusted mean LC_{50} of sensitive farms were much higher than those of resistant farms. Similarly, mean LC_{50} of SR farms was higher than mean LC_{50} of resistant farms, and there was a shift to the right in the dose response curve for the sensitive population whereas a right shift would be expected for the resistant population. In addition, tests for between assay consistencies using Population S demonstrated non-overlapping 95% CI for LC_{50} in two of five assays, although one of these assays was questionable in quality. These data suggest that repeatability with LEV is moderate to poor. Overall, these data provide strong evidence that the LDA using LEV is not useful in detecting resistance to PYR when results are evaluated on the basis of LC_{50} or LC_{95}, and that these measurements do not give results consistent with PYR in the FECRT. However, it is interesting to note that the LC_{50} values for LEV in our study tended to be higher than those reported in other studies (Table 3.4) while at the same time the prevalence of resistance we found for PYR using the FECRT was higher than in any previous report.

Results from Population S demonstrated consistency for IVM-2 in the four assays that were viewed as giving representative results (Fig. 3.5). A fifth assay (assay 2) showed inconsistency

with the other four, but this assay is believed to be of poor quality based on a low rate of larval development in control wells. In contrast, assay results for IVM-1 were not consistent owing to the disparate results of assay 5. In the IVM-1 row of assay 5, the proportion of larvae developing to the L_3 stage was extremely high at all drug concentrations tested. All drugs in this particular assay plate other than IVM-1 yielded data that was consistent with the other Population S assays, suggesting that IVM-1 does not yield consistent results in the DrenchRite® LDA.

Dose response curves of mean values demonstrated a distinct shift to the right for the IVM exposed populations when compared to the IVM naïve population suggesting that the DrenchRite® assay may be useful for resistance detection (Fig 3.1). However, there was a very wide range of LC_{50} values among the 15 farms; LC_{50} of some farms were extremely high while the LC_{50} of other farms were substantially lower than those calculated for the IVM-naïve Population S. The three farms with the highest LC_{50} values for IVM-2 all had LC_{50} greater than 100 nM, and a resistance ratio (RR) of 82.5 was calculated by comparing the mean LC_{50} value of these three farms to the mean LC_{50} values for the four IVM-naïve Population S assays (of good quality). If results of these three farms are evaluated in a clinical context with a goal of establishing sensitivity or resistance to ivermectin, then one would almost certainly classify these three farms as resistant. Yet all three of these farms demonstrated 100% reduction in FEC following treatment with IVM. In the FECRT portion of this study, there were no farms designated as resistant to IVM, and the range in FECR for these 15 farms was 99.8–100%. Consequently, there is no way to know if differences seen in LC_{50} actually correlate to any real differences in IVM sensitivity among the different worm populations. Nevertheless, given the extraordinarily large range in LC_{50} in the face of uniform in vivo efficacy of nearly 100%, it is difficult to imagine how this assay can yield meaningful results for ivermectin sensitivity.

Though some of the data suggests that the DrenchRite® assay with additional optimization may have some usefulness in detecting resistance to IVM, our results fail to demonstrate any real value of this assay in detecting IVM resistance in cyathostomins at this time. However, before the LDA can be properly evaluated in this capacity, known IVM resistant populations will be needed for testing.

Delineating doses (Fisher et al., 2001, Kenworthy et al., 2003) may be a more reliable measure for the detection of resistance in helminths because small numbers of resistant individuals within a population may be identified much earlier in the evolution of resistance than is possible using LC_{50}. In this study, we used 5% DD for TBZ and LEV because we felt that given the high prevalence of resistance to BZ drugs and PYR, this value was appropriate. In contrast, we used a 1% DD for IVM because clinical resistance has not yet been recognized to occur. Delineating doses for IVM-2 showed some noticeable differences between IVM naïve and exposed populations (Fig. 3.4 c) that could be indicative of resistance. However, with IVM-1 DD did not indicate any distinction between these populations (Fig. 3.4 b). Using DD to evaluate the presence of resistance for LEV suggested that there was a high degree of resistance, because for most of the farms, a small number of larvae survived the highest concentration. This is an interesting finding because the prevalence of resistance to PYR determined by FECRT was also much higher than in previous reports and may suggest that DD but not LC_{50} is the most appropriate measure to use for this drug. Further evaluation of the assay will be needed to determine this. These results contrast with those for TBZ, where despite the fact that all farms in our study had BZ resistance as determined by FECRT using FBZ, and 10 of 14 farms had resistance to OBZ, the DD for TBZ tended to be fairly low and only 4 farms had DD greater than 1 μM. This differs from what is seen with BZ-resistant populations in sheep and goats where L_3

are routinely found in high numbers at the 10 μM concentration. More data will be needed before proper interpretation of DD results can be made. Although the DrenchRite® LDA continues to be a cost-effective, practical, and fairly accurate method for detecting anthelmintic resistance in gastrointestinal nematodes of sheep and goats, evidence that this method is useful for detecting resistance in cyathostomins is scarce. Correlations between in vitro and in vivo studies for anthelmintic resistance were found to be poor by Craven et al. (1999) and they were not able to use the outcome of one test to predict the result of another. Pook et al. (2002) suggested careful evaluation, further testing and refinement of assay for cyathostomins. Young et al. (1999) found that the DrenchRite® assay was unable to quantify efficacies for the horse parasites but the authors felt it showed promise in determining future anthelmintic resistance in horse parasite populations.

Differences in biology between sheep/goat gastrointestinal nematodes and the cyathostomins are likely responsible for differences in the results obtained using the LDA. In our experience cyathostomin larvae appear to be more sensitive to temperature, humidity, and nutritional conditions as compared to sheep/goat nematode larvae. Another important factor, which may impact LDA results is the makeup of cyathostomin populations, which are composed of a heterogeneous mixture of numerous species of worms. Different species may differ in their innate susceptibility to drugs in a LDA and may require slightly different conditions for optimal development. This feature of cyathostomins may significantly confound the ability to measure resistance using a LDA. Since there is no ready morphologic means to distinguish these species, it is possible that some of the disparate results may due to differences in the relative numbers of eggs of the various species in a particular sample. Whatever the causes for irregularities in results, based on this study it appears that the DrenchRite® LDA as currently sold and used is not

a useful or effective means for detecting IVM or PYR in resistance in horses. Although LDA may have some usefulness in detecting BZ resistance in cyathostomins, the wide variation of LC_{50} values seen in BZ-resistant populations within a relatively narrow range of drug concentrations suggest that it would be very difficult to place confidence in the results of a single assay performed for diagnostic purposes. This finding combined with the high prevalence of BZ resistance and the inadequacy of the assay in detecting resistance against the other major drug classes makes recommending use of the LDA in horses difficult to justify. Further studies are needed to improve our ability to properly interpret DD data to determine if LDA assay conditions for cyathostomins can be optimized to improve the quality of results.

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Table 3.1 $LC_{50,}$ LC_{95}, and resistance status for TBZ and LEV for 15 farms and Population S. LC_{50}/LC_{95}: anthelmintic concentration where L_3 development in 50%/95% of the larvae is blocked. TBZ: thiabendazole, LEV: levamisole, PS: Population S, ND: Resistance status of farm as not determined for these drugs, μM: micromoles.

Farm	Drug[‡,†]	Status[£]	LC_{50} (μM)	LC_{95} (μM)
A	TBZ	R	0.09153	1.07784
B	TBZ	SR	0.22210	0.90574
C	TBZ	R	0.04151	0.83337
D	TBZ	R	0.29060	0.92903
E	TBZ	R	0.16380	0.97873
F	TBZ	SR	0.17920	0.94861
G	TBZ	R	0.23840	0.76225
H	TBZ	R	0.23880	2.89095
I	TBZ	R	0.21460	0.51519
J	TBZ	S	0.13550	1.69449
K	TBZ	R	0.21510	0.93465
L	TBZ	R	0.19700	0.59738
M	TBZ	R	0.32320	1.51106
N	TBZ	SR	0.08609	0.27926
O	TBZ	ND	0.13020	0.55751
PS-1	TBZ	R	0.03417	ND
PS-2	TBZ	R	4.60900	ND
PS-3	TBZ	R	0.15700	ND
PS-4	TBZ	R	0.96360	ND
PS-5	TBZ	R	0.09759	ND
			Mean = 0.43140	
A	LEV	SR	0.94840	10.11799
B	LEV	SR	1.71000	9.84996
C	LEV	SR	0.60550	11.93139
D	LEV	R	0.51640	5.14880
E	LEV	R	2.14600	08.22443
F	LEV	R	0.88830	7.87219
G	LEV	S	1.53300	19.13101
H	LEV	R	0.61600	2.91137
I	LEV	S	2.31100	7.87812
J	LEV	SR	3.09100	8.56512
K	LEV	S	1.15200	7.46729
L	LEV	R	4.42500	91.04418
M	LEV	S	1.32300	7.39643
N	LEV	S	0.83260	4.48371
O	LEV	ND	1.38000	19.30631
PS-1	LEV	R	0.78260	ND
PS-2	LEV	R	0.00880	ND
PS-3	LEV	R	2.29000	ND
PS-4	LEV	R	4.10700	ND
PS-5	LEV	R	3.85500	ND
			Mean = 1.72608	

Table 3.2 LC_{50}, LC_{95} and resistance status for IVM-1 and IVM-2 for 15 farms and Population S. LC_{50}/LC_{95}: anthelmintic concentration where L_3 development in 50%/95% of the larvae is blocked. IVM-1 and IVM-2: two different analogs of ivermectin, PS: Population S, ND: Resistance status of farm was not determined for these drugs, nM: nanomoles. b: not possible to calculate.

Farm	Drug	Status[£]	LC_{50} (nM)	LC_{95} (nM)
A	IVM-1	S	10.11257	16.39106
B	IVM-1	S	6.54363	3.27417
C	IVM-1	S	54.59846	111.27339
D	IVM-1	S	1.46190	16.65143
E	IVM-1	S	0.53845	2.13706
F	IVM-1	S	0.93914	220.6777
G	IVM-1	S	11.40050	4.45556
H	IVM-1	S	0.17772	4.79577
I	IVM-1	S	1.69836	7.39203
J	IVM-1	S	10.71723	3.30432
K	IVM-1	S	0.38997	5.09860
L	IVM-1	S	2.07542	4.01103
M	IVM-1	S	6.39105	1.03601
N	IVM-1	S	5.08029	0.28830
O	IVM-1	S	1.46678	0.32403
PS-1	IVM-1	S	1.59625	ND
PS-2	IVM-1	S	0.05359	ND
PS-3	IVM-1	S	2.72495	ND
PS-4	IVM-1	S	1.20618	ND
PS-5	IVM-1	S	b	ND
			Mean = 6.27223	
A	IVM-2	S	0.02857	5.5827e-37
B	IVM-2	S	28.70873	23.95585
C	IVM-2	S	834.69665	126710.0
D	IVM-2	S	283.30493	13.79809
E	IVM-2	S	0.84474	0.32983
F	IVM-2	S	0.54707	5.29701
G	IVM-2	S	2.28840	11.81715
H	IVM-2	S	2.35005	1.64973
I	IVM-2	S	2.94492	2.72637
J	IVM-2	S	114.22242	4.49942
K	IVM-2	S	3.13292	4.16156
L	IVM-2	S	8.77800	7.22513
M	IVM-2	S	20.94217	2.84278
N	IVM-2	S	8.47130	0.87175
O	IVM-2	S	8..31701	3.33130
PS-1	IVM-2	S	2.79166	ND
PS-2	IVM-2	S	0.0056	ND
PS-3	IVM-2	S	9.61998	ND
PS-4	IVM-2	S	2.19228	ND
PS-5	IVM-2	S	5.25963	ND
			Mean = 66.9723	

Table 3.3 *F*-test results for farm, drug and status effects on LC_{50} and LC_{95}

	LC_{50}		LC_{95}	
Effect	F value	P value	F value	P value
Drug	235.51	<0.0001*	9.10	0.0002*
Status	11.82	0.0002*	1.05	0.3626
Drug with Status	29.14	<0.0001*	0.54	0.5885[†]

Table 3.4 Comparison of LC_{50} of 15 farms in current study with LC_{50} reported in other studies using LDA in horses. S: susceptible, SR: suspected resistant, R: resistant, ND: not determined, TBZ: thiabendazole, LEV: levamisole, IVM: ivermectin, μM: micromoles, nM: nanomoles.

Drug		LC_{50}		
	(15 farms) Current	Craven et al., 1999	Pook et al., 2002 (Pre-treatment)	Pook et al., 2002 (Post-treatment)‡
TBZ (μM)	Overall Mean = 0.1845 Range: S = 0.1355 (1 farm) SR = 0.0221 to 0.1792 R = 0.04151 to 0.3232	Overall Mean = 0.3490 Range: 0.09 to 1.01	Overall Mean = 0.1375 Range: 0.0623 to 0.19	Overall Mean = 0.2024 Range: 0.155 to 0.324
LEV (μM)	Overall Mean = 1.5652 Range: S = 0.8326 to 2.311 SR = 0.6055 to 3.091 R = 0.5164 to 4.425	Overall Mean = 0.6148 Range: 0.14 to 1.7	Overall Mean = 0.5412 Range: 0.11 to 1.0	Overall Mean = 0.5327 Range: 0.369 to 0.558)
IVM-1 (nM)	Overall Mean = 7.5727 Range: S =0.17772 to 54.59846 No R or SR farms	ND	Overall Mean = 1.8633 Range: 0.71 to 3.63	ND
IVM-2 (nM)	Overall Mean = 87.971833 Range: S = 0.02857 to 834.69665 No R or SR farms	ND	Overall Mean = 3.9467 Range: 1.90 to 7.07	ND

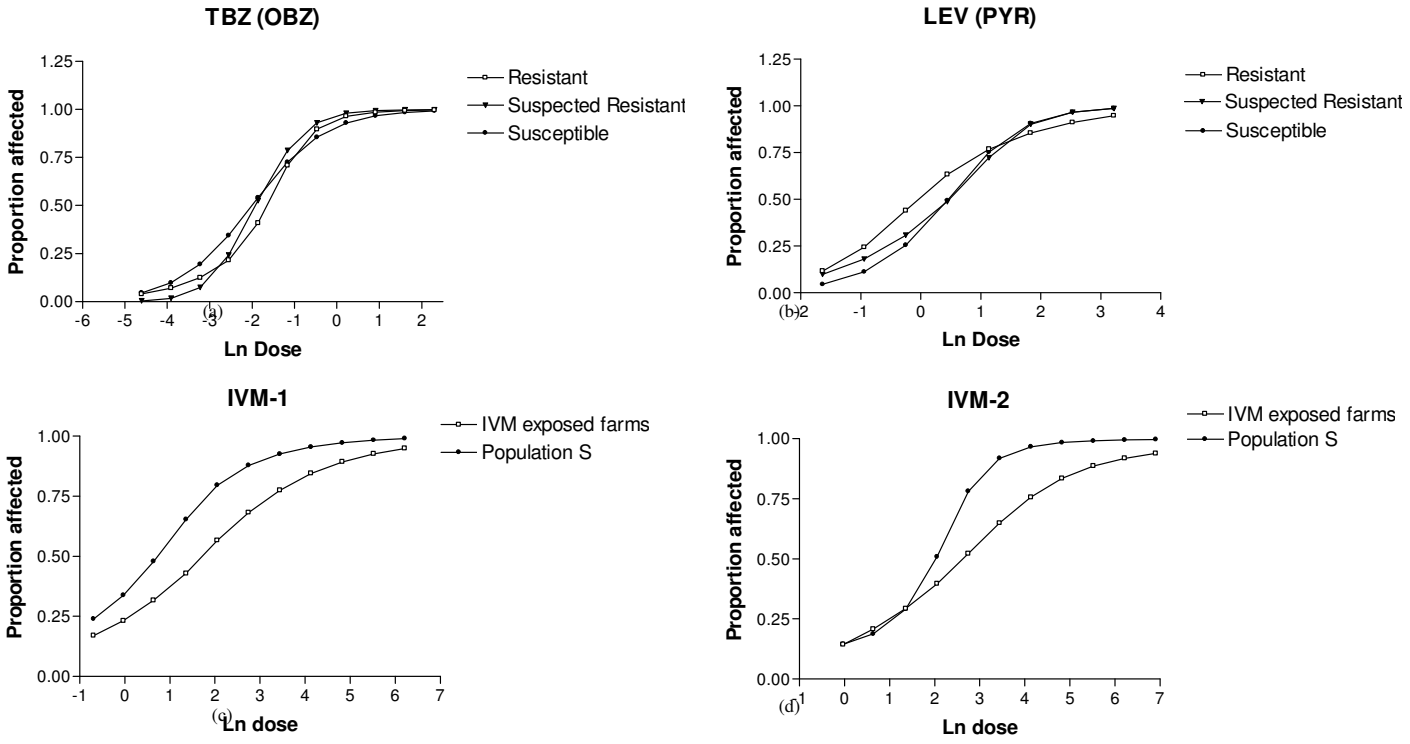

Fig. 3.1 Means of responses from the logistic analysis model for susceptible, suspected resistant and resistant farms were plotted and compared between groups for OBZ and PYR and between IVM naïve and exposed populations for IVM-1 and IVM-2.

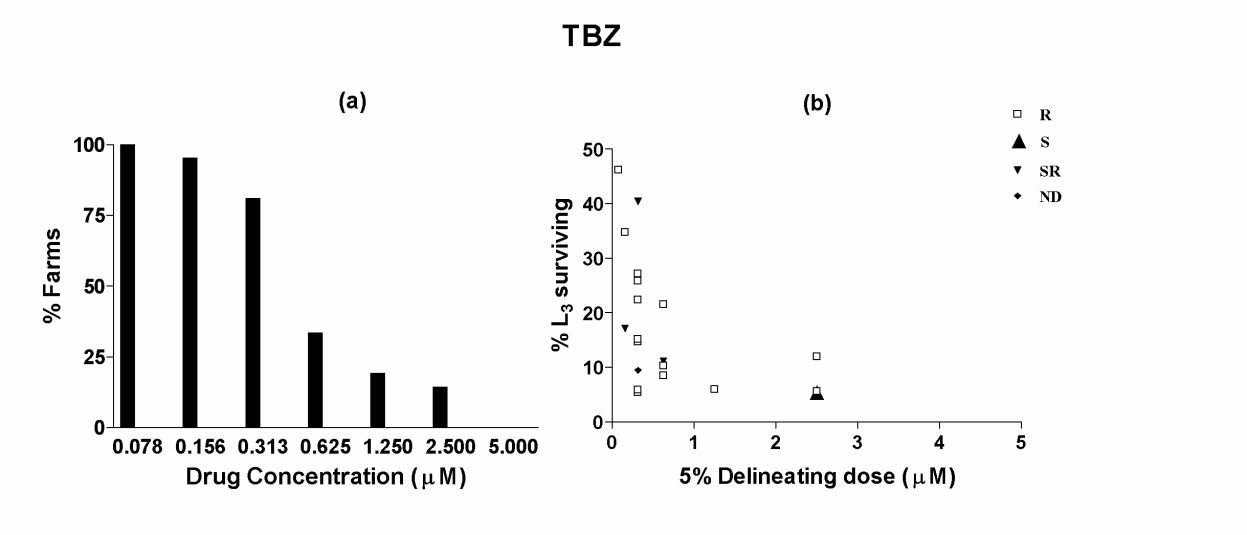

Fig. 3.2 Graphic representation of 5% delineating doses for thiabendazole (TBZ). (a) Percentage of farms having greater than or equal to 5% of L_3 surviving at the corresponding drug concentration. (b) Percentage of L_3 surviving at each delineating dose. Each symbol in the graph represents results of an individual DrenchRite® plate. R: resistant, S: susceptible, SR: suspected resistant, ND: resistance status not determined.

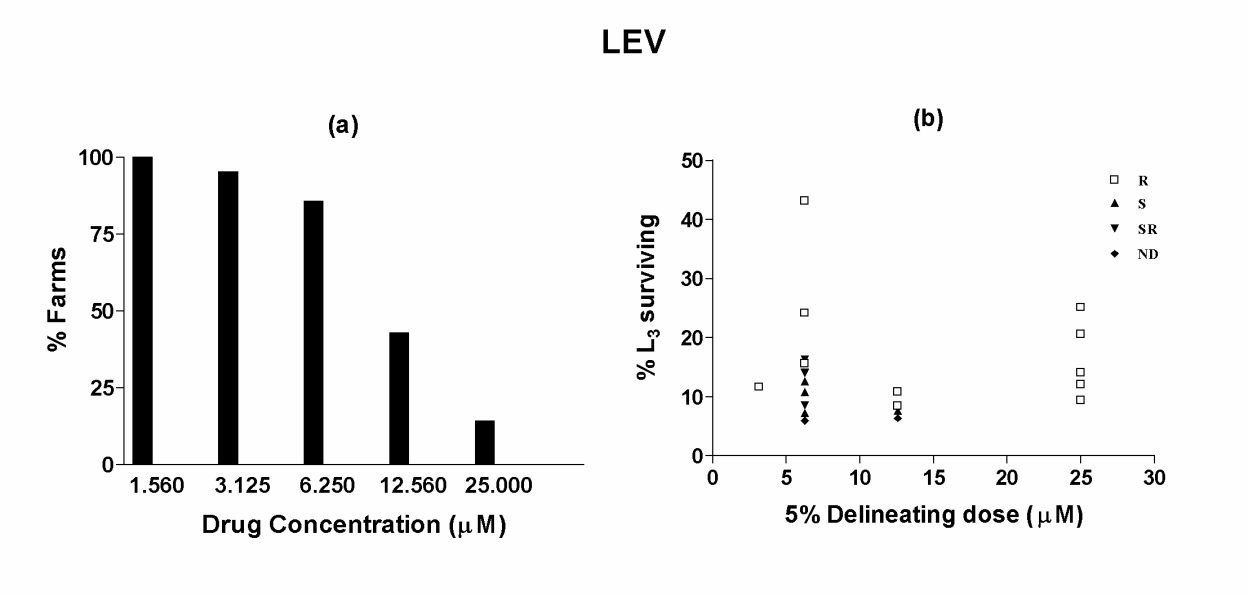

Fig. 3.3 Graphic representation of 5% delineating doses for levamisole (LEV). (a) Percentage of farms having greater than or equal to 5% of L_3 surviving at the corresponding drug concentration. (b) Percentage of L_3 surviving at each delineating dose. Each symbol in the graph represents results of an individual DrenchRite® plate. R: resistant, S: susceptible, SR: suspected resistant, ND: resistance status not determined.

