Mechanisms of anthelmintic resistance in *Cooperia oncophora*, a nematode parasite of cattle

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December 2003

A thesis submitted to McGill University in partial fulfillment of the requirements of the degree of Doctor of Philosophy

Your file Votre référence
ISBN: 0-612-98337-4
Our file Notre référence
ISBN: 0-612-98337-4

ABSTRACT

Anthelmintic resistance is a major problem in livestock, and while it has been slower to emerge in cattle, there are reports of its occurrence. Three broad-spectrum anthelmintics are available for use, and one mechanism of resistance that is common to all is target site alteration. Glutamate-gated chloride channels (GluCls) are an important target for macrocyclic lactone anthelmintics (MLs), while beta-tubulin represents the benzimidazole (BZ) target. The objectives of this thesis were to determine whether GluCls are involved in ML resistance in the cattle parasite *Cooperia oncophora*, and whether beta-tubulin is involved in BZ and ML resistance. Two isolates of *C. oncophora* were used. In a fecal egg-count reduction test, ivermectin was found to be 100% effective against one isolate (IVS), and only 77.8% effective against the second isolate (IVR). Two full-length GluCl cDNAs, encoding GluClα3 and β subunits, were cloned. These subunits share high sequence identity with similar GluCl subunits from *Haemonchus contortus* and *Caenorhabditis elegans*. Genetic variability analysis of the two genes showed significant differences in allele frequencies between IVS and IVR worms at the GluClα3 gene, but not the GluClβ gene, suggesting that the GluClα3 gene is involved in ivermectin resistance. Sequencing of full-length GluCl subunit cDNAs from IVS and IVR worms revealed the presence of mutations in the N-terminal domains. Mutations in the GluClα3 caused modest but significant reductions in glutamate, ivermectin and moxidectin sensitivity, while mutations in the GluClβ abolished glutamate sensitivity. Of the three mutations identified in the IVR GluClα3, the L256F mutation accounted for the difference in glutamate and ivermectin response between IVS and IVR GluClα3 channels. Two beta-tubulin isotypes cloned from *C. oncophora* were found to share a high homology with beta-tubulin isotypes from other trichostrongylids. Genetic variability analysis of the two isotype genes revealed no association between beta-tubulin and ivermectin resistance. However, a small proportion of the IVR worms were found to carry the BZ resistance allele. The results of this thesis demonstrate the involvement of the GluCls in ivermectin resistance in *C. oncophora*, and the presence of a BZ resistance allele similar to that identified in trichostrongylids of sheep.

AGRÉGÉ

La résistance anthelminthique est un problème d'importance et quoique ce phénomène ait été plus lent à apparaître chez les bovins, on retrouve des comptes rendus de son occurrence. Trois anthelminthiques à spectre étendue sont disponibles dont le mécanisme de résistance commun est l'altération de récepteurs cibles. Les canaux de chlorure glutamate dépendants (GluCls) sont une cible importante des anthelminthiques à lactones macrocycliques (MLs), tout comme l'est la tubuline-bêta pour les benzimidazoles (BZ). Les objectifs de cette thèse étaient de démontrer que les GluCls jouent un rôle au niveau de la résistance aux MLs du parasite des bovins, *Cooperia oncophora*, et que la tubuline-bêta est impliquée dans la résistance aux BZ et MLs. Deux isolats de *C. oncophora* ont été utilisés. Lors d'un test coproscopique, *Feacal egg count reduction test*, l'ivermectine a eu une efficacité de 100% contre un des isolats (IVS), et de seulement 77,8% contre l'autre isolat (IVR). L'ADNc complet des GluCls qui encodent GluClα3 et les sous-unités β ont été clonés. Ces sous-unités partagent une grande identité séquentielle avec des sous-unités GluCls similaires d'*Haemonchus contortus* et *Caenorhabditis elegans*. L'analyse de la variabilité génétique de ces deux gènes a démontré des différences significatives dans la fréquence des allèles du gène GluClα3 entre des vers adultes IVS et IVR mais non avec le gène GluClβ. Ceci suggère que le gène GluClα3 joue un rôle dans la résistance à l'ivermectine. Le séquençage de l'ADNc complet de la sous-unité GluCl de vers adultes IVS et IVR a révélé la présence de mutations dans le domaine N-terminal. Les mutations de GluClα3 causent de modestes réductions significatives à la sensibilité au glutamate, à l'ivermectine et à la moxidectine. Cependant les mutations chez GluClβ ont uniquement supprimé la sensibilité au glutamate. Des trois mutations identifiées chez GluClα3 IVR, la mutation L256F explique le type de réaction des GluClα3 IVS et IVR à l'ivermectine et le glutamate. Deux isotopes tubuline-bêta clonés de *C. oncophora* partagent une forte homologie avec les isotypes tubuline-bêta d'autres trichostrongles. L'analyse de la variabilité génétique de ces deux gènes isotypes n'a révélée aucune association entre la tubuline-bêta et la résistance à l'ivermectine. Cependant, une petite proportion des vers adultes IVR présentait des allèles de résistance aux BZ. Les résultats

de cette thèse démontrent la participation des GluCls à la résistance à l'ivermectine chez *C. oncophora* et la présence d'un allèle de résistance aux BZ similaire à celle qui a été identifiée chez les trichostrongles du mouton.

ACKNOWLEDGEMENTS

I would like to thank my advisor, Roger Prichard, for giving me the opportunity to study in Canada, where I've learned a great deal, both in and out of the lab. I am truly grateful for his mentorship and financial support. Other people I'd like to thank: Christiane, Marcelo and Yaroslav for their help with identifying and isolating the *Cooperia* eggs and larvae; Christiane, for translating my abstract; Shirley, for help with administrative matters; Gordy, for looking after the calves for all those years; Yeu-jin, for patiently teaching me the basics of molecular biology; Jeff, for being a great person to work with, for the useful discussions we've had over the years, and for sharing some valuable 'quick and easy' techniques; Dr. David Zadworny and his student, Babu, for help with the SSCP technique: Dayle, for her encouragement, for being a great friend, and a joy to be around; Xiao-peng and Bernie, for their insight, support and friendship- I am indebted to Bernie for kindly hosting me during the defense period; Sean and Bill, for taking the time to share their expertise when I started; Guan hua, Cat, Meredith, Jie, Yemisi, Megan, Anne, Amy, Mike, and Alain, for their friendship, and for the many discussions and laughs that we have shared; Sean and Alain, for helping me with the data analysis; Peter and Aws, for their friendship and kindness; Dr. Jim Smith, for help with computer programs; Dr. Jon Hayashi, for his tireless determination to get the McGill-FMC Agreement signed, and, with Lyle, for making electrophysiology such an interesting subject; Dr. Armando Jardim, for suggestions regarding the functional analysis of the GluCl receptors: Dr. Paula Ribeiro, for giving me the opportunity to be a TA for the biotech course, where I learnt a lot through interacting with the students; Mum, Dad, my siblings Larry, Jennifer,

Tony, Nana and Kaari, for showing an interest in my work, for their encouragement, love and prayers; and my husband, Trevor, for his patience, love and support.

In memory of my late grandfather,

Paul Njagi

CONTRIBUTIONS OF AUTHORS

All experiments were designed and performed by the author, under the supervision of Prof. Roger Prichard. All data presented were obtained by the author, and all analyses were performed by the author. For the electrophysiology experiments (Chapter 4), Dr. Xiao-Peng Feng, who co-authored the paper, made key suggestions on the experimental design. The experiments were carried out at the FMC labs in New Jersey, under the expert guidance of Dr. Jon Hayashi and Lyle Kinne, who helped with frog dissections and recordings. The degenerate primers used for the initial isolation of a GABAβ subunit fragment (Appendix A) were designed by Dr. Xiao-Peng Feng. All other primers were designed by the author. Animal infections and treatments were carried out by the author, with the assistance of Dayle Eshelby. This thesis was written by the author with editorial contributions from Prof. Roger Prichard.

CONTRIBUTION TO KNOWLEDGE IN THE FIELD

In this thesis, we report the cloning of six full-length *C. oncophora* cDNAs, which have not previously been reported. The sequences have all been deposited in GenBank. This is the first study of mechanisms of anthelmintic resistance in a cattle nematode.

Glutamate-gated chloride channels (GluCls) have been implicated in ivermectin resistance in *Caenorhabditis elegans* and *Haemonchus contortus*, and here, we demonstrate that they are also involved in ivermectin resistance in *C. oncophora*.

Functional analysis of heterologously expressed *C. oncophora* GluCl subunits demonstrated that mutations affect agonist sensitivity. The findings that *C. oncophora* GluCl channels appear to be more sensitive to moxidectin than ivermectin, though ivermectin resistance selects for moxidectin resistance, are significant. Because of the higher sensitivity of moxidectin, this drug may be useful against ivermectin-resistant parasites.

Analysis of *C. oncophora* beta-tubulin isotype 1 demonstrated the presence of a mutation that has previously been shown to confer benzimidazole resistance in nematodes of sheep.

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APPENDIX A

INTRODUCTION

The benzimidazoles and the macrocyclic lactone (ML) anthelmintics are broad spectrum anthelmintics that are widely used to treat helminth infections in domestic animals. The benzimidazoles act by binding to beta-tubulin, thereby interfering with microtubule-based processes such as cell division. The MLs target chloride channels gated by glutamate and gamma aminobutyric acid (GABA) and interfere with neurotransmission. These chloride channels belong to the cys-loop superfamily of receptors, which mediate fast chemical neurotransmission, and include the nicotinic acetylcholine receptors and the mammalian glycine receptors.

The effectiveness of anthelmintics in controlling helminth infections is threatened by the development of resistance. Resistance is widespread in nematodes of sheep, and has been slower to develop in cattle parasites. However, there are reports of anthelmintic resistance in cattle. Benzimidazole resistance has been found in several cattle nematode species, including *Cooperia oncophora*, *Ostertagia ostertagi* and *Trichostrongylus axei*. With ML resistance, all cases that have been reported so far involve *Cooperia* species. The mechanisms of benzimidazole resistance are well established, and are known to involve the target, beta-tubulin. ML resistance mechanisms are less well understood. The main objective of this thesis is to determine the role of the glutamate-gated chloride channel (GluCl) genes in ML resistance in *Cooperia oncophora*. As well, the role of beta-tubulin in benzimidazole and ML resistance is explored.

A review of what is currently known about the modes of action and mechanisms of resistance to the benzimidazoles and MLs is presented in Chapter 1. Information

relating to anthelmintic use in cattle is also provided. Chapter 2 describes the ivermectin susceptibility and resistance status of the two *C. oncophora* field isolates used throughout the study. The two isolates differ in their sensitivities to ivermectin, with one being 100% susceptible, and the other, resistant, providing a basis for comparison of the two at the genetic and functional levels for changes which can be linked to ivermectin resistance. Chapter 3 describes the cloning of two GluCl genes from *C. oncophora*, and the genetic variability data presented provides indirect evidence that one of these genes, GluClα3, may be involved in ivermectin resistance in this nematode. Previous studies have shown that the *C. elegans* orthologue of this subunit has a binding site for ivermectin. Chapter 4 provides direct evidence that ivermectin interacts with receptors formed by the *C. oncophora* GluClα3 subunit in a heterologous expression system, and that mutations found in GluCl subunits of ivermectin-resistant worms significantly reduce agonist sensitivity.

Beta-tubulin has been implicated in ivermectin resistance in *Haemonchus contortus*, a nematode parasite of sheep. Chapter 5 describes the cloning of two *C. oncophora* beta-tubulin isotypes cDNAs. While no association was found between beta-tubulin and ivermectin resistance in *C. oncophora*, the benzimidazole resistance-associated mutation was found in one of the field isolates. In Chapter 6, the findings presented in this thesis are discussed in light of what is currently known about cys-loop receptors, as well as anthelmintic resistance.

CHAPTER 1

LITERATURE REVIEW

1.1 *Cooperia oncophora*

Cooperia oncophora is a parasitic gastrointestinal nematode of cattle belonging to the superfamily *Trichostrongyloidea.* It is a common and economically important nematode in temperate regions (Coop et al. 1979; Kloosterman et al. 1984; De Graaf et al. 1992; Parmentier et al. 1995). It is found in the small intestine, and has a direct life cycle, with free-living first and second larval stages, and an infective third larval stage (L3) (Bowman et al. 2003). The first and second stage larvae feed on bacteria, while third-stage larvae, which retain the cuticle of the second-stage larvae during the second molt, do not feed. Infection is by ingestion of L3, which exsheath in the abomasum before reaching their predilection site. Once in the small intestine, they penetrate the crypts between the villi, and while in the crypts, molt twice to reach the immature adult stage (L5). Maturation to adult stage (from ingestion) takes 17-22 days (Isenstein 1963). *C. oncophora* is a lumen-dwelling nematode that, as an adult, lives in the crypts between the villi, or attaches to the mucus lining the intestine (Herlich 1965). However, the fourth-stage larvae penetrate the intestinal mucosa during development, contributing to the pathology of infection (Armour et al. 1987; Parkins et al. 1990). While worms are typically restricted to the anterior part of the small intestine during infection, they can also be found in the ileum and jejunum when infection levels are high (Armour et al. 1987; Parkins et al. 1990; Bowman et al. 2003).

Immunity to *Cooperia* infections develops with age, and young animals up to two years of age are most at risk of succumbing to infection (Armour 1989; Kloosterman et al. 1991; Satrija and Nansen 1993). However, mature cattle that have not been exposed to sufficient contact with worms remain susceptible to infection. Mature animals that

have acquired immunity harbor small populations of nematode parasites that are a source of constant pasture contamination (Corwin 1997). *C. oncophora* infections are usually sub-clinical, limiting weight gains in young growing calves, and can lead to poor production in later years (Herlich 1965; Ploeger et al. 1990; Hawkins 1993). In older dairy animals, sub-clinical parasitism reduces milk production (Thomas et al. 1984; Bisset et al. 1987a, b; Gross et al. 1999). Massive infections can, however, cause clinical disease, and the severity of the pathological changes and clinical signs correlates directly with the level of infection (Herlich 1965; Armour et al. 1987). A tentative diagnosis of helminth infections in general can be made based on the clinical signs, and confirmed by demonstrating strongyle-type eggs in feces (Fraser 1991). While *Cooperia* eggs can usually be differentiated from those of other trichostrongylid species by their shape, a definitive diagnosis of *Cooperia* infection can only be made by culturing feces and identifying the L3 stage (Fraser 1991). However, for treatment purposes, and when anthelmintic resistance is not an issue, this is not warranted, since the broad spectrum anthelmintics used are effective against all trichostrongylid species (Fraser 1991).

1.2 Control of Gastrointestinal Helminths Using Anthelmintics: Its Effect on the Development of Immunity

Control of gastrointestinal helminths, among them *C. oncophora*, relies on the use of broad-spectrum anthelmintics. These drugs are often used prophylactically to limit production loss caused by gastrointestinal parasites, and to reduce pasture contamination by limiting fecal egg output (Vercruysse et al. 1994, 1995). With the strategic use of these highly effective drugs, clinical parasitism is rare in the developed world, and

economic losses are mostly associated with sub-clinical parasitism (Corwin 1997; Gasbarre et al. 2001; Vercruysse and Claerebout 2001). However, suppression of nematode infections through intensive treatment of calves reduces host-parasite contact and interferes with the development of immunity during the first grazing season (FGS) (Kloosterman et al. 1991; Ploeger and Kloosterman 1993; Vercruysse et al. 1994, 1995; Claerebout et al. 1999; Claerebout 2002). Consequently, unexposed animals have heavier worm burdens in their second grazing season (Vercruysse et al. 1994; Satrija et al. 1996; Claerebout et al. 1999). Unexposed animals are susceptible to infection when they are older, and when anthelmintics are not usually being used to control helminth infections (Ploeger and Kloosterman 1993; Dorny et al. 2000). Reducing the number of treatments during the grazing season affords some exposure to parasites, thus allowing immunity to develop (Vercruysse et al. 1995). While one or two treatments a year are sufficient to protect FGS calves against the effects of parasitism, reports show that anthelmintic use tends to be excessive (Ploeger and Kloosterman 1993; Ploeger et al. 2000; Claerebout 2002), and that on average, farmers give up to four treatments a year (Vercruysse and Dorny 1999). Bisset et al. (1987a, b) have shown that, in an all-year grazing management system, fewer curative calf treatments have more positive production effects on herds than regular preventive treatments. As well, fewer treatments cost less, and reduce the selection for anthelmintic resistance (Vercruysse and Claerebout 2001; Claerebout 2002).

While chemoprophylaxis may interfere with the development of immunity, some studies have reported that the effects of reduced immunity are limited, since animals in their second grazing season are exposed to light infections on pasture (Conder et al. 1983;

Guldenhaupt and Burger 1983; Jacobs et al. 1987; Claerebout et al. 1998). Under low levels of challenge, previously treated (unexposed) animals rapidly acquire immunity with exposure, and appear not to suffer the loss-producing effects of parasitism (Satrija et al. 1996; Suarez et al. 2001). However, with high larval challenge, untreated animals that have developed resistance to infection show significantly higher live-weight gains than previously treated (unexposed) animals (Suarez et al. 2001). Additional treatment of previously treated (unexposed) animals may be necessary to prevent sub-clinical losses and to limit pasture contamination, as these animals also have higher fecal egg counts (Suarez et al. 2001).

1.3 Control of Gastrointestinal Helminths: Anthelmintics Used in Cattle

Three classes of broad spectrum anthelmintics are used to control gastrointestinal helminth infections in cattle:- the benzimidazoles, the macrocyclic lactones, and the tetrahydropyrimidines and imidazothiazoles. Only the first two groups, which are dealt with in this thesis, will be discussed.

1.4 The Benzimidazoles and their Target Site

The benzimidazoles were introduced in the early 1960s and have been widely used in the human, veterinary and agricultural fields (Campbell 1990; Cook 1990; Horton 1990; Baraldi et al. 2003). They were the first group of anthelmintics on the market to show a broad spectrum of activity, a wide margin of safety, and efficacy against immature stages, and were used in strategic control programs to increase performance and maximize profits (Campbell 1990). Several benzimidazoles are available as oral formulations for use in cattle, including albendazole, oxfendazole and febendazole (Bowman et al. 2003).

The benzimidazoles exert their anti-parasitic effect by binding directly to beta-tubulin and preventing its polymerization into microtubules, thereby affecting various microtubule-based processes, which include cell division and intracellular transport (Lacey 1988). Initial studies on the mode of action of benzimidazoles suggested various mechanisms, including inhibition of fumarate reductase (Prichard 1973), inhibition of monoamine oxidase activity (Moreno and Barrett 1979), interference with glucose uptake (Van den Bossche and De Nollin 1973) and altered metabolic pathways (Sangster and Prichard 1984). These are thought to result from failure in microtubule function. The inhibitory effect of benzimidazoles on tubulin polymerization was first indicated by studies in fungi (Davidse 1973; Hammerschlag and Sisler 1973), and confirmed by cytological studies in mammalian cell lines and in rats (Styles and Garner 1974), and in the nematode *Ascaris suum* (Borgers and De Nollin 1975). Subsequently, Friedman and Platzer (1980) provided the first evidence of the higher affinity, and hence selective toxicity, of benzimidazoles for nematode tubulin as compared to mammalian tubulin.

Six beta-tubulin genes have been identified in the free-living nematode *Caenorhabditis elegans* (Gogonea et al. 1999). One of these, mec-7 (tbb1), is expressed in the touch neurons, and, along with the alpha-tubulin isotype mec-12, is thought to influence the unique microtubule architecture in these neurons (Savage et al. 1989; Savage et al. 1994; Fukushige et al. 1999). In *Drosophila melanogaster*, the beta 2 isotype is expressed only in the testis, and its spermatogenic-specific functions cannot be performed by another *D. melanogaster* isotype, beta-3, or by a beta-2 ortholog from the moth *Heliothis virescens* (Hoyle and Raff 1990; Raff et al. 1997).

Among trichostrongylids, two distinct beta-tubulin isotypes have been described which can be differentiated based on the sequence at the carboxy terminal (Geary et al. 1992; Elard et al. 1996; Grant and Mascord 1996). The expression pattern and functional significance of these two isotypes are unknown. In vertebrates, seven beta-tubulin isotypes have been identified which show tissue-specific distribution and different relative stabilities (Luduena 1993; Roach et al. 1998; Schwarz et al. 1998). This suggests that isotypic differences may be important in regulating microtubule function *in vivo* (Schwarz et al. 1998). The isotype-defining carboxy termini of the different isotypes are located on the surface of the tubulin molecule, and may interact differently with cellular factors (Nogales et al. 1998). Tubulin performs a wide variety of functions, and the various isotypes may differ in their abilities to fulfill these functions (Roach et al. 1998; Burkhart et al. 2001).

1.5 The Macrocyclic Lactones and their Target Sites

1.5.1 The Macrocyclic Lactones

The macrocyclic lactones (MLs) are broad spectrum anthelmintics that are widely used to control nematode infections in humans and animals (Campbell et al. 1983). They include the avermectins and milbemycins. The avermectins are natural fermentation products of the soil-dwelling actinomycete *Streptomyces avermectilis* (Campbell et al. 1983). Avermectins produced by this organism are a mixture of eight compounds, designated A_1a, A_1b, A_2a, A_2b, B_1a, B_1b, B_2a and B_2b (Campbell 1989). The B_1 homologs are the most potent and also have the broadest spectrum of activity among nematodes. The a and b homologs have almost identical activities, although the former are produced in greater amounts (Campbell 1989).

Ivermectin (22, 23-dihydroavermectrin B1) was the first ML to be developed for commercial use. Following its introduction in 1981, ivermectin has been widely used to treat nematode infections in domestic animals, and has been described as 'the most commercially successful veterinary parasiticide ever produced' (Raymond and Sattelle 2002). With its novel mode of action, ivermectin was effective against helminths of sheep that showed resistance to existing compounds (Shoop et al. 1995). In humans, ivermectin is used to control onchocerciasis and lymphatic filariasis (Campbell 1991; Brown et al. 2000). Ivermectin itself is a semi-synthetic derivative of avermectin B1 (Chabala et al. 1980). It is highly effective at a low dose, has a wide safety margin, and a broad spectrum of activity against a range of ecto- and endo-parasites, including nematodes (Table 1) (Campbell et al. 1983). However, these parasites develop at

different times, and ML's are used more frequently for effectiveness against endo- and ecto-parasites (Gasbarre et al. 2001). Ivermectin is not effective against trematodes and cestodes.

Table 1. Ivermectin given subcutaneously at 200 μg kg^{-1} is effective against a wide range of endo- and ecto-parasites in cattle. Adapted from Shoop et al. (1995).

NEMATODA	ARTHROPODA
Gastrointestinal worms	**Cattle grubs**
Bunostomum phlebotomum	*Dermatobia hominis*
Cooperia oncophora	*Hypoderma bovis*
Cooperia pectinata	*Hypoderma lineatum*
Cooperia punctata	
Cooperia species	**Screwworm fly larvae**
Haemonchus placei	*Chrysomya bezziana*
Mecistocirrus digitatus	*Cochliomyia hominivoraz*
Nematodirus helvetianus	
Nematodirus spathiger	**Sucking lice**
Oesophagostomum radiatum	*Haematopinus eurysternus*
Ostertagia lyrata	*Linognathus vituli*
Ostertagia ostertagi	*Solenopotes capillatus*
Strongyloides papillosus	
Toxocara vitulorum	**Biting lice**
Trichostrongylus axei	*Damalinia bovis*
Trichostrongylus colubriformis	
Trichuris spp.	**Mange mites**
	Psoroptes ovis
Lungworms	*Sarcoptes scabiei var. bovis*
Dictyocaulus viviparous	*Chorioptes bovis*
Skin worms	**Ticks**
Parafilaria bovicola	*Boophilus microplus*
	Boophilus decoloratus
Eyeworms	*Ornithodorus savignyi*
Thelazia spp.	

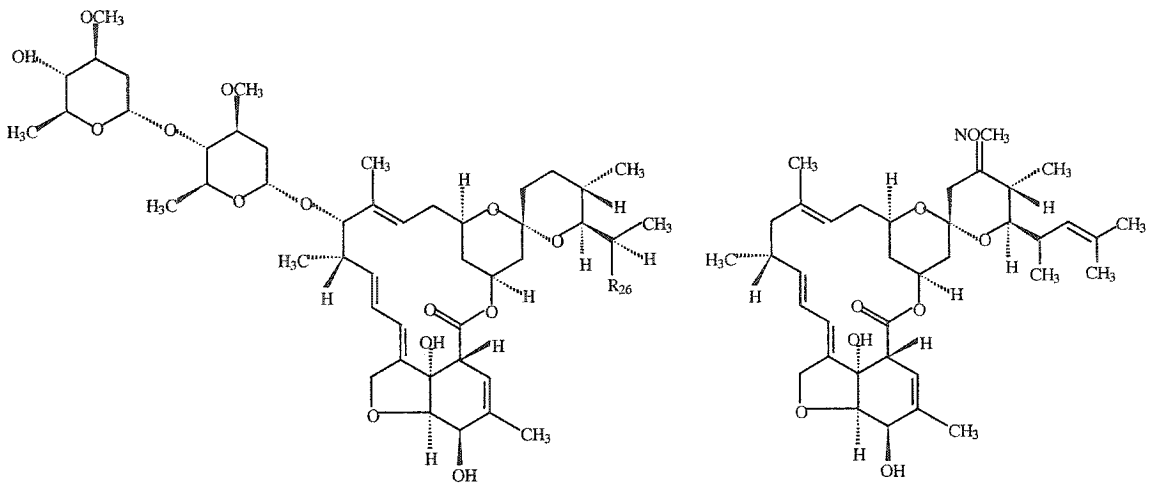
Ivermectin is available in topical, injectable (subcutaneous), and oral (liquid, paste, and slow-release bolus) formulations for use in cattle (West et al. 1994; Vermunt et al. 1995). Injectable and oral formulations are given at a dosage level of 0.2 mg/kg body weight, while the topical formulation is given at 0.5 mg/kg body weight (Campbell 1989). Cattle seem to tolerate up to a maximum dose of 1 mg/kg ivermectin, above which animals show signs of central nervous system depression, including ataxia, which progress to paresis, recumbency, decreased lip and tongue tone, drooling, coma and death (Campbell 1989). Administration of propylene glycol, in which ivermectin is diluted, at 30 times the use level (6 mg/kg) causes no ill effects (Campbell 1989).

Although ivermectin has broad spectrum activity against gastrointestinal nematodes, its efficacy varies with target species (Shoop et al. 1996a). Ivermectin has better activity against large intestinal- and abomasal- dwelling parasites than against those in the small intestine of sheep and cattle. The topical formulation has been found to have lower-than-expected efficacy against *Cooperia* species in cattle (Bisset et al. 1990; Eagleson and Allerton 1992; West et al. 1994; McKenna 1995). *C. oncophora* and *Nematodirus helvetianus*, both small intestinal parasites, are considered the dose limiting species for ivermectin in cattle, and the recommended dosage is determined based on efficacy against these species (Egerton et al. 1981; Shoop et al. 1995). Ivermectin's lower efficacy against small intestinal nematodes may not be related to the drug concentration available at the target site, since concentrations of ivermectin in abomasal and in small intestinal mucus are similar (Bogan and McKellar 1988). It is possible that small intestinal nematodes may recover from ivermectin's temporary paralytic effects and

re-establish in a more distal location in the small intestine (Bogan and McKellar 1988; Bogan et al. 1988).

Other avermectins that are available for treatment of internal and external parasites in cattle include doramectin and eprinomectin (Shoop et al. 1996b; Bowman et al. 2003). Eprinomectin is unique among avermectins in that it can be used in lactating animals, since it exhibits minimal residue in milk (Shoop et al. 1996a). It is available as a convenient topical formulation, and retains the high potency and wide spectrum of activity that is characteristic to the avermectins (Shoop et al. 1996b). The availability of such anthelmintics with zero withholding time, which can be used in lactating dairy cattle to increase production by alleviating sub-clinical parasitism, has consequences for the development of anthelmintic resistance (Reinemeyer 1995; Morris et al. 2002).

The milbemycins are closely related to the avermectins. Moxidectin, the only milbemycin used in cattle, is available in topical (0.5 mg/kg) and injectable (0.2 mg/kg) formulations (Rock, 2001). It is a chemically altered derivative of F-alpha, a fermentation product of *Streptomyces cyaneogriseus noncyanogenus* (Bowman et al. 2003). While ivermectin and moxidectin are similar in structure, differences include a dissacharide at carbon-13 unique to ivermectin, a methoxine moiety at carbon-23 found only in moxidectin, and different side chains in the two compounds at carbon-25 (Figure 1). Moxidectin's range of activity is similar to that shown by ivermectin. However, compared to ivermectin, moxidectin shows longer persistence and higher efficacy against tissue-dwelling nematodes, while ivermectin is more potent against ectoparasites (Shoop et al. 1995). As with ivermectin, the two dose-limiting species for moxidectin in cattle are *C. oncophora* and *N. helvetianus* (Ranjan et al. 1992; Scholl et al. 1992).



Ivermectin Moxidectin

Figure 1. Chemical structures of ivermectin and moxidectin. Adapted from Rock, 2001.

1.5.2 Biological Effects of Macrocyclic Lactones on Nematodes

The effects of MLs have been described in several nematode species. Most experiments have been carried out using ivermectin or avermectin. Avermectin induces irreversible paralysis of somatic musculature in *A. suum*, *C. elegans* and *Haemonchus contortus* (Kass et al. 1980; Geary et al. 1993). In *H. contortus*, the ivermectin-induced paralysis is restricted to the mid-body of the worm, with the head and tail sections showing normal movement (Geary et al. 1993). Ivermectin has also been shown to inhibit motility of the free-living stages of several trichostrongylid species, including *H. contortus*, *Trichostrongylus colubriformis* and *Ostertagia circumcincta* (Ibarra and Jenkins 1984; Gill et al. 1991). Ivermectin's inhibitory effect on motility is the basis of an *in vitro* larval motility assay designed to detect resistance in *H. contortus* (Gill et al. 1991).

Ivermectin also inhibits feeding in adult nematodes, including *T. colubriformis*, *H. contortus* and *C. elegans* (Bottjer and Bone 1985; Avery and Horvitz 1990; Geary et al. 1993). The inhibition of feeding is due to specific inhibition of pharyngeal pumping by ivermectin (Martin 1996). The pharyngeal muscle is far more sensitive to the effects of ivermectin than somatic musculature, and concentrations of ivermectin required to inhibit pharyngeal pumping are 100-fold lower than those required to inhibit motility in *H. contortus* (Geary et al. 1993). This suggests that the pharynx is the main target for the MLs. Inhibition of pharyngeal pumping has also been demonstrated in *H. contortus* larval stages (Kotze 1998). Ivermectin is also a potent inhibitor of larval development (Gill et al. 1995). This occurs at the L1 stage at a concentration of ivermectin comparable to that which inhibits pharyngeal pumping. Therefore, it is possible that inhibition of pharyngeal pumping caused by ivermectin results in starvation, which then leads to inhibition of larval development (Gill et al. 1995).

Several anthelmintics, including ivermectin, levamisole and oxfendazole, suppress oviposition in *T. colubriformis* (Bottjer and Bone 1985). Ivermectin was found to have the most pronounced effect in both *in vivo* and *in vitro* tests (Bottjer and Bone 1985). Ivermectin's effect on fertility has also been observed in *C. elegans*, where it not only inhibits the release of eggs already present in the uterus, but it also suppresses the production of new eggs (Grant 2000). Ivermectin also suppresses the production of new microfilaria by *Onchocerca volvulus* females for up to 12 months after treatment (Awadzi et al. 1985; Duke et al. 1991). A similar effect has also been observed in the dog filarid *Dirofilaria immitis* following treatment with milbemycin D (Sasaki and Kitagawa 1993).

Among filarial nematodes, therefore, the reproductive tract appears to be an important target for MLs.

1.5.3 Ivermectin Sites of Action- the GABACls

Several anthelmintic compounds, including the MLs, organophosphates, imidazothiazoles, tetrahydopyrimidines and piperazine, exert their nematocidal effects by interrupting neuromuscular transmission (Sangster 1996). One of the earliest studies on ivermectin's mode of action suggested that the drug acted on a chloride channel, since it induced paralysis of lobster stretcher muscle by increasing the membrane permeability to chloride ions (Fritz et al. 1979). Ivermectin's effect was blocked by the γ-aminobutyric acid (GABA) antagonist picrotoxin, and the authors suggested that the drug was acting on chloride channels gated by GABA. Other electrophysiological studies in the nematodes *A. suum* and *C. elegans* showed that avermectin blocked transmission between interneurons and excitatory motor neurons, leading to paralysis of the somatic musculature (Kass et al. 1980). Similar effects were produced by the GABAergic agonists muscimol and piperazine, and as with avermectin, the effects were reversed by picrotoxin (Kass et al. 1984). These results suggested that avermectin was acting as a GABA agonist at a GABA receptor. The experiments of Holden-Dye and Walker (1990) suggested that ivermectin was acting as a GABA antagonist on somatic muscle cells of *A. suum*. In all these studies, the high (micromolar) concentrations of ivermectin required to elicit an effect were inconsistent with the high *in vivo* potency of this drug (Geary et al. 1993). Work by Duce and Scott (1985) showed that, in addition to activating GABA-sensitive chloride channels, low ivermectin concentrations increased chloride

conductance in GABA-insensitive muscle bundles. The glutamate analog ibotenate produced a dose-dependent increase in chloride conductance in these GABA-insensitive bundles, and demonstrated that ivermectin was interacting with chloride channels that were gated by glutamate. These receptors, which were originally identified as H- (hyperpolarization) receptors (Cull-Candy and Usherwood 1973; Lea and Usherwood 1973a, b), are widely distributed on locust muscle cells (Cull-Candy 1976).

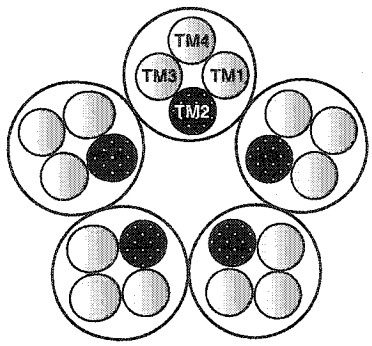
1.5.4 GABA- and Glutamate-gated Chloride Channels Belong to the 'cys loop' Superfamily of Ligand Gated Ion Channels

GABA- and glutamate-gated chloride channels (GABA-Cls and GluCls) are ligand gated ion channels of the 'cys loop' superfamily of receptors, which mediate fast chemical neurotransmission (Raymond and Sattelle 2002). Members of this superfamily include nicotinic acetylcholine (nAch), serotonin (5-HT_3), $GABA_A$ and glycine receptors, and the recently characterized serotonin (MOD-1) and histamine-gated chloride receptors (Ortells and Lunt 1995; Ranganathan et al. 2000; Gisselmann et al. 2002; Zheng et al. 2002). The nAch and 5-HT_3 receptors are excitatory cation channels, and the GluCls, glycine, $GABA_A$, MOD-1 and histamine receptors are inhibitory chloride channels. GluCls are found only in invertebrates, while glycine-gated chloride channels have been found only in vertebrates (Vassilatis et al. 1997b). MOD-1 and histamine chloride channels have been found only in *C. elegans* and arthropod photoreceptors, respectively (Hardie 1989; Ranganathan et al. 2000; Gisselmann et al. 2002; Zheng et al. 2002). Phylogenetic analysis shows that the GluCls are a distinct group within the ligand-gated ion channel

superfamily, and are most closely related to the vertebrate glycine-gated chloride channels (Vassilatis et al. 1997b).

The cys-loop receptors are believed to be structurally similar, and are made up of five subunits that are arranged together around a central pore (Figure 2A). This characteristic image was first derived from electron microscopic images of nAchR from the electric fish, *Torpedo* (Brisson and Unwin 1985). Native receptors are heteropentameric in structure, though some nAchR subunits like α7 and α8 can form homopentameric receptors *in vitro* which display pharmacological properties resembling those of the native receptor (Karlin et al. 1983; Bechade et al. 1994; Gerzanich et al. 1994; Chang et al. 1996). The different subunits are encoded by closely related genes, and the diversity of native receptors is increased by alternative splicing of these genes (Whiting et al. 1990). Each subunit is made up of a number of domains; an N-terminal region, which is located extracellularly and carries the ligand binding domain, four transmembrane domains, with the second transmembrane domain lining the channel pore, a long intracytoplasmic loop between the third and fourth transmembrane domains, and an extracellular carboxy terminal (Figure 2B) (Unwin 1989; Galzi et al. 1992). Two cysteines, separated by 13 amino acid residues, are found in the extracellular N-terminal domain of all subunits belonging to the cys-loop superfamily (Kao and Karlin 1986; Ortells and Lunt 1995). As well, in GluCls, glycine- and histamine-gated chloride channels, a second pair of cysteine residues, separated by 10 residues, is found before the first transmembrane domain (Vassilatis et al. 1997b; Zheng et al. 2002).

A.



B.