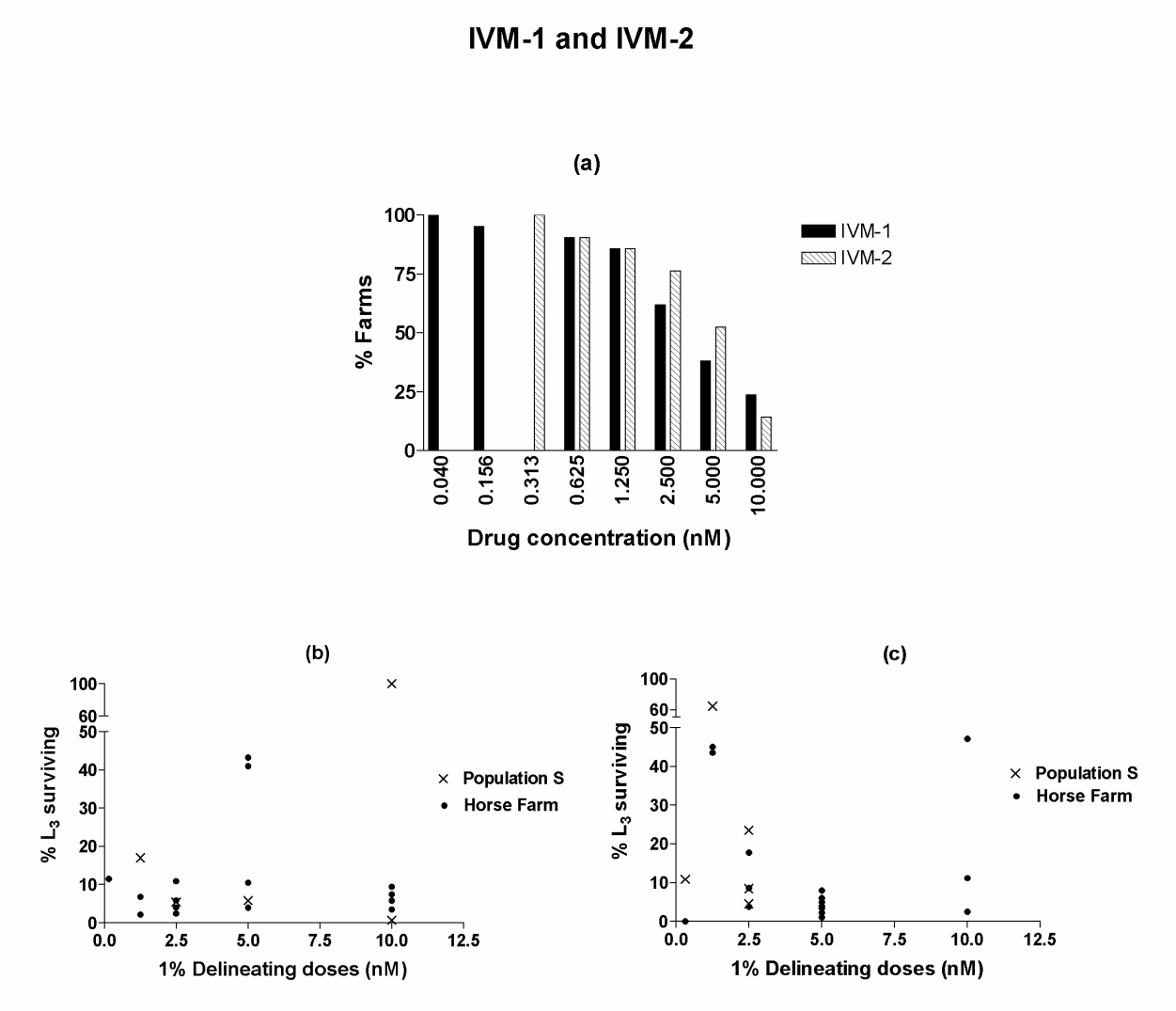

Fig.3.4 Graphic representation of 1% delineating doses for ivermectin (IVM). (a) Percentage of farms having greater than or equal to 1% of L_3 surviving at the corresponding drug concentration for either IVM-1 or IVM-2. (b) Percentage of L_3 surviving at each delineating dose for IVM-1. (c) Percentage of L_3 surviving at each delineating dose for IVM-2. Each symbol in the graph represents results of an individual DrenchRite® plate. R: resistant, S: susceptible, SR: suspected resistant, ND: resistance status not determined, Population S: Population S ponies (see text for description).

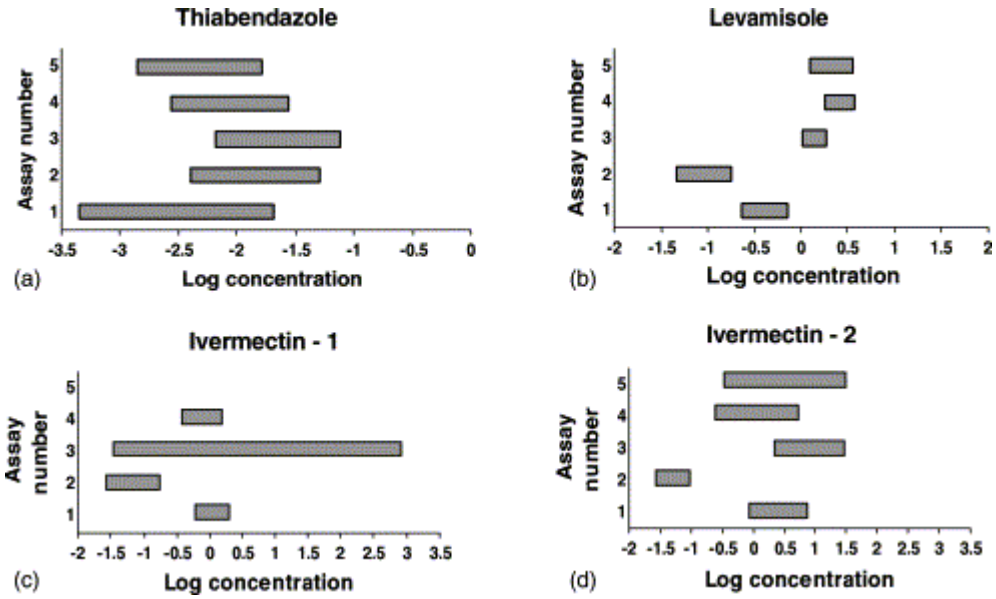

Fig. 3.5 Graphic representation of between-assay consistency showing overlap of 95% confidence intervals for the natural response rate estimate. Five DrenchRite® assays were performed on three separate occasions over a 2-year period using Population S cyathostomins. A confidence limit could not be calculated for IVM-1 assay 5.

CHAPTER 4

EFFECT OF MOXIDECTIN SELECTION ON THE GENETIC VARIATION WITHIN *CYLICOCYCLUS NASSATUS* BASED ON AMPLIFIED FRAGMENT LENGTH POLYMORPHISM (AFLP)*

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Abstract

Cyathostomins are among the most important intestinal nematodes of horses, yet, the literature on the molecular genetics of these worms is scarce. In this study, the technique of amplified fragment length polymorphism (AFLP) was applied to study the genetic diversity as well as to determine the effect of moxidectin selection on the population genetic diversity for *Cylicocyclus nassatus,* one of the most common cyathostomin species. Genomic DNAs from thirty individual male worms were used from each of two populations: an avermectin-milbemycin (AM) - naïve population (Population-S) and a population derived from Population S following 21 treatments with moxidectin (Population-Mox). Three selective primer pairs were used for each worm**,** yielding a total of 229 AFLP markers. Calculation of average pair wise Jaccard indices revealed a high degree of genetic variation within both populations using all three primer combinations. In addition, selection by moxidectin during a three year period caused a significant decrease in the level of genetic diversity as evidenced by analysis of AFLP markers for two primer combinations but not for the third. A dendrogram of relationships among individuals based on AFLP markers did not show a clear classification of individuals in separate groups. It was concluded that a high degree of genetic intrapopulation variation exists in *C. nassatus* and that moxidectin selection has a significant effect on the genetic composition of *C. nassatus*.

Keywords

Cyathostomin, *Cylicocyclus nassatus*, Genetic variation, Anthelmintic resistance,

AFLP, Moxidectin.

1. Introduction

Cyathostomins (small strongyles) are among the most important intestinal nematodes of horses (Lyons et al., 1999). Infection with cyathostomins produces an inflammatory enteropathy, which results in impaired intestinal microcirculation and motility (Love et al., 1999). Adult cyathostomins typically cause mild disease symptoms such as weight loss, poor hair coat**,** and intermittent diarrhea (Murphy and Love, 1997). However, heavily infected young horses may develop a severe life-threatening disease known as larval cyathostominosis characterized by severe diarrhoea, protein losing enteropathy**,** and severe weight loss (Mirck, 1977). Control of cyathostomins for the past 40 years has been based predominantly on the frequent use of anthelmintics (Love, 2003). About 50% of all horses in the United States are treated four or more times a year, and it is not uncommon for horses to be treated as often as every 6 to 8 weeks (Kaplan, 2002). This control paradigm is now being threatened by the development of resistance to these drugs. Cyathostomins are reported to be resistant to all classes of anthelmintics except the avermectin-milbemycin (AM) group (Kaplan, 2002) and the prevalence of resistance is beginning to reach alarming levels (Kaplan et al., 2004). Although no resistance to ivermectin or moxidectin in the cyathostomins has been reported to date, many years of frequent intensive treatments are likely to have placed heavy selection pressure on cyathostomin populations. It is probable that AM- resistance alleles are accumulating in these parasites; however, lack of knowledge regarding these alleles prevents measurement of such genetic changes in cyathostomin populations.

Amplified fragment length polymorphism (AFLP) is a DNA fingerprinting technique which allows the scanning of multiple loci across the whole genome for the presence of polymorphism/s produced by point mutations, insertions, deletions**,** and/or other genetic

rearrangements (Vos et al. 1995). Prior sequence knowledge is not necessary to conduct AFLP experiments; therefore, this method is particularly applicable to organisms for which no substantive DNA sequence data are available (Masiga and Turner, 2004). This technique has been used widely to investigate organisms ranging from very simple unicellular bacteria to complex multicellular organisms (Barker et al., 1999; Hayashi et al., 2004; Hill et al., 2004; John et al., 2004). AFLP has good reliability and reproducibility between laboratories, (Gorni et al., 2004) and has a wide range of polymorphism detection (Ajmone-Marsan et al., 1997). AFLP was first described by Vos et al. (1995) and since then has been used extensively to study plant genomes. Recently, AFLP has been used in genetic studies of several parasitic nematodes (Otsen et al., 2001; Hoglund et al., 2004; van der Veer and de Vries, 2003).

Here, the use of AFLP is reported as a tool to study the genetic diversity of *Cylicocyclus nassatus* as well as to determine the effects of moxidectin selection on this diversity.

2. Materials and Methods

2.1. Selection protocol and parasite material

Scientists at the University of Kentucky have maintained a closed herd of ponies (Population-S) for more than thirty years that has never been exposed to AM drugs (Lyons et al. 1996). In January, 2000, a portion of this herd was moved to a new cyathostomin-free pasture and a moxidectin-selection protocol was applied. Three days prior to the move, the ponies were treated with a sub-therapeutic dose of moxidectin (50 μg/kg) that was selected based on data (unpublished) demonstrating that this dose will reduce the mean fecal egg count by 95%. Between January, 2000 and May, 2003, a total of 21 moxidectin treatments were administered to this group of ponies (Population-Mox). The individual Population-Mox pony foal sacrificed for

this study was never treated with moxidectin, but was born on this pasture to a mare that was part of the original Population-Mox herd. Worms infecting this pony were acquired from the infested pasture grazed by the Population-Mox pony herd, and therefore, it is expected that the worms would have been exposed to differing numbers of the moxidectin treatments. Yearling pony foals from both the Population-S and Population-Mox herds were euthanized and worms were recovered from the dorsal colon and cecum. Immediately following necropsy, worms were washed twice in phosphate buffered saline (PBS) and identified to species (Lichtenfels et al., 1998; Tolliver, 2000) using a binocular dissecting microscope Individual male *C. nassatus* worms were placed in microfuge tubes containing 100% ethyl alcohol, transported on ice to University of Georgia**,** and frozen at –20˚C until used for genomic DNA extraction.

2.2. Genomic DNA extraction from adult worms:

Genomic DNA was extracted from individual male *C. nassatus* worms collected at necropsy. Briefly, ethyl alcohol was removed and 500 µl of DNA extraction buffer (50 mM Tris pH 8.0, 100 mM NaCl, 50 mM EDTA pH 8.0, and 1% SDS) was added to rinse the worms. Worms were then transferred into a clean tube containing 200 µl of DNA extraction buffer with 500 µg/ml of proteinase-k and were ground using a micro pestle. Two µl of 98% 2-mercaptoethanol (BME) was then added and the tubes were incubated at 65˚C for 2 h. Following this, phenol/chloroform/isoamyl alcohol extraction was performed, 2 µl of poly acryl carrier (Molecular Research Center, Inc., Cincinnati, OH) was added**,** and DNA was precipitated with 2.5 volumes of ice-cold 100% ethyl alcohol. DNA pellets were rinsed with 75% ethyl alcohol, allowed to air dry**,** and then were reconstituted in 20 µl of TE (10 mM Tris-HCl and 1mM EDTA). DNA concentration was determined by measuring the absorbance at 260 nm and the integrity of genomic DNA was confirmed by gel electrophoresis. RNA was present in the

extracted genomic DNA samples but it was assumed that the RNA would degrade during the heat activation step of Hotstar® Taq DNA polymerase (Qiagen Inc., Valencia, CA). Several samples were treated with RNAse A and used in AFLP reactions to confirm that the RNA did not alter observed AFLP banding patterns.

2.3. Restriction enzyme digestion and adaptor ligation

Approximately 200 ng of genomic DNA from each individual worm was digested with 1 unit of *Mse1* and 5 units of *EcoR1* restriction enzymes (New England Biolabs, Beverly, MA, USA). Fifty picomoles of *Mse1* adaptor (5´GACGATGAGTCCTGAG/ 3´TACTCAGGACTCATA) and five picomoles of *EcoR1* adaptor (5´CTCGTAGACTGCGTACC/3´CATCTGACGCATGGTTAA) were ligated in the same reaction as the digestion, using 1 weiss unit of T4 DNA ligase in a total reaction volume of 20 µl containing 90 mM Tris–HCl, 10 mM $MgCl_2$, 10 mM dithiothreotol (DTT), 1 mM adenosine triphosphate (ATP), 2.0 µg bovine serum albumin (BSA) and 50 mM NaCl. This restriction-ligation reaction was incubated for 3 h at 37º C. The reaction was then diluted (1:20) to a total volume of 200 µl with TE (10 mM Tris–HCl, 0.1 mM EDTA, pH 8.0) and either used immediately or frozen at –20˚C for future use.

2.4. Preselective amplification

All the polymerase chain reactions (PCR) were performed using HotStarTaq® polymerase (Qiagen Inc., Valencia, CA) on a PTC-200 thermocycler (MJ Research, Inc, Waltham, MA). Four µl of diluted ligation-digestion reaction was used as a template for amplification in a 25 µl reaction containing 20 pmoles of each preselective primers: *EcoR1*-A (GACTGCGTACCAATTCA, Tm = 51.9ºC) and either *Mse1*-C (GATGAGTCCTGAGTAAC, Tm = 45.9ºC), *Mse1*-A (GATGAGTCCTGAGTAAA, 45.0ºC) or *Mse1*-T

(GATGAGTCCTGAGTAAT, Tm = 44.7ºC). Cycle parameters were: 15 min at 95˚C, 30 sec at 94˚C, 30 sec at 50˚C, 1 min at 72˚ C for 30 cycles total. Agarose gel electrophoresis was used to verify the production of the preselective amplification products. The remaining amplification products were diluted 20 fold with deionized water.

2.5. Selective Amplification

Four µl of the diluted pre-amplification product was used as a template in a 25µl selective amplification reaction containing 20 pmoles of fluorescently (5´FAM) labeled *EcoR1* selective primer along with 20 pmoles of non-labeled *Mse1* selective primer. Cycle parameters were: 95ºC for 15 min, 94ºC for 30 sec, 65ºC for 30 sec (decreasing by 0.7ºC every cycle for 19 cycles), and 72ºC for 1 min with 21 additional cycles at an annealing temperature of 50ºC. Primer combinations were tested using three 5´ fluorescent-labeled *EcoR1* primers [*EcoR1*-ACT (GACTGCGTACCAATTCACT, Tm = 53.5), *EcoR1*-AGC and *EcoR1*-AGG] and 7 *Mse1* unlabeled primers (*Mse1*-CTA, *Mse1*-CAG, *Mse1*-CAC, *Mse1*-CTC, *Mse1*-C, *Mse1*-A, *Mse1*-T) on genomic DNA template from two worms. Of all the combinations tested, three combinations designated as A (*EcoR1*-ACT and *Mse1*-C), B (*EcoR1*-ACT and *Mse1*-A) and C (*EcoR1*-ACT and *Mse1*-T) were adjudged best for use in AFLP experiments based on the uniform distribution of bands between 50 and 500 bp and appreciable number of bands produced. These three primer combinations were used for all 60 genomic DNA samples to generate AFLP patterns.

2.6. Electrophoresis

AFLP products were separated on a 6% denaturing poly-acrylamide gel using an ABI PRISM® 377 DNA sequencer following manufacturer's protocols. Briefly, 1 µl of the product was mixed with 3 µl of loading buffer [deionized formamide containing ET-ROX 400 size

standard (Amersham Biosciences, Piscataway, NJ, USA)]. Samples in loading buffer were denatured at 95ºC for 3 min, 1.25 µl was loaded in each well**,** and the gels were run for 2.5 h.

2.7. Data Analysis

Analysis of the output was performed using the ABI GeneScan® Analysis Software (Applied Biosystems**,** Foster City, CA, USA) which automatically detects the bands and calls the molecular weight of the bands obtained based on the internal standard dye. Individual lanes were extracted as ABI trace files. These files were exported to Genographer Version 1.6.0 (available from program's website: http://hordeum.oscs.montana.edu/software/ genographer/) and a virtual gel was constructed by combining all lane files for a particular primer pair combination on a single virtual gel. These gels were scored in Genographer using a bin size of +/- 0.5 bases. Output was converted to the binary format using Microsoft Excel 2002. Jaccard indices (Jaccard, 1908) of differences were calculated in PHYLTOOLS version 1.32 (http://www.dpw.wau.nl/PV). An unpaired two-tailed student's t test was performed using an online tool (http://bardeen.physics.csbsju.edu/stats/t-test.html) to examine the significance of differences of means of Jaccard distances for the two populations under study. Unweighted Pair Group Method with Arithmatic Mean (UPGMA) trees were constructed in Winboot based on Jaccard indices (Yap and Nelson, 1996) with 500 bootstraps.

3. Results

Sixty genomic DNA extractions**,** consisting of 30 individuals from each of the two worm populations**,** were used for amplifications. Different banding patterns were evident within and between populations for each primer pair combination tested (Fig. 4.1). A total of 229 bands were scored in Genographer using three primer combinations. Majority of bands were

polymorphic in both the populations. There were 7 bands in Population-Mox and 14 bands in Population-S that were unique to the individuals. Also 2 bands in Population-S and 4 bands in Population-Mox were polymorphic in only the respective populations in 2 to 3 individuals. Overall the polymorphic bands ranged from 88.6 % to 98.38 % of the total bands scored for Population-Mox and 92.4 % to 100 % for Population-S (Table 4.1). These values provide an estimate of the number of bands that were polymorphic and not the degree of polymorphism in two populations. Means of Jaccard distances for Population-S worms were 0.80736, 0.8160, and 0.790896 for primer combinations A, B**,** and C, respectively; whereas, for Population-Mox**,** these values were 0.780909, 0.7750**,** and 0.78394, respectively (Fig. 4.2). Differences between means of Jaccard distances for the two populations were significant for primer combinations A and B ($p = 0.0130$ and 0.0018, respectively) but not for C ($p = 0.9000$). A dendrogram of relationships among individuals based on AFLP markers constructed using UPGMA approach (Fig. 4.3) did not show a clear classification of individuals in separate groups. Even when the individuals from population were found to group together, the bootstrap values were not very high, signifying a low level of confidence on these associations (Fig. 4.3).

4. Discussion

In the present study, AFLP is used to study the genetic diversity as well as the effects of moxidectin**-**selection on population genetic diversity for *C. nassatus*. The results obtained suggest that moxidectin selection is associated with reduced genetic polymorphism in these worms. The data also demonstrated that there is a substantial amount of inherent polymorphism present within a particular population of worms, which is in agreement with earlier studies on

Haemonchus contortus, *Cooperia oncophora* and *Dictyocaulus viviparus* (Otsen et al., 2001; Roos et al., 2004; van der Veer and de Vries, 2003).

Cyathostomins have a very different biology compared to protozoan parasites or the non-parasitic nematode *C. elegans* and this has a great impact on the way that drug selection affects population genetic diversity and the development of drug resistance. Horses are typically infected with many thousands of cyathostomins and it is not uncommon for a single horse to harbor more than 100,000 worms (Chapman et al, 2003). Cyathostomin eggs are passed in the feces and develop into infective third stage larvae within the feces. Infective larvae escape the feces onto pasture as the fecal piles break apart and disintegrate due to physical disruption, invasion by numerous invertebrates, and by rainfall. This causes widespread dispersal and mixing of infective larvae on a pasture so that it is assured that horses will become infected with eggs shed from other horses. Infective larvae are ingested by the host while grazing and then penetrate host large intestine where they encyst in the intestinal wall for a variable length of time. Larvae then emerge from the intestinal wall into the lumen, mature into female and male worms, mate, and the cycle is completed when the eggs produced in large numbers by female worms are shed into the feces. Completion of this lifecycle can occur in as few as 5 weeks, but larvae may remain encysted in the intestinal mucosa for many months or even years, where they remain unaffected (and therefore also unselected) by anthelmintic treatments. Repetition of this process during each worm generation ensures that there is an extremely efficient mixing of larvae on the pasture and between horses. No evidence exists that individual worms display specific antigens that affect establishment within particular hosts, thus all horses sharing a common pasture are expected to harbor worms with a similar overall genetic structure. Furthermore, genetic differences between individual nematodes within the same host or other

hosts on the same pasture remain high due to sexual reproduction being the only means of reproduction.

It would be preferable to study population genetic changes occurring in parasitic nematodes under drug selection by comparing a susceptible isolate with a derived resistant isolate of a single species both before and after drug selection. However, in the case of cyathostomins, no laboratory lines are maintained, monospecific infections have not been established yet and no AM-resistant populations exist. Therefore, in order to minimize background genetic differences, a well-characterized AM naïve population (Population-S) was used and compared with an isolate derived from Population-S (Population-Mox) that had undergone a selection protocol using moxidectin. A dose of moxidectin was chosen that caused a 95% reduction in fecal egg counts, because worm reductions at this level are expected to select for all alleles conferring any selective advantage (Prichard, 2001). In addition, use of a field selection protocol ensured that our population of worms would more closely resemble a natural population of drug-selected (resistant) worms than would a selection protocol utilizing worm-passaging between hosts.

It is important to note that using this type of selection protocol on a parasite with a life cycle such as a cyathostomin, will result in a fairly slow selection process because for any given treatment of drug (moxidectin), many worms in the population would not be exposed to the drug because they either were encysted in the intestinal wall at the time of treatment (against which moxidectin at the dose administered has negligible activity) or they existed as egg or larval stages on pasture. As a result, only a relatively small percentage of worms in the total population are exposed to the drug during each round of treatment (selection). The dynamics of this process have not been properly modeled, so it is difficult to predict what the average number of worm

exposures to moxidectin would be after 21 treatments (for a particular worm and its descendants). But it is likely that even after 21 treatments, many worms would still have had only a few exposures to the drug. Thus, the results of our analysis demonstrating a significantly reduced level of polymorphism in the moxidectin selected population is of great interest.

The means of Jaccard distances were significantly different in the two populations for two of the three primer combinations tested and Jaccard distances were less in the moxidectin-selected population compared with the AM-naïve population. These data suggest that the level of moxidectin selection applied to these worms was sufficient to reduce genetic polymorphism. Also, decreased number of loci were found to be polymorphic in the Population-Mox worms as compared to Population-S, but it was not possible to test these differences statistically due to the nature of the data. To further examine the genetic differences between these two populations, dendrograms were constructed using UPGMA approach (Fig. 4.3). Results were not indicative of grouping of individuals from one particular population but a tendency to aggregate with other members of the same population was observed. This can be attributed to the high amount of inherent polymorphism present in both the populations.