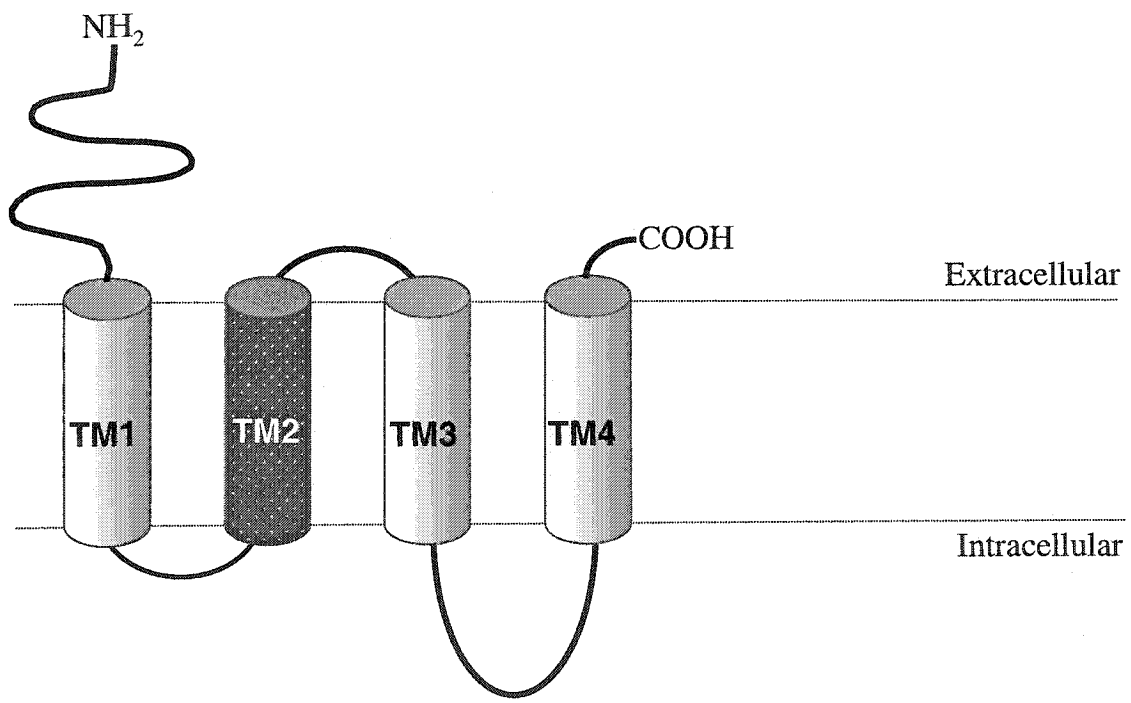


Figure 2. Topological organization of cys-loop receptors. **A.** The five subunits that make up the receptor are arranged around a central pore, with the TM2 of each subunit lining the channel pore. **B.** Each subunit is made up of a large N-terminus, four transmembrane domains (TM1-4), an intracytoplasmic loop between TMs 3 and 4, and a short C terminus. Adapted from Cherubini and Conti (2001).

The nAchR is the most extensively characterized member of the ligand gated ion channel superfamily, and much of what is known about the agonist binding sites of the cys-loop receptors comes from studies of this receptor (Miyazawa et al. 1999; Sine 2002). In vertebrates, five distinct subunit types (α, β, γ, δ and ε) contribute to the formation of nAchRs (Karlin 1993). Two adjacent cysteine residues found in α subunits, but not in non-α subunits (β, γ ε or δ), are essential for acetylcholine binding. Both α and non-α subunits (or only α subunits in the case of the α7 nAchR) contribute to ligand binding, and the acetylcholine binding sites occupy the boundary between individual subunits (Figure 3) (Changeux and Edelstein 1998). The binding site is formed by several amino acid residues located in distinct regions of the N-terminal extracellular domain known as 'loops' (Changeux and Edelstein 1998; Corringer et al. 2000). Loops A, B and C, termed the principle components, are contributed by the α subunits (Dennis et al. 1988; Galzi et al. 1990; Fu and Sine 1994), while loops D, E and F, the complementary components, are contributed by the neighboring subunit residues (Figure 3) (Czajkowski et al. 1993; Corringer et al. 1995; Martin et al. 1996; Chiara and Cohen 1997; Martin and Karlin 1997). Several residues from loops A, B and C are highly conserved in α subunits (Changeux and Edelstein 1998; Corringer et al. 2000). Opening of the channel occurs when binding of Ach initiates small rotations of the subunits, which then cause a conformational change of the transmembrane domains lining the channel pore, resulting in channel opening (Unwin 1995).

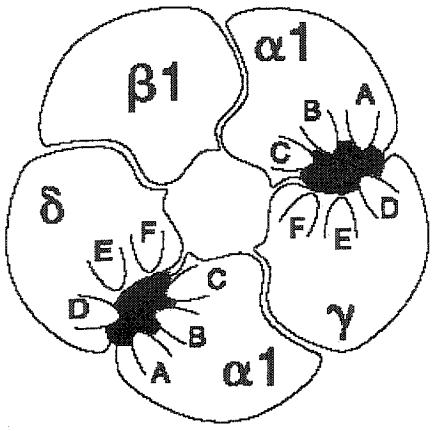


Figure 3. Schematic drawing of the muscle-type $(\alpha 1)_2\beta 1\gamma\delta$ nAchR. This receptor has two Ach-binding sites per receptor complex, corresponding to the two α subunits. The Ach binding sites are located at the interface between α and δ, and α and γ subunits. Loops A, B and C, the 'principal component', are contributed by the α subunits, and loops D, E and F, the 'complementary component', are contributed by the adjacent δ and γ subunits. Adapted from Corringer et al. (2000).

Until recently, knowledge of the three-dimensional structure of LGICs was based on electron microscopy studies on nAchR of *Torpedo californica* (Unwin 1993, 1995). These studies revealed the organization of the ion channel, while mutagenesis and affinity labeling studies were used to identify the location of the ligand binding sites. Recently, Smit et al. (2001) identified an acetylcholine binding protein (AchBP) in the snail *Lymnaea stagnalis* which is produced by glial cells and modulates cholinergic transmission by binding Ach. This protein has a high degree of homology with the extracellular domains of nAchR subunits. Its structure has been solved at high resolution, and provides detailed information on the arrangement of the ligand binding sites (Brejc et

al. 2001). With a high resolution structural model of a receptor ligand binding domain now available, the determinants of ligand binding of members of the cys-loop superfamily can now be examined in a three dimensional context. Models for the extracellular domains of chick nAchR $(\alpha 7)_5$ and the human $GABA_A$ $(\alpha_2\beta_2\gamma)$ receptor have been developed on the basis of the AchBP crystal structure (Cromer et al. 2002; Le Novere et al. 2002). These models show that residues implicated in ligand binding by photoaffinity labeling and mutagenesis experiments do indeed form the ligand binding pocket.

1.5.5 The GluCls

The GluCls are important targets for the MLs (Duce and Scott 1985). These channels are distinct from the excitatory glutamate-gated cation channels found in both vertebrates and invertebrates. They are found only in invertebrates, making them ideal drug targets (Duce and Scott 1985; Arena et al. 1992).

C. elegans is highly sensitive to MLs, and has been widely used as a model organism for studying the mode of action of these drugs (Kass et al. 1980; Schaeffer and Haines 1989; Arena et al. 1991; Cully and Paress 1991; Arena et al. 1992; Cully et al. 1994; Arena et al. 1995; Dent et al. 1997; Dent et al. 2000). Specific high-affinity avermectin binding sites have been identified in *C. elegans*, and the binding affinities of different avermectin analogs correlate well with their biological activity (Schaeffer and Haines 1989; Cully and Paress 1991). In experiments to identify the site and mechanism of action of avermectins, Arena et al. (1991) expressed *C. elegans* mRNA in *Xenopus laevis* oocytes, and demonstrated avermectin-sensitive (EC_{50} = 90 nM), GABA-

insensitive chloride currents. Further experiments showed that the avermectin-sensitive currents were directly activated by glutamate (EC_{50} = 350 μM), and that avermectin potentiated the effects of glutamate (Arena et al. 1992). The first GluCl subunits to be cloned were the GluClα1 and GluClβ subunits of *C. elegans* (Cully et al. 1994). Other GluCl subunits were subsequently cloned from *C. elegans* (Dent et al. 1997; Laughton et al. 1997a; Vassilatis et al. 1997a; Dent et al. 2000), the parasitic nematodes *H. contortus* (Delany et al. 1998; Forrester et al. 1999; Jagannathan et al. 1999; Cheeseman et al. 2001), *A. suum* (Jagannathan et al. 1999), *D. immitis* and *O. volvulus* (Cully et al. 1996a), and the insect *D. melanogaster* (Cully et al. 1996b).

In *C. elegans*, five genes have been identified that encode GluCl subunits (Cully et al. 1994; Dent et al. 1997; Laughton et al. 1997a; Vassilatis et al. 1997a; Dent et al. 2000; Horoszok et al. 2001). Two of these genes, *avr-14* (*GluCla3*) and *avr-15* (*GluCla2*) are alternatively spliced to yield two different subunits (Dent et al. 1997; Laughton et al. 1997a; Dent et al. 2000). The two *avr-14* splice variants, GluClα3A and GluClα3B, share the same N-terminal extracellular domain but different membrane-associated domains. Interestingly, the membrane-associated domain of GluClα3B lies in the 3' untranslated region (UTR) of the GluClα3A cDNA, and may have arisen as a result of partial gene duplication (Laughton et al. 1997a). The *avr-15* gene also encodes two alternatively spliced channel subunits, GluClα2A and GluClα2B (Dent et al. 1997). The predicted protein sequences of the two subunits are identical, except at the N-terminal, where GluClα2A and 2B consist of 202 and 23 amino acids, respectively.

The properties of the different *C. elegans* GluCl subunits have been examined by expression in *Xenopus* oocytes. GluClα1 and GluClβ form homomeric channels with

distinct properties. The GluClβ channel responds to glutamate (EC_{50} = 380 μM) and is insensitive to ivermectin, while the GluClα1 channel responds to ivermectin (EC_{50} = 140 nM), but shows no response to glutamate (Cully et al. 1994). The glutamate response activates rapidly, and is completely reversible. In contrast, the ivermectin response activates slowly, and is irreversible. Co-expression of GluClα1 and GluClβ results in the formation of heteromeric channels that are gated by both glutamate (EC_{50} = 1.36 mM) and ivermectin (EC_{50} = 190 nM). Ivermectin also potentiates the effect of glutamate on the heteromeric channels, indicating that it acts as an allosteric modulator of the channel. The reversal potentials for the ivermectin- and glutamate-sensitive channels are consistent with these receptors being permeable to chloride ions. These channels are also insensitive to the excitatory glutamate receptor agonists NMDA, kainate, quisqualate, AMPA and aspartate. As well, they are insensitive to the chloride channel agonists glycine and GABA. Ibotenate, a glutamate agonist, activates the heteromeric receptor, and the glutamate and ivermectin responses of this channel are blocked by picrotoxin. The ability of several avermectin analogs to activate current in the heteromeric channel correlates with their nematocidal efficacy, suggesting that the effects of the avermectins are mediated through interaction with the GluCls (Cully et al. 1994; Arena et al. 1995).

Other *C. elegans* GluCl subunits have been examined and shown to have pharmacological properties similar to those of GluClα1 (Dent et al. 1997; Vassilatis et al. 1997a; Dent et al. 2000; Horoszok et al. 2001). Like GluClα1, the *avr-15* splice variants GluClα2A and 2B form ivermectin-sensitive channels (EC_{50} = 108 nM for GluClα2B) when expressed in *Xenopus* oocytes (Dent et al. 1997; Vassilatis et al. 1997a). However, unlike GluClα1, the GluClα2A and 2B channels are also gated by glutamate (EC_{50} = 2

mM and 208 μM, respectively). Other *C. elegans* subunits which form ivermectin-sensitive, glutamate gated channels include GluClα3B and GluClα4 (glutamate and ivermectin EC_{50} = 1.9 mM and 400 nM, respectively) (Dent et al. 2000; Horoszok et al. 2001). Despite its inability to form glutamate-sensitive channels, GluClα1 possesses the determinants for glutamate binding on its N-terminal extracellular domain, since a chimera between the GluClα1 N-terminal domain and the GluClβ C-terminal domain forms glutamate-sensitive homomeric channels (Etter et al. 1996).

Co-expression of GluClα2B with GluClβ results in formation of heteromeric channels that are sensitive to both glutamate (EC_{50} = 62 μM) and ivermectin (EC_{50} = 103 nM) (Vassilatis et al. 1997a). Unlike the GluClα1 + GluClβ heteromeric channels which are less sensitive to glutamate than the GluClβ homomeric channels, the GluClα2B + GluClβ heteromeric channels are more sensitive to glutamate than the respective homomeric channels. It is likely that GluClα2B and GluClβ co-assemble *in vivo*, since they are both expressed in the pharyngeal muscle (Dent et al. 1997; Laughton et al. 1997b). The expression sites of GluClα1 and GluClα4 have not been reported. *avr-14,* which encodes GluClα3A and 3B, is expressed in extrapharyngeal neurons, and can inhibit pharyngeal pumping in response to ivermectin (Dent et al. 2000). This action is mediated via gap junctions between pharyngeal and extrapharyngeal neurons.

Like *C. elegans*, the parasitic nematode *H. contortus* is highly sensitive to the effects of the MLs, and the GluCls are also thought to be a major target (Geary et al. 1993; Cheeseman et al. 2001; Forrester et al. 2002; Forrester et al. 2003). Glutamate and ivermectin binding sites have been identified in *H. contortus* crude membrane preparations (Rohrer et al. 1994; Paiement et al. 1999; Hejmadi et al. 2000). Three genes

have been identified in *H. contortus* that encode GluCl subunits (Delany et al. 1998; Forrester et al. 1999; Jagannathan et al. 1999). Two of these genes, *HcGluCla* and *HcGluCla3*, are alternatively spliced to yield two subunits each. Phylogenetic analysis shows that HcGluCla is not orthologous to any of the GluCl subunits that have so far been identified in nematodes (Forrester et al. 1999). However, it is most closely related to the GluClα3-type subunits. HcGluClβ and the *HcGluClα3* splice variants, HcGluClα3A and 3B, are orthologs of *C. elegans* GluClβ, GluClα3A and 3B, respectively (Delany et al. 1998; Jagannathan et al. 1999). When expressed in COS-7 cells, HcGluCla and HcGluClα3B bind ivermectin with high affinity, similar to what is observed in whole membrane preparations. This suggests that GluCls represent the high affinity ivermectin binding site. While direct glutamate binding was not demonstrated in GluCls expressed in COS-7 cells, it was found to potentiate ivermectin and moxidectin binding (Forrester et al. 2002). Recently, Forrester et al. (2003) have demonstrated that HcGluCla forms functional homomeric receptors when expressed in *Xenopus* oocytes. These receptors are activated by glutamate (EC_{50} = 8.4 μM), ivermectin (EC_{50} = 131 nM) and moxidectin. As in *C. elegans*, therefore, the GluCls of this parasitic nematode are a target for MLs.

The expression patterns of the four *H. contortus* GluCl subunits have been reported. HcGluCla is expressed in motor neuron commissures, and the receptor it forms is likely to mediate the effects of the MLs on locomotion (Portillo et al. 2003). HcGluClβ, which is orthologous to *C. elegans* GluClβ, is also expressed in motor neuron commissures, and appears to co-localize with HcGluCla, suggesting that in the motor neurons innervating the body-wall muscle of *H. contortus*, the GluCl receptors contain

HcGluCla and HcGluClβ (Delany et al. 1998; Portillo et al. 2003). The splice variants HcGluClα3A and 3B are also expressed in the motor neuron commissures (Jagannathan et al. 1999; Portillo et al. 2003). In other regions, these two splice variants have unique expression patterns (Portillo et al. 2003). HcGluClα3A is found in a pair of lateral neurons in the head of the worm, which are most likely amphidial neurons. HcGluClα3B is expressed in nerve cords, as well as three cell bodies on the pharynx, which are most likely pharyngeal neurons M1 and M2. This suggests that pharyngeal receptors containing HcGluClα3B are involved in mediating ivermectin's inhibitory effects on pharyngeal pumping. The expression patterns of orthologous subunits of *H. contortus* and *C. elegans* therefore appear to be different, even though the two are closely related phylogenetically (Blaxter et al. 1998). Nonetheless, MLs have similar effects in both species, and the differences in subunit distribution may reflect their adaptation to different life styles (Portillo et al. 2003).

1.5.6 Ivermectin's Interaction with Mammalian Host Targets

Apart from interacting with invertebrate GluCls and GABA-Cls, the MLs have also been shown to interact with vertebrate ion channel receptors. While ivermectin does not directly activate current in heterologously expressed chick or human α7 nAch receptors, it potentiates the response to Ach in these receptors, causing a 20-fold increase in affinity to Ach (Krause et al. 1998). Ivermectin has also been shown to directly activate mammalian recombinant $GABA_A$ receptors and to potentiate GABA-induced chloride currents in cultured mammalian hippocampal neurons (Krusek and Zemkova 1994; Adelsberger et al. 2000). In the mouse seizure model, ivermectin acts as an

anticonvulsant, and this effect is mediated through $GABA_A$ receptors (Dawson et al. 2000). As well, ivermectin has a potentiating effect on ATP-gated $P2X_4$ cation channels (Khakh et al. 1999). Shan et al. (2001) have also demonstrated that ivermectin directly activates glycine-gated chloride channel receptors, and also potentiates the response to glycine. In these receptors, the ivermectin binding site is thought to be different from the glycine binding site, since mutations of known glycine binding sites have little effect on ivermectin sensitivity. Ivermectin therefore interacts with a wide range of host receptors. However, Schaeffer and Haines (1989) showed that the affinity for vertebrate receptors is 100-fold lower than the affinity for nematode receptors. This, and the fact that MLs do not readily cross the blood brain barrier, may account for the low toxicity of these compounds to the mammalian host (Krusek and Zemkova 1994). However, disruption of the *mdr1a* gene, which encodes a drug transporting P-glycoprotein (P-gp) in the blood brain barrier, can interfere with the protective capacity of the blood-brain barrier (Schinkel et al. 1994). P-gp acts as an efflux pump and prevents the accumulation of a variety of chemicals, including ivermectin, in the brain. Mice that are homozygous for a disruption of the *mdr1a* gene are 50- to 100-fold more sensitive to ivermectin than wild-type mice (Schinkel et al. 1994). Some collie dogs are also extremely sensitive to the effects of ivermectin (Paul et al. 1987). This phenotype is caused by a deletion mutation in the *mdr1* gene (Mealey et al. 2001; Roulet et al. 2003). The 4 bp deletion in the fourth coding exon causes a frameshift and introduces a premature stop codon 16 codons downstream of the mutation (Roulet et al. 2003). The mutation is recessive, since only dogs that are homozygous for the deletion mutation show the ivermectin-sensitive phenotype, while dogs that are heterozygous do not (Mealey et al. 2001). In normal

individuals, therefore, functional P-gp-encoding genes limit ivermectin's access to the central nervous system, where the ivermectin-sensitive mammalian receptors are restricted.

1.6 Anthelmintic Resistance

The currently available broad spectrum anthelmintics have provided farmers with safe, easy to use, and effective means of controlling helminth infections in domestic animals (Waller 1994). However, the heavy reliance on anthelmintics has led to the development of resistance, which limits the usefulness of these drugs in domestic animals (Coles et al. 1994; Le Jambre et al. 2000) Although anthelmintic resistance has been reported in horses, cattle, goats and pigs, it is most prevalent in nematode parasites of sheep, and the situation is worst in the sheep-raising countries of the Southern Hemisphere, namely South Africa, New Zealand and Australia (Coles et al. 1994; Waller 1994). While intensive use of anthelmintics is a major factor in resistance development in these countries, the use of inferior generic products has been cited as an important factor in increasing the selection pressure for resistance in poorer developing countries (Waller 1994, 1997).

Worms in natural populations show considerable variability in response to anthelmintics, with some being highly sensitive while others are highly resistant (Le Jambre 1993; Sangster 1996). Alleles conferring resistance are present in an anthelmintic-naïve population, albeit at low frequencies (Kelly et al. 1978; Prichard et al. 1980; Jackson 1993). At this stage, most resistant alleles occur in heterozygous (RS) worms, and homozygous resistant worms (RR) are rare (Barnes et al. 1995). Following

treatment with anthelmintic at the recommended dose rate, susceptible parasites are eliminated, while the small proportion of worms carrying resistance alleles survive and contribute their gene pool to the next generation (Prichard et al. 1980; Martin 1987; Waller 1994). Over time, and with continued selection pressure, the frequency of resistance alleles in the population increases, eventually manifesting as treatment failure. The rate at which resistance develops will be determined by the initial frequency of resistance alleles in the worm population, the degree of dominance and the number of genes involved (Martin 1987; Barnes et al. 1995; Le Jambre et al. 2000). Resistance will evolve faster if the initial frequency of resistance- associated alleles is high, and if it is determined by a single gene. As well, resistance will also develop more rapidly when it is dominant, since homozygous resistant (RR) and heterozygous (RS) worms are able to survive treatment.