The assumption in AFLP analysis is that individual bands of the same migratory characteristics are the same in sequence. However, co-migration of the bands originating from different loci is not likely to affect these results because the similar co-migration should be observed in all the samples tested.

Given that AM- resistance has not yet been reported in cyathostomins even after many years of intensive use of this class of drugs, it is unknown what level of selection for AM resistance exists in natural populations. In this study, we report the first evidence of genetic alterations imposed by AM (moxidectin)-selection on a population of cyathostomins. However,

it is still unknown whether the genetic changes found in the moxidectin-selected worms are directly associated with particular loci that are important in the development of resistance. It is possible that the observed decrease in genetic diversity has little to do with resistance, but it is also possible that we are detecting some important early changes in the evolution of resistance.

Acknowledgements

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Table 4.1. A summary of the number of bands scored and the percentage polymorphism detected using three primer combinations.

Mse1-C/*EcoR1*-ACT (A)

Population	Total bands scored	Polymorphic Markers	Percentage of polymorphism
Population-Mox	62	61	98.38 %
Population-S	62	62	100 %

Mse1-A/*EcoR1*-ACT (B)

Population	Total bands scored	Polymorphic Markers	Percentage of polymorphism
Population-Mox	88	83	94.32 %
Population-S	88	84	95.45%

Mse1-T/*EcoR1*-ACT (C)

Population	Total bands scored	Polymorphic Bands	Percentage of polymorphism
Population-Mox	79	70	88.6%
Population-S	79	73	92.4 %

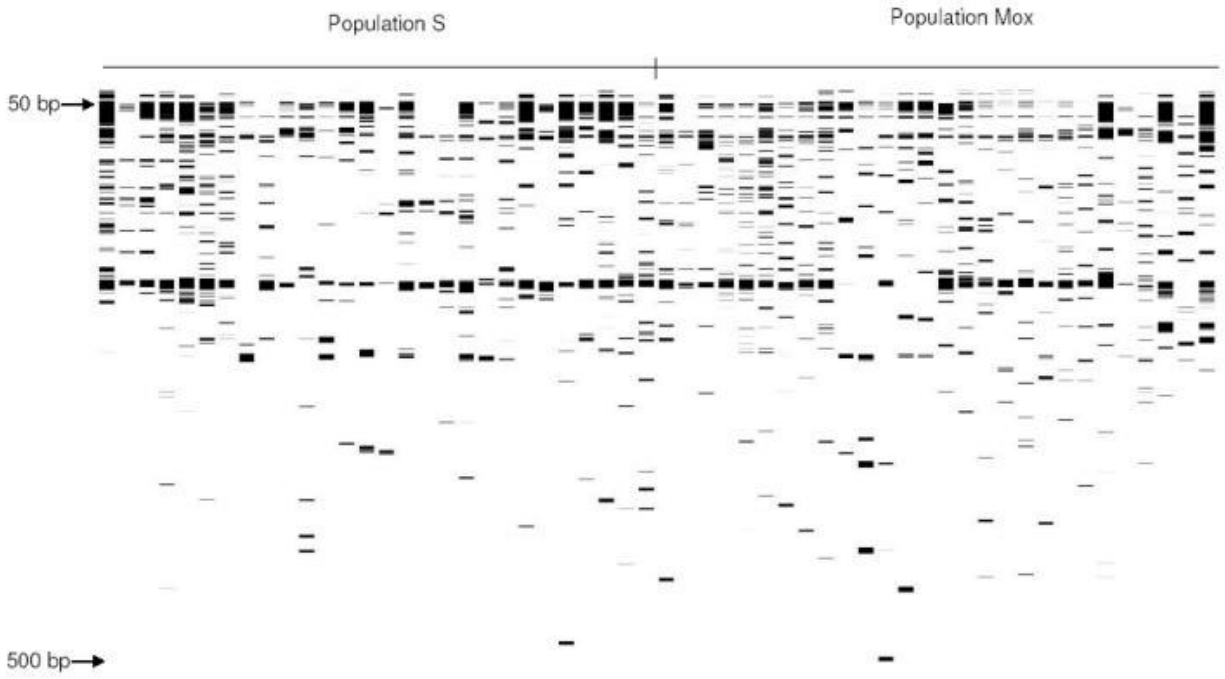


Fig. 4.1. Reconstructed image of an AFLP gel using Genographer® ver.1.6.0. Individual lanes represent individual worms. Fragments shown here were obtained using *EcoR1*-ACT/ *Mse1*-C selective primer combination.

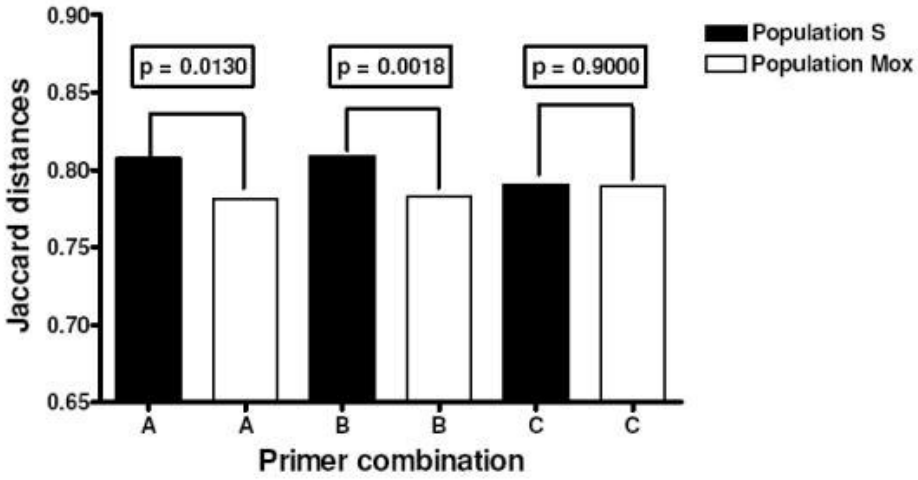
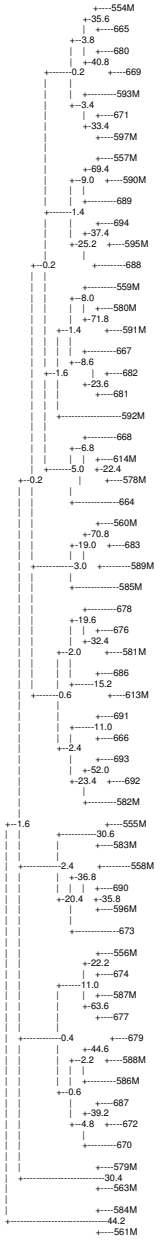
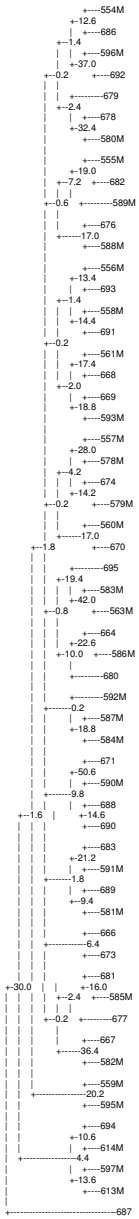


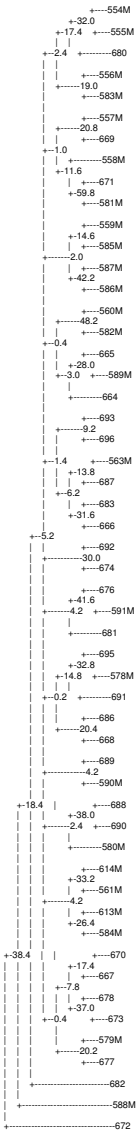
Fig. 4.2. Jaccard distances were calculated for all pair wise comparisons and averaged for a particular primer combination and population.



(A)



(B)



(C)

Fig. 4.3 Unrooted UPGMA trees generated using AFLP binary data. Trees were constructed in Winboot (Yap and Nelson, 1996). M = Individuals belonging to Population-Mox. Unlabeled individuals belong to Population-S. Primer combinations used were *Mse1*-C/*EcoR1*-ACT (A), *Mse1*-A/*EcoR1*-ACT (B) or *Mse1*-T/*EcoR1*-ACT (C). The numbers at the forks show the percentage of times the group consisting of the individuals that are to the right of that fork occurred (bootstrap values).

CHAPTER 5

CLONING AND CHARACTERIZATION OF GENES ENCODING α AND β SUBUNITS OF GLUTAMATE-GATED CHLORIDE CHANNEL (*GLUCL*) PROTEIN IN *CYLICOCYCLUS NASSATUS*

Tandon, R., LePage, K. and Kaplan, R.M. To be submitted to *Molecular and Biochemical Parasitology.*

Abstract

The invertebrate glutamate-gated chloride channels (*GluCl*) are putative receptor molecules and target for the avermectin-milbemycin group of drugs. *GluCl*s have been associated with ivermectin resistance in the soil dwelling nematode *Caenorhabditis elegans* as well as the parasitic nematode *Cooperia oncophora*. In this study, full-length cDNAs encoding α and β subunits of *GluCl* (*Cn-GluCl-α* and *Cn-GluCl-β*) were cloned and sequenced in *Cylicocyclus nassatus*, a common cyathostomin species infecting horses. The length of *Cn-GluCl-α* cDNA was 1593 base pairs encoding a 439 amino acid protein and the *Cn-GluCl-β* cDNA was 1424 base pairs encoding a 447 amino acid protein. Both of these genes possess the typical sequence characteristics of glutamate-gated chloride channels. Phylogenetic analysis confirmed that these genes are evolutionarily closely related to glutamate-gated chloride channels of other nematodes. Two different clades were evident in both NJ and MP trees: a clade containing *GluCl*-β subunit genes (*C. nassatus GluCl-β, H. contortus GluCl-β*, *C. oncophora GluCl-β and C. elegans glc-2* gene) and a bigger clade containing *GluCl-α* subunit genes from different species. Both trees grouped *C. nassatus GluCl-α* with the *glc-3* protein (NP_504441) of *C. elegans*. COS-7 cells expressing *Cn-GluCl-α* were found to bind to [^{3}H]ivermectin with a high amount of displaceable binding (IC_{50} = 208 pM) whereas *Cn-GluCl-β* expressing COS-7 cells showed little binding to [^{3}H]ivermectin. Compared to the mock transfected COS-7 cells, the means of [^{3}H]ivermectin binding were significantly different for *Cn-GluCl-α* and *HcGluCla* (p = 0.018 and 0.023, respectively) but not for *Cn-GluCl-β* (p = 0.370). This is the first report of invertebrate orthologs of *GluCl* genes and *in vitro* expression of an ivermectin-binding gene in a cyathostomin species.

Keywords: Glutamate-gated chloride channel, Avermectin-Milbemycin, *Cylicocyclus nassatus*, Cyathostomin, cDNA cloning, Phylogenetic analysis, Expression, COS-7

1. Introduction

Cyathostomins (small strongyles) are the most important intestinal nematode parasites of horses. A majority of horses over the world harbor these worms in their large intestines. Infections are usually sub-clinical but may result in weight loss, poor hair coat, and intermittent diarrhea (Murphy and Love, 1997). Cyathostomins are also responsible for a life-threatening syndrome known as larval cyathostominosis, which is more common in young horses (Love et al., 1999). The drugs used to control these parasites fall under three major groups: benzimidazoles, tetrahydropyrimidines and avermectin-milbemycins (AM). Cyathostomins are commonly resistant to the first two classes of anthelmintics (Kaplan, 2002; 2004) leaving the AM group of drugs as the only effective group of drugs on many farms. Avermectin-milbemycin resistance is reported in many trichostrongylid nematodes of ruminants (*H. contortus, C. oncophora, T. colubriformis, and T. circumcincta*) (Gopal et al., 1999; Wooster et al., 2001; Fiel et al., 2001; Sutherland et al. 2002; 2003, Chandrawathani et al., 2003). Although no reports of resistance to ivermectin or moxidectin in the cyathostomins have been published to date, intensive use of these drugs has placed a selection pressure on cyathostomin populations (Tandon et al., 2005). There is a likelihood of development of resistance to avermectin-milbemycins in cyathostomins at some future time (Kaplan, 2002). It is wise to develop tools to detect resistance at the molecular level before resistance is detected clinically so that appropriate management practices can be employed to monitor and prevent the development and spread of resistance. Probable target genes of AM drugs in cyathostomins must be cloned and characterized before relevant mutations can be identified to develop molecular diagnostic tools.

Glutamate-gated chloride channels (*GluCl*s) are the putative target sites for the action of the AM group of drugs in the free-living nematode *C. elegans* (Arena et al., 1995). *GluCl*s have

been found only in invertebrates (Cully et al., 1994), which explains why AM drugs are selectively toxic to nematode parasites and not to the host. *GluCl*s are expressed in certain muscle cells and neurons in the pharynx of the free-living nematode *Caenorhabditis elegans* (Dent et al., 1997; Laughton et. al., 1997).

The crystal structure of invertebrate *GluCl* has not been explored yet. It is important to understand the subunit composition of these ion channels in order to gain insights into mechanism of action of AM drugs as well as to focus on target genes with probable involvement in the development of resistance. Vassilatis et al, (1997) reported that *C. elegans GluCl*-α and *GluCl*-β genes belong to a discrete ligand-gated ion channel family orthologous to the vertebrate GABA- and glycine-gated channels. This close phylogenetic relationship suggests a pentamer composition for *GluCls* (Vassilatis et al., 1997). Six *GluCl* genes have been reported to date in *C. elegans* forming at least eight possible subunits due to alternative splicing reported in two of these genes (reviewed by Yates et al. 2003). In *H. contortus,* three genes encoding four *GluCl* subunits have been identified (Delany et al., 1998, Forrester et al., 1999, Jagannathan et al., 1999 and Cheeseman et al., 2001). The fact that one of these subunits (*HcGluCl*α) does not seem to be the ortholog of any of the *C. elegans* genes (Cheeseman et al., 2001) indicates that there are differences in the genetics of the *GluCl*s between these closely related nematode species.

In *C. elegans*, *GluCl*-α and *GluCl*-β genes were found to form functional homomeric channels in *Xenopus* oocytes, which were selectively responsive to ivermectin or glutamate, respectively (Cully et al., 1994). A *GluCl* subunit from *H. contortus* (*HcGluCla*) has been shown to bind [^{3}H]ivermectin with high affinity (Kd = 110 $\pm$ 0.021 pM) (Forrester et al., 2002). Also, an ivermectin-sensitive, glutamate-gated chloride channel subunit has recently been reported

from *Dirofilaria immitis* (Yates and Wolstenholme, 2004*)*. These studies confirm the presence of invermectin binding GluCl genes in free living as well as parasitic nematode species.

Studies on *GluCls* in cyathostomins are important because AM drugs are the only effective class of drugs on many horse farms and *GluCls* were shown to be associated with the development of AM resistance in several nematode species. (Dent et al., 2000; Njue and Prichard, 2004).

In this study, two full length cDNAs encoding α and β subunits of *GluCl* in *Cylicocyclus nassatus* were cloned and translated protein sequences were characterized. Individual genes were transiently expressed in COS-7 cells to characterize the binding affinity of *Cn-GluCl-α* and *Cn-GluCl-β* homomeric proteins to [^{3}H]ivermectin.

2. Materials and Methods

2.1 Parasite material

Worms used in this study were collected from the Population-S ponies at the University of Kentucky. Immediately following necropsy, worms were washed twice with phosphate buffered saline (PBS) and identified to species (Lichtenfels et al., 1998; Tolliver, 2000) using a binocular dissecting microscope. *C. nassatus* worms were collected and stored in Trizol® (Invitrogen, Carlsbad, CA, U.S.A.) at -20º C. Pooled *C. nassatus* worms were homogenized in Trizol® and total RNA was extracted following manufacturer's protocols. RNA was precipitated using isopropanol and the pellet was washed with 75% v/v ethyl alcohol, air-dried and dissolved in 50 µl RNAse free distilled water. Five µl of this RNA was run on an agarose gel to check the quality of extracted RNA and the rest was stored at –80˚ C until use.

2.2 Degenerate PCR

Cn-GluCl-α: *C. elegans* and *H. contortus GluCl-α* sequences (accession numbers S50864 and S50865, respectively) were retrieved from the NCBI protein database and aligned in Vector NTI ver. 9.0 using the ClustalW algorithm. Degenerate primers B1F and B3R (Table 5.1) were designed manually in the region of the protein that was conserved only in the *GluCl-α* subunit of *C. elegans* and *H. contortus*.

Cn-GluCl-β: *C. elegans* and *H. contortus GluCl-β* sequences (AAD13405 and CAA70929, respectively) were retrieved from NCBI protein database and degenerate primers DegB1 and DegC1 (Table 5.1) were designed in the region that was conserved only in *GluCl-β* subunit protein sequence of *C. elegans* and *H. contortus*.

All amplifications were performed in a PTC-200 thermocycler (MJ Research, Inc, Waltham, MA) in a 25 µl reaction. *C. nassatus* total RNA template was amplified using degenerate primers in a one-step RT-PCR reaction (Qiagen Inc., Valencia, CA) following the manufacturer's protocols and the following cycle parameters: 30 sec at 50º C, 15 min at 95º C, 45 sec at 94˚ C, 1min at 50˚ C, 1 min at 72˚ C for 34 cycles. Reactions were run on a 1% Seakem® LE agarose gel (Cambrex, Baltimore, MD) in 1X TAE buffer. Amplified fragments were excised from the gel and DNA was purified using Qiaquick Gel Extraction Kit (Qiagen, Valencia, CA, USA). Purified DNA fragments were cloned into the pDrive vector using Qiagen PCR Cloning Kit (Qiagen, Valencia, CA, USA). Clones that were positive in a blue-white screening were sequenced in an ABI PRISM® 3700 DNA Analyzer (Applied Biosystems, Foster City, CA) using T7 or SP6 universal primers. Sequences were searched for similarity on a NCBI blast server using the blastx algorithm.

2.3 cDNA library screening and Rapid Amplification of cDNA ends (RACE)

Cn-GluCl-α: A 140 base pair fragment of *Cn-GluCl-α* cDNA was obtained in a one-step RT-PCR (Qiagen Inc., Valencia, CA) using degenerate primer pair B1F-B3R and *C. nassatus* total RNA as a template (Table 5.1). The sequence information from this fragment was utilized in designing gene-specific primers (2F and 3R, Fig. 5.1), which were used for PCR screening of a *C. nassatus* cDNA library constructed using a ZAP-cDNA® Gigapack® III Gold Cloning Kit (Stratagene, La Jolla, CA). A 1258 bp fragment of *Cn-GluCl-*α cDNA sequence containing the 3' end of the gene was obtained using this approach. To obtain the full length cDNA for *Cn-GluCl-α* an adaptor ligated cDNA library was made (Marathon® cDNA Amplification kit, Clontech, Mountain View, CA) following the manufacturer's protocols. Primary amplification to obtain the 5' end was done using the adaptor specific primer AP1 and *Cn-GluCl-α* specific primer R1 using adaptor ligated cDNA library as template. A nested PCR using adaptor specific primer AP2 and *Cn-GluCl-α* specific primer R6 was performed using the amplicon obtained in primary reaction as template. Full length *Cn-GluCl-α* cDNA was amplified using primers AF1-AR1.

Cn-GluCl-β: Degenerate primers DegC1 and DegB1 (Table 5.1) were used in a one-step RT-PCR (Qiagen Inc., Valencia, CA) using *C. nassatus* total RNA as a template. A 350 bp cDNA fragment of *Cn-GluCl-β* was obtained, which was cloned and sequenced as described for *Cn-GluCl-α.* The sequence information from this fragment was utilized to design gene-specific primers 6R, 7R, 7F and 8F. The 5' end of *Cn-GluCl-β* was obtained using the AP1-7R primer pair for the primary and the AP2-6R primer pair for the secondary (nested) PCR using an adaptor ligated cDNA library of *C. nassatus* as template (Marathon® cDNA Amplification kit, Clontech, Mountain View, CA). Three prime end was obtained using the AP1-8F primer pair for the

primary and the AP2-7F primer pair for the secondary (nested) PCR. Full length *Cn-GluCl-β* cDNA was amplified using the BF1 and BR3 primer pair.

2.4 Sequence and Phylogenetic analysis

Full length *Cn-GluCl-α* and *Cn-GluCl-β* gene sequences were translated in Vector NTI ver. 9.0 (Invitrogen, Carlsbad, CA, USA). Secondary structure of the translated protein was predicted using the PredictProtein server (http://www.embl-heidelberg.de/predictprotein/predictprotein.html) using default parameters. This prediction is based on a homology based modeling (Rost et al., 1996). *GluCl* protein sequences from other organisms were retrieved from NCBI protein database. Expressed Sequence Tags (ESTs) showing strong alignment (E value < 0.01) with *GluCl* proteins of *C. elegans*, *H. contortus* and *C. nassatus* were retrieved from www.nematode.net and were translated in Vector NTI. The phylogenetic and molecular evolutionary analyses were done in PAUP version 4.0 (Sinauer associates, MA, USA). Total length of the alignment of *GluCl* proteins from different species was 752 amino acids. Three blocks of alignment (1 through 240, 277 through 286, and 581 through 752) were ignored in phylogentic analysis based on the quality of alignment. Ten thousand bootstraps were used for both the Maximum parasimony and the Neigbor-Joining trees. Thousand random addition replicates were used in Maximum Parsimony analysis. Mean character distances were used to construct the Neighbor-Joining tree. Both type of trees were rooted using *GluCl* proteins from flies (AAG40735, AAC31949 and BAD16657) as the outgroup.

2.5 Cell Culture

Ivermectin was purchased from Sigma-Aldrich corporation (St. Louis, MO, USA) and [22,23(n)-^{3}H]-Ivermectin (49 Ci/mmol) was a gift from Pfizer Inc. (manufactured by Amersham Biosciences UK limited). The complete coding sequence of *Cn-GluCl-α* (AY727925) and *Cn-*

GluCl-β (AY727924) were subcloned into a pTL1 mammalian expression vector (Leid et al., 1992). The constructs were sequenced to confirm correct ligation and orientation. An HcGluCla clone previously shown to bind [^{3}H]ivermectin was used as a positive control (Forrester et al., 2002). Cell culture and transfections were done following the protocols described earlier (Forrester et al., 2002) with slight modifications. The pTL1-*GluCl*-α, pTL1-*GluCl*-β and *HcGluCla* (a gift from Dr. Adrian Wolstenholme, University of Bath, UK) were transiently transfected individually into COS-7 cells in 10 cm tissue culture dishes using GeneFECTOR (Venn Nova Inc., FL, USA) lipid transfection reagent, according to the recommendations of the manufacturer. Briefly, 16µg of construct was mixed with 64 µg of lipid and added to dishes that had been seeded with approximately 10^6 cells 24 hr earlier. These cells were grown in Dulbecco's Modified Eagle's Medium (DMEM) without fetal bovine serum (FBS) or Penicillin/Streptomycin (PS). The medium was supplemented with 10% FBS 24 hr post-transfection, and the cells were harvested 48 hr later. Total RNA was extracted from COS-7 cells using Trizol reagent (Invitrogen Corporation, Carlsbad, CA, USA.) following manufacturer's protocols and was used as a template in RT-PCR reactions to detect the presence of transcripts. Gene-specific primers for *Cn-GluCl* α, *Cn-GluCl* β and *HcGluCla* (Forrester et al., 2002) were used for RT-PCR. Mock transfected COS-7 cells served as negative controls for these reactions. Membranes were prepared by homogenization of COS-7 cells using a dounce homogenizer and the protein concentration was determined by Bradford assay. Membranes (5 µg per reaction) were incubated with a range of [^{3}H]ivermectin concentrations (10^{-7} to 10^{-12} moles) in 250 µL of 50 mM HEPES buffer (pH 7.4). All competitive binding experiments were done in the presence of 10µM glutamic acid. Reactions were terminated by passage through Whatman GF-B glass fiber filters previously soaked in 0.1% polyethyleneimine. Filters were washed three times with

5 mL of ice-cold H_2O. Non-specific binding in one point competition assays was measured in the presence of a 1000-fold molar excess of unlabelled ivermectin. Further competition assays were done using 100-fold molar excess of unlabelled ivermectin. Specific binding was measured as a difference between total and non-specific binding. Sample radioactivity was assessed using a Beckman Coulter liquid scintillation counter. Binding data were analyzed using a non-linear regression analysis and the data was fitted to a one-site competition binding model (Prism 4.0, Graph Pad Software, Inc, San Diego, CA USA.)

3. Results

3.1 Cloning of Cn-GluCl- α and Cn-GluCl-β genes and sequence analysis

Full length cDNAs for two genes *Cn-GluCl-α* and *Cn-GluCl-β* (deposited under accession numbers: AY727925 and AY727924, respectively) were cloned and sequenced using a *C. nassatus* cDNA as a template in PCR and RACE. The length of *Cn-GluCl-α* cDNA was 1593 base pairs encoding a 439 amino acid protein and the *Cn-GluCl-β* cDNA was 1424 base pairs encoding a 447 amino acid protein. The full length of the sequences at the 5' end was confirmed by the presence of a Kozak (Kozak 1981) sequence in both genes as well as the presence of the nematode splice leader sequence (SL1) in the case of *Cn-GluCl-α*. Poly A tails were found in both cDNA sequences at the 3' end. Similarity searches of the NCBI blast server for both genes returned hits with a high degree of similarity to other nematode as well as arthropod *GluCl* genes. Alignment of *Cn-GluCl-α* and *Cn-GluCl-β* with *H. contortus* and *C. elegans GluCl* proteins revealed a high degree of overall similarity (Table 5.2) The secondary structures predicted for both the *C. nassatus GluCl* proteins (Fig. 5.2) were similar to the structure predicted for *C. elegans GluCl*s (Cully et al., 1994). Both the *Cn-GluCl-α* and *Cn-GluCl-β*

protein sequences were found to possess characteristic features of the ligand-gated ion channel (LGIC) superfamily, including the signature pair of cysteine residues within the N-terminal region and four proposed transmembrane regions (TM 1–4) in the C-terminal region. A second pair of N-terminal cysteine residues diagnostic for the *GluCl*s and glycine receptors (Yates and Wolstenholme, 2004) was also found conserved in these subunits (Fig. 5.3).

3.2 Phylogenetic analysis

Phylogenetic analysis confirmed that *Cn-GluCl-α and Cn-GluCl-β* form an evolutionarily close relationship with other nematode *GluCls* (Fig. 5.4 and Fig. 5.5). Maximum Parsimony analysis generated 39 phylogenetic trees. The shortest length for a tree was 926. These real trees differed from the 50% majority rule consensus tree only at the cluster containing Di CAE46429, Di CAE46430, Mh BM952376, Ov AAB03404 and Sr BI741994. Maximum Parsimony and Neighbor Joining trees were different at the cluster containing Ce AAC25481, Hc CAA74622, Ce AAK68666, Di CAE46430, Ov AAB03404, Mh BM952376, Di CAE46429, Sr BI741994, Hc AAD13405 and Mh BQ83757. Two different clades were evident in both NJ and MP trees: a clade containing *GluCl*-β subunit genes [*Cn-GluCl-β, H. contortus GluCl-β* (CAA70929), *C. oncophora GluCl*-beta (AAR21856) *and C. elegans* glc-2 gene (AAB42252)] and a bigger clade containing *GluCl-α* subunit genes from different species. Both trees grouped *Cn-GluCl-α* with the *glc-3* protein (NP_504441) of *C. elegans* (Horoszok et al., 2001). Protein sequences of *GluCls* from flies (AAG40735, AAC31949 and BAD16657) were used as an out-group for rooting these trees.

3.3 Radioligand binding assays

A high amount of ivermectin binding was detected (Fig. 5.6) for COS-7 cells expressing the homomeric *Cn-GluCl-α* subunit in one-point competition binding assays (0.0701

picomoles/mg protein), which was comparable to the binding observed for COS-7 cells expressing *HcGluCla* (0.0521 picomoles/mg). A low amount of binding was observed for COS-7 cells expressing the homomeric *Cn-GluCl-β* subunit (0.0208 picomoles/mg) as well as for mock transfected COS-7 cells (0.0253 picomoles/mg). Compared to the mock transfected COS-7 cells, the means of [^{3}H]ivermectin binding were significantly different for *Cn-GluCl-α* and *HcGluCla* ($p = 0.018$ and 0.023, respectively) but not for *Cn-GluCl-β* ($p = 0.370$).

The binding to mock transfected and *Cn-GluCl-β* expressing COS-7 cells can be explained by the fact that high amount of unlabelled ivermectin (1000 fold molar excess than [^{3}H]ivermectin) was used in the one-point competition assays. Binding to *Cn-GluCl-β* can also be attributed to a low affinity binding site for ivermectin on this subunit. This noise was later reduced in the competition assays by using only 100-fold molar excess of unlabelled ivermectin than [^{3}H]ivermectin in competition assays for *Cn-GluCl-α* (Fig. 5.7). The application of 10 μM concentration of L-glutamate has been shown to enhance [^{3}H]moxidectin binding to *in vitro* expressed *H. contortus* GluCl subunit (*HcGluCla*) by 69±15% (Forrester et al., 2004). In competition assays for *Cn-GluCl-α,* 10 μM concentration of L-glutamate was added in binding reactions to increase the efficiency of ivermectin binding to expressed *Cn-GluCl-α* and thereby reduced nonspecific binding. An IC_{50} of 208 pM was established for *GluCl-α* in competition experiments by fitting the data to a one site competition model (Fig. 5.7).

Discussion

We have cloned, sequenced and characterized the genes encoding α and β subunits of the *GluCl* channel in *Cylicocyclus nassatus*. The close relationship of *Cn-GluCl-α* and *Cn-GluCl-β* with other nematodes and fly *GluCl*s as well as conservation of signature sequences suggests

very similar biological activity for these genes. Also the predicted secondary structures of the protein coded by both *Cn-GluCl-α* and *Cn-GluCl-β* genes are very similar to the structure predicted for other *GluCl* genes (Fig. 5.2). Not surprisingly, in our phylogenetic analysis all the *GluCl*-α subunits including *Cn-GluCl-α* grouped in one clade and all the *GluCl*-β subunits including *Cn-GluCl-β* grouped in another clade. Moreover, bootstrap values for clustering of α and β type GluCls were strong and both the Neighbor Joining and Maximum Parsimony trees agreed upon relationships of *GluCl* subunits from *C. elegans, H. contortus, C. oncophora* and *C. nassatus*. Consensus tree for Maximum Parsimony differed and weak associations were found only for the partial sequences or translated ESTs used in phylogenetic analysis. This supports evolutionarily conserved relationships of *GluCl* genes from different species.

GluCls have been characterized from *C. elegans* as well as *H. contortus* for their binding affinities to ivermectin, moxidectin or glutamate on *in vitro* expression. *H. contortus* Hcgbr-2B and HcGluCl (both alpha subunit genes) when expressed in COS-7 cells showed a single site saturable binding to [^{3}H]ivermectin with a K_d of 70±16 pM and 26±12 pM, respectively (Cheeseman et al., 2001). Another gene from *H contortus* (HcGluCla) bound [^{3}H]ivermectin and [^{3}H]moxidectin with K_d values of 0.11±0.021 and 0.18±0.02 nM, respectively in similar experiments (Forrester et al., 2002). We found that *Cn-GluCl-α* binds with [^{3}H]ivermectin (0.0701 pmoles/mg) and this binding is displaceable by unlabelled ivermectin with an IC_{50} of 0.208 +/- 0.047 nM . These results are similar to the results reported for *H contortus* (*HcGluCla*), where IC_{50} values of 0.24+/-0.035 nM and 0.21+/-0.005 nM were reported for ivermectin and moxidectin, respectively (Forrester et al., 2002). One important difference in our experiments compared to these experiments is the addition of glutamate (10 μM) in competition assays. Glutamate is an allosteric modulator of [^{3}H]moxidectin and [^{3}H]ivermectin binding. It was found

to increase the affinity of [^{3}H]moxidectin for *HcGluCla* by more than 50% and that of [^{3}H]ivermectin by more than 7-fold (Forrester et al., 2002). Glutamate was used in our competition curves to enhance the specific binding signal because we detected a high amount of non-specific binding when experiments were done without the addition of glutamate. This addition may have resulted in slightly increased estimates of *GluCl* binding in competition assays.

In *Cooperia oncophora*, *GluCl* cDNAs, encoding *GluClα3* and *GluClβ* subunits were cloned from ivermectin-susceptible (IVS) and resistant (IVR) adult worms. GluClα3 subunits formed ivermectin and moxidectin sensitive glutamate-gated channels in case of both the IVS and IVR genes when expressed in *Xenopus* oocytes. It was reported that mutations in the IVR GluClα3 subunit cause a 2.5-fold reductions in sensitivity to ivermectin and moxidectin whereas mutations in the IVR GluClβ subunit abolish responsiveness to glutamate (Njue et al., 2004). These results suggest presence of binding site for AM drugs on GluCl-α subunit and also show the involvement of GluCl-α subunits in AM resistance development. Based on these results and also the studies done in *C. elegans, GluCl α type* subunits appear to play important role in the development of AM resistance in nematodes. The β type *GluCls* appear to be the binding site of glutamate which is the natural ligand for *GluCls* and probably these subunits have lesser or no involvement in AM drugs binding and development of resistance. The presence of high [^{3}H]ivermectin binding affinity for *Cn-GluCl-α* and low binding affinity for *Cn-GluCl-β* indicate the similar biological properties for *C. nassatus GluCls*.

In contrast to the studies looking at the binding properties of radioactive AM drugs to *GluCls*, Paiement et al., (1999) studied the glutamate binding properties of unselected and ivermectin-selected adult and larvae of *H. contortus* by preparing the crude membranes from

these stages. Resistant adults showed a 2 fold increase in B_{max} and the larvae a 2 fold decrease in Kd for glutamate binding suggesting that resistant parasites overcome the effects of ivermectin by enhancing glutamate binding at this binding site, either through an increase in binding affinity, in the case of the larvae, or by making more receptor sites available for glutamate binding in adults. Rohrer et al. prepared membranes from ivermectin-sensitive and -resistant *H. contortus* L_3 larvae and examined for the presence of [^{3}H]ivermectin binding sites. Both tissue preparations displayed high affinity drug binding sites (Kd = 0.13 nM). Receptor density was the same in both the ensitive and resistant nematodes suggesting that target site modification was not involved in the development of drug resistance in this particular strain of *H. contortus*. These studies suggest that mechanisms other than the decrease in binding affinity of *GluCls* for AM drugs may be involved in the development of AM resistance in nematodes. These type of studies have not been done yet for any cyathostomin species and would be interesting in seeking insights into the development of AM resistance.

GluCls have been implicated in the development of resistance against AM drugs in several nematode species (Paiement et al, 1999; Dent et al, 2000; Njue and Prichard, 2004, Yates and Wolstenholme, 2004) as well as in flies (Kane et al, 2000). Dent et al., (2000) demonstrated that simultaneous mutation of three genes, avr-14, avr-15, and glc-1, encoding glutamate-gated chloride channel (*GluCl*) α-type subunits in *C. elegans* conferred high-level resistance to ivermectin. Interestingly, mutating any two channel genes conferred modest or no resistance. Based on these results it was proposed that ivermectin sensitivity in *C. elegans* is mediated by genes affecting parallel genetic pathways defined by the family of *GluCl* genes. Njue and Prichard (2004) reported that mutations in the N-terminal extracellular region in *Cooperia*

oncophora correlate with ivermectin resistance. These studies indicated that *GluCl* genes in different species may be associated with the development of AM resistance.

Cylicocyclus nassatus is one of the most prevalent and abundant species of cyathostomin. The fact that no AM resistance has been reported in horse cyathostomins even after more than 20 years of use of AM drugs raises a question about the differences in biology of cyathostomins compared to other nematode species as well as the molecular similarity of target genes in these species. According to Yates (2003), differences between the *GluCl*s from different nematodes may explain species-specific variations in anthelmintic action. The presence of *GluCls* with evolutionary common sequence characteristics in *C. nassatus* as well as the presence of high affinity binding site in *C. nassatus GluCl*-α suggests the possibility of similar mechanism of action of AM drugs and suggests that resistance mechanisms may also be similar.

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Table 5.1. Nucleotide sequences and properties of the primers used:

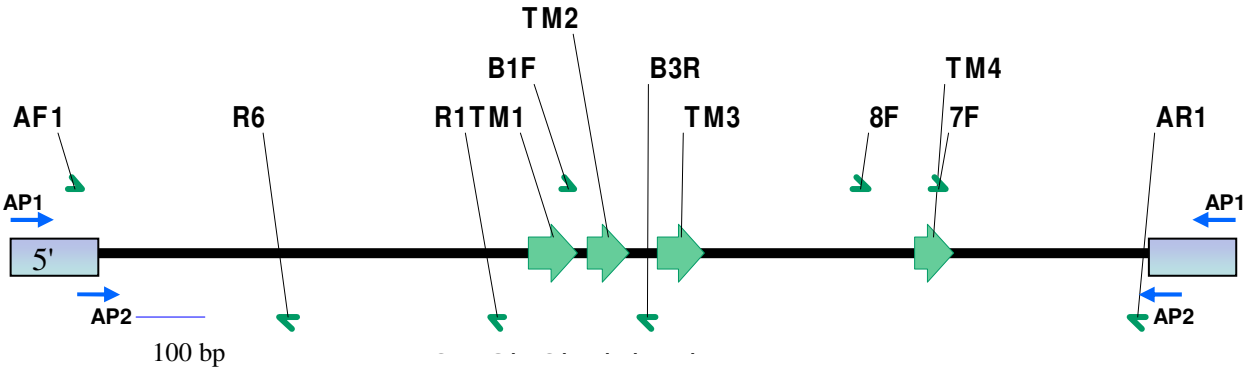
Name	**Sequence 5' to 3'**	**Tm (ºC)**	**Specificity**	**Property**
B1F	GTIWSITGGGTIWSITTYTGG	51.5	*Cn-GluCl-α*	Degenerate
B3R	GCYTTIRTRTAISWIACIGGIGG	52.0	*Cn-GluCl-α*	Degenerate
2F	GTGTCATGGGTGTCGTTCTCGG	55.0	*Cn-GluCl-α*	Gene- specific
3R	GGGGCAATTTCGCATTTATA	52.0	*Cn-GluCl-α*	Gene- specific
1F	TATCCCATCTTCTATGCTAGTC	45.0	*Cn-GluCl-α* and *Cn-GluCl-β*	Gene- specific
DegB1	GCWGGDAGRTARTCWGGY	52.9	*Cn-GluCl-β*	Degenerate
DegC1	GTYACYACYCTYCTYACYAT	51.7	*Cn-GluCl-β*	Degenerate
7F	GCGACACGAAAGGCTCAGAAACGTCGAG	66.4	*Cn-GluCl-β*	Gene- specific
8F	TGCGTTCGTTTCCTACAAGGACACATCG	64.4	*Cn-GluCl-β*	Gene- specific
7R	CGTTTGACAGGCTCCTAACCATACGTCC	64.2	*Cn-GluCl-β*	Gene- specific
6R	GGCAGCTTGCATTGTTGTCATGGTAAGG	64.2	*Cn-GluCl-β*	Gene- specific
AF1	ATGGCCGATGCGACATCAGAC	64.5	*Cn-GluCl-α*	Gene- specific
AR1	CAGCAATTCGATTCATTACACATA	57.73	*Cn-GluCl-α*	Gene- specific
BF1	TTGCTCACTTCTTCTGACATGG	56.4	*Cn-GluCl-β*	Gene-specific
BR3	CAGTATACAGCTGACATTCC	51.1	*Cn-GluCl-β*	Gene-specific
AP1	CCATCCTAATACGACTCACTATAGGGC	71.0	Adaptor	Adaptor-specific
AP2	ACTCACTATAGGGCTCGAGCGGC	77.0	Adaptor	Adaptor-specific

Table 5.2. Percentage similarity in amino acid composition of glutamate-gated chloride channel subunits from *C. elegans, H. contortus and C. nassatus*. *Cn-GluCl-α* and *Cn-GluCl-β* subunit proteins and their highest similarity values are highlighted.

	***C. nassatus* GluCl-α**	*C. elegans GluCl*-α S50864	*H. contortus GluCl*-α AAD13405	***C. nassatus* GluCl-β**	*C. elegans GluCl*-β S50865	*H. contortus GluCl*-β CAA70929
***C. nassatus* GluCl-α**	100	**54**	52	43	45	46
C. elegans GluCl-α S50864		100	49	41	43	44
H. contortus GluCl-α AAD13405			100	44	47	46
***C. nassatus* GluCl-β**				100	74	**82**
C. elegans GluCl-β S50865					100	78
H. contortus GluCl-β CAA70929						100

a)

Cn-GluCl-α (1593 bp)



b)

Cn-GluCl-β (1424 bp)

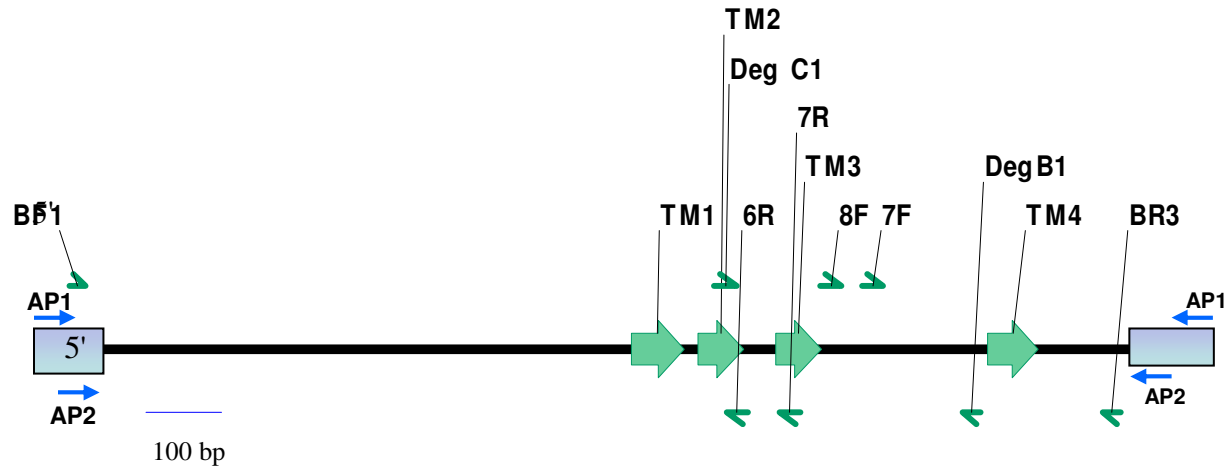


Fig. 5.1. Scheme for amplification of (a) *Cn-GluCl-α* and (b) *Cn-GluCl-β* subunit cDNAs from *C. nassatus.* Locations of transmembrane domains (TM1 through TM4) are indicated on both genes. Blue arrows: adaptor specific primers, Green arrows: Gene-specific or degenerate primers.

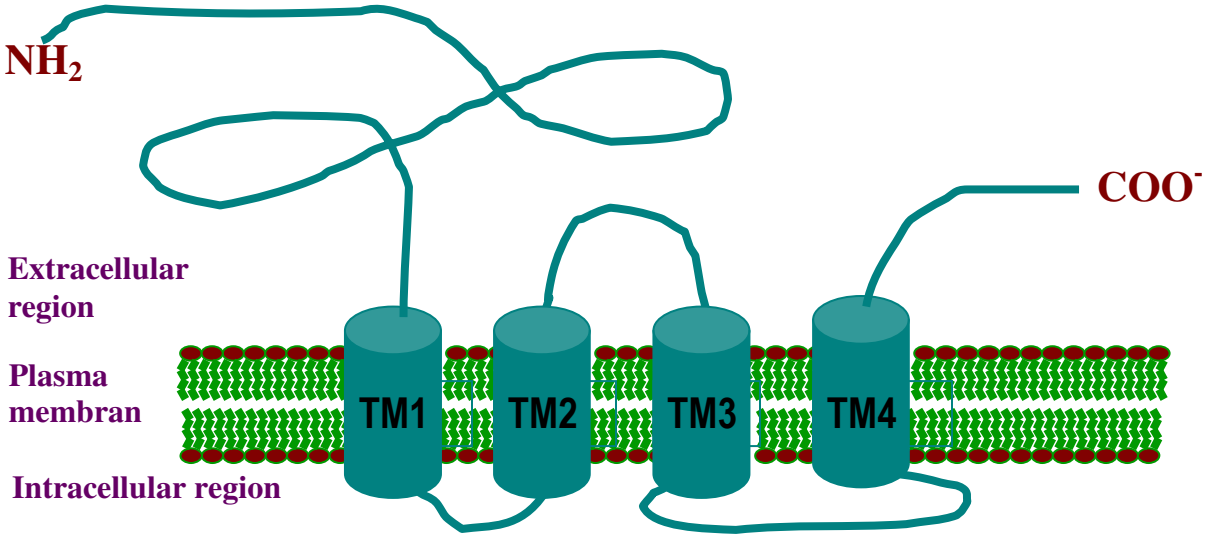
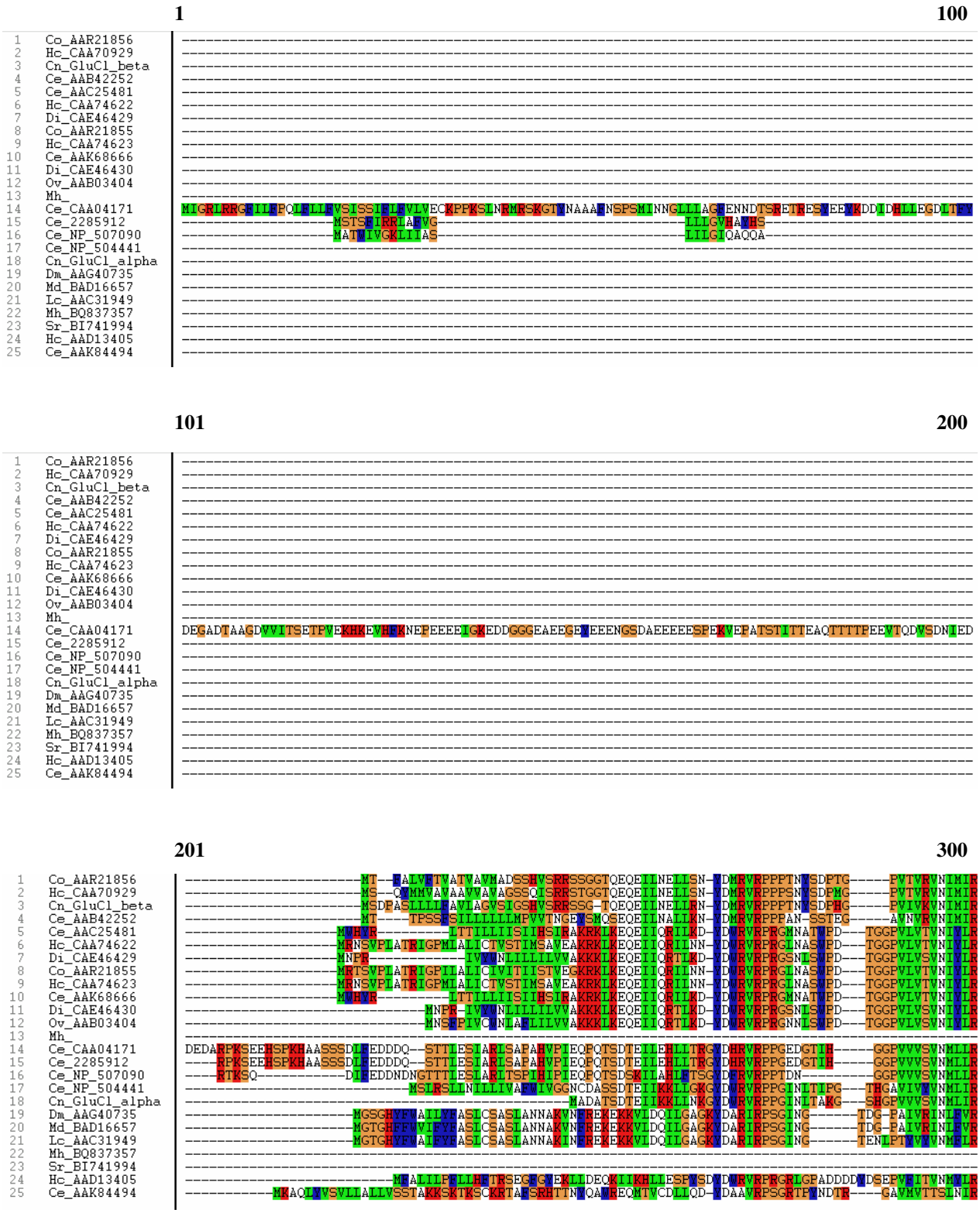