Drug resistance is a growing problem in the cattle industry. In recent years, resistance to all three broad spectrum anthelmintic classes has been reported in several countries, including Australia, New Zealand, Argentina, Brazil, the UK and Belgium. Benzimidazole resistance has been found in *Trichostrongylus axei* (Eagleson and Bowie 1986), *C. oncophora* (Jackson et al. 1987; McKenna 1991), *Cooperia punctata* (Mejia et al. 2003), *Ostertagia ostertagi* (Hosking et al. 1996; Mejia et al. 2003), and *Haemonchus* species (McKenna 1991; Mejia et al. 2003). Levamisole and morantel resistance have been reported in *O. ostertagi* (Geerts 1986; Geerts et al. 1987; Borgsteede 1988, 1991) and *Haemonchus placei* (Yadav and Verma 1997), while ivermectin resistance has been reported in *Cooperia* species (Vermunt et al. 1996; Coles et al. 1998; Stafford and Coles 1999; Anziani et al. 2001; Coles et al. 2001; Familton et al. 2001; Fiel et al. 2001).

Multiple resistance to the benzimidazoles and avermectins in *Cooperia* species has also been reported (Vermunt et al. 1995; Mejia et al. 2003).

Anthelmintic resistance has been slower to emerge in cattle than in small ruminants (Waller 1994; Coles 2002a, b). Several factors may account for this. With sufficient exposure, cattle build up a good level of immunity to helminth infections, therefore, as adult animals, they often do not require treatment (Reinemeyer 1995; Coles 2002a). In cattle enterprises, therefore, the practice has been to treat only first-year calves, leaving older animals untreated, so the selection pressure for resistance has been low (Stafford and Coles 1999; Vercruysse and Dorny 1999; Coles 2002a). As well, unlike sheep fecal pellets which disintegrate rapidly, cattle dung pats provide a safe environment in which the free-living stages can develop. Parasites which survive on pasture and are unexposed to anthelmintic will serve to dilute any parasites that survive treatment, thereby slowing down the development of resistance (Armour et al. 1987; Coles 2002a, b). However, the availability of easy-to-use drug formulations, and the treatment of older animals to increase performance will most likely lead to an increase in the incidence of anthelmintic resistance in cattle (Reinemeyer 1995; Vercruysse and Claerebout 2001; Coles 2002a; Morris et al. 2002).

In the field, anthelmintic resistance is usually first suspected when animals that have been treated with the recommended drug dose fail to respond to treatment (Prichard et al. 1980; Taylor and Hunt 1989; Fraser 1991; Coles et al. 1998; Sangster 2001). For broad-spectrum claims, anthelmintics are marketed at dose rates determined by efficacy against the least sensitive (dose-limiting) species (Sangster 1996). The recommended dose rates are therefore much higher than doses required to eliminate the more sensitive

target species. Ivermectin, for example, is >95% effective against susceptible *H. contortus* when administered orally at 0.02 mg/kg body weight, which is ten times less than the recommended dose rate (Shoop et al. 1995). At the early stages of resistance, therefore, the drug will remain effective, since the use level is well above the 95% efficacy level against this species (Sangster 1999). Consequently, by the time resistance becomes apparent, a 10-fold decrease in efficacy will have occurred (Shoop et al. 1995). However, for the dose-limiting species, a smaller decrease in sensitivity will manifest as treatment failure, since this species requires the full dose for complete efficacy (Shoop et al. 1995).

The relative fitness of the resistance genes is an important factor in determining the maintenance of resistance in a parasite population, once it has been selected for (Prichard et al. 1980; Martin 1987). For the benzimidazole class of anthelmintics, several studies have been conducted to determine whether reversion from resistance to susceptibility is likely to occur following removal of the selection pressure. Simpkin and Coles (1978) reported slight reversion to susceptibility after two generations in the absence of drug challenge. However, other studies found that once resistance had developed, reversion to susceptibility did not occur following removal of the anthelmintic selection pressure (Hall et al. 1982; Le Jambre et al. 1982; Herd et al. 1984; Martin 1987; Martin et al. 1988; Jackson and Coop 2000). As well, counter-selecting with a different anthelmintic, levamisole, did not result in reversion to benzimidazole susceptibility (Martin et al. 1988; Borgsteede and Duyn 1989). For reversion to occur, the resistance alleles would have to confer a selective disadvantage in the absence of treatment (Le Jambre et al. 1982). Elard et al. (1998) examined several fitness-related traits in

benzimidazole-susceptible and -resistant *Teladorsagia circumcincta*, and found no significant difference between the two groups, suggesting that benzimidazole resistance does not confer a fitness disadvantage, and that once it occurs, it is irreversible. Whether this is the case with ivermectin resistance is not known. The reversion reported by Simpkin and Coles (1978) may have been due to dilution of the resistant parasites following ingestion of susceptible larvae present on pasture (Hall et al. 1982). While the deliberate introduction of susceptible parasites would be an impractical means of slowing down the spread of resistance, reducing the frequency of treatment would reduce the selection pressure for resistance, and prolong the shelf-life of existing anthelmintics (Prichard et al. 1980; Hall et al. 1982; van Wyk and van Schalkwyk 1990; Sangster 1999).

1.7 Benzimidazole Resistance Mechanisms

In nematodes, studies into the mechanisms of benzimidazole resistance have focused on the role of beta-tubulin, which is known to be the target for this class of anthelmintics (Lacey 1990). In *C. elegans*, benzimidazole resistance is associated with the deletion of a benzimidazole-susceptible beta-tubulin gene (Driscoll et al. 1989). Apart from resistance to benzimidazoles, worms lacking this gene show the same phenotype as susceptible worms, suggesting that this gene is not essential, and can be replaced by other members of the beta-tubulin family (Driscoll et al. 1989). In *H. contortus*, restriction fragment length polymorphism (RFLP) analysis on individual worms showed a reduction in diversity of beta-tubulin isotype 1 and 2 genes with benzimidazole resistance (Roos et al. 1990; Kwa et al. 1993b; Kwa et al. 1993a; Beech et al. 1994; Lubega et al. 1994).

Selection of isotype 1 and 2 variants occurs at lower degrees of resistance, while at higher degrees of resistance, individuals carrying isotype 2 genes are eliminated from the population (Kwa et al. 1993b). Analysis of beta-tubulin isotype 1 sequences from susceptible and resistant worms revealed the presence of a phenylalanine-to-tyrosine (Phe-Tyr) mutation at position 200 in all resistant worms examined (Kwa et al. 1994). Other studies found that the same Phe-Tyr mutation at position 200 conferred benzimidazole resistance in fungi (Jung et al. 1992; Koenraadt et al. 1992). The role of this mutation in benzimidazole resistance in *H. contortus* was confirmed in a heterologous transformation study (Kwa et al. 1995). The *H. contortus* beta-tubulin isotype 1 gene with a Phe-Tyr mutation at position 200 did not alter the drug phenotype of the benzimidazole-susceptible wild-type *C. elegans* or the benzimidazole-resistant *ben-1* mutant. In contrast, transformation with the *H. contortus* benzimidazole-susceptible gene (with Phe at position 200) conferred benzimidazole sensitivity in the *ben-1* mutant, indicating the functional importance of the codon 200 position in benzimidazole sensitivity and resistance.

Identification of the genetic determinant of benzimidazole resistance in nematodes led to the development of an allele-specific polymerase chain reaction (PCR) test for genotyping individual adult worms based on the amino acid at position 200 (Elard et al. 1999). The test has been adapted for use on L3 larval stages of *T. circumcincta*, *T. colubriformis* and *H. contortus*, which can easily be obtained from fecal cultures, as opposed to sacrificing animals to obtain adult worms (Silvestre and Humbert 2000). These molecular tests are useful for detecting the emergence of resistance, when the frequency of resistant worms in the population is low (Elard et al. 1999).

The mutation of residue 200 of beta-tubulin isotype 1 may not be the only determinant of benzimidazole resistance in nematodes. A Phe-Tyr mutation on isotype 2 has also been found to confer benzimidazole resistance in *H. contortus* (Prichard 2001). As well, mutagenesis studies show that a Phe-Tyr mutation at position 167 reduces binding of benzimidazole to heterologously expressed *H. contortus* beta-tubulin (Prichard et al. 2000). This mutation has also been identified in a benzimidazole-resistant cyathostome population (Kaplan et al. 2000). These studies suggest that a diagnostic test that only identifies the mutation at position 200 of isotype 1 may give false negative results (Prichard 2001). However, the position 167 mutation appears to be rare in benzimidazole-resistant field isolates of *H. contortus*, *T. colubriformis* and *T. circumcincta* (Silvestre and Cabaret 2002). These authors found the position 200 mutation in all benzimidazole-resistant worms examined, suggesting that this is the major determinant of benzimidazole resistance.

1.8 Macrocyclic Lactone Resistance Mechanisms

While the genetic basis of benzimidazole resistance is well established, the mechanisms underlying ivermectin resistance are less well understood. Resistance to the MLs is thought to involve alterations in the target sites, as well as a reduction in the drug concentration at the site of action. *In vitro* assays have been developed to investigate differences between avermectin-susceptible and –resistant parasite isolates (Gill et al. 1998). Avermectin-resistant isolates of *H. contortus* are less sensitive to the effects of avermectins on feeding, larval development and motility (Gill et al. 1998; Kotze 1998). However, not all avermectin-resistant isolates of *H. contortus* show the same phenotype.

While some resistant isolates are resistant to avermectin's inhibition of larval development and motility, other resistant isolates show no reduction in sensitivity to the effects of avermectins (Gill et al. 1998). These differences may reflect the differences in selection pressures used to produce the different isolates. As well, the differences may reflect the relative contributions of different genes which contribute to the resistance phenotype in different geographic worm populations.

In other studies, Rohrer et al. (1994) compared the binding of ivermectin to membrane preparations from ivermectin-susceptible and –resistant *H. contortus* L3. The authors found no difference in binding affinity or density between the two strains, suggesting that ivermectin resistance was not due to target site alteration. However, Paiement et al. (1999) demonstrated that ivermectin resistance was associated with an increase in low-affinity glutamate binding sites. These results were confirmed by Hejmadi et al. (2000). Ivermectin was also found to inhibit glutamate binding in membrane preparations from ivermectin-susceptible *H. contortus*, but had no effect on membrane preparations from ivermectin-resistant worms (Paiement et al. 1999). These authors concluded that ivermectin resistance was associated with changes at a glutamate binding site. Indeed, genetic variability analysis of ivermectin-susceptible and –resistant strains of *H. contortus* revealed that there was selection on a GluCl gene, suggesting that this gene is involved in ivermectin resistance (Blackhall et al. 1998a). The involvement of GluCls in ivermectin resistance has also been demonstrated in *C. elegans*, where simultaneous mutation of three GluCl genes, *glc1*, *avr-14* and *avr-15*, conferred high-level (4,000-fold) resistance (Dent et al. 2000). In contrast, simultaneous mutation of two of these genes, *avr-14* and *avr-15*, conferred modest (~14-fold) resistance.

In *H. contortus*, the HG1 gene, which encodes a GABA receptor, appears to be involved in ivermectin resistance. Genetic variability analysis of this gene revealed significant differences in allele frequencies between ivermectin-susceptible and –resistant strains of *H. contortus* (Blackhall et al. 2003). One allele, e, increased substantially in frequency in the resistant strain relative to the ivermectin-susceptible strain. The full-length cDNA sequence of this resistance-associated allele was determined, as was the sequence of the most common allele in the susceptible strain, allele A (Feng et al. 2002). These two alleles differ at four amino acid positions, two of which are located in the N-terminal domain. The functional properties of these two alleles were examined by co-expressing them in *Xenopus* oocytes with a *C. elegans* GABA-β subunit (Feng et al. 2002). While ivermectin potentiated the GABA response of the GABA-β/Allele A receptor, it attenuated the response of the GABA-β/allele e receptor. Mutations in the resistance-associated allele (e) therefore seem to affect ivermectin's interaction with the receptor.

Apart from target site alteration, ivermectin resistance may also be mediated by mechanisms that alter the drug concentration at the sites of action. Several studies have provided evidence for the involvement of P-gp in ML resistance in nematodes. A P-gp homolog isolated from *H. contortus* is upregulated in ivermectin-resistant strains of *H. contortus* (Xu et al. 1998). Genetic variability analysis of this same gene in ivermectin-susceptible and –resistant strains of *H. contortus* showed a significant difference in allele frequencies between the two strains (Blackhall et al. 1998b). As well, verapamil, a multidrug-resistance-reversing agent, significantly increased the efficacy of ivermectin against the ivermectin-resistant strain in jirds (Molento and Prichard 1999). These

studies indicate that P-gp-mediated drug efflux plays a role in ML resistance in *H. contortus*.

1.9 Anthelmintic Resistance Detection Methods

Once resistance has developed to a particular drug, it is no longer useful in protecting animals against parasite infections, and change to a drug with a different mode of action is necessary (McKenna 1990; Prichard 1990). In order to delay the onset of resistance, early detection through regular monitoring is important (Jackson and Coop 2000). Unfortunately, testing for resistance is often carried out when drug inefficacy becomes apparent, by which time the frequency of resistance alleles in the population is high. The presence of anthelmintic resistance can be determined using *in vivo* and *in vitro* assays. The assays include the fecal egg count reduction test (FECRT), the egg hatch assay (EHA), the larval paralysis/motility assays, and the larval development assay (Taylor et al. 2002).

Of the tests available, the FECRT is the one that is routinely used for detecting resistance in the field (Waller 1994, 1997). This test provides an estimate of anthelmintic efficacy by comparing the fecal egg counts of a group of animals before and after treatment, and can be used to evaluate the efficacy of any anthelmintic (McKenna 1990). Guidelines have been recommended by the World Association for the Advancement of Veterinary Parasitology (WAAVP) to standardize the efficacy evaluation process, so that results obtained in different studies can be compared (Coles et al. 1992). Based on the guidelines, resistance is suspected if the percentage reduction in egg count is less than

95%, since the efficacy of modern broad-spectrum anthelmintics is greater than 95% (McKenna 1990).

The FECRT is easy to perform, and does not require sophisticated equipment or highly trained personnel (Waller 1994). It does however, have limitations. Several nematode genera that infect ruminants produce strongyle-type eggs, including *Haemonchus*, *Ostertagia/Teladorsagia*, *Trichostrongylus* and *Cooperia* (Fraser 1991). These strongyle-type eggs cannot easily be identified to genus level (McKenna 1996). In a mixed infection, only one species may be resistant, and an egg count cannot determine which species is involved (Prichard et al. 1980). As well, the occurrence of resistance in low-fecundity genera may be masked by reduction in egg counts of susceptible genera that have a high biotic potential (McKenna 1997). Larval cultures of feces collected before and after treatment are therefore recommended for identification of resistant genera (McKenna 1996, 1997).

The major drawback of the FECRT is its lack of sensitivity (Martin et al. 1989). It can only detect resistance when the proportion of resistant worms in the population is at least 25%. Sensitive tests would aid in the early detection of resistance. Such tests have been developed for the benzimidazole class of anthelmintics (Elard et al. 1999; Silvestre and Humbert 2000). No such test exists for the ML anthelmintics. Such a test would allow not just the early detection of ML resistance, but also a survey of its prevalence (Coles et al. 2001). ML resistance in cattle nematodes is still not widespread, and elucidating mechanisms of ML resistance will shed light on possible markers of ML resistance that could be used in designing molecular tests. This thesis focuses on examining several genes that have been implicated in ML resistance.

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CHAPTER 2

Inefficacy of ivermectin against a resistant *Cooperia oncophora* field isolate

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Submitted, Parasitology Research

Abstract

Since 1999, two *Cooperia oncophora* isolates, originally obtained from the UK, were maintained by regular passage through calves at the Macdonald Campus, McGill University farm. One isolate, IVS, was susceptible to ivermectin, while the IVR isolate was resistant to ivermectin. These two isolates were used to study the mechanisms of ivermectin resistance. To confirm the susceptible/ resistant status after four years of passaging through calves, a controlled study was performed in which calves infected with IVS and IVR infective larvae were treated with ivermectin. Ivermectin at the recommended dose was 100% effective at eliminating the IVS isolate, since no eggs were found in feces, and no adult worms were found in the small intestine of the treated IVS-infected calf. In contrast, the IVR-infected calf continued to pass eggs in feces even after treatment with ivermectin, and adult worms were found in the small intestine at necropsy.

2.1 Introduction

Ivermectin is a highly potent broad-spectrum anthelmintic that is widely used in cattle. It is available in injectable, oral and topical formulations for use in cattle (Vermunt et al. 1995). *Cooperia* is one of the target species for ivermectin, though the topical and injectable formulations have been shown to have lower than expected efficacy against this species (Benz et al. 1989; Bisset et al. 1990; West et al. 1994; McKenna 1995). *Cooperia* is the dose-limiting species for ivermectin, and the lower efficacy against this species may exert a high selection pressure for resistance (Vermunt et al. 1995).

Cooperia species resistant to ivermectin have been identified under field conditions in New Zealand (Vermunt et al. 1995, 1996; Familton et al. 2001), the UK (Coles et al. 1998, 2001), and Argentina (Anziani et al. 2001; Fiel et al. 2001). Our work focuses on understanding the mechanisms of ivermectin resistance in *Cooperia oncophora*. An ivermectin-resistant isolate (IVR), originally identified by Coles et al. (1998), has been central to our work. A second isolate, designated as ivermectin-susceptible (IVS), has also been used. The parasites have been maintained at the McGill University Macdonald campus farm by regular passage through male Holstein calves. To establish the susceptible/ resistant status of the two isolates after four years of passaging, they were subjected to a controlled efficacy test.

2.2 Materials and Methods

2.2.1 Parasites

Two *C. oncophora* isolates (IVS and IVR) were kindly provided by Dr. G. C. Coles (University of Bristol, UK). The IVS isolate had been maintained without anthelmintic

pressure at Weybridge Experimental Station, UK, while IVR represents a field isolate originally obtained from a farm in Somerset, UK, where ivermectin resistance was reported (Coles et al. 1998).

2.2.2 Animals

Four three-month old calves were used in the study. They were housed indoors in individual pens at the Macdonald Campus, McGill University farm. The animals were fed hay and water *ad libitum*, along with restricted grain supplement. Fourteen days prior to the start of the experiment, the calves were drenched with fenbendazole at a dose rate of 10 mg per kg body weight to clear any existing helminth infections. Prior to infection with IVS or IVR infective larvae, animals were checked for general health, and feces collected to test the worm-free status of the animals.

2.2.3 Fecal egg count reduction test (FECRT)

To determine the efficacy of ivermectin against IVS and IVR worms, two calves were infected with 10,000 IVS infective larvae (L3), and two others were infected with 10,000 IVR L3. In each group, one animal was treated with ivermectin (Ivomec®, Merial) at the recommended dose rate of 200 µg per kg body weight in cattle, while the second animal was left untreated to act as control. The ivermectin dose was calculated based on the weight of the animal. Treatments were administered as subcutaneous injections.