Fig. 5.2. A model of the structure of *C. nassatus GluCl* α and β proteins as predicted by PredictProtein software. TM: Transmembrane region.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Hc_CAA74623
Ce_AAK68666
Di_CAE46430
Ov_AAB03404
Mh_
Ce_CAA04171
Ce_2285912
Ce_NP_507090
Ce_NP_504441
Cn_GluCl_alpha
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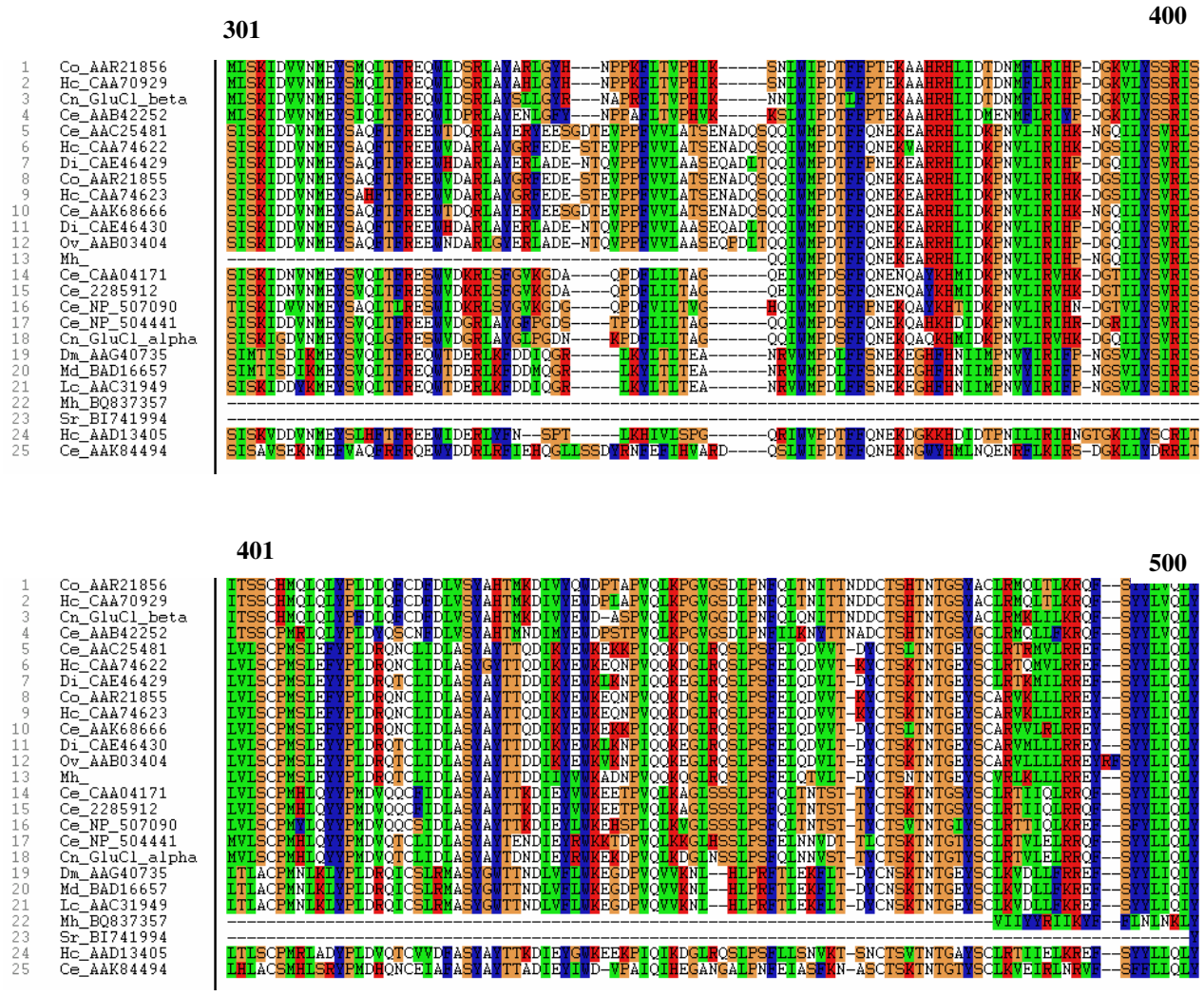
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Di_CAE46429
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Hc_CAA74623
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Mh_
Ce_CAA04171
Ce_2285912
Ce_NP_507090
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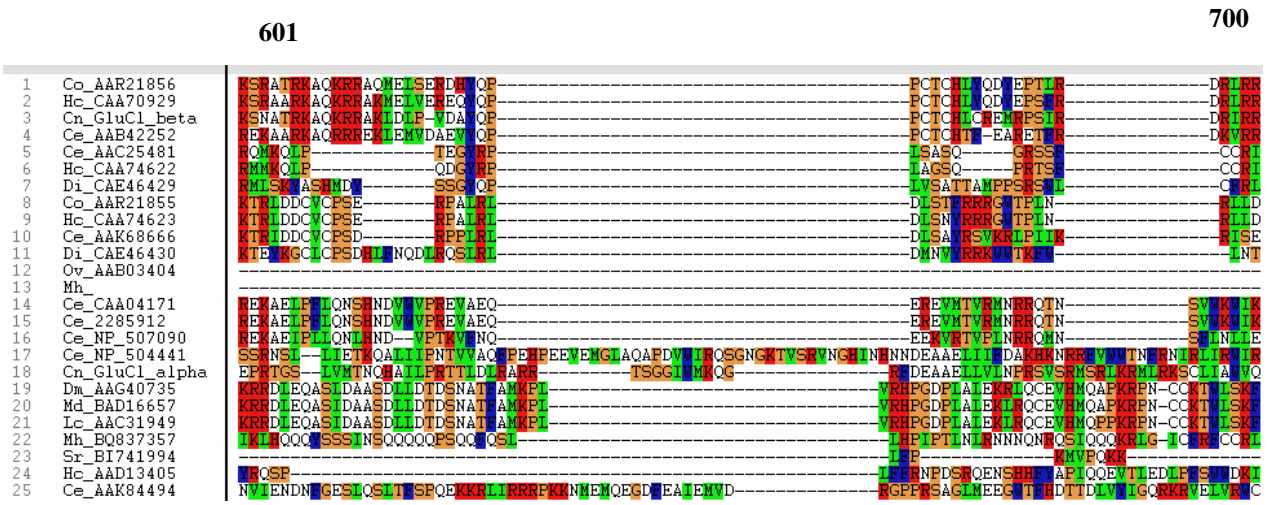
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Ce_AAK68666
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Ov_AAB03404
Mh_
Ce_CAA04171
Ce_2285912
Ce_NP_507090
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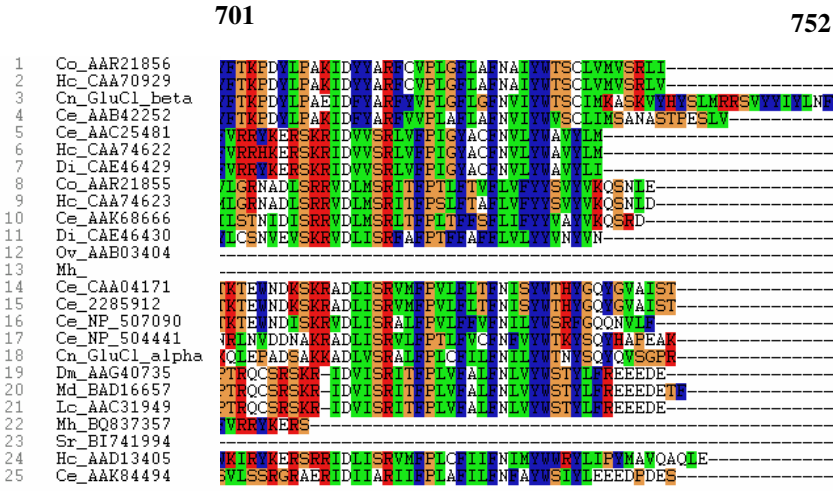
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Hc_CAA74623
Ce_AAK68666
Di_CAE46430
Ov_AAB03404
Mh_
Ce_CAA04171
Ce_2285912
Ce_NP_507090
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Cn_GluCl_alpha
Dm_AAG40735
Md_BAD16657
Lc_AAC31949
Mh_BQ837357
Sr_BI741994
Hc_AAD13405
Ce_AAK84494

Fig. 5.3. Alignment of *GluCl* genes from different nematode and fly species for the purpose of constructing a phylogenetic tree. Protein sequences were retrieved from NCBI protein database and were aligned in Vector NTI (Informax Inc.) Accession number of sequence is listed after its name. Abbreviations: Co: *Cooperia oncophora*, Hc: *Hemonchus contortus,* Cn: *Cylicocyclus nassatus,* Ce: *Caenorhabditis elegans,* Mh: *Meloidogyne hapla,* Di: *Dirofilaria immitis,* Ov: *Oncocerca volvulus, Sr: Strongyloides ratti,* Lc: *Lucilia cuprina,* Dm: *Drosophila melanogaster,* Md: *Musca domestica.* Accession numbers are listed after the taxon name. Accesssion number for Mh is BM952376.

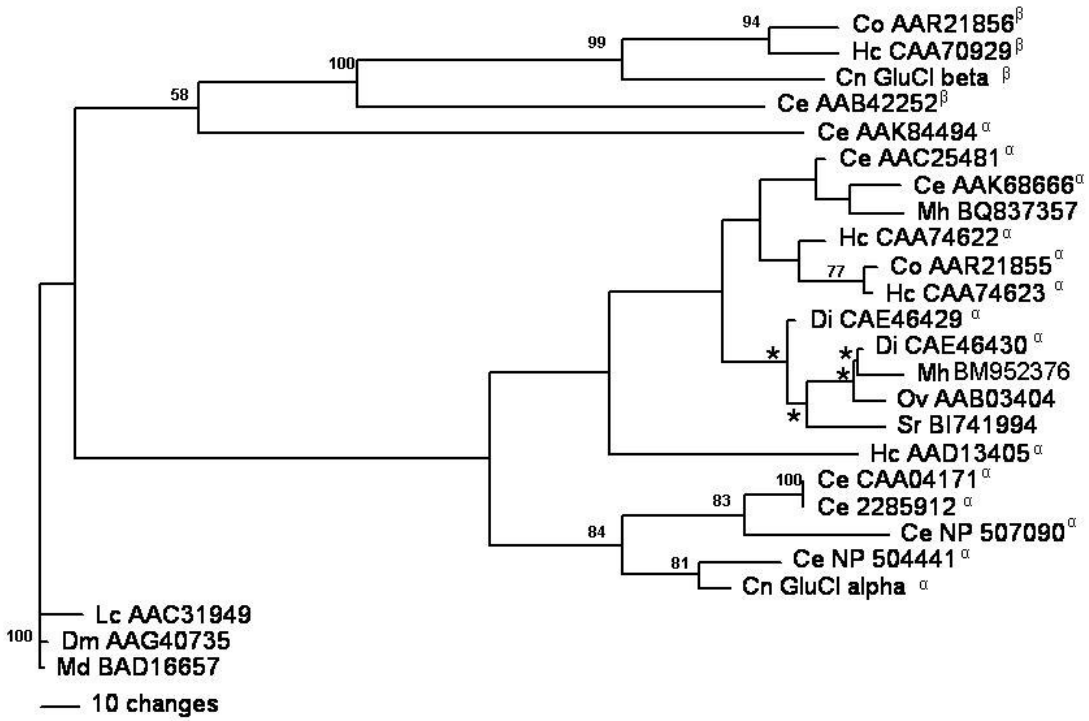

Fig. 5.4. Maximum parsimony tree illustrating the relationships of *Cn-GluCl-α* and *Cn-GluCl-β* translated proteins with *GluCl* proteins from other species. GluCl-β proteins are marked with β and GluCl-α proteins are marked with an α. Unmarked proteins are partial sequences available from GenBank. Branches that are different from 50% majority rule consensus tree are marked (*). Only bootstrap values that are greater than 50 are displayed. Abbreviations: Co: *Cooperia oncophora*, Hc: *Hemonchus contortus,* Cn: *Cylicocyclus nassatus,* Ce: *Caenorhabditis elegans,* Mh: *Meloidogyne hapla,* Di: *Dirofilaria immitis,* Ov: *Oncocerca volvulus, Sr: Strongyloides ratti,* Lc: *Lucilia cuprina,* Dm: *Drosophila melanogaster,* Md: *Musca domestica.* Accession numbers are listed after the taxon name.

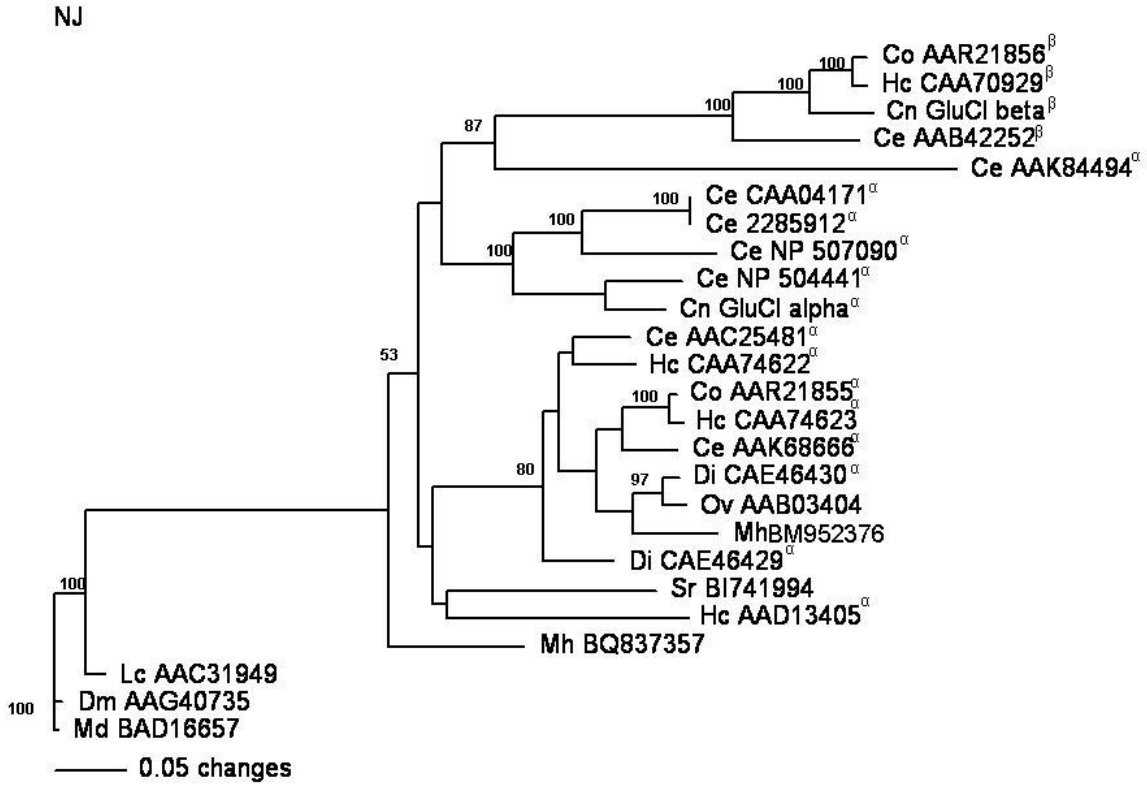

Fig. 5.5. Neighbor-joining tree illustrating the relationships of *Cn-GluCl-α* and *Cn-GluCl-*β translated proteins with *GluCl* proteins from other species. GluCl-β proteins are marked with β and GluCl-α proteins are marked with an α. Unmarked proteins are partial sequences available from GenBank. Only bootstrap values that are greater than 50 are displayed. Abbreviations: Co: *Cooperia oncophora*, Hc: *Hemonchus contortus,* Cn: *Cylicocyclus nassatus,* Ce: *Caenorhabditis elegans,* Mh: *Meloidogyne hapla,* Di: *Dirofilaria immitis,* Ov: *Oncocerca volvulus, Sr: Strongyloides ratti,* Lc: *Lucilia cuprina,* Dm: *Drosophila melanogaster,* Md: *Musca domestica.*. Accession numbers are listed after the taxon name.

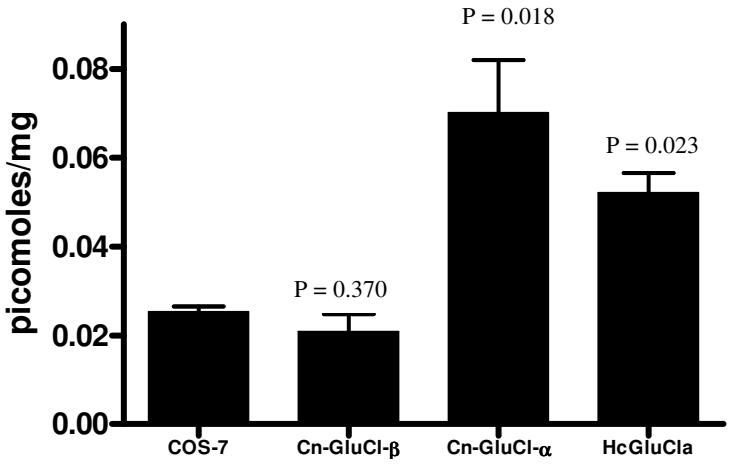

Fig. 5.6. One-point competition binding assay. Average specific binding to [^{3}H] ivermectin to the membranes prepared from COS-7 cells expressing *Cn-GluCl*-β, *Cn-GluCl-α* or *Hemonchus contortus GluCl*a (*HcGluCla*). Mock transfected COS-7 cells were used as negative control. P values from a paired t-test testing the significance of differences between mean mock transfected COS-7 cells [^{3}H]ivermectin binding and mean [^{3}H]ivermectin of *Cn-GluCl*-β, *Cn-GluCl-α* or *HcGluCla* are shown.

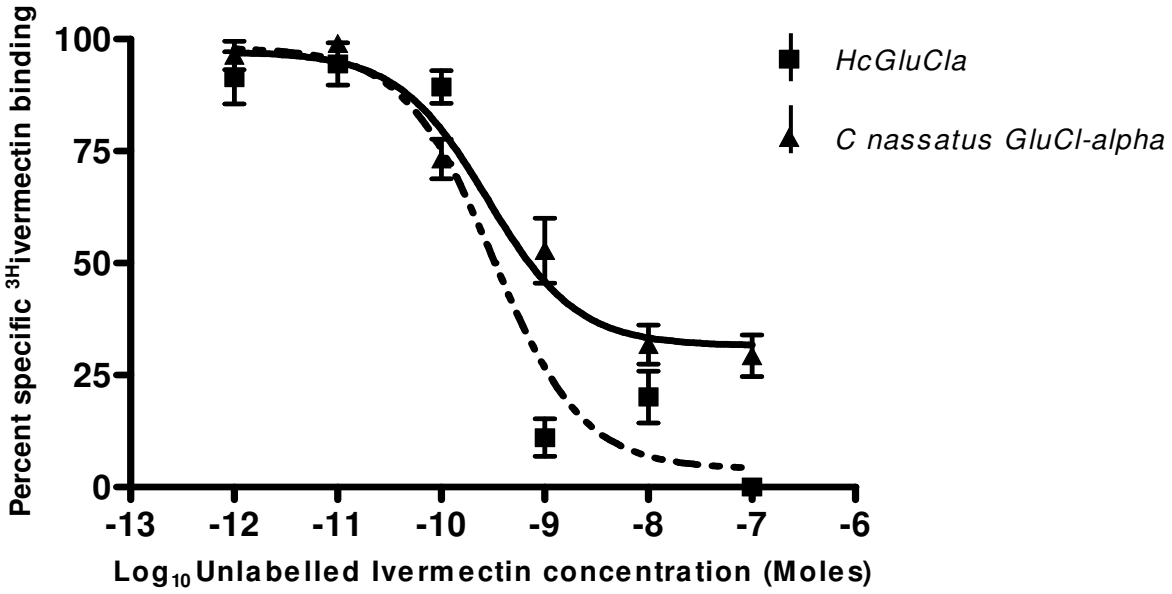

Fig. 5.7. Competition for [^{3}H]ivermectin binding by unlabelled ivermectin to membranes prepared from COS-7 cells expressing *C. nassatus GluCl*-α (■) or *HcGluCla* (▲). Experiments were done in the presence of 10μM glutamic acid.

CHAPTER 6

CONCLUSIONS AND SUMMARY

This dissertation presents important findings related to the feasibility of using Drenchrite® bioassay for the diagnosis of anthelmintic resistance in horse parasitic nematodes, effects of drug (moxidectin) selection on the genetic composition of cyathostomins and possible molecular targets of avermectin-milbemycin (AM) action and resistance in *Cylicocyclus nassatus*.

Project 1: Evaluation of the Drenchrite® bioassay for the diagnosis of anthelmintic resistance under the current situation of anthelmintic resistance in horse nematodes.