Fecal samples were collected *per rectum* from all animals at the time of treatment, and seven and 14 days post-treatment. Egg counts were performed on fecal samples using a modified McMaster technique (Ministry of Agriculture Fisheries and Food.

1986). 4.5 grams of feces were thoroughly mixed in 40.5 ml of water. The suspension was sieved through a piece of gauze. The strained fluid was poured into a centrifuge tube, and centrifuged for 2 min at 1500 r.p.m. The supernatant was discarded, and the sediment loosened by stirring. The tube was then filled with saturated sodium chloride solution to the same level as before. The tube contents were thoroughly mixed by inverting the tube five or six times, and a McMaster slide chamber filled using a Pasteur pipette. After mixing again, the second chamber on the slide was filled. All the eggs in both chambers were counted, and the total number multiplied by 10 and expressed as eggs per gram of feces.

The anthelmintic efficacy was calculated based on the percent reduction in fecal egg counts using the formula:

$$(C - T) / C \times 100 = \% \text{ efficacy},$$

where C and T are the fecal egg counts of the control (untreated) and treated animals, respectively. Ivermectin resistance was defined as less than 95% reduction in fecal egg counts post-treatment (Coles et al. 1992).

2.2.4 Calculating Reduction in Worm Burden

Necropsy of all four animals was performed on days 22 and 23 post-treatment. The contents of the small intestine were emptied into a sampling bucket, and the small intestine split open longitudinally using scissors. The mucosal surface was washed thoroughly with warm water (38°C). 1 liter aliquots were then processed for worm

recovery and enumeration by a modification of the agar gel technique of Slotved et al. (1996). A 2% agar solution (Bacto-Agar) was prepared by mixing the agar with tap water. This was heated to boiling point and cooled to 45°C. One liter of intestinal washings was mixed with 1 liter of the 2% agar solution at 45°C. The mixture was then poured into eight trays in which disposable J-cloths (22 x 28 cm) had been placed. The agar gel solidified after a few minutes, and the cloths with the adhering gel were clipped to a stick and placed in a plastic container, with the sticks being used to suspend the cloths in the container. The container was then filled with RPMI medium at 38°C, and incubated at 38°C for three hours. The worms migrated out of the gel and collected at the bottom of the container. These were picked and counted. Worms remaining in the agar gel were also picked and included in the total count. Percent reduction in adult worm burden was calculated using the formula listed above.

2.3 Results

To determine the efficacy of ivermectin against IVS and IVR worms, two calves were infected with 10,000 IVS L3, and two others infected with 10,000 IVR L3. Calves infected with the IVS isolate were in good body condition, and did not show any clinical signs of parasitic gastroenteritis (PGE). Following treatment of one of the IVS-infected animals with ivermectin, no eggs were observed in the feces (Table 1). In contrast, the two calves infected with the IVR isolate had diarrhea, rough hair coats, and were unthrifty. Even after one of the IVR-infected calves was treated with ivermectin, it continued to pass eggs in feces (Table 1), and remained in poor condition.

Table 1. Pre- and post-treatment fecal egg counts from untreated calves and calves treated with injectable ivermectin. Day 0 = day of treatment, and Day 7 and 14 = days post-treatment.

Animal	Day 0	Day 7	Day 14
IVS treated	170	0	0
IVS untreated	110	110	90
% reduction	-	**100**	**100**
IVR treated	190	30	40
IVR untreated	180	130	180
% reduction	-	**76.9**	**77.8**

The efficacy of ivermectin was measured by the percentage reduction in post-treatment fecal egg counts in treated animals compared with untreated animals. The fecal egg counts were determined on days 0, 7 and 14 post-treatment, and are shown in Table 1. The values for the treated animals were compared with those for the untreated animal in the same group. Treatment with the recommended dose of ivermectin (200 μg / kg body weight) was 100% effective against the IVS isolate, since no eggs were seen in the treated IVS animal on days 7 and 14 post-treatment. For the IVR isolate, ivermectin reduced fecal egg counts by 76.9% and 77.8% at days 7 and 14 post-treatment, respectively.

Table 2 shows adult worm counts for the four calves. Adult worms were found in untreated IVS- and IVR-infected animals. No adult worms were found in the small intestine of the IVS-infected animals following treatment with ivermectin. In contrast, ivermectin reduced the worm burden of the IVR-infected animal by only 70.5%.

Table 2. Post-treatment worm burdens in treated and untreated calves

Animal	**Post-treatment Adult Worm Burden**
IVS treated	0
IVS untreated	1330
% reduction	**100**
IVR treated	250
IVR untreated	848
% reduction	**70.5**

2.4 Discussion

The present study compared the efficacy of ivermectin against IVS and IVR isolates of *C. oncophora*, and confirmed the ivermectin-resistant status of the IVR isolate, which was previously reported by Coles et al. (1998). While ivermectin was 100% effective against the susceptible IVS isolate, efficacy against the IVR isolate was 77.8% on day 14 post-treatment. FECRT results below 95% are indicative of anthelmintic resistance (Coles et al. 1992). These findings were supported by the adult worm counts, where treatment of

IVR-infected animals resulted in a 70.5% reduction in adult worms, while no worms were found in the treated IVS-infected animal.

Consistent with the results of Coles et al. (2001), the IVR-infected animal remained in poor body condition even after treatment, though the egg count was low (40 EPG on day 14 post-treatment). At slaughter, adult worms were found in the small intestine of this animal, suggesting that ivermectin was ineffective at eliminating the adult IVR worms. Of interest is the observation that the untreated IVS-infected animal had a higher worm burden that the IVR-infected animals, yet the IVS-infected animal did not show any clinical signs of PGE. As stated by Coles et al. (2001), ivermectin-susceptible isolates appear to be of low pathogenicity compared to ivermectin-resistant isolates. Calves infected with 10,000 to 20,000 ivermectin-susceptible L3 did not show any clinical signs of disease (Borgsteede and Hendriks 1979; Coop et al. 1979), while animals infected with 15,000 or 20,000 ivermectin-resistant L3 showed edema, weight loss and diarrhea (Coles et al. 2001; Anziani et al. 2001). *Cooperia* is generally considered a parasite of low pathogenicity, and young calves develop immunity to this parasite following exposure in the first grazing season (Coop et al. 1979; Vermunt et al. 1995; Claerebout et al. 1999). However, at high infection levels (a single dose of 200,000 L3, or 10 000 larvae per day for six weeks), *Cooperia* can cause clinical disease (Borgsteede and Hendriks 1979; Armour et al. 1987). The ivermectin-resistant isolate appears to be more pathogenic than the susceptible isolate, with infected animals showing clinical signs of parasitic gastroenteritis (Anziani et al. 2001; Coles et al. 2001), including failure to thrive, that could impact negatively on productivity (Coop et al. 1979; Armour et al. 1987; Vermunt et al. 1995).

Doramectin and moxidectin were found to be ineffective against ivermectin-resistant *C. oncophora* (Vermunt et al. 1995, 1996; Anziani et al. 2001), and this suggests that ivermectin-resistant *C. oncophora* show side resistance to doramectin and moxidectin, both members of the macrocyclic lactone class of anthelmintics. Levamisole, fenbendazole and oxfendazole were used successfully against ivermectin-resistant *C. oncophora* (Anziani et al. 2001; Coles et al. 1998, 2001). Levamisole belongs to the imidazothiazole class of anthelmintics, while fenbendazole and oxfendazole are benzimidazoles. These anthelmintics act via mechanisms that are distinct from those of the macrocyclic lactones. The imidazothiazoles act on worm acetylcholine receptors, while the benzimidazoles act on tubulin (Kwa et al. 1993, 1995; Sangster 1999). Chloride channels gated by the neurotransmitters glutamate and gamma-aminobutyric acid (GABA) have been identified as targets for the macrocyclic lactones (Holden-Dye and Walker 1990; Cully et al. 1994; Arena et al. 1995; Martin 1996; Dent et al. 2000; Feng et al. 2002).

The FECRT is the most widely used test for detecting the presence of anthelmintic resistance (Taylor et al. 2002). It compares egg counts in treated and non-treated animals at the time of treatment and 10-14 days later, which is less than the minimum prepatent periods of important nematode species infecting ruminants (Bowman et al. 2003). While the FECRT is easy to perform, its major drawback is that it can only detect resistance if more than 25% of the worms in the population are resistant (Martin et al. 1989). No other technique is used routinely for detecting anthelmintic resistance, and there is a need for developing sensitive techniques if low-level ivermectin resistance is to be detected. This would allow action to be taken before a high proportion of worms in

the population are resistant. Knowledge of the molecular mechanism of benzimidazole resistance has made possible the development of a sensitive molecular tool for detecting benzimidazole resistance in individual worms (Kwa et al. 1994; Elard and Humbert 1999; Silvestre and Humbert 2000). Studying the differences in the genes thought to be involved in ivermectin resistance in the IVS and IVR worms will help identify possible markers for ivermectin resistance, which could be used in the development of sensitive diagnostic tests.

Acknowledgements

The authors thank G. Bingham and D. Eshelby for technical assistance. This study was funded by Fort Dodge Animal Health and NSERC, Canada.

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CONNECTING STATEMENT I

In the previous chapter, we confirmed that the two isolates used in this study, IVS and IVR, differ in their sensitivity to ivermectin. Based on studies in *C. elegans* and *H. contortus*, the glutamate-gated chloride channels are thought to be major targets for ivermectin. To determine their role in ivermectin resistance in *C. oncophora*, we first cloned two full-length GluCl cDNAs from this nematode, since these sequences have not previously been reported. We then analyzed the genetic variability of the GluCl genes in IVS and IVR worms. The results are presented in Chapter 3.

CHAPTER 3

Genetic variability of glutamate gated chloride channel genes in ivermectin-susceptible and -resistant strains of *Cooperia oncophora*

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Submitted, Parasitology

Abstract

The glutamate-gated chloride channels (GluCls) are members of the ligand-gated ion channel (LGIC) superfamily that are thought to be involved in ivermectin's mode of action and mechanism of resistance. Using reverse-transcriptase PCR techniques, two full-length GluCl cDNAs, encoding GluClα3 and GluClβ subunits, were cloned from *Cooperia oncophora*, a nematode parasite of cattle. The two sequences show a high degree of identity to similar subunits from other nematodes. The *C. oncophora* GluClα3 subunit is most closely related to the *Haemonchus contortus* GluClα3B subunit, while *C. oncophora* GluClβ subunit shares high sequence identity with the *H. contortus* GluClβ subunit. Using single-strand conformation polymorphism, the genetic variability of these two genes was analyzed in ivermectin-susceptible and -resistant isolates of *C. oncophora*. Statistical analysis revealed an association between the *C. oncophora GluClα3* gene and ivermectin resistance. No such association was seen with the *GluClβ* gene.

3.1 Introduction

Gastrointestinal helminth infections can severely limit the productivity of domestic ruminants, causing serious economic losses even at sub-clinical infection levels (Le Jambre 1993). Control relies heavily on the use of anthelmintics, among them ivermectin. Unfortunately, the development of resistance threatens the usefulness of these drugs. While resistance is primarily a problem in sheep and goats, reports of its occurrence in cattle, though not as common, are emerging (Williams 1997; Coles 2002a, b). Because they are genetically diverse, parasitic helminths are able to respond to selective pressure (Grant 1994; Otsen et al. 2001). Parasites carrying a resistance gene or genes are able to survive drug treatment and pass on their resistance genes to their offspring (Prichard 1990; Jackson 1993; Jackson and Coop 2000). Over time, the frequency of resistant individuals in the population increases, resulting in treatment failure.

Ivermectin resistance involving *Cooperia* species has been reported by several authors (Vermunt et al. 1995; Coles et al. 1998; Anziani et al. 2001; Coles et al. 2001; Familton et al. 2001; Fiel et al. 2001), highlighting the need for research into the mechanisms of resistance with a view to monitoring and limiting its spread. Studies on the mode of action of ivermectin have focused largely on the free- living *Caenorhabditis elegans*, where the GluCls have been identified as major targets (Arena et al. 1992; Cully et al. 1994; Arena et al. 1995; Dent et al. 1997; Dent et al. 2000). These channels have also been identified in parasitic nematodes, including *Haemonchus contortus* (Delany et al. 1998; Forrester et al. 1999; Jagannathan et al. 1999) and *Ascaris suum* (Martin 1996; Jagannathan et al. 1999). As members of the LGIC superfamily, GluCls are thought to be

hetero-pentameric in structure, with the five membrane-spanning subunits assembled around a central pore (Barnard 1996). Binding of ivermectin opens the channel, which is permeable to chloride ions, leading to inhibitory action in the nervous system (Arena et al. 1995; Martin 1996; Dent et al. 1997). Ivermectin also exerts its effects on other members of the LGIC superfamily, including GABA- gated chloride channels (Holden-Dye and Walker 1990; Feng et al. 2002) and nicotinic acetylcholine receptors (Krause et al. 1998). As well, ivermectin is a substrate for P-glycoproteins (P-gps), transmembrane proteins involved in transporting compounds across membranes (Schinkel et al. 1994; Pouliot et al. 1997).

Ivermectin resistance is thought to involve multiple mechanisms (Gill et al. 1998; Prichard 2001). Genetic variability studies suggest a role for P-gp in ivermectin resistance in *Haemonchus contortus* (Blackhall et al. 1998b; Sangster et al. 1999). Xu et al. (1998) have also shown that P-gp is over-expressed in ivermectin-selected strains of *H. contortus*. As well, selection at an α subunit GluCl gene is associated with ivermectin resistance in *H. contortus* (Blackhall et al. 1998a). In *C. elegans*, simultaneous mutations in three α-subunit GluCl genes results in high-level resistance (Dent et al. 2000). Whether the GluCls also play a role in ivermectin resistance in *C. oncophora* is not known. To determine this, we have cloned two GluCl subunit cDNAs (Co GluClα3 and Co GluClβ) from this nematode, and examined their genetic variability in ivermectin-susceptible (IVS) and -resistant (IVR) worms. The results suggest a role for Co GluClα3, but not GluClβ, in ivermectin resistance.

3.2 Materials and Methods

3.2.1 Parasites

IVS and IVR *C. oncophora* isolates were provided by Dr. Coles, University of Bristol, UK. The IVS isolate was passaged through calves without drug treatment at the Weybridge Experimental Station, UK, while IVR is an ivermectin-resistant field isolate (Coles et al. 1998). The parasites were maintained at the Macdonald Campus (McGill University) farm by passaging through male Holstein calves every four months. Adult *C. oncophora* were collected live at necropsy from the small intestine, and stored in liquid nitrogen until used for RNA and DNA extraction.

3.2.2 Isolation of two full-length C. oncophora GluCl cDNAs using RT-PCR

Using an oligo-dT primer (GibcoBRL) and murine Moloney leukemia virus reverse transcriptase (GibcoBRL), first strand cDNA was synthesized from total RNA isolated from bulk adult worms. The cDNA served as template for the initial amplification of a GluCl fragment using degenerate primers and the Advantage 2 PCR kit (ClonTech). For a nested PCR approach, four degenerate primers were designed based on an alignment of known GluCl sequences from *H. contortus* and *C. elegans*. In the first-round PCR reaction, the primers used were: outer sense primer 5' TGGATGCCNGAYACNTT 3' and the outer antisense primer 5' CCARTANRRDATRTTRA 3'. The PCR product from the first round was then used as template for a second amplification using the nested primers 5' ATHGAYAARCCNAAYGT 3' (sense) and 5' GCNCCRAADATRAANGC 3' (antisense). For both rounds, amplification conditions were 94°C for 30 s to denature, and 30 cycles of 94°C for 20s, 50°C for 30s, and 72°C for 1 min, followed by a final

extension at 72°C for 5min. PCR products from the second round were examined on a 1% agarose gel stained with 0.5 μg/ml ethidium bromide, and the fragment of the expected size (~550 bp) was purified using the Nucleospin Gel Extraction Kit (ClonTech). The purified PCR products were cloned into a TA vector (Invitrogen) and sequenced. Using the sequence information of this fragment, gene-specific primers were designed for 5' and 3' RACE reactions.

For the 5' RACE reaction, the Advantage 2 cDNA kit (ClonTech) was used. Two 5' RACE primers, 5' RACE1 and 5' RACE2 (5' CGCCGATCCACACGTCCACCGCCTTTAT 3' and 5' CGAGCCAGAATGAAACCCAAGAGACGAC 3', respectively) were designed and used in a semi-nested PCR reaction with the nematode splice leader sequence SL1 (5' GGTTTAATTACCCAAGTTTGAG 3'). Amplification conditions were 94°C for 1 min, followed by 30 cycles of 94°C for 30s, 54°C for 30s, and 72°C for 1 min, followed by a final extension at 72°C for 5min. The fragment obtained after the second round of PCR was purified, ligated into a TA cloning vector (Invitrogen) and subsequently sequenced from both directions with M13 forward and reverse vector primers.

To isolate the 3' end of the *C. oncophora* GluCl cDNA, the Marathon cDNA Amplification kit (ClonTech) was used. Adaptor-ligated double-stranded cDNA was prepared according to the manufacturer's recommendations and used as template. Two gene-specific sense primers 3' RACE1 (5' CTGGTGTTGTCCTGCCCGATGTCGTTGG 3') and 3' RACE2 (5' CCAGCAAAACCAATACGGGAGAATACAG 3') were used in a nested PCR reaction with the antisense adaptor primers AP1 and AP2, respectively. A

touchdown PCR was employed as recommended by the manufacturer. The resulting product was purified, cloned and sequenced from both directions.

A second full-length GluCl cDNA, encoding a β subunit, was also isolated from *C. oncophora* using the same procedures outlined above, except for the primers. The degenerate primers used for the initial isolation were designed based upon an alignment of the amino acid sequences of GluCl β subunits from *H. contortus* (Delany et al. 1998) and *C. elegans* (Cully et al. 1994). Four degenerate primers were used in a nested PCR approach. In the first round of PCR, the sense and antisense primers used were 5' GTYCGKGTYAAYATYATGAT 3' and 5' GACRAAYGCGTATTCSAGMA 3', respectively. The first-round reaction was then used as template for further amplification using the nested primers 5' GAYGTMGTYAAYATGGARTA 3' (sense) and 5' TTGRCASGCTCCRAGCCASA 3' (antisense). The 736-bp product from the second round was cloned and sequenced, and gene-specific primers were then designed for 5' and 3' RACE reactions.

To amplify the 5' end of the GluClβ cDNA, two antisense primers, 5' RACE1 (5' GGTACAGTGAGAAACTTTGGAGGG 3') and 5' RACE2 (5' CATATGCCAGACGCGAATCGAGCC 3'), were used in a semi-nested PCR reaction with SL1 and Advantage 2 cDNA polymerase (ClonTech). For the 3' RACE reactions, two sense primers, 3' RACE1 (5' AGCAGCCGAATTAGTATCACGAGC 3') and 3' RACE2 (5' CCAGTGCAGCTCAAACCGGGCGTC 3') were used with the antisense adaptor primers AP1 and AP2 in a nested PCR reaction using the Marathon cDNA amplification kit (ClonTech).

3.2.3 PCR amplification of genomic DNA from individual male worms for single-strand conformation polymorphism (SSCP) analysis

Genomic DNA was isolated from individual adult male worms as described by (Beech et al. 1994). To ensure that the genomic DNA sample obtained represented one worm, only male worms were used. For the first GluCl subunit, 150 genomic DNA samples were examined (75 from each group), while 160 samples (80 from each group) were tested for GluClβ subunit. The DNA samples were amplified in a standard PCR reaction using Taq polymerase (GibcoBRL) with the gene specific primers listed in Table 1. All primers were exonic, spanning a predicted intron in both genes (Table 1). The primers were designed using the OLIGO® primer analysis software (Version 4.06). The intron position was predicted based on the *C. elegans* GluCl cosmid sequence (Accession Number U97196). All PCR amplifications were carried out in a 25-µl reaction volume using 2 µl (~2 ng) genomic DNA from individual worms as template, 3 mM $MgCl_2$, 0.2 mM dNTPs, 0.5 U Taq polymerase and 0.4 µM of each primer. Negative (no DNA) controls were included. PCR conditions were optimized individually to each region to ensure specificity. For the first GluCl subunit gene, the thermal cycling conditions included an initial incubation at 94°C for 4 min, followed by 35 cycles of 94°C for 30 sec, 48°C for 30sec, and 72°C for 1 min, with a final extension at 72°C for 5 min. The same conditions were used to amplify a fragment of the *C. oncophora* GluClβ gene from individual worms, except that the annealing temperature was 53°C. Prior to SSCP, a 5 µl aliquot of each PCR product was checked by agarose gel electrophoresis to confirm the size and specificity of the products.

Table 1. Primers used to amplify fragments of Co *GluClα3* and Co *GluClβ* genes from individual male worms for SSCP. The primers were exonic, and the position of the intron was predicted based on a *C. elegans* GluCl cosmid sequence (Accesssion Number U97196).

Gene	Primer	Sequence (5' -3')	Intron size (bp)	Fragment size (bp)
Co *GluClα3*	Sense	TGGATCGACAAAATTGCCT	93	228
	Antisense	ACTTGGTAACGACATCTTG		
Co *GluClβ*	Sense	AGCTCGTGCCACATGCAGC	68	278
	Antisense	ATTGGTGTGACTAGTGCAG		

3.2.4 SSCP analysis

For SSCP screening of the first GluCl subunit gene samples, 1 µl of each PCR product was mixed with 15 µl of loading buffer containing 95% formamide, 10 mM NaOH, 0.25% bromophenol blue, and 0.25% xylene cyanol. The samples were then denatured at 95°C for 5 min, and immediately placed on ice to stabilize the single strands of DNA. 10 µl of each sample were loaded onto a 15% nondenaturing polyacrylamide gel and electrophoresed in 1XTBE for 18 hours at room temperature and 110V. The conditions for SSCP electrophoresis were standardized for the optimal resolution of bands. This involved testing gels of different polyacrylamide concentrations (8%, 12%, 15%, 20%), as well as voltage settings, product:dye ratios, and gel running times. For the GluClβ gene, the PCR samples were mixed with the loading dye in the same product: dye ratio of 1:15, and electrophoresed on a 12% polyacrylamide gel for 24 hrs at 90V and room

temperature. Following electrophoresis, the gels were stained with ethidium bromide and visualized using the Biorad Molecular Imager FX with its corresponding Quantity One (Version 4.2.1) software. Alleles were identified based on their unique banding patterns. A chi-square analysis was performed to test for differences in allele frequencies between the two groups, IVS and IVR. As well, genotypic frequencies for both groups were analyzed for Hardy-Weinberg equilibrium using a chi-square test.

3.2.5 Sequence analysis

All alleles were investigated by direct sequencing. PCR products amplified from genomic DNA of individual worms were purified using the Nucleospin Gel Extraction kit (ClonTech) and sequenced using PCR primers. Nucleic acid sequences were analyzed using MacVector 7.0 software (Oxford Molecular Groups, England). The exact size and position of the intron was determined by comparative alignment of the alleles with the corresponding full-length *C. oncophora* GluCl cDNA sequences.

C. oncophora GluCl predicted proteins were aligned with other GluCl sequences using CLUSTALW on the SDSC Biology Workbench (Version 3.2). Phylogenetic analyses were then carried out using MEGA Version 2.1 (Kumar et al. 2001). The strength of the tree nodes was assessed by bootstrap resampling.

3.3 Results

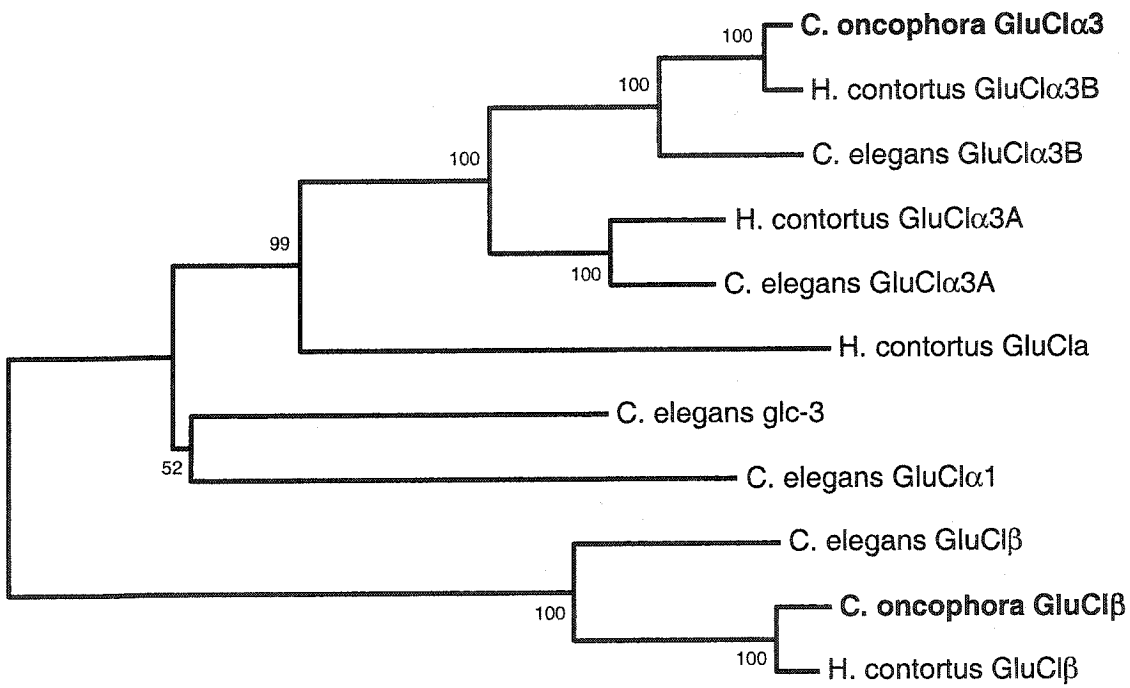
Using degenerate primers and RT-PCR, two partial cDNA fragments encoding different GluCls were obtained from *C. oncophora*. 5' and 3' RACE techniques were then used to amplify the full-length sequences. The spliced leader sequence SL1, which is found on

many nematode mRNAs, facilitated isolation of the 5' end. The two cDNAs were 1818 and 1480 nucleotides long, with short 5' untranslated regions of 97 and 12 nucleotides, respectively. The predicted protein sequences were 438 and 432 amino acids (aa) long, respectively. A database search showed the 438-aa sequence to have highest identity to the *H. contortus* and *C. elegans* GluClα3B subunits (88% and 82% identity, respectively), while the 432-aa long predicted protein sequence showed 90% and 76% identity to *H. contortus* and *C. elegans* GluClβ subunits (Table 2). Because of the high homology to GluClα3 and GluClβ subunit classes, the two cDNAs cloned from *C. oncophora* were assigned to these groups, and are referred to as *C. oncophora* (Co) GluClα3 and Co GluClβ. These two sequences shared 54% identity at the amino acid level. A phylogenetic tree constructed by the Neighbor-Joining method placed Co GluClα3 predicted protein with GluClα3B sequences from *H. contortus* and *C. elegans*, while CoGluClβ grouped with *H. contortus* and *C. elegans* GluClβ sequences (Figure 1). These groupings were highly supported by bootstrap analysis. The nucleotide sequence data for Co GluClα3 and GluClβ have been submitted to GenBank under Accession Numbers AY372756 and AY372757, respectively (Appendices B.1 and B.2, respectively).

Table 2. Amino acid sequence identities of the Co GluClα3 and GluClβ subunits with other GluCl sequences reported in GenBank. Partial sequences are indicated by an asterisk. Highest sequence identity to a different full-length sequence is shown in bold type.

	C. oncophora GluClα3 (438 aa)	*C. oncophora* GluClβ (432 aa)
C. oncophora GluClβ	54	-
H. contortus GluClα3B	**88**	51
C. elegans GluClα3B	82	51
**O. volvulus* GluClX	88	52
**D. immitis* GluClX	85	56
**A. suum* gbr-2	76	44
H. contortus GluClα3A	74	44
C. elegans GluClα3A	71	44
C. elegans glc-3	69	55
H. contortus GluCla	63	42
D. melanogaster GluCl	59	40
H. contortus GluClβ	55	**90**
C. elegans GluClβ	54	76
C. elegans GluClα2A	53	46
C. elegans GluClα2B	53	46
C. elegans GluClα1	52	45

Figure 1. Neighbor-joining tree showing relationships of the *C. oncophora* GluCl subunits with full-length GluCls from *H. contortus* and *C. elegans*. The two *C. oncophora* sequences are highlighted in bold. The Co GluClα3 sequence is most closely related to GluClα3B sequences from *H. contortus* and *C. elegans* (100% bootstrap value). The Co GluClβ subunit groups with the two β subunits from *H. contortus* and *C. elegans*, and this grouping is also highly supported by bootstrap analysis.



An alignment of the *C. oncophora* GluClα3 polypeptide with GluClα3B sequences from *H. contortus* and *C. elegans* is shown in Figure 2A, while an alignment of GluClβ polypeptide sequences from *C. oncophora*, *H. contortus* and *C. elegans* is shown in Figure 2B. The *C. oncophora* GluClα3 and GluClβ predicted protein sequences contain features common to ligand-gated ion channel subunits- a long N-terminal extracellular domain containing four cysteine residues, four predicted membrane-spanning domains (TM1-4), a long intracellular loop between TM3 and TM4, and a very short C-terminal extracellular domain. The estimated molecular weights of the Co GluClα3 and GluClβ predicted proteins were 50,667 and 49,651 respectively. Putative signal peptides were identified at the amino termini of both sequences, and these were predicted to be cleaved between amino acids 30 and 31 for Co GluClα3 (Figure 2A), and 16 and 17 for Co GluClβ (Figure 2B). Co GluClα3 contains one predicted N-linked glycosylation site at amino-acid residue 57, as well as consensus sites for phosphorylation by casein kinase II and protein kinase C in the intracellular loop between TM's 3 and 4 (Figure 2A). These are conserved in *H. contortus* and *C. elegans* GluClα3B. The Co GluClβ sequence contains two conserved glycosylation sites at asparagines residues 52 and 209, and phosphorylation sites for protein kinase C and casein kinase II at amino acid residues 337 and 392 respectively (Figure 2B).

Figure 2. Alignment of GluClα3 (**A**) and GluClβ (**B**) deduced amino acid sequences of *C. oncophora, H. contortus*, and *C. elegans*. Completely conserved residues are shaded grey. Transmembrane domains are indicated (TM1- TM4). Predicted phosphorylation (protein kinase C (●) and casein kinase 2 (○)) and N- linked glycosylation sites (◇) are shown. The glycosylation and phosphorylation sites are conserved among similar subunits. The putative signal peptide cleavage sites are indicated by (▼). The four extracellular cysteine residues are indicated by (♦). The first two cysteines, separated by 13 residues, are common to all members of the ligand-gated ion channel superfamily, and this region forms the neurotransmitter signature sequence. The second cysteine pair, separated by 10 residues, is unique to glutamate-, glycine- and histamine- gated chloride channel subunits.

Figure 2A

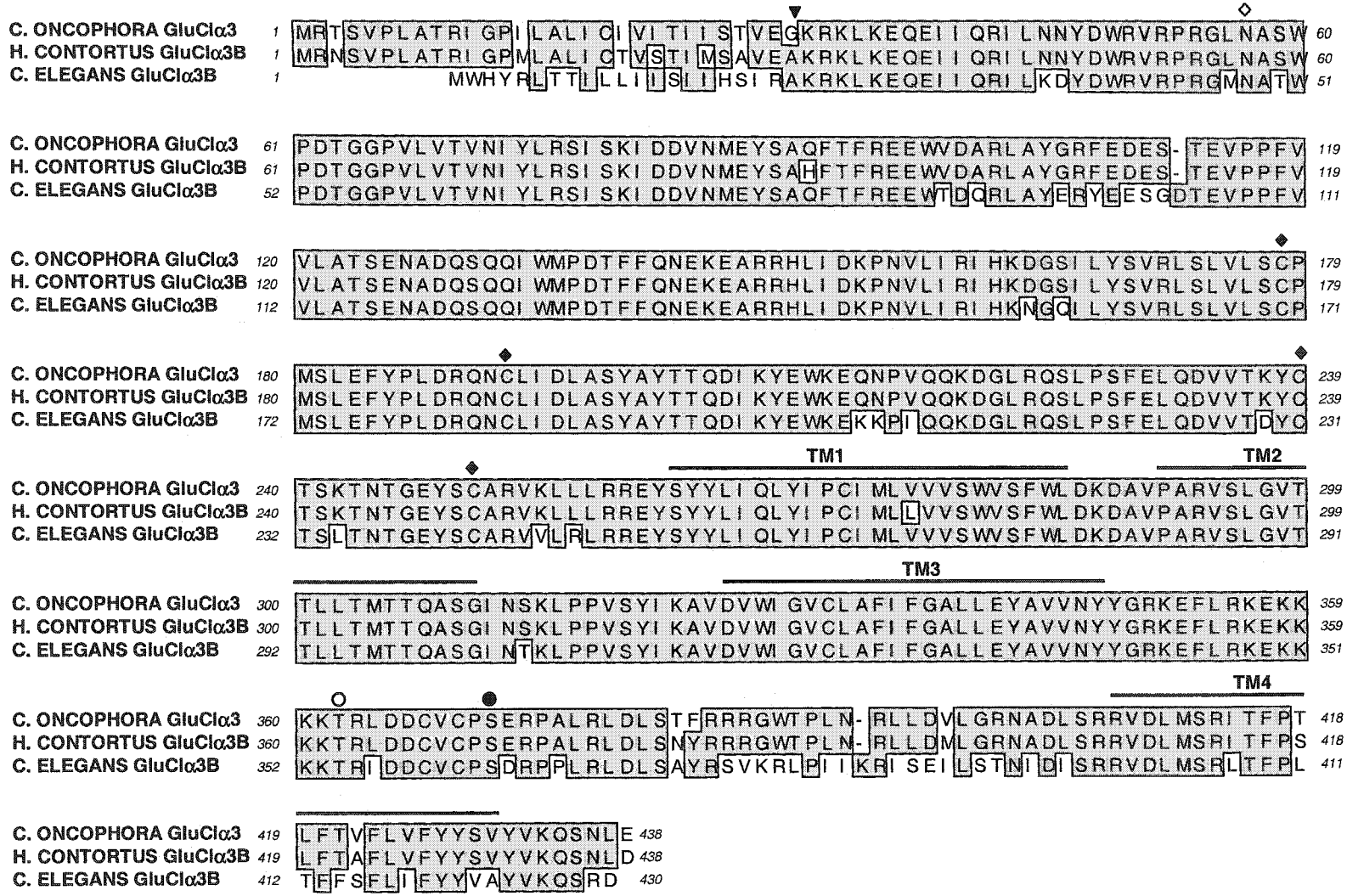

C. ONCOPHORA GluClα3 1 MRTSVPLATRIGPILALICIVITIISTVEGKRKLKEQEIIQRILNNYDWRVRPRGLNASW 60
H. CONTORTUS GluClα3B 1 MRNSVPLATRIGPMLALICTVSTIMSAVEAKRKLKEQEIIQRILNNYDWRVRPRGLNASW 60
C. ELEGANS GluClα3B 1 MWHYRLTTILLIISIIHSIRAKRKLKEQEIIQRILKDYDWRVRPRGMNATW 51
C. ONCOPHORA GluClα3 61 PDTGGPVLVTVNIYLRSISKIDDVNMEYSAQFTFREEWVDARLAYGRFEDES-TEVPPFV 119
H. CONTORTUS GluClα3B 61 PDTGGPVLVTVNIYLRSISKIDDVNMEYSAHFTFREEWVDARLAYGRFEDES-TEVPPFV 119
C. ELEGANS GluClα3B 52 PDTGGPVLVTVNIYLRSISKIDDVNMEYSAQFTFREEWTDQRLAYERYEESGDTEVPPFV 111
C. ONCOPHORA GluClα3 120 VLATSENADQSQQIWMPDTFFQNEKEARRHLIDKPNVLIRIHKDGSILYSVRLSLVLSCP 179
H. CONTORTUS GluClα3B 120 VLATSENADQSQQIWMPDTFFQNEKEARRHLIDKPNVLIRIHKDGSILYSVRLSLVLSCP 179
C. ELEGANS GluClα3B 112 VLATSENADQSQQIWMPDTFFQNEKEARRHLIDKPNVLIRIHKNGQILYSVRLSLVLSCP 171
C. ONCOPHORA GluClα3 180 MSLEFYPLDRQNCLIDLASYAYTTQDIKYEWKEQNPVQQKDGLRQSLPSFELQDVVTKYC 239
H. CONTORTUS GluClα3B 180 MSLEFYPLDRQNCLIDLASYAYTTQDIKYEWKEQNPVQQKDGLRQSLPSFELQDVVTKYC 239
C. ELEGANS GluClα3B 172 MSLEFYPLDRQNCLIDLASYAYTTQDIKYEWKEKKPIQQKDGLRQSLPSFELQDVVTDYC 231
TM1
TM2
C. ONCOPHORA GluClα3 240 TSKTNTGEYSCARVKLLLRREYSYYLIQLYIPCIMLVVVSWVSFWLDKDAVPARVSLGVT 299
H. CONTORTUS GluClα3B 240 TSKTNTGEYSCARVKLLLRREYSYYLIQLYIPCIMLLVVSWVSFWLDKDAVPARVSLGVT 299
C. ELEGANS GluClα3B 232 TSLTNTGEYSCARVVLRLRREYSYYLIQLYIPCIMLVVVSWVSFWLDKDAVPARVSLGVT 291
TM3
C. ONCOPHORA GluClα3 300 TLLTMTTQASGINSKLPPVSYIKAVDVWIGVCLAFIFGALLEYAVVNYYGRKEFLRKEKK 359
H. CONTORTUS GluClα3B 300 TLLTMTTQASGINSKLPPVSYIKAVDVWIGVCLAFIFGALLEYAVVNYYGRKEFLRKEKK 359
C. ELEGANS GluClα3B 292 TLLTMTTQASGINTKLPPVSYIKAVDVWIGVCLAFIFGALLEYAVVNYYGRKEFLRKEKK 351
TM4
C. ONCOPHORA GluClα3 360 KKTRLDDCVCPSERPALRLDLSTFRRRGWTPLN-RLLDVLGRNADLSRRVDLMSRITFPT 418
H. CONTORTUS GluClα3B 360 KKTRLDDCVCPSERPALRLDLSNYRRRGWTPLN-RLLDMLGRNADLSRRVDLMSRITFPS 418
C. ELEGANS GluClα3B 352 KKTRIDDCVCPSDRPPLRLDLSAYRSVKRLPIIKRISEILSTNIDISRRVDLMSRLTFPL 411
C. ONCOPHORA GluClα3 419 LFTVFLVFYYSVYVKQSNLE 438
H. CONTORTUS GluClα3B 419 LFTAFLVFYYSVYVKQSNLD 438
C. ELEGANS GluClα3B 412 TFFSFLIFYYVAYVKQSRD 430

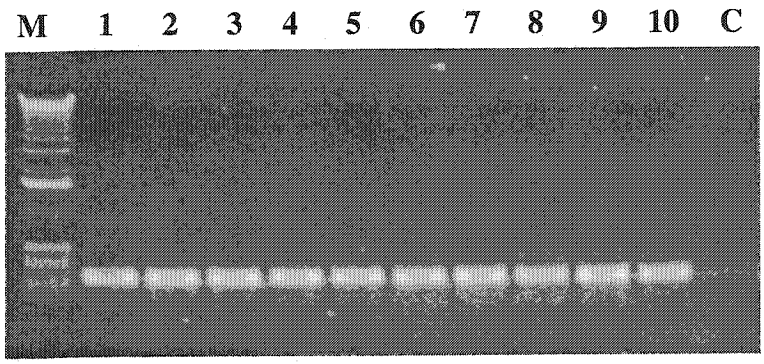
Figure 2B


C. ONCOPHORA GluClβ
H. CONTORTUS GluClβ
C. ELEGANS GluClβ
TM1
TM2
TM3
TM4

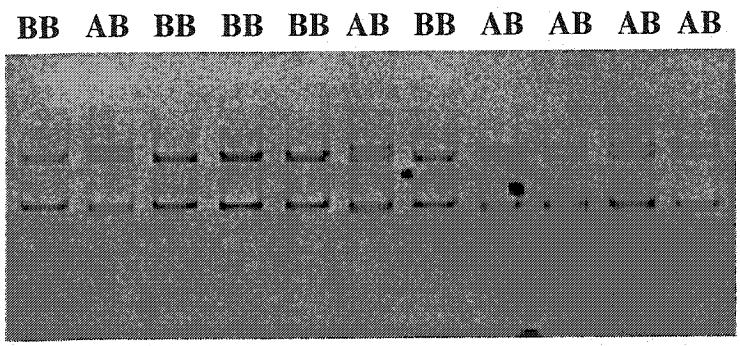
To examine genetic variation of the Co *GluClα3* and *GluClβ* genes, genomic DNA was prepared from individual IVS and IVR male worms. *Co GluClα3* and *GluClβ* gene fragments were amplified separately and subjected to agarose gel electrophoresis. While no size variations were detected on agarose gels, SSCP revealed differences among the samples (Fig. 3).