We explored the possibilities and relevance of using an *in vitro* larval development bioassay (Drenchrite®) for detecting anthelmintic resistance in cyathostomins. Tests for between assay consistencies with thiabendazole (TBZ) demonstrated that the DrenchRite® LDA gave repeatable results for this drug and may be a reasonably good assay for detecting benzimidazole resistance in cyathostomins among a group of farms. Drenchrite® using levamisole is not useful in detecting resistance to pyrantel (PYR) when results are evaluated on the basis of LC_{50} or LC_{95}. Our results failed to demonstrate any real value of this assay in detecting ivermectin (IVM) resistance in cyathostomins at this time. However, before Drenchrite® can be properly evaluated, known IVM-resistant populations will be needed for testing. In the present scenario of

anthelmintic resistance in horses worldwide, we cannot recommend the use of Drenchrite® for its diagnosis .

Project 2. Study the genetic diversity of cyathostomin populations and the effects of AM selection on this diversity and also search for probable genetic markers for AM resistance in *Cylicocyclus nassatus*.

Cyathostomins are the most important parasitic pathogens in horses, yet no studies were done to characterize the molecular genetic composition of any species of cyathostomin before the work described in this dissertation. Using the amplified fragment length polymorphism (AFLP) technique, we discovered that cyathostomins are very diverse genetically and a moxidectin selection protocol has significant effect on this genetic diversity. A high genetic diversity seems to be the common feature of animal parasitic nematodes (Blouin et al., 1992) and it provides a good background for drug selection because of the increased possibility of presence of an allele that may provide some advantage under selection pressure. Selection of existing alleles although considered as the major mechanism of development of drug resistance in animal parasitic nematodes (Grant 2000), it is not the only explanation for development of resistance. Introduction of novel mutations in the population due to errors in DNA replication, unequal crossing over, environmental factors and viral invasion of host genome can also contribute to the development of anthelmintic resistance.

AFLP is a genome based approach, which may be able to detect particular molecular markers for drug resistance. In our studies we found one of the polymorphic bands, which was present only in the moxidectin selected population was similar in DNA sequence to C44E4 region of *C. elegans*, which is located on Chromosome 1: 4,605,612 to 4,640,880 (Fig. 6.1). This location is close to the location of glc-2 in *C. elegans* (Chromosome 1: 4,885,188 to 4,887,942)

indicating a probability of genetic linkage. Also, C44E4 region adjoins unc-57 (location 1: 4,675,140 to 4,678,853) and *unc-11* genes (location: 1: 3,799,207 to 3,805,706) in *C. elegans*. A genetic linkage can be expected among C44E4, glc-2, unc-11 and unc-57 based on their location. *Unc-57* seems to have the highest probability of genetic linkage with C44E4 because of the close location of these two loci.

Some of the *unc* genes (*unc-7* and *unc-9*) have been implicated in the mechanism of ivermectin resistance in *C. elegans* (Dent et al., 2000). *Unc-11* codes for the proteins that are linked to the process of pharyngeal pumping in *C. elegans* (Nonet et al., 1999). Since inhibition of pharyngeal pumping is one of the major effect of ivermectin on nematodes (Brownlee et al., 1997) the possibility of genetic linkage between C44E4 and *unc-11* may provide valuable information in understanding the process of development of AM resistance in *C. nassatus*. *Unc-57* gene encodes the *C. elegans* ortholog of endophilin A (Schuske et al., 2003). Endophilin is a membrane-associated protein required for endocytosis of synaptic vesicles. Endophilin localizes synaptojanin to synapses, which is a polyphosphoinositide phosphatase regulating clathrin-mediated endocytosis at the synapse of neurons throughout the nervous system in *C. elegans* (Lagnado, 2005). A mutation in *unc-57* can have an effect on the transmission of nerve impulse (Schuske et al., 2003) affecting the activity of pharynx. The possible linkage between C44E4 region and *unc-57* may be interesting in terms of development of AM resistance in cyathostomins since pharyngeal paralysis is believed to be the major mechanism of ivermectin action in nematodes.

Project 3. Clone and sequence the orthologs of the invertebrate glutamate gated chloride channel (*GluCl*) genes from *Cylicocyclus nassatus* and characterize their pharmacological

properties by recombinant gene expression in mammalian cells and radioligand binding studies.

We found that *Cylicocyclus nassatus* possesses at least two genes *Cn-GluCl-α* and *Cn-GluCl-β* that encode for two different glutamate gated chloride channel (*GluCl*) subunit proteins. The DNA sequences of these genes were highly conserved evolutionarily and the translated protein clustered with *GluCl* protein from other species in phylogenetic trees constructed using Maximum-Parsimonay as well as Neighbor-Joining approaches. One of these genes, *Cn-GluCl-α*, when expressed *in vitro* in mammalian cells (COS-7), showed high amount of displaceable binding to [^{3}H]ivermectin (IC_{50} = 210 pM). *GluCl*s are important targets for the mechanism of action of AM drugs and have also been implicated in the mechanism of development of resistance against AM drugs. Characterization of these genes from cyathostomin species provide an opportunity for the discovery of specific molecular markers for the diagnosis of AM resistance in cyathostomins in the future.

Discussion

A. Factors contributing to the delay in onset of AM resistance in cyathostomins: *Cylicocyclus nassatus* is one of the most common species and a good representative of cyathostomins. The fact that no AM resistance has been reported in horse cyathostomins even after more than 24 years of use of AM drugs raises a question about the differences in biology of cyathostomins compared to other nematode species as well as the molecular similarity of target genes in these species. There are several factors that can contribute to the delay in onset of AM resistance in cyathostomins .

1. Efficacy of the drug

It is believed that the lack of resistance to ivermectin in cyathostomins is due to the less efficiency of this drug to kill encysted mucosal larval stages. A huge number of larvae encyst in mucosa and therefore provide a large refugia (proportion of a parasite population that is not exposed to a particular drug, thereby escaping selection for resistance) (Sangster and Gill 1999; Kaplan 2004). Inefficacy of drugs against these mucosal stages may be due to inability of the drug to reach inside the fibrous cyst or the inefficacy of drug for the particular stage of life-cycle of this parasite. Different life-cycle stages may have different expression patterns for particular genes that can influence the activity of drug. Benzimidazole and tetrahydropyrimidine classes of drugs are also ineffective at removing the encysted larval stages at the normal dose rate but resistance is widely prevalent against benzimidazoles in cyathostomins and is becoming increasingly common against tetrahydropirimidines (pyrantel). This undermines the role of effectiveness of drugs on these stages in the rate of development of AM resistance. The influence of efficacy is a complex issue. At very high efficacy there are very few survivors and resistance will develop very slowly but on the other hand the only worms carrying resistance alleles survive and reproduce in this case making the process of selection fast. A balance between these two aspects of high efficacy drugs such as AMs can determine the rate of selection for a particular species. Percent efficacy is a determinant of the rate of selection of resistance and lower doses (50%) of the same drug fail to generate resistant worms in a given time-period (Sangster and Bjorn, 1995), the survivors in these cases carry susceptible alleles which results in dilution of the resistant alleles and thereby reduced rate of selection. A drug with 99.0% efficacy would kill most of the susceptible worms and the only ones that survive would be the ones carrying resistant alleles making the process of selection faster. A drug with 99.9% efficacy although

would kill all the susceptible worms, the proportion of worms surviving and therefore carrying resistance allele would be too small and would result in a slow rate of selection.

2. *Multiple species*

Fifty two species of cyathostomins have been reported to date. In a typical infection, five to ten species constitute more than 90% of the worm burden in an individual horse. The more the number of species the more chances are there for the presence of an individual with an allele that can confer selective advantage. This is expected because interspecies genetic differences are normally higher than the intraspecies genetic differences. It can be hypothesized that there will be a bias in prevalence of cyathostomin species when particular species are found to be more or less resistant to the effect of AM drugs.

3. Means of inheritance of the resistance trait

It was shown by computer modeling that resistance evolves fastest when it is inherited as dominant trait, more slowly when co-dominant, and slowest when it is recessive (Barnes et al 1995). The pattern of inheritance of a trait can only be determined after that trait is expressed phenotypically. Because there are no reports of resistance against AM drugs in cyathostomins at present, there is no way to predict or establish what the pattern of inheritance will be. If inheritance of the ivermectin resistance trait in cyathostomes is recessive, then this would also greatly decrease the rate of the selection process toward resistance (Kaplan 2002). No efforts were made in our study to establish the pattern of inheritance because we were unable to generate an AM resistant phenotype for *C. nassatus*.

4. *Number of genes involved and their expression patterns*

Involvement of more than one gene in the development of resistance against a particular drug can delay the rate of development of resistance. Dent et al (2000) demonstrated that simultaneous mutation of three genes, avr-14, avr-15, and glc-1, encoding glutamate-gated chloride channel (*GluCl*) α-type subunits in *Caenorhabditis elegans* conferred high-level resistance to ivermectin whereas mutating any two channel genes conferred modest or no resistance. In *C. elegans*, *avr-15* gene encoding an α type *GluCl* subunit is expressed in pharynx as well as somatic motor neurons. Although expression patterns for all the *GluCl* genes in *C. elegans* have not been completely characterized expression of the same *GluCl* protein in multiple organs hint towards more than one target organ for AM drugs in this organism. Increased expression of a receptor through promoter changes can also influence the rate of development of drug resistance.

Several other genes (*unc-7*, *unc-9*, and the *Dyf*) were also found to modulate ivermectin sensitivity in *C. elegans* Which supports a multigenic nature of AM resistance in *C. elegans*. In *H. contortus,* changes in allele frequencies of the *GluCl*α-gene, p-glycoprotein gene as well as GABA-receptor gene, HG1 are associated with ivermectin/moxidectin reistance (Xu et al., 1998; Blackhall et al. 1998a, 1998b). These results hint towards a multigenic nature of AM resistance in parasitic nematodes too. Although it is possible that the nature of resistance in cyathostomins is different from *C. elegans* and *H.contortus* a multigenic nature may provide explanations for its slow onset. In AFLP experiments, we established that the genetic locus linked with moxidectin selection in *C. nassatus* has the probability of genetic linkage with unc-57, unc-11 and glc-2 genes based on location of these genes in *C. elegans* genomic DNA sequence. These studies though do not provide with a statistically significant association of these genes with moxidectin

selection, they hint towards involvement of more than one gene in the process of AM selection in cyathostomins. Orthologs of p-glycoprotein, GABA-receptor, *unc* genes and *dyf* genes have not been reported for any of the cyathostomin species. Future studies in the field of genetics of AM resistance in cyathostomins should be aimed towards finding these orthologs and their possible roles in AM resistance.

5. *Copy number of genes involved and structure of the protein*

It is possible that more than one copy of a particular gene exist in the genome of a particular organism. In this situation the development of resistance will be slower because the normal copy of the gene can compensate a mutated copy of the gene. It is highly desirable that the sequence information of *GluCl* genes from *C. nassatus* obtained in this study is utilized in future for the determination of copy number of *GluCl* genes in *C. nassatus*. Structure of the target protein can also influence the mechanism of resistance. As in case of benzimidazole resistance in trichostrongylids where an isotype of tubulin with lower affinity for benzimidazoles predominates in resistant populations. The number of alleles for *GluCl* existing in cyathostomin populations should also be investigated.

B. Forward and reverse genetic approaches to understand the development of AM resistance in cyathostomins: In the model nematode *C. elegans*, resistance to AM drugs is thought to involve alterations in the target sites, as well as a reduction in the drug concentration at the site of action. The complexity of the development of AM resistance in cyathostomins can be addressed by the traditional forward and reverse genetic approaches. We started a drug (moxidectin) selection protocol in an effort to generate the AM resistant phenotype in

cyathostomins and investigated the genetic alterations posed by this selection. The method available to examine the resistant phenotype (fecal egg count reduction test) in parasitic nematodes is not very sensitive and it can only detect resistant worms when they exceed more than 25% of the worm burden (Martin et al., 1989). Although we were unable to produce a resistant phenotype based on fecal egg count reduction test, we hypothesized that the heavy moxidectin selection placed on a particular population of cyathostomins for more than 3 years would result in some genetic alterations. In our AFLP studies, we found significant differences in genetic distances between the moxidectin-selected and AM-naïve population of worms and these distances were less in the drug-selected population signifying reduced polymorphism in this group. Reduction in polymorphism may be due to selection of particular alleles of a particular gene. Further examination of the AFLP results for the presence of loci linked to AM resistance, resulted in the identification of at least one locus (C44E4 in *C. elegans*) that could be linked to resistance. Although we were unable to identify specific genes linked to AM resistance in cyathostomin in these studies it is conceivable that we are detecting some important early changes in the evolution of resistance.

We also used a reverse genetic approach where we utilized the information gained from AM resistance studies on other nematode species. Glutamate-gated chloride channels (*GluCls*) are the putative target sites of AM action in *C. elegans* (Cully et al., 1994) and have also been associated with AM resistance in nematodes (Dent et al., 2000, Njue and Prichard, 2004). We investigated the presence of genes encoding *GluCls* in *C. nassatus,* one of the most common cyathostomin species and were able to clone the full length cDNAs of *Cn-GluCl-alpha* and *Cn-GluCl-beta* genes. On the examination of ivermectin binding affinities of these genes on *in vitro* expression in mammalian cells, we found that homomeric channels formed of *Cn-GluCl-alpha*

protein bind [^{3}H]ivermectin with high affinity whereas homomeric *Cn-GluCl-beta* protein has very low binding affinity to [^{3}H]ivermectin. These results support the earlier studies in *C. elegans* and *H. contortus* that showed that *GluCl-alpha* subunits are responsible for binding ivermectin. Phylogenetic analysis of *C. nassatus GluCl* showed that *Cn-GluCl-alpha* and *Cn-GluCl-beta* form evolutionarily close relationship with other nematode *GluCls* signifying conservation of structure and properties. Based on these results, it is possible that *GluCls* play an important role in the development of AM resistance in cyathostomins. Cyathostomins have a complex biology and it probably influences the development of AM resistance up to a great extent. The most interesting part of cyathostomin life cycle is the encysted stage in host large intestine. This stage is believed to be resistant to the action of ivermectin as well as benzimidazoles and pyrantel at the normal dose rate. Because resistance is detected in cyathostomins against benzimidazoles and pyrantel, it is conceivable that inefficacy at this stage probably does not offer a huge advantage in terms of dilution of resistance alleles. Another important observation is the survival of cyathostomin larvae at high concentrations of ivermectin but not the benzimidazole or pyrantel class of drug in *in vitro* larval development assays (Young et al., 1999; Tandon and Kaplan., 2004). This hints towards inefficacy of ivermectin against larval stages of cyathostomins. In our experiments we were able to demonstrate the presence of *GluCl* transcripts (data unpublished) in adult as well as larval cyathostomins but the functional expression of *GluCls* in cyathostomin larvae is not confirmed yet. Also, tissue-specific expression of *GluCls* in cyathostomins is also unknown. *GluCls* have not been established as the only molecular targets for the action of AM drugs in cyathostomins and it is possible that some other genes play a major role in AM action and resistance in these nematodes. Although results of our AFLP studies hint towards the multigenic nature of AM resistance in cyathostomins the

actual involvement of other genes in AM action and resistance can only be established after the possible target genes are cloned from cyathostomin species and are characterized in terms of their involvement in AM resistance.

Suggested future directions

Drenchrite assay: Results of our studies indicated that it is not possible to use the Drenchrite® assay in its present commercially available form for anthelmintic resistance detection in horses. The results were consistent in case of thiabendazole (TBZ) and Drenchrite® was able to differentiate susceptible, suspected-resistant and resistant farms based on LC_{50} values for benzimidazole drug class. Our data indicated that Drenchrite® may be a reasonably good assay for detecting resistance in cyathostomins among a group of farms. But the resistance to benzimidazole drug class is very prevalent over the world (Lyons and Tolliver, 2003; Cirac et al., 2004) signifying no practical value of this test in benzimidazole resistance detection at present or in foreseeable future. Our data clearly demonstrated that Drenchrite® using levamisole is not useful in detecting resistance to pyrantel (PYR) when results are evaluated on the basis of LC_{50} or LC_{95}. For ivermectin, we found survival of larvae even at the highest concentration of drug for both the ivermectin analogs used. These results are surprising considering the high efficacy of ivermectin in the field. Although the means of responses for ivermectin exposed farms showed a statistical difference from the ivermectin naïve farm, the range of LC_{50} for exposed farms were very wide. These results question the value of using Drenchrite® for detection of ivermectin resistance in cyathostomins. It may be possible to reanalyze the results of Drenchrite® for ivermectin resistance detection in cyathostomins in future when the resistant isolates of a cyathostomin species become available, but based on our data the current analogs of ivermectin

used in Drenchrite® would hardly be successful in detecting this resistance. A representative of pyrantel to use in Drenchrite® that is better than levamisole in estimating the resistance to pyrantel in cyathostomins can also be investigated.

Glutamate-gated chloride channel and selection for AM resistance: We cloned two genes *Cn-GluCl-α* and *Cn-GluCl-β* in *C. nassatus* coding for α and β *GluCl* subunit, respectively. It is possible that a number of *GluCl* genes exist in *C. nassatus.* This can be studied by designing degenerate primers using the amino acid sequence information from the most conserved region of multiple aligned *GluCl* genes of *C. elegans*, *H. contortus and C. nassatus* and using these primers for amplification of *C. nassatus* genomic and complementary DNAs. A nucleotide probe from the conserved region of *GluCl* sequences can also be utilized to ascertain the number of *GluCl* genes and their copy numbers in *C. nassatus*. Our preliminary experiments using the degenerate primer approach suggest that there may be more than two different *GluCl* genes in *C. nassatus*. All of these genes have not been cloned and explored yet for their functional properties and expression. It will be interesting to know the exact number of *GluCl* genes present in *C. nassatus* and the roles these genes play in the mechanism of AM action as well as in the development of AM resistance. These studies are particularly important because more than one α-type of *GluCl* genes have been implicated in the development of AM resistance in *C. elegans*. We found that *Cn-GluCl-α* gene when expressed *in vitro* binds to [^{3}H]ivermectin with high affinity but *Cn-GluCl-β* shows only a negligible binding to [^{3}H]ivermectin. It is possible to investigate the ivermectin/moxidectin binding properties of other *GluCl* genes discovered in future in *C. nassatus* using this approach.

In our experiments we were unable to generate an AM resistant phenotype of *C. nassatus* even after more than three years of intensive drug selection. But, we did find a significant decrease in genetic diversity in the AM-selected population. The data suggests that this population is at the early stages of selection and in near future it should be able to show the AM resistant phenotype. Once the resistant isolate of *C. nassatus* is generated, it can be compared with the susceptible isolates for amino acid or DNA sequence differences in multiple genes that have probable involvement in AM resistance based on our studies and the studies done in *C. elegans*. *C. nassatus* orthologs of *GluCl*, *pgp, unc* and GABA-gated chloride channels can be cloned using the degenerate primer strategy utilized by us and searched for any sequence differences in resistant versus susceptible isolates.

These mutations can be identified by the direct sequencing of the whole or partial gene sequences from individual nematodes or mutation scanning techniques such as Single Stranded Confirmational Polymorphism (SSCP), Heteroduplex Analysis or Mutation Specific PCR. If particular mutations are found to be significantly increased in frequency among resistant isolates, these can be studied further by site-directed mutagenesis of cloned genes and *in vitro* expression of these mutants. A difference in binding affinities of wild type and mutant genes will ascertain the role of these mutations in AM resistance in *C. nassatus*. In our attempts to find particular mutations in GluCl-α gene that could be associated with resistance, we sequenced a 300 bp fragment of *Cn-GluCl-α* from 20 worms from each of the two populations: AM naïve Population-S and moxidectin-selected Population-Mox. This region was chosen for direct sequencing because it is the proposed ligand binding domain of *GluCls* (Etter et al., 1996) and particular mutations in similar regions were found to confer ivermectin resistance in *C. oncophora* (Njue et al., 2004). Analysis of single nucleotide polymorphisms showed the

presence of a total of 93 mutations in Population-Mox. Out of these 35 mutations were present in the exon regions and 8 of these exon mutations were non-synonymous. For Population-S, a total of 65 mutations were detected, 30 of them were located in exons and 2 out of these exon mutations were non-synonymous. Particular mutation could not be linked to resistance because the frequency of non-synonymous mutations was very low.

As we have learned from earlier studies done on different nematode species, it is highly probable that a number of genes together play roles in the development of AM resitance in *C. nassatus.* Looking at one gene at a time may not be the best option in this case because of the complexity of interplay of several genes. In this scenario, it would be wise to compare the resistant and susceptible isolates by genome based approaches such as microsatellite analysis, amplified fragment length polymorphism, restriction landmark genome scanning (RLGS) or genomic microarrays to search for specific markers. Genetic crosses of ivermectin-resistant and susceptible *C. nassatus* should be helpful in determining if the resistance is mediated by a single gene or gene complex, but establishing monospecific infections in cyathostomins is challenging. Overexpression or underexpression of particular genes should also be studied in resistant isolate compared to the susceptible isolate of *C. nassatus* because this will influence the activity of drug against this organism. A cDNA microarray or Realtime-PCR based approach can be utilized for these studies. It is also important to study the tissue-specific expression of particular genes that have implications in AM resistance development in *C. nassatus.* It is difficult to obtain amplifiable RNA from specific tissues of *C. nassatus*, therefore tissue-specific PCR is not feasible. Techniques like *in-situ* hybridization can be utilized to detect the tissue-specific expression of a particular gene. These experiments will help in understanding the mechanism of

action of AM drugs in *C. nassatus* as well as the strategies used by this organism to counter the effects of AM drugs.

In our AFLP studies, we discovered that *C. nassatus* is very diverse genetically and a moxidectin selection protocol has significant effect on this genetic diversity. The level of genetic diversity in other cyathostomin species can be studied in future to investigate interspecies differences in genetic diversity and its effects on anthelmintic selection. We show that it is possible to reduce *C. nassatus* genetic diversity by moxidectin selection. The selection protocol we used should be utilized further to obtain highly AM resistant populations of cyathostomins that can be used for candidate gene association studies.

Given the time and resources, first thing I would suggest to do is to search for the *C. nassatus* orthologs of all the *GluCl-α* genes reported to date in *C. elegans*, *H. contortus* and *C. oncophora*. I will start out by searching for orthologs of *C. oncophora GluCl-α3* gene because particular mutations in this gene were linked to the AM resistance in *C. oncophora* (Njue et al., 2004). In the meantime I would direct the efforts towards generating an AM resistant phenotype by continuing the moxidectin selectin protocol. Generation of an AM resistant phenotype would be a milestone in the search of AM resistance mechanism in cyathostomins. If an ortholog of *C. oncophora GluCl-α3* gene is found in *C. nassatus* and if the AM resistance phenotype is generated, I would scan the proposed ligand binding region (Etter et al., 1999) of this gene from a number of AM resistant and sensitive *C. nassatus* by single stranded conformational polymorphism technique to check the presence of mutations. If the frequency of a particular allele is found to be high in AM resistant population, it would indicate its involvement in AM resistance. In this case, I will clone and express the wild type allele as well as the resistant allele in a mammalian expression systme and look for differences in their binding affinities to AM

drugs. If the resistant allele contains more than one mutation, individual mutations will be expressed separately and studied for their binding affinities. A decreased binding affinity will ascertain the involvement of particular mutations in the development of AM resistance in *C. nassatus.* In future these studies will provide framework for the desing of molecular kits for the detection of AM resistance in cyathostomins. If these experiments fail to find particular mutations associated with AM resistance in cyathostomins, I'll contintue to search for allelic differences in AM resistant and sensitive populations in other *GluCl-α* orthologs if found in *C. nassatus.*

We have already established the protocols for amplified fragment length polymorphism analysis of *C. nassatus* species. I would like to use this technique to scan individuals from AM resistant cyathostomins and compare them with the AM sensitive individuals. If particular bands are found to be present in a number of individuals in only one population, sequence information from these bands will be obtained and searched for similarity on NCBI sequence database. It is expected that whole genome sequences of a number of parastic nematodes will be available in near future. It would be easier then to locate particular fragments found in AFLP experiments to a specific chromosome and examine the probability of genetic linkage. If these genes with high probability of linkage turn out to be the genes that were earlier implicated in AM resistance in other species, it will indicate a stong proability of involvement of these genes in AM resistance development in *C. nassatus*. These genes will then be used in a reverse genetic approach for studying their role in AM resistance as described before. These gene fragments if found to confer significant resistance in *in vitro* studies will serve as molecular markers for AM resistance in cyathostomins.