Figure 3.

A.



B.



Single-strand conformation polymorphism analysis of Co *GluClβ* PCR products. (A). Agarose gel showing the quality of the PCR products derived from 10 individual male worms. M= molecular weight marker, C= No-DNA control. (B). SSCP patterns of 11 individual IVS worms at the Co *GluClβ* gene. Worms shown here are homozygous BB (2 bands) or heterozygous AB (three bands).

For the Co *GluClα3* gene, 75 IVS and 75 IVR individual male worms were analyzed. A total of nine different alleles (A-J) were identified (Fig. 4). Allele A was the most common allele in the IVS group, with a frequency of 0.406. The frequency of allele A in the IVR group was 0.21. Allele E was the most common allele in the IVR group, with a frequency of 0.27. This allele was also found in the IVS group, but at a lower frequency of 0.18. As with allele A, alleles C and G were found at a higher frequency in the IVS group, while alleles D, F and H were found at higher frequencies in the IVR group. Allele J was found only in the IVR group. Chi-square analysis revealed a significant difference in allele frequencies between IVS and IVR groups ($p < 0.001$). Genotypic frequencies did not differ significantly from Hardy-Weinberg equilibrium.

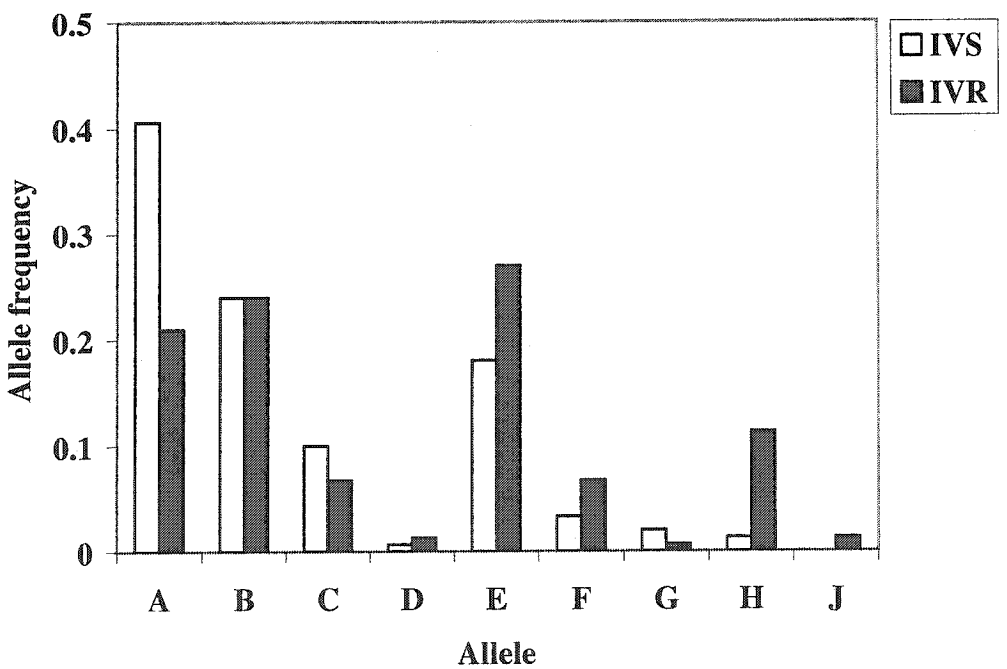


Figure 4. Co *GluClα3* allele frequencies in IVS and IVR worms

For the Co *GluClβ* gene, 160 worms (80 IVS and 80 IVR) were examined. Only two alleles, A and B, were detected by SSCP (Figs. 3 and 5). Allele A had frequencies of 0.137 and 0.1 in the IVS and IVR groups, respectively, while allele B had frequencies of 0.863 and 0.9 in the same respective groups. Allele frequencies were not significantly different between IVS and IVR groups, and genotypic frequencies did not differ significantly from Hardy-Weinberg equilibrium.

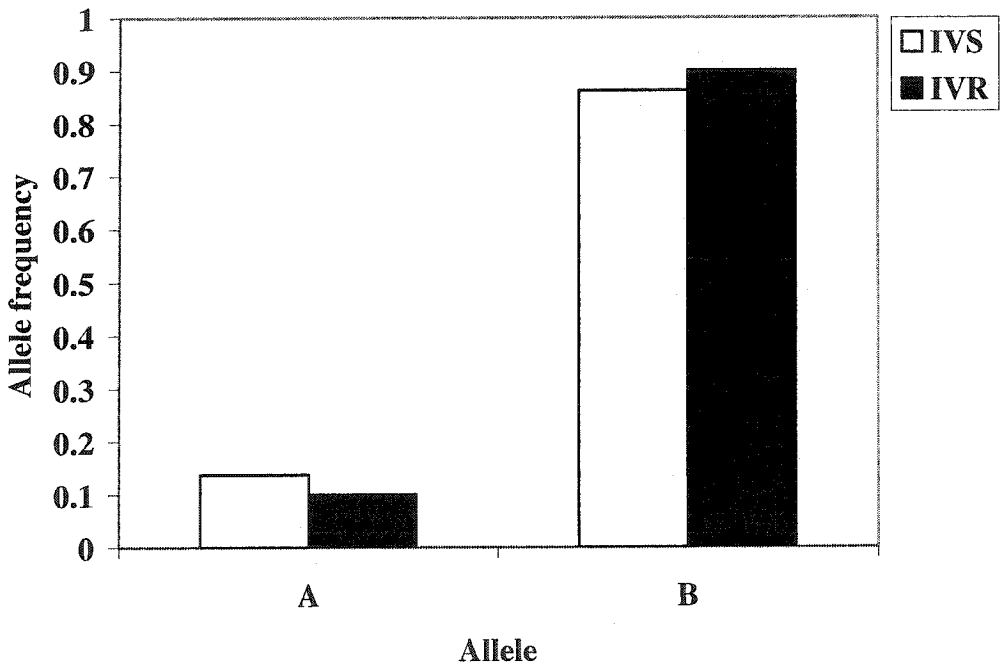


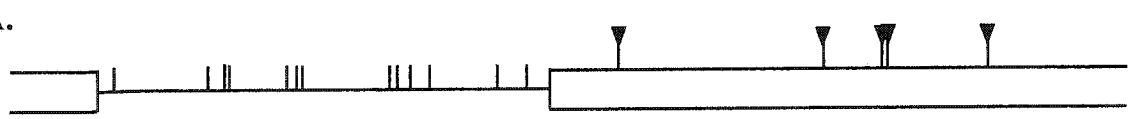
Figure 5. Co *GluClβ* allele frequencies in IVS and IVR worms

To determine the nucleotide differences among the different alleles, amplicons representing all Co *GluClα3* and *GluClβ* alleles were subjected to direct sequencing. For the Co *GluClα3* gene, 228 bp of sequence was obtained for each allele, with a 93-bp

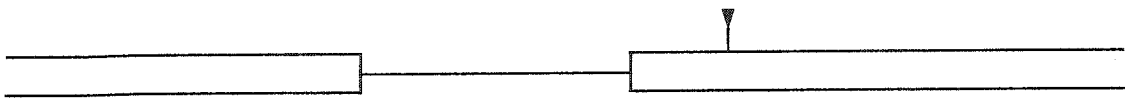
intron flanked by exons. No length polymorphisms were detected. Alignment of the alleles revealed 18 polymorphic sites, distributed across the length of the sequence (Fig. 6A). Of these, 13 were singletons, while five were found two or more times among the nine allele sequences. All were single base substitutions, with 14 (77.8%) being transitions (A↔G, n=9; C↔T, n=5) and 4 (22.2%) being transversions (C↔G, n=2; A↔T, n=1; A↔C, n=1). 13 of the polymorphic sites were found in the intron region. Of the five nucleotide changes found in the exon, four were at the third codon position, and one was at the first codon position. All exon variants were silent mutations, consisting of single nucleotide substitutions with no amino acid changes.

Figure 6.

A.



B.



Positions of polymorphic sites in the region of the Co *GluClα3* and Co *GluClβ* genes analyzed. Polymorphic sites are highlighted as vertical bars. (**A**). 18 positions were found to be polymorphic for the Co *GluClα3* gene. Five of these were in the exon, and are marked by arrows. All were silent mutations. (**B**). Only one polymorphic site was identified in the Co *GluClβ* gene in an exon region. The nucleotide substitution at this position did not result in amino acid change.

For the Co *GluClβ* gene, 278 bp of sequence was obtained for both alleles A and B. The intron was 68 bp long. Figure 6B shows that only one polymorphic site was identified following alignment of the alleles. This nucleotide difference, a C↔T transition, was found in an exon region, and represented a silent mutation at the third codon position.

3.4 Discussion

GluCls are members of the 'cys-loop' class of LGICs that are found only in invertebrates (Cleland 1996). Among nematodes, full-length GluCl cDNAs have been cloned from *C. elegans* (Cully et al. 1994; Cully et al. 1996; Dent et al. 1997; Vassilatis et al. 1997; Dent et al. 2000; Horoszok et al. 2001), and *H. contortus* (Delany et al. 1998; Forrester et al. 1999; Jagannathan et al. 1999). Partial GluCl cDNA sequences have also been obtained from *A. suum* (Jagannathan et al. 1999), *Dirofilaria immitis* and *Onchocerca volvulus* (Cully et al. 1996). In the present study, two full-length GluCl cDNAs, encoding GluClα3 and GluClβ subunits, were cloned from *C. oncophora*. The Co GluClα3 predicted protein sequence showed high identity to GluClα3 sequences from *H. contortus* (88%), *O. volvulus* (88%), *C. elegans* 82%), *A. suum* (76%) and *D. immitis* (85%) (Table 2). The conservation of sequence among GluClα3 subunits from different nematodes, which was previously highlighted by Jagannathan et al. (1999), suggests that these subunits may play an important role in the function of nematode nerve cells.

The Co GluClα3 and GluClβ subunits exhibit features that are characteristic of cys-loop LGICs- a long hydrophobic N-terminal extracellular domain containing a pair of cysteine residues separated by 13 amino acids, and four transmembrane domains. A second pair of cysteine residues, separated by 10 amino acids, is also present in the N-

terminal extracellular region. This is conserved in GluCls, vertebrate glycine-gated chloride channels (Vassilatis et al. 1997), and the recently characterized histamine-gated chloride channels (Zheng et al. 2002).

Studies of *C. elegans* GluCls have shown that these receptors are targets of the avermectin class of endecocides (Arena et al. 1992, 1995; Cully et al. 1994, 1996; Vassilatis et al. 1997: Dent et al. 1997, 2000: Horoszok et al. 2001). When expressed in *Xenopus* oocytes, the *C. elegans* GluClα subunit forms homomeric channels that are activated by ivermectin (Cully et al. 1994). Other *C. elegans* GluCls that are sensitive to ivermectin include AVR-15/ GluClα2 (Dent et al. 1997), AVR-14B/ gbr-2B/ GluClα3B (Dent et al. 2000) and glc-3 (Horoszok et al. 2001). While ivermectin sensitivity appears to be a feature of α-like GluCl subunits, the *C. elegans* AVR-14A subunit, which is a splice variant of the *avr-14/ GluClα3* gene, does not respond to ivermectin (Dent et al. 2000). As well, the *C. elegans* GluClβ subunit is sensitive to glutamate, but not ivermectin (Cully et al. 1994).

GluCls have also been implicated in the mechanism of resistance to ivermectin. In *C. elegans*, simultaneous mutation of three GluCl genes, *glc-1, avr-15* and *avr-14*, confers high-level ivermectin resistance (Dent et al. 2000). In *H. contortus*, changes in allele frequencies as a result of ivermectin selection have been observed in a GluCl gene encoding a putative α-subunit (Blackhall et al. 1998a). In the present study, genetic variability of two *C. oncophora* GluCl genes, Co *GluClα3* and Co *GluClβ*, was analyzed in IVS and IVR worms using SSCP in combination with direct sequencing. Amplicons of Co *GluClα3* and Co *GluClβ* genes analyzed were ~228 and 278 bp in length, respectively, well within the high sensitivity range of 100-350 bp for SSCP (Benkwitz et

al. 1999). A significant difference in allele frequencies was observed between IVS and IVR worms at the Co *GluClα3* gene locus, suggesting that this gene may be involved in ivermectin resistance. Two alleles, E and H, were found at higher frequencies in the IVR group, while allele A had a higher frequency in the IVS group. Differences in allele frequencies between the two groups at the less variable Co *GluClβ* gene locus were not significant, and this gene may not be involved in ivermectin resistance. Similar analyses were performed on the gene encoding actin in the same group of worms. All 32 IVS and 32 IVR worms examined were heterozygous for two alleles, A and B. At the variable beta tubulin isotypes 1 and 2 loci, where ten alleles were identified, allele frequencies were not significantly different between the two groups (Njue and Prichard, in press). These findings suggest that the differences seen at the Co *GluClα3* locus were most likely due to ivermectin selection, and not population bottlenecking, which would have affected the other genes.

While a significant difference in Co *GluClα3* allele frequencies was evident, there was no reduction in genetic variability in the IVR group. Similar results were also obtained by Blackhall et al. (1998a), who found five alleles of a putative GluClα gene in ivermectin-selected and unselected *H. contortus*. In other studies, low levels of benzimidazole resistance did not result in loss of genetic diversity of beta tubulin isotype 1 and 2 genes (Kwa et al. 1993). However, at higher levels of benzimidazole resistance, significant reductions in genetic variability have been reported in *H. contortus*, *Trichostrongylus colubriformis* and *Teladorsagia circumcincta* (Kwa et al. 1993; Beech et al. 1994; Grant and Mascord 1996; Elard and Humbert 1999). With no detectable loss of genetic variability, selection at the Co *GluClα3* gene may be at the early stages.

For both Co *GluClα3* and *GluClβ* genes, the region analyzed by SSCP lay between the two dicysteine loops. Sequencing of the two *GluClβ* alleles revealed that they were identical at the amino acid level. As well, all nine Co *GluClα3* alleles had identical amino acid sequences, suggesting that this region may not directly be involved in ivermectin resistance. For both *GluClα3* and *GluClβ* genes, a second region, the intracytoplasmic loop, was also analyzed by SSCP and sequencing of alleles, and no amino acid changes were seen among *GluClα3* alleles or the *GluClβ* alleles identified (Appendix B). SSCP cannot detect mutations located outside the region being analyzed, and it is possible that mutations in other regions of the gene may be involved in ivermectin resistance.

The expression sites of the Co GluClα3 and GluClβ subunits remain to be determined. In *C. elegans*, the avr-14/ GluClα3 subunits, which are homologous to Co GluClα3, are expressed in extrapharyngeal neurons (Dent et al. 2000). Activation of extrapharyngeal neurons by ivermectin can inhibit pharyngeal pumping, an action mediated via gap junctions (Dent et al. 2000). The *C. elegans* GluClβ subunit is expressed in the pharynx (Laughton et al. 1997). In *H. contortus*, the GluClβ subunit is expressed in motor neuron commissures, and may therefore have some effect on body wall muscle (Delany et al. 1998; Portillo et al. 2003). The splice variants HcGluClα3A and 3B are also expressed in the motor neuron commissures (Jagannathan et al. 1999; Portillo et al. 2003). In other regions, these two splice variants have unique expression patterns (Portillo et al. 2003). HcGluClα3A is found in a pair of lateral neurons in the head of the worm, which are most likely amphidial neurons. HcGluClα3B is expressed in nerve cords, as well as three cell bodies on the pharynx. This suggests that pharyngeal

receptors containing HcGluClα3B are involved in mediating ivermectin's inhibitory effects on pharyngeal pumping.

The two isolates used in this study, IVS and IVR, were originally obtained from different locations in the UK, and their geographical separation, albeit not too great, may have contributed to the difference in allele frequencies at the Co *GluClα3* gene. However, no significant differences in allele frequencies were seen at actin and beta tubulin genes. These two genes have no known association with ivermectin's mode of action. As well, the same Co *GluClα3* alleles were found in the IVS and IVR groups, suggesting that the significant differences observed in Co *GluClα3* allele frequencies were most likely due to ivermectin selection. In *H. contortus*, selection with benzimidazoles has been shown to select for the same allelic types in independently derived field isolates, suggesting that geographical separation does not preclude relevant comparison of the allele frequencies of such isolates (Kwa et al. 1993; Beech et al. 1994). However, further work is required to determine whether there are any functional differences between IVS and IVR Co GluClα3 and GluClβ subunits. Studies are underway to characterize these subunits by expressing them in *Xenopus* oocytes.