I would also do a mRNA differential display to check if particular genes are transcribed more in an AM resistant or sensitive cyathostomin population. If differences are detected, particular mRNAs will be sequenced and studied for their role in AM resistance. I would be interested in finding the differences in genes that have probable implications in AM resistance as well as the transcription factors that may influence the rate of expression of particular genes. The biological explanation of differential effect of AM drugs on different life-cycle stages of cyathostomins is not established till date. Historically, it has been difficult to identify the eggs and larval stages of a particular cyathostomin species. With the advancement of molecular-technology, the tools are now becoming available to overcome this identification hurdle. I would also use mRNA from eggs, larvae and adult *C. nassatus* for the detection of particular genes that are expressed more or less in particular life-cycle stage of *C. nassatus* using the differential display approach in an effort to explain the differences in activity of AM drugs against these stages.

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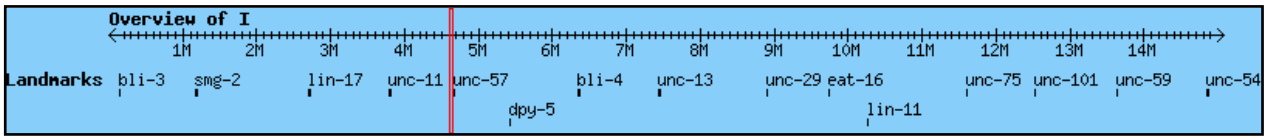


Fig. 6.1 Overview of the *C. elegans* chromosome 1. Location of C44E4 region is indicated by the double red line.

CHAPTER 7

ADDITIONAL STUDIES

A. Cloning and sequencing of the AFLP bands

AFLP selective reactions were run on a 25 X 20 cm 3% Metaphor® agarose gel (Cambrex Bio Science Rockland, Inc., Rockland, ME, USA) at 120V for 10 hours at 4ºC. Gel was stained using SYBR® Gold nucleic acid stain (Molecular Probes, Oregon, USA) and visualized under UV light. Appropriate sized bands were cut from the gel (Fig. 7.1), DNA was purified using Zymoclean gel DNA recovery kit (Zymo research, Orange, CA, USA) and cloned into pDrive vector (Qiagen Inc., Valencia, CA). Sequencing was performed on an ABI3100 sequencer using vector specific primer T7 with dye termination protocol. An attempt was made to clone bands #58, #30, #44, #47, #54, #77, #34, #70, #73, #76, #87, #78, #83, #85 and #88 (Table 7.1).

Results: We selected to clone bands from the primer combinations that showed significant differences between Population-S and Population-Mox. A total of eight of polymorphic and unique bands were cloned and sequenced to determine if any of these bands correspond to a locus which may be linked to loci of genes in *C. elegans* with probable involvement in AM resistance. Sequences were searched for similarity with *C. elegans* genomic DNA sequence (wormbase.org) or parasitic nematode ESTs (NemaBlast, Nematode.net). Most of these

sequences showed no similarity to any of the sequences in these two databases as well as NCBI nucleotide database which can be explained by the fact that genomic DNA sequence data for *C. nassatus* is not available at present and also it is possible that a great number of these fragments are derived from the intron regions. One of the bands present in two individuals only in Population-S showed sequence similarity to a hypothetical *C. elegans* protein (CBG17224) and one other band present in two individuals only in Population-Mox showed similarity to the *C. elegans* C44E4 genomic DNA sequence.

C. elegans C44E4 genomic DNA sequence is located on chromosome 1 (between 4,605,612 and 4,640,880), which is close to the *glc-2* gene (located between 4,885,188 and 4,887,942). *Glc-2* encodes a glutamate-gated chloride channel (GluCl) β subunit in *C. elegans*. Also, C44E4 region adjoins *unc-57* (location 1: 4,675,140 to 4,678,853) and *unc-11* genes (location: 1: 3,799,207 to 3,805,706) in *C. elegans.* Several *GluCl* and *unc* genes have been found to be linked to AM resistance in *C. elegans*. *Unc-11* itself has been found involved in the process of pharyngeal pumping. Blocking of pharyngeal pumping is one the major effects of AM action in nematodes. This finding though does not provide a statistical support for the involvement of a particular gene or locus in selection for AM resistance it does provide an interesting finding in terms of probable involvement of more than one gene in the process of selection for AM resistance in cyathostomins. Orthologs of any *unc* gene have not been characterized from cyathostomins and it may be interesting to study their properties and involvement in AM resistance.

B. Testing the presence of multiple *GluCl* genes in *C. nassatus*

Degenerate primers were designed using the amino acid sequence information from the most conserved region of multiple aligned *GluCl* genes of *C. elegans, H. contortus* and *C. nassatus.* These primers were used for amplification of *C. nassatus* genomic and complementary DNAs. We were able to amplify a number of fragments from *C. nassatus* as well as *C. elegans* gemomic DNA using one of the degenerate primer combinations. We tested if these bands represent real *GluCl* genes or not in three ways:

1. Using the same primer combination used for genomic DNA amplification, in a *C. nassatus* cDNA amplification reaction (lane 2, Fig.7.2), which resulted in amplification of smaller sized fragments, although same number of fragments were not amplified. This may be explained by the fact that this primer-pair may span an intron region for some of these genes.
2. Using a seminested PCR using the products of genomic DNA amplification as template. This resulted in amplification of a number of fragments, some of them were similar in size as in the genomic DNA reaction.
3. Extracting individual bands and using them as templates in a PCR using one of the *Cn-GluCl-α* gene specific primer combination (Fig. 7.3), Some bands when used as template gave a band at expected 200 base pair size, supporting the idea that these are real *GluCl* genes, while some fragments failed to amplify. Although this experiment does not disregard the bands that failed to amplify (because this may be due to splitting of primer sequence by an intron) it supports the genuinity of bands that were amplified.

The bands that were positive in Fig. 7.3 are being cloned and sequenced to determine if they are real *GluCl* genes. The sequence information obtained from these fragments will be utilized to design gene specific primers and amplify the full-length *GluCl* genes from *C. nassatus* using the rapid amplification of cDNA ends (RACE) approach.

C. Single Nucleotide Polymorphism Analysis

A summary of the single nucleotide polymorphisms detected in a 280 base pair region of *Cn-GluCl-α* gene sequenced using direct-PCR-sequencing (Polymorphic DNA Technologies Inc., Alameda, CA, USA) is reported (Table 7.2). This region was chosen for direct sequencing because it is the proposed ligand binding domain of *GluCls* and particular mutations in similar regions of *GluCl-α3* gene were found to confer ivermectin resistance in *C. oncophora*. Position, type and number of individuals possessing particular mutations are listed in Table 7.2. Mutations were detected from 20 worms in each of the two populations (Population-Mox and Population–S).

Table 7.1 Summary of bands that were polymorphic or unique in a specific population.

	Population - S		Population - Mox	
Primer Combination	**Polymorphic**	**Unique**	**Polymorphic**	**Unique**
Mse1-C/EcoR1-ACT (A)	**#58**			
Mse1-A/EcoR1-ACT (B)		**#34, #70, #73, #76, #87**		**#78, #83, #85, #88**
Mse1-T/EcoR1-ACT (C)	**#30**	**#45, #51, #56, # 71, #74, #75, # 76, #78, #79**	**#44, #47, #54, #77**	**#57, #63, #67**

Table 7.2 Positions and types of mutations. Number of individuals possessing particular mutations are also listed.

Position in sequence	Type of Mutation	Population-Mox Total	Population-Mox Synonymous	Population-Mox Non-Synonymous	Population-S Total	Population-S Synonymous	Population-S Non-Synonymous
7	C to T	3	3		7	7	
21	A to M	0	0		1	1	
36	A to R	3	3		1	1	
45	G to R	1	1		0	0	
52	T to Y	1			0		
55	T to Y	1			0		
56	T to K	2			0		
57	T to Y	1			0		
58	C to T	1			1		
58	C to S	0			1		
59	T to Y	1			0		
61	C to S	1			0		
61	C to M	1			0		
63	T to Y	0			1		
67	C to Y	3			2		
67	C to T	3			0		
69	C to Y	3			0		
70	C to Y	1			0		
71	C to S	1			1		
71	C to G	1			0		
72	A to W	1			0		
76	A to R	1			0		
79	A to M	0			1		
83	G to R	0			1		
84	C to Y	1			0		
86	G to K	0			1		
87	T to K	2			1		
88	T to W	1			0		
89	C to Y	0			1		
90	G to R	1			1		
91	T to Y	1			1		
92	A to W	1			0		
99	T to K	1	1		0		
102	T to C	1	1		0		
102	T to Y	1	1		0		
102	T to K	0	1		1	1	
105	A to W	1	1		2	2	
108	T to Y	0	0		1	1	
111	T to W	1		1	0		

111	T to C	0			1	1
113	C to S	1	1		0	
126	T to Y	3	3		0	
132	T to Y	0			1	1
135	A to R	1	1		0	
144	T to k	1		1	0	
145	T to C	0			1	1
145	T to Y	1	1		0	
146	T to Y	1		1	0	
153	A to R	3	3		1	1
153	A to W	0			2	2
153	A to G	1	1		0	
154	G to K	1		1	0	
177	C to S	1		1	0	
188	A to G	0			1	
195	T to G	0			1	
196	T to G	0			1	
197	T to C	0			1	
198	C to T	0			1	
198	C to Y	1			1	
198	C to S	0			1	
199	T to G	0			1	
200	C to G	0			1	
200	C to Y	1			0	
200	C to S	1			0	
201	C to A	0			1	
201	C to Y	3			0	
202	A to R	0			1	
205	T to W	1			0	
205	T to Y	1			0	
208	A to G	0			1	
208	A to R	1			0	
209	C to M	0			1	
211	A to C	0			1	
213	C to S	1			0	
213	C to Y	1			0	
214	T to A	0			1	
219	T to W	1			0	
221	T to G	0			1	
221	T to Y	1			0	
221	T to K	1			0	
222	G to R	0			1	
222	G to K	2			0	
223	T to W	2			0	
225	T to Y	1			0	
225	T to K	1			0	

226	T to K	1			0			
227	G to K	2			0			
229	T to W	1			0			
230	T to K	0			2			
231	A to M	0			1			
232	T to Y	1			1			
232	T to W	0			1			
232	T to K	1			0			
233	A to W	1			0			
234	G to K	1			0			
235	T to W	1			0			
237	A to M	0			1			1
237	A to R	1		1	0			
241	T to Y	0			1	1		
249	A to W	0			1	1		
260	C to Y	3	3		3	3		
260	C to M	0			1	1		
260	C to T	0			2	2		
260	C to W	1	1		0			
274	T to W	1		1	0			
276	G to R	1		1	0			
280	T to K	0			1			1
287	A to R	1	1		0			
287	A to G	0			1	1		
		93	27	8	65	28		2

Summary

Population- Mox

Total mutations = 93,
In Exons = 35 (Synonymous: 27, Non Synonymous: 8), In Introns (shown in pink): 58

Population S

Total mutations = 65,
In Exons = 30 (Synonymous: 28, Non Synonymous: 2), In Introns (shown in pink): 35

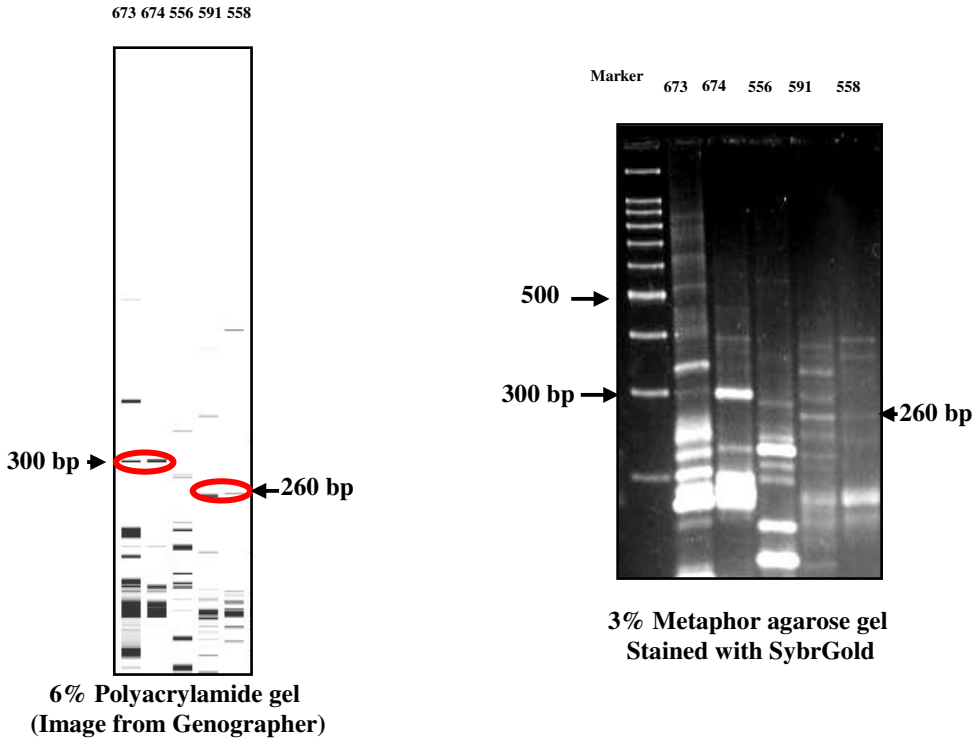
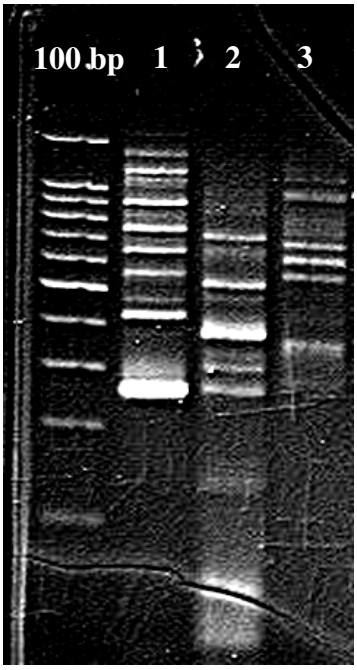
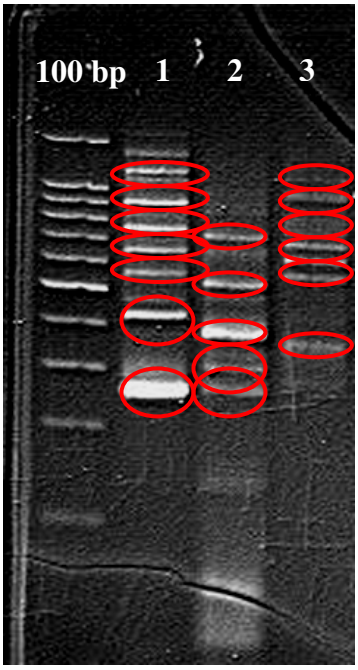
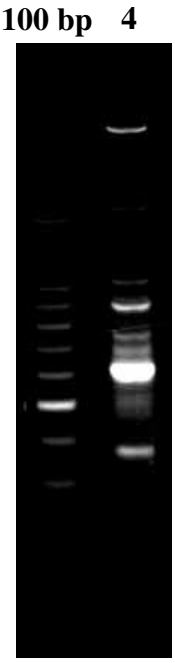


Fig. 7.1 Cloning of individual bands from AFLP reactions. (Band #58 and band #54 are shown)



a) 2% Metaphor agarose gel





b) 2% Metaphor agarose gel (bands that were excised are shown in red circles.

1. *C. nassatus* genomic DNA template

2. *C. nassatus* RNA template

3. Seminested PCR on the products of lane #1 as template

4. *C. elegans* genomic DNA as template

Fig. 7.2. Degenerate PCR. Degenerate primers DegF2 (CADSARATYTGGATGCCHG, Tm = 59.44) and DegR2 (GGYSKMCKRACWCAHCAGTA, Tm = 62.11) were used for amplifications in lane 1, 2 and 4. Seminested PCR was performed using DegF2 and DegR3 (AGNTHGGTYTTDCTDTGGGT, Tm = 58.35). Bands were named as 1A through 1G, 2A through 2E and 3A through 3F from bottom to top in each lane. 100 bp: Promega 100 bp DNA ladder (Catalog no. G2101, Promega US, Madison, WI, USA)

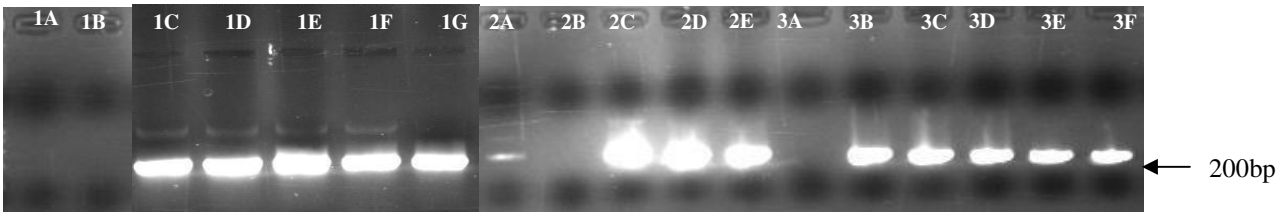


Fig.7.3. Testing the genuineness of bands amplified in degenerate PCR. Bands that were excised in figure 1 were used as a template in a PCR using *Cn-GluCl-α* specific primers [Glcf (gacgggctaagctcctcactacc) and Glcr (atccagccagaacgacaccc)] to confirm the genuinity of these bands. Lanes are labeled by the name of templates used. 2% Metaphor agarose gel was used.

APPENDICES

A. Genomic DNA Extraction from worms

1 For worms stored in ethanol at –20˚C, spin the microfuge tube at 14,000 rpm for three minutes. Pour off the ethanol, add 0.5 mL of extraction buffer (see recipe below) without 2-mercaptoethanol (BME) and Proteinase K to rinse off more of the ethanol, and spin again at 14,000 rpm. Carefully remove the supernatant.

(Skip this step for worms stored in –80˚C)

2 Grind the worms using a micropestle and add 200 µL of the Pro-K and BME extraction buffer in parts during grinding. Put the tubes into a 65˚C block for two hours.

3 Add 200 µL of phenol/chloroform/isoamyl alcohol (25:24:1) to the extraction buffer, and shake vigorously for 15 seconds. Centrifuge at 14,000 rpm for five minutes at room temperature. Remove the top aqueous layer and put it into a new tube. Put phenol/chloroform /isoamyl alcohol into the waste bottle.

4 Put about 200 µL of chloroform into the saved aqueous layer and shake it vigorously for 15 seconds. Spin at 14,000 rpm for 1 minute, then remove and save the top aqueous layer in a new tube. Put the chloroform into a waste bottle.

5 Measure the volume of the sample—it should be about 150 to 190 µL. Add 1 µL of polyacryl carrier. Mix. Add enough 5M NaCl to make 0.2 M (8 µL in 200 µL). Mix again. Add 2.5 volumes of 100% ethanol, and mix again. Incubate at room temp for ten minutes. Spin at 3000 G for 10 minutes at room temperature.

6 Remove the supernatant and save the little white pellet. Add about a mL of 70% ethanol (EtOH), vortex briefly, and spin again at 14,000 rpm for 5 min. Remove the supernatant. Repeat the 70% EtOH rinse. After removing the supernatant, spin the pellet again and use a pipetter to remove the residual EtOH. Air dry for 5 minutes or until no EtOH is left. Resuspend in a small volume of 10 mM Tris, pH 7.5 or 8.

100 mL Extraction Buffer:

100 mM NaCl (0.58 grams, or 2 mL of a 5 M solution)

5 mL of 1 M Tris, pH 7.5 or 8 (50 mMol final conc)

10 mL of 0.5 M EDTA, pH 8 (50 mMol final conc)

10 mL of 10% SDS, or 1 gram (1% final conc)

Immediately prior to use, add Proteinase K to make a 500 µg/mL solution. 10 mLs (5 mg per 10 mL, or 500 µg/mL). Add 100 µL of 2-mercaptoethanol (BME) to 10ml of the extraction buffer.

B. Total RNA Extraction from adult nematodes using TRIzol® (Invitrogen)

1. Place the worm/s in a 1.5 ml microcentrifuge tube containing 100µl TRIzol® reagent and crush them using a micro-pestle. (Worms can be stored directly in TRIzol® at –20˚C after washing twice with 1X PBS (Phosphate buffered saline) during necropsy).

2. Add 650 µl of TRIzol® to the tube, bringing the total volume of the tube to 750 µl. Incubate for 5 minutes at 15 to 30°C to permit the complete dissociation of nucleoprotein complexes.

3. Add 0.2 ml of chloroform. Cap the sample tubes securely. Shake the tubes vigorously by hand for 15 seconds and incubate them at 15 to 30°C for 2 to 3 minutes. Centrifuge the samples at 12,000× *g* for 15 minutes at 2 to 8°C. Following centrifugation, the mixture separates into a lower red, phenol-chloroform phase, an interphase, and a colorless upper aqueous phase. RNA remains exclusively in the aqueous phase. The volume of the aqueous phase is about 60% of the volume of TRIzol® reagent used for homogenization.

4. Transfer the aqueous phase to a fresh tube. Precipitate the RNA from the aqueous phase by mixing with 0.5 ml of isopropyl alcohol. Incubate samples at 15 to 30°C for 10 minutes and centrifuge at 12,000 × *g* for 10 minutes at 2 to 8°C. The RNA precipitate,

often invisible before centrifugation, forms a gel-like pellet on the side and bottom of the tube.

5. Discard the supernatant. Wash the RNA pellet once with 1ml 75% ethanol, mix the sample by vortexing and centrifuge at no more than 7,500 × *g* for 5 minutes at 2 to 8°C.

6. At the end of the procedure, briefly dry the RNA pellet (air-dry or vacuum-dry for 5-10 minutes). Do not dry the RNA by centrifugation under vacuum. It is important not to let the RNA pellet dry completely as this will greatly decrease its solubility. Partially dissolved RNA samples have an A260/280 ratio < 1.6. Dissolve RNA in RNase-free water and incubate for 10 minutes at 55 to 60°C. Store at –80˚C in multiple aliquots.

C. Rapid Amplification of cDNA ends using Marathon® cDNA amplification kit (Stratagene)

First Strand cDNA synthesis

1. Combine the following in a sterile 0.5-ml microcentrifuge tube:

   1 µg (1–4 µl) RNA sample (Total RNA extracted from worms)

   1 ul cDNA Synthesis Primer (10 mM) (Oligo dT)

2. Add sterile H_2O to a final volume of 5 µl.
3. Mix contents and spin the tube briefly in a microcentrifuge.
4. Incubate the tube at 70°C for 2 min.
5. Cool the tube on ice for 2 min.
6. Spin the tube briefly to collect the contents at the bottom.
7. Add the following to each reaction tube:

   2 µl 5X First-Strand Buffer

   1 µl dNTP Mix (10 mM)

   1 µl AMV Reverse Transcriptase (20 units/ml)

   10 µl Total volume

8. Mix the contents of the tube by gently pipetting.
9. Spin the tube briefly to collect the contents at the bottom.
10. Incubate the tube at 42°C for 1 hr in an air incubator.
11. Place the tube on ice to terminate first-strand synthesis.

12. Proceed to second-strand synthesis.

Second-Strand cDNA Synthesis

1. Combine the following components in the reaction tube

    10 µl First-strand reaction

    48.4 µl Sterile H_2O

    16 µl 5X Second-Strand Buffer

    1.6 µl dNTP Mix (10 mM)

    4 µl 20X Second-Strand Enzyme Cocktail

2. Mix contents thoroughly with gentle pipetting.
3. Spin the tube briefly to collect the contents at the bottom.
4. Incubate the tube at 16°C for 1.5 hr.
5. Add 2 µl (10 units) of T4 DNA Polymerase and mix thoroughly with gentle pipetting.
6. Incubate the tube at 16°C for 45 min.
7. Add 4 µl of the EDTA/Glycogen Mix to terminate second-strand synthesis.
8. Add 100 µl of phenol:chloroform:isoamyl alcohol (25:24:1).
9. Vortex thoroughly.
10. Spin the tube in a microcentrifuge at 14,000 rpm for 10 min to separate phases.
11. Carefully transfer the top aqueous layer to a clean 0.5-ml microcentrifuge tube. Discard the interface and lower phase.
12. Add 100 µl of chloroform:isoamyl alcohol (24:1) to the aqueous layer and vortex thoroughly.
13. Spin the tube in a microcentrifuge at 14,000 rpm for 10 min to separate the phases.

14. Remove the top aqueous layer and place it in a clean 0.5-ml microcentrifuge tube.

15. Add one-half volume of 4 M Ammonium Acetate.

16. Add 2.5 volumes of room-temperature 95% ethanol.

17. Vortex the mixture thoroughly.

18. Spin the tube immediately in a microcentrifuge at 14,000 rpm at room temperature for 20 min.

19. Remove the supernatant carefully.

20. Gently overlay the pellet with 300 µl of 80% ethanol.

21. Spin in a microcentrifuge at 14,000 rpm for 10 min.

22. Carefully remove the supernatant.

23. Air dry the pellet for approximately 10 min to evaporate residual ethanol.

24. Dissolve the precipitate in 10 µl of H_2O and store at –20°C.

a. Analyze 2 µl of experimental ds cDNA and 2 µl of the positive control ds cDNA on a 1.2% agarose/EtBr gel with suitable DNA size markers.

Adaptor Ligation

1. Combine the following reagents in a 0.5-ml microcentrifuge test tube at room temperature and in the order shown:

5 µl ds cDNA

2 µl Marathon cDNA Adaptor (10 mM)

2 µl 5X DNA Ligation Buffer

1 µl T4 DNA Ligase (400 units/ml)

10 µl Total volume

2. Mix by vortexing and spin briefly in a microcentrifuge.

3. Incubate at either:

    • 16°C overnight; or

    • room temperature (19–23°C) for 3–4 hr.

4. Heat at 70°C for 5 min to inactivate the ligase.

6. Dilute 1 µl of the positive control reaction mixture with 250 µl of Tris-EDTA Buffer.

7. Store the undiluted adaptor-ligated cDNA at –20°C for future use.

8. Heat the diluted ds cDNA at 94°C for 2 min to denature it.

9. Cool the tube on ice for 2 min.

10. Briefly spin the tube in a microcentrifuge to collect the contents at the bottom of the tube. Store at –20°C until ready for RACE PCR.

PCR

1. Prepare enough master mix for all PCR reactions and 1 extra reaction to ensure sufficient volume. For each 50-ml PCR reaction, mix the following reagents:

    36 µl H2O

    5 µl 10X cDNA PCR Reaction Buffer

    1 µl dNTP Mix (10 mM)

    1 µl Advantage 2 Polymerase Mix (50X)

D. Electroporation for Electrocompetent cells

1. Thaw a tube of cells (XL1 blue) on ice.
2. Add no more than 1µl of ligation mix.
3. Mix gently.
4. Chill a cuvette on ice, then put the cells + ligation mix in cuvette.
5. Keep the cuvettes on ice.
6. Set up electroporator (follow directions for *E. coli* on electroporator) . Wipe off the cuvette so it's dry but still cold.
7. Zap the cells + ligation mix.
8. Immediately add 0.5ml SOC, mix gently, then put it into a culture tube. Put tubes at 37˚ C for around 1 hr at 200rpm in shaking incubator.
9. Plate out various amounts. Save 100µl, so that if you only get blue colonies you can try it without X-gal + IPTG.
10. Put the plates in a 37˚C incubator overnight.

E. Preparation of COS-7 Cell Media

1. For every liter (1000ml) of media to be made, add 1 packet of DMEM media (Gibco BRL, Cat. No. 12100-046).
2. Add 3.7 gm $NaHCO_3$ and pH the solution to 7.20 using dilute HCl. Media can also be prepared using DMEM containing L-glutamine but low glucose (Add 3.5 g glucose and 2.2 gm $NaHCO_3$).
3. Filter sterilize the media using bottle cap filter (0.2 μm).
4. Add 100 ml fetal bovine serum (FBS) (Cat. S-100-05, Lot 03D42, Summit) per 1000 ml DMEM just before use and be sure that it is thoroughly mixed since heavier proteins will settle to the bottom after thawing.
5. Add 10 ml penicillin-streptomycin (P/S) (Cat. 15070-030, Gibco) to each 1000 ml bottle of media.

F. Thawing COS-7 cell stock and cell culture

1. Warm a bottle of DMEM media + FBS + P/S to 37°C. Get one T25 (25 cm^2 flask) and put 5 ml of media into it, remove the desired cell stock from the LN_2 and roll it rapidly between your hands to thaw it.
2. Use the media in the flask to help thaw the cells by removing a small amount, about 0.3 to 0.5 ml, and add it to the vial. This will quickly thaw the remaining frozen portion. Draw that off and put it in the flask.
3. Repeat these steps until the cells are completely thawed and have been added to the flask. Rock the flask a few times to disperse the cells and DMSO.
4. Place in a 37º C, 5% CO_2 incubator. Cells should not be used above a passage of about 20.
5. After the flask has incubated for 24 hours, change the media to remove the DMSO. Monitor cell growth. The media may need to be changed at 48 and 72 hours.

Splitting cells

1. When the COS-7 cells in the flask are > 50% confluent and healthy, it is time to split the cells (usually in a day or two). Prepare enough media (DMEM + FBS + P/S) for 17 ml per 75 cm^2 flask; cells from one 25 cm^2 flask will be transferred to a 75 cm^2 flask.

2. Warm the media and trypsin-EDTA to 37° C in the water bath. Place 15 ml media into each flask. Aspirate the old media. Add 5 ml Trypsin/EDTA, put it into a 37ºC incubator for 5 min.
3. When the cells are loose, aspirate the trypsin. Firmly hit the side of the flask about 5 times with the side of your hand to help loosen the cells. If cloudy areas are visible, hit the flask again a couple of times. Immediately add 6 ml media and mix gently, washing the cell surface with the pipette.
4. Dispense this cell solution into the prepared flasks, 2ml / flask, being sure to gently draw the solution up and down each time to maintain a well mixed solution.

Plating the cells

1. When the flasks are confluent and healthy, the cells are ready to be plated. Have about 0.5 liter DMEM-P/S-FBS media ready, enough for 10 ml per 50 plates. Warm the DMEM and trypsin in the 37° water bath. Place 50 Nunc tissue culture plates under the hood. Add 10 ml media to each plate.
2. Aspirate the old media. Add 5 ml Trypsin/EDTA to each flask, and be sure it coats the bottom of the flask. Incubate in 37º C incubator for 5 min or until the cells come loose in Trypsin, hit the flask wall to detach the cells, and collect the trypsin from all the flasks into one 50 ml falcon tube sitting on ice.
3. When trypsin from all the flasks has been collected, spin the falcon tube at 4000 rpm for 10 min. Aspirate the trypsin, leaving the cell pellet intact and add 5 ml of DMEM-P/S-FBS. Vortex briefly to mix.

4. Prepare 3 plastic 2ml tubes with 890 µl DMEM first and then put 900 µl DMEM in two other tubes. Then add 10µl of Tryptan blue dye in the first tube. Add 100 µl of the 5 ml cell suspension in the first tube and vortex; add 100 µl from the first tube to the second and vortex; and then add 100 µl from the second to the third tube. Count the cells from the first tube using a hemocytometer: (If cells are too numerous to count, use a second or third tube and multiply by the dilution factor). Clean the hemocytometer with 70 % alcohol. Add a well mixed sample to the hemocytometer, just enough to fill the space under the cover slip. Count the square grid in the middle of both sides. Take an average of the two sides:

----If the cell count is ------ 72

72 cells /mm^3 or 7.2 X 10^6 cells/ ml of original suspension (when samples were drawn from first tube)

Add 0.5 million cells per tissue culture plate. Depending upon the concentration, add the appropriate amount of cell suspension to each plate containing 8 ml of DMEM-P/s-FBS, being sure to maintain a well-mixed solution. Mix using a figure-8 motion. Put in the 37º C incubator.

G. Lipid mediated transfection of COS-7 cells

1. When the cells are confluent, it's time to transfect. Wash the cells with 10 ml Ser/Ab free DMEM. Then, add 10ml Ser/Ab free DMEM to each plate.
2. 1: 4 ratio of DNA: Lipid usually works best. I have tried 1:1, 1:2, 1:3 also.
3. For one 100 X 15 mm plate, use 16 µg of DNA and 64 µg of lipid.
4. Based on how many plates you are planning to transfect with a particular kind of DNA, multiply the amount of DNA and Lipid.
5. Eg: for 10 plates: DNA = 160 µg, Lipid = 640 µg
6. Lipid is usually 1µg/µl (check the label), so 640 µl lipid. Calculate the volume of plasmid DNA sample based on the concentration and volume required. Eg. 1µg/µl, 160µl sample required.
7. Add 3 ml transfection reaction / 100mm X 15 mm plate. For 10 plates 30 ml transfection reaction is needed.
8. Add 14,460 µl DMEM (No PS, No FBS) to a 50 ml falcon tube. Add 640µl lipid to it.
9. Add 14,840 µl DMEM (No PS, No FBS) to a 50 ml falcon tube. Add 160 µl DNA to it.
10. Mix these falcon tubes by inverting and then combine both of them in one. Incubate at RT for 15-30min.

11. Add 3 ml of this transfection reaction to each plate, incubate in a 37º C incubator. Check after 4 hours for any signs of cell death. After 24 hrs, change the media to DMEM-PS-FBS. Harvest the cells at 48 hrs.

H. Collecting cells and making pellets for binding experiments

1. When the plates from step 4 are confluent and healthy, the cells are ready to be collected. Put 2 beakers (200ml) into a bucket of ice. In one beaker, put enough 4°C 25 mM Tris for 3ml per plate. The other beaker is for collecting the scraped membranes and Tris. Other items needed are a rubber policeman, 1ml repeater tip and repeater, unplugged glass pipette, Dounce homogenizer, and approximately 6 polypropylene centrifuge tubes.
2. To collect cells, aspirate media from the plates. Add 3 ml cold buffer to the plate. Scrape thoroughly with a cell scraper. Pour the solution into the next plate, and scrape. Do this for 4-5 plates, and then pour this collection into the empty beaker. Scrape all the plates this way.
3. When all plates are scraped, dounce the membrane solution well (using a glass douncer). Evenly divide the cells from 10 plates into 1 centrifuge tube. Spin at 18,000 rpm for 20 min. Discard the supernatants.
4. Two more douncings and more re-suspensions should be done before the cells are used for experiments. Always dounce on ice, and use the buffer to the capacity of the douncer. Do a Bradford assay on the membrane solution to determine the protein content.

5. When the last wash has been completed, discard the supernatants. Label the tubes with the receptor type and date prepared. Cover tubes with 2 or 3 pieces of Parafilm (these break off easily in the freezer) and store the pellets at -70^{o}C.

I. Preparing cell stocks in DMSO (freezing down cells)

1. Prepare new cell stocks from confluent, healthy flasks which have been carried (split) a minimal number of times, i.e., use as low a passage number as possible. The following things are needed: ice bucket, prelabeled cryovials (date, cell line, and initials), a blue cryovial freezie holder, trypsin, media w/ FBS and pen-strep only and sterile DMSO.
2. Place the cryovials in the freezie tray, or on ice, and the media on ice.
3. Aspirate the old media and add 5ml trypsin. Remove the trypsin after a few minutes. Knock the cells loose, add 3.5 ml cold media to the cells. Wash the cells to the bottom of the flask with the media. If there is more than one flask, combine them all into one.
4. Add 10% DMSO (for 3.5ml, add 350µl), mix, and add 1ml to each cryovial, keeping it on ice. This must be carried out quickly, once the cells are exposed to the DMSO, as they start to die. They must be put in the -20°C freezer as quickly as possible.
5. It is necessary to gradually freeze the cell mixture. After a couple of hours in the -20^{o}C freezer, transfer the tubes (on ice) to the -70^{o}C freezer. The tubes need to stay in the -70 for at least a few hours or overnight. Then store the vials with the rest of its kind in the liquid nitrogen carboy.

6. After a new batch of cell stocks has been prepared, it is necessary to test one vial from the batch to be sure that the batch is ok.

J. Bradford Assay

1. Prepare the BSA standard by dissolving 20 mg BSA powder in 1 ml nanopure water. The lysis buffer is HEPES or any other buffer in which the membranes are suspended.
2. To 4 plastic tubes, add the following:

A. 50 µl stock BSA to950 µl Lysis Buffer

B. 125 µl stock BSA to 875 µl Lysis Buffer

C. 250 µl stock BSA to 750 µl Lysis Buffer

D. 375 µl stock BSA to625 µl Lysis Buffer

Vortex the tubes and add 3 µl of each standard to 2 ml diluted Bradford reagent (1:5).Then, add 3µl of each sample to 2 ml diluted Bradford reagent (1:5). Divide the concentration by 3. Multiply the concentration by the amount of sample.

K. Drenchrite® assay set-up procedure

Samples from animals that have not been treated for a period of at least 4–8 weeks should be used.

1. Weigh the amount of feces to be processed. Add an equal amount of tap water to the feces and mix well to break up the pellets or feces using a tongue depressor and pestle.

2. After the sample is mixed well, add another equal volume of water and mix well. If the sample was very moist to start with and is a good homogeneous mixture, 2 volumes of water may be enough. If the feces was very dry to start with, you may need to add a total of 3 volumes of water to get a homogeneous mixture. Record the final amount of water added to calculate a correct egg per gram ratio for the egg calculation. Make sure all fecal pellets are dissolved.

3. Take a sample of the slurry equivalent to 2 gms of feces and do an egg count using the McMaster's technique. For example, if you have added two volumes of water to one volume of feces, take 6 gm of slurry to get 2 gms of feces.

4. Use 10 grams of feces (30-40 grams of slurry) for a culture using the vermiculite culture technique (you can use less than 10 grams if short on feces). Proceed to set up a culture following the usual protocol. This is for observation of the type of larvae present in the sample.

5. Using the McMaster's egg count, calculate the amount of feces slurry required to obtain the eggs needed for the assay. Generally, you want to obtain about 50,000 eggs for the assay.

- For example, if the epg is 1500,you need to take 50,000/1500 = 33.33 grams feces = 99.99 grams slurry.

6. When you are sure you have enough eggs to proceed with the test, take out a DrenchRite plate from the refrigerator and allow it to warm to room temperature. Be sure the plate is properly hydrated. Observe the plate for moisture content and add distilled water, if the plate has dried. Add 10 to 20 µl sterile water to all wells. NOTE: Older plates are generally dried out and need additional water whereas newer plates may be hydrated. Use your judgment as to whether water should be added, but moist agar is essential for the assay to develop properly.

7. Use a 1-liter flask for every 50 grams of fecal slurry, however it is easiest to deal with just one 1-liter flask. Take the slurry mixture to be used and add water to the top of the flask. Allow the solution to "settle" for about 30–60 seconds for the largest chunks to fall to the bottom. Using a #40 sieve (425 µm.) over a 5 qt. bucket, pour the supernatant through the sieve to filter out the largest debris. Using a water bottle, squirt tap water over the debris to wash the eggs through that may be trapped.

8. Repeat this step using a #80 sieve (180 µm.). Again, use the water bottle to wash the debris. Pour the solution into a clean cylinder flasks and allow it to stand for 30 minutes for the eggs to fall to the bottom of the container.

9. Prepare the pump/ filter apparatus for the next filtering step. Use the pump in 2213 and set up a 4000 ml flask to a "trap" flask to ensure no fluid gets sucked into the pump during the next filtering step. Use a Buckner funnel with an 85 μm nylon filter for this next step. Be sure there is a tight seal with the funnel so the filtering will proceed in a timely fashion.

10. After the 30 minute settling time has elapsed, slowly pour off the top half to 2/3rd of the supernatant to discard the excess fluid. CAUTION: Once you stop pouring off the supernatant the eggs get mixed up in the solution. DO NOT continue to pour additional fluid off once you stop pouring. Turn on the pump apparatus and slowly pour the solution through the 85 μm filter, a little at a time. At this step the eggs will pass through the filter and smaller debris will be collected on the filter. You will need to use a tongue depressor to gently scrap the filter as the collected debris will clog up the filter and hinder to passage of the water containing the eggs through to filter. CAUTION: There is a potential for losing a lot of eggs in this step, so the debris collected should be thoroughly washed using the water bottle to wash trapped eggs through the filter.

11. Next, replace the 85 μm. filter with a 30 μm. nylon filter. The 30 μm. filter will allow the passage of the excess water but will trap the eggs. This filtering step may be lengthy as the egg slurry quickly clogs the filter. Again, use the tongue depressor to gently scrap the filter and break the surface tension so the water may pass though. After all the water has filtered through and you are left with the thick slurry containing the eggs, use distilled water to wash the eggs out into a beaker, trying to keep the total volume down to 50 ml. Pour the solution into a 50 ml. Centrifuge

tube and centrifuge at 2000 rpm for 5 minutes. Use a plastic pipette to remove the supernatant so that you are left with a total volume of about 10-15 ml egg slurry for the sucrose gradient step.

12. Prepare the sucrose gradient by taking 10 ml of Yellow sugar solution with a syringe and needle and transferring it into a 50 ml Centrifuge tube. Then add 10 ml Blue sugar solution by placing the tip of the needle at the bottom of the tube and slowly releasing the blue liquid so that it stays below the yellow solution. Then add 15 ml of Red solution as described above so that it remains below the blue sugar solution. The end result should have three distinct colored liquids in the centrifuge tube: yellow on top of blue and red on the bottom. (Reference Kaplan Lab Procedures manual for directions on making the Yellow, Blue, and Red sugar solutions)

13. Carefully place the egg slurry from step 11 over the Sucrose gradient by using a syringe and needle. Centrifuge at 3500 rpm for 7 minutes at 4C using slow acceleration (1 on scale) and deceleration times (1 on scale).

14. Retrieve the layer of eggs from the gradient by using a Pasteur pipette. To ensure that you have actually retrieved the eggs, take two 20 µl aliquots on a glass slide and count the eggs to estimate the actual number of eggs recovered.

- For example, the mean egg count in 20 µl = 60 X 50 = 3000 eggs/ml. If 8 ml is recovered from the gradient, then 3,000 X 8 = 24,000 total eggs recovered.

15. After retrieval of eggs, wash off the sucrose solution. Prepare a small 30 µm. filter apparatus and pour the sucrose solution containing the eggs through it. Using a wash bottle with distilled water, wash the eggs well and rinse off the eggs into a 15 ml tube, using a small funnel.

16. Centrifuge at 2500 rpm for 7 minutes and aspirate off the water leaving a 3 ml volume. Vortex and count two 20µl aliquots. Add additional water as needed so that there are approximately 50 to 70 eggs in each 20 µl of final sample. NOTE: 20 µl of egg sample is needed for each of the 96 wells on the plate. In order to have enough eggs to hatch, you should have about 50 eggs in each well.

- For example, if there is total of 35 eggs in 20µl aliquot, centrifuge the tube again and concentrate the eggs by discarding the excess water. If there are 85 eggs in the 20µl aliquot, dilute the eggs so that there is no crowding of the eggs in the wells.

Calculation – 85 X 20 = 70 X V (V = required diluted volume per 20µl of sample)

V = 85 X 20/70 = 24.28

Therefore dilute every 20 µl of sample to 24.28 µl. If there is a total of 3ml,dilute it to 3 X 24.28/20 = 3.64 ml.

17. Also, “fungizone” should be added to the egg sample to inhibit growth of fungus during the incubation time. Add 90 µl. of fungizone per ml. of egg solution.

18. Add the sample to all wells of the DrenchRite plate. Vortex before taking sample each time to ensure an equal distribution of eggs on the plate.

19. After the sample is added to the plate, label and seal it well with parafilm to avoid excess drying during the incubation time.

20. The next day, check the control wells to ensure that more than 80% of eggs are hatched. Add 20µl of Nutritive Media to each well and reseal them with Para film.
Note- Nutritive Media needs to be diluted by 50% with distilled water.

21. Check the plate daily for drying. Add 10µl distilled water to wells that look dry.

22. End assay on Day 7 by adding 20µl of 50% lugols iodine.

Drench Rite "Washing" Procedure

This is a washing procedure to enhance the reading of the larvae after the iodine has been added to preserve the worms and end the assay after the incubation is completed. Washing can be completed directly following the iodine addition or a few days afterwards.

1. Use a clean 96-well plate that has been scored into quadrants for easier counting.

2. Using the multichannel pipette "auto-mix" setting, "pick-up" 50 µl of distilled water and use it to wash one column in the old DrenchRite wells by "rinsing up and down" several times to dislodge and retrieve the larvae.

3. Transfer the washings to the appropriate column of wells on the clean plate. Aspirate 2 or 3 times to transfer as much fluid containing larvae as possible.

4. Before moving on to the next column, repeat steps 2 and 3 once more with 50 µl of distilled water to transfer any larvae left behind from the first washing.

5. After transferring all the wells, observe the old plate under the microscope to ensure all the larvae are transferred to the new plate. (If more than 5 larvae are left, add about 50 µl of additional water and repeat washing process to retrieve all possible larvae.)

6. Check the new plate under the microscope to observe the wells. Seal the edges with parafilm to prevent drying and label appropriately. The plate may be held at 4 ˚C until counting can be completed.

Agar Culture

When possible, if there are extra eggs after the DrenchRite® assay has been completed, set up an agar culture on days 1-7 using the same amount of eggs in order to observe the hatch rate over time. When setting up agar, add 10 µl of fungizone per 1ml of egg solution. Then add an equal amount of 50% nutritive media as egg solution.

Drenchrite data analysis

1. Count and identify larvae in all control wells to calculate the % development. Then average the number of L1 and L2 larvae. For the L3's, identify them by species and then calculate the averages.

2. Scan the entire plate and count and identify the critical wells in each column as well as the wells on either side of the critical well.

3. Count and identify all L3's on all wells above the critical well to determine the presence of drug resistance.