Acknowledgements

The authors thank Dr. G. Coles for providing the parasite isolates used in this study. The technical assistance of D. Eshelby and G. Bingham is greatly appreciated. This study was supported by grants from Fort Dodge Animal Health and NSERC, Canada.

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CONNECTING STATEMENT II

Results from the previous chapter demonstrate an association between the *C. oncophora* *GluClα3* gene and ivermectin resistance. However, no amino acid changes were found in the short exon regions examined. Full-length GluCl cDNA sequences amplified from IVS and IVR worms revealed amino acid differences. The following chapter describes the experiments carried out to determine the effects of these mutations on channel function.

CHAPTER 4

Mutations in the extracellular domains of glutamate-gated chloride channel α3 and β subunits from ivermectin-resistant *Cooperia oncophora* affect agonist sensitivity

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Journal of Neurochemistry- In press

Abstract

Two full-length glutamate-gated chloride channel (GluCl) cDNAs, encoding GluClα3 and GluClβ subunits, were cloned from ivermectin-susceptible (IVS) and -resistant (IVR) *Cooperia oncophora* adult worms. The IVS and IVR GluClα3 subunits differ at three amino acid positions, while the IVS and IVR GluClβ subunits differ at two amino acid positions. The aim of this study was to determine whether mutations in the IVR subunits affect agonist sensitivity. The subunits were expressed singly and in combination in *Xenopus laevis* oocytes. Electrophysiological whole-cell voltage-clamp recordings showed that mutations in the IVR GluClα3 caused a modest but significant threefold loss of sensitivity to glutamate, the natural ligand for GluCl receptors. As well, a significant decrease in sensitivity to the anthelmintics ivermectin and moxidectin was observed in the IVR GluClα3 receptor. Mutations in the IVR GluClβ subunit abolished glutamate sensitivity. Co-expressing the IVS GluClα3 and GluClβ subunits resulted in heteromeric channels that were more sensitive to glutamate than the respective homomeric channels, demonstrating co-assembly of the subunits. In contrast, the heteromeric IVR channels were less sensitive to glutamate than the homomeric IVR GluClα3 channels. The heteromeric IVS channels were significantly more sensitive to glutamate than the heteromeric IVR channels. Of the three amino acids distinguishing the IVS and IVR GluClα3 subunits, only one of them, L256F, accounted for the differences in response between the IVS and IVR GluClα3 homomeric channels.

4.1 Introduction

Ivermectin (22, 23- dihydroavermectin B_{1a}) and moxidectin are potent endectocides that are widely used to control parasite infections in domestic animals (Campbell 1989; McKellar and Benchaoui, 1996). Ivermectin is believed to exert its antiparasitic effects by activating glutamate-gated chloride channels (GluCls) (Cully et al. 1994; Cully et al. 1996; Dent et al. 1997; Vassilatis et al. 1997; Dent et al. 2000). These inhibitory receptors are found only in invertebrates (Cleland 1996), and belong to the superfamily of ligand-gated ion channels. The GluCls of *Caenorhabditis elegans* have been studied extensively. Four GluCl subunits, GluClα, GLC-3, AVR-14 (GluClα3) and AVR-15, form ivermectin-sensitive channels when expressed in *Xenopus* oocytes (Cully et al. 1994; Dent et al. 1997; Laughton et al. 1997a; Dent et al. 2000; Horoszok et al. 2001). A fifth subunit, GluClβ, forms glutamate-sensitive channels that do not respond to ivermectin (Cully et al. 1994). A GluClα subunit cloned from *Drosophila melanogaster* forms ivermectin-sensitive channels in *Xenopus* oocytes (Cully et al. 1996). A strong correlation between ivermectin's nematocidal effect and the potentiation and activation of an ivermectin-sensitive chloride current in *Xenopus* oocytes has been demonstrated (Arena et al. 1995). Ivermectin also acts on nematode γ-aminobutyric acid (GABA)-gated chloride channels, and this may also contribute to the antiparasitic effects of macrocyclic endectocides (Holden-Dye and Walker 1990; Feng et al. 2002).

Cooperia oncophora is a nematode that belongs to the superfamily Trichostrogyloidea, and is a common parasite of cattle in temperate climates (Parmentier et al. 1995). It is one of the dose-limiting species for ivermectin in cattle (Shoop et al. 1995). Ivermectin resistance involving *Cooperia* species has been reported (Vermunt et

al. 1996; Coles et al. 1998; Anziani et al. 2001; Coles et al. 2001; Familton et al. 2001; Fiel et al. 2001), and highlights the need to understand ivermectin's mode of action and mechanisms of resistance. Milbemycins, a class of compounds structurally related to avermectins, also activate GluCls (Arena et al. 1995). While some reports suggest that moxidectin, a milbemycin, is effective against ivermectin-resistant nematodes (Craig et al. 1992; Pankavich et al. 1992), other reports suggest that ivermectin resistance leads to loss of sensitivity to moxidectin (Conder et al. 1993; Shoop et al. 1993). Moxidectin has been shown to be ineffective against ivermectin-resistant *C. oncophora* (Vermunt et al. 1996).

In an attempt to understand the role of GluCls in ivermectin resistance in *C. oncophora*, we have cloned two full-length cDNAs, encoding GluClα3 and β subunits (Njue and Prichard, submitted). These subunits show high homology to related sequences in *C. elegans*; at the amino acid level, *C. oncophora* GluClα3 shares 80% identity with the *C. elegans* AVR-14B/GluClα3B, while *C. oncophora* GluClβ shares 76% identity with *C. elegans* GluClβ subunit. Genetic variation analysis of short fragments of the *C. oncophora* GluCl genes in ivermectin-susceptible (IVS) and –resistant (IVR) worms showed the *GluClα3* gene to be polymorphic, and the *GluClβ* gene less so (Njue and Prichard, submitted). Initial isolation of the IVR *C. oncophora* was reported by Coles et al. (1998). Here, we report the cloning of full-length GluClα3 and β subunit alleles from IVS and IVR worms. Three non-synonymous mutations were identified in the N-terminal extracellular domains of IVR GluClα3. As well, two mutations were identified in the N-terminal domain of IVR GluClβ. To determine whether mutations in the IVR GluCl subunits affect receptor function, the subunits were

expressed in *Xenopus* oocytes and responses examined. We demonstrate that *C. oncophora* GluCls form functional homomeric and heteromeric channels when expressed in *Xenopus* oocytes, and show that mutations in the two GluCl subunits affect agonist sensitivity.

4.2 Materials and methods

4.2.1 Generation of full-length cDNAs, and cRNA synthesis

Total RNA extracted from bulk IVS and IVR adult worms using Trizol Reagent™ was reverse-transcribed using an oligo-dT primer (GibcoBRL). This was used as template for PCR amplification of the *C. oncophora* GluClα3 and GluClβ cDNAs. Subunit cDNAs generated were subcloned into the pT7TS vector, which contains 5' and 3' untranslated *Xenopus* β-globin regions (Dent et al. 1997). The clones were sequenced from both directions using the vector primers T7 and SP6.

The IVS and IVR GluClα3 subunits differ at three amino acid positions (Fig. 1). To determine the effect of each mutation on channel properties, site-directed mutagenesis was used to construct three mutants (MUT 1-3) each bearing one of the amino acid substitutions that distinguish IVS and IVR GluClα3 (Table 1). All three mutations required a single base substitution. Site-directed mutagenesis was performed on pT7TS-IVS GluClα3 clones using the QuickChange mutagenesis kit (Stratagene), and the successful incorporation of mutations was confirmed by sequencing.

All cDNA/pT7TS plasmids were linearized using *Xba* 1 immediately downstream of the 3' untranslated β-globin sequence, and cRNA was transcribed using the T7 mMessage mMachine Kit (Ambion). Quality and quantity of cRNA were checked by

agarose gel electrophoresis and absorption spectroscopy. Samples were stored at -80°C until use.

Table 1. *C. oncophora* GluClα3 mutants tested. To determine the effect of each amino acid substitution on channel properties, three mutants, each representing one of the amino acid substitutions, were constructed. The letter preceding the position number refers to the amino acid in the IVS GluClα3 subunit, and the letter following the number refers to the amino acid in the IVR GluClα3 subunit. For each mutant, the remainder of the sequence was identical to that of the IVS GluClα3 subunit.

GluClα3 MUTANT	NUCLEOTIDE MUTATION	AMINO ACID CHANGE
MUT 1	GAG to GGG	E114G
MUT 2	GTT to GCT	V235A
MUT 3	CTT to TTT	L256F

4.2.2 Oocyte preparation and injection

Ovarian tissue was isolated from mature female *Xenopus laevis* by a simple surgical procedure. The frogs were anaesthetized by immersion in neutralized ethyl m-aminobenzoate solution (tricaine, methanesulphonate salt, 0.2% solution w/v; Sigma). The isolated ovarian lobes were placed in a dish containing OR2 calcium-free buffer (82.5 mM NaCl, 2 mM KCl, 1mM $MgCl_2$, 5 mM HEPES, pH adjusted to 7.5 with NaOH) and dissected into smaller clumps of oocytes. These were rinsed several times, and gently shaken in OR2 containing 4 mg/ml collagenase type 1A (Sigma) for 1 hour at

17°C. The oocytes were rinsed thoroughly in OR2 and manually defolliculated before being placed in ND96 buffer (96 mM NaCl, 2 mM KCl, 1mM $MgCl_2$, 1.8 mM $CaCl_2$, 5 mM HEPES, pH adjusted to 7.4 with NaOH) supplemented with 100 units/ml penicillin and 100 μg/ml streptomycin. 9.2 nl of cRNA (~1ng/subunit) in RNAse-free water were injected cytoplasmically. To express the heteromeric GluClα3/GluClβ receptors, the two subunit cRNAs were mixed at a ratio of 1:1 before injection into oocytes. Injected oocytes were maintained at 17°C in ND96 supplemented with penicillin and streptomycin, and electrophysiological recordings were performed at room temperature (22-25°C) 2-3 days after injection.

4.2.3 Electrophysiological recordings

Whole-cell currents were recorded from oocytes using the two-electrode voltage-clamp technique. Oocytes were voltage-clamped at a holding potential of -80 mV using Axoclamp 2B voltage clamp amplifier (Axon Instruments). Recordings were made in ND96. Recording microelectrodes were filled with 3M potassium acetate, and had tip resistances of 1-3 MΩ. Oocytes were held in a 0.13 ml bath, and continually superfused with ND96 at a rate of 0.8 ml/min. Drugs were applied by local microperfusion using an electronic digital pipette, and the duration of application was 4 sec. For the dose-response experiments, recordings were made sequentially from the lowest to the highest drug concentration, with an interval of 2 min between applications. For the glutamate responses, a second application of the same concentration was applied every third response to ensure reproducibility. Glutamate was dissolved in ND96. Ivermectin and

moxidectin were prepared as 10 mM stock solutions in dimethyl sulfoxide (DMSO) and diluted in ND96.

Data acquisition and analysis were conducted using the pCLAMP suite of programs (Axon Instruments). The current-voltage relationship of the glutamate-induced response was determined over a voltage range of -80 to +10 mV. 250 μM glutamate was first applied at -80 mV. This was followed by repeated glutamate application at holding potentials of -40, -20, 0 and 10 mV. The peak amplitude of the glutamate-induced currents at various potentials was normalized by assigning 100% to the value at -80 mV. The glutamate dose-response relationships were measured by applying a series of glutamate concentrations to each oocyte. For each oocyte, measurements for the glutamate dose-response curves were normalized by assigning 100% to current activated by saturating concentrations of glutamate in the same oocyte. Because the effects of ivermectin and moxidectin were irreversible, the responses to successive drug administration were taken to be additive (Arena et al. 1995). The ivermectin and moxidectin responses were normalized to the maximal response elicited with 5 μM ivermectin or moxidectin, respectively. Dose-response data were analyzed using GraphPad Prism (GraphPad Software). Dose-response curves were fitted to the equation:

$$I / I_{max} = 1 / [1 + (EC_{50} / [D])^{h}],$$

where I_{max} is the maximal response, [D] is the drug concentration, EC_{50} is the concentration of agonist necessary to elicit half the maximum response, and h is the Hill

coefficient. Data are given as means ± S.E. Statistical analyses were performed using Student's unpaired two-tailed *t* test, with a significance level of $P < 0.05$.

4.2.4 Drugs

Except for moxidectin, all drugs were purchased from Sigma. Moxidectin was a gift from Fort Dodge Animal Health.

4.3 Results

Full-length cDNAs encoding *C. oncophora* GluClα3 and GluClβ subunits were obtained from ivermectin-susceptible (IVS) and ivermectin-resistant (IVR) worms. The predicted protein sequences were aligned using Clustal W and are shown in Figure 1. Comparison of the IVS and IVR GluClα3 subunits showed three amino acid differences at positions 114, 235 and 256. The first two mutations (E114G and V235A) were a result of transitional substitutions at the second codon position (Table 1), while the position 256 mutation (L256F) was caused by a substitution at the first codon position. All three mutations were located in the N-terminal extracellular domain. Alignment of the IVS and IVR GluClβ subunits also revealed two mutations (V60A and R101H) in the N-terminal extracellular domain, both resulting from transitional substitutions at the second codon position (Fig. 1B).

Figure 1. Comparing the predicted amino acid sequences of the *C. oncophora* GluClα3 (A) and GluClβ (B) subunits from IVS and IVR worms. Conserved residues are shaded grey. The putative transmembrane domains, TM 1 – 4, are overlined. **A.** The IVS and IVR GluClα3 sequences differ at three amino acid positions (highlighted by ▼), all located in the N-terminal extracellular domain. **B.** The IVS and IVR GluClβ sequences differ at two amino acid positions, both found in the N-terminal extracellular domain.

Figure 1A.


IVS GluClα3
IVR GluClα3
MRTSVPLATRIGPILALICIVITIISTVEGKRKLKEQEIIQRILNNYDWRVRPRGLNASW
MRTSVPLATRIGPILALICIVITIISTVEGKRKLKEQEIIQRILNNYDWRVRPRGLNASW
PDTGGPVLVTVNIYLRSISKIDDVNMEYSAQFTFREEWVDARLAYGRFEDESTEVPPFVV
PDTGGPVLVTVNIYLRSISKIDDVNMEYSAQFTFREEWVDARLAYGRFEDESTGVPPFVV
LATSENADQSQQIWMPDTFFQNEKEARRHLIDKPNVLIRIHKDGSILYSVRLSLVLSCPM
LATSENADQSQQIWMPDTFFQNEKEARRHLIDKPNVLIRIHKDGSILYSVRLSLVLSCPM
SLEFYPLDRQNCLIDLASYAYTTQDIKYEWKEQNPVQQKDGLRQSLPSFELQDVVTKYCT
SLEFYPLDRQNCLIDLASYAYTTQDIKYEWKEQNPVQQKDGLRQSLPSFELQDVATKYCT
TM 1
TM 2
SKTNTGEYSCARVKLLLRREYSYYLIQLYIPCIMLVVVSWVSFWLDKDAVPARVSLGVTT
SKTNTGEYSCARVKLFLRREYSYYLIQLYIPCIMLVVVSWVSFWLDKDAVPARVSLGVTT
TM 3
LLTMTTQASGINSKLPPVSYIKAVDVWIGVCLAFIFGALLEYAVVNYYGRKEFLRKEKKK
LLTMTTQASGINSKLPPVSYIKAVDVWIGVCLAFIFGALLEYAVVNYYGRKEFLRKEKKK
TM 4
KTRLDDCVCPSERPALRLDLSTFRRRGWTPLNRLLDVLGRNADLSRRVDLMSRITFPTLF
KTRLDDCVCPSERPALRLDLSTFRRRGWTPLNRLLDVLGRNADLSRRVDLMSRITFPTLF
IVS GluClα3
IVR GluClα3
TVFLVFYYSVYVKQSNLE
TVFLVFYYSVYVKQSNLE

Figure 1B.


10 20 30 40 50 60
IVS GluClβ MTFALVFTVATVAVMADSSHVSRRSSGGTQEQEILNELLSNYDMRVRPPPTNYSDPTGPV
IVR GluClβ MTFALVFTVATVAVMADSSHVSRRSSGGTQEQEILNELLSNYDMRVRPPPTNYSDPTGPA
70 80 90 100 110 120
IVS GluClβ TVRVNIMIRMLSKIDVVNMEYSMQLTFREQWLDSRLAYARLGYHNPPKFLTVPHIKSNLW
IVR GluClβ TVRVNIMIRMLSKIDVVNMEYSMQLTFREQWLDSRLAYAHLGYHNPPKFLTVPHIKSNLW
130 140 150 160 170 180
IVS GluClβ IPDTFFPTEKAAHRHLIDTDNMFLRIHPDGKVLYSSRISITSSCHMQLQLYPLDLQFCDF
IVR GluClβ IPDTFFPTEKAAHRHLIDTDNMFLRIHPDGKVLYSSRISITSSCHMQLQLYPLDLQFCDF
190 200 210 220 230 240
IVS GluClβ DLVSYAHTMKDIVYQWDPTAPVQLKPGVGSDLPNFQLTNITTNDDCTSHTNTGSYACLRM
IVR GluClβ DLVSYAHTMKDIVYQWDPTAPVQLKPGVGSDLPNFQLTNITTNDDCTSHTNTGSYACLRM
TM 1
TM 2
300
IVS GluClβ QLTLKRQFSYYLVQLYGPTTMIVIVSWVSFWIDMHSTAGRVALGVTTLLTMTTMQAAINA
IVR GluClβ QLTLKRQFSYYLVQLYGPTTMIVIVSWVSFWIDMHSTAGRVALGVTTLLTMTTMQAAINA
TM 3
310 340 350 360
IVS GluClβ KLPPVSYVKVVDVWLGACQTFVFGALLEYAFVSYQDSQRQTDQAKSQATRKAQKRRAQME
IVR GluClβ KLPPVSYVKVVDVWLGACQTFVFGALLEYAFVSYQDSQRQTDQAKSQATRKAQKRRAQME
TM 4
370 380 390 400
IVS GluClβ LSERDHYQPPCTCHLYQDYEPTLRDRLRRYFTKPDYLPAKIDYYARFCVPLGFLAFNAIY
IVR GluClβ LSERDHYQPPCTCHLYQDYEPTLRDRLRRYFTKPDYLPAKIDYYARFCVPLGFLAFNAIY
430
IVS GluClβ WTSCLVMVSRLI
IVR GluClβ WTSCLVMVSRLI

cRNAs corresponding to the IVS and IVR GluClα3 and GluClβ subunits were expressed in *Xenopus* oocytes as homomers and heteromers. Initially, 5 ng of cRNA (in 26 nl) were injected into oocytes. This led to a dramatic increase in holding current, which may have been caused by spontaneous opening of channels in the absence of agonist. This was particularly so in oocytes expressing the IVS GluClα3-GluClβ heteromeric channels. As well, this 'leakiness' was more evident when recordings were done three or more days post-injection. As a result, the amount of RNA injected in ooctyes was reduced to ~1 ng, and recordings were done from as early as 24 hours post-injection and continued on day two post-injection.

The IVS and IVR GluClα3 subunits were expressed in *Xenopus* oocytes as homomers, and as heteromers with the IVS and IVR GluClβ subunits, respectively. Figure 2 illustrates the responses evoked in homomeric and heteromeric channels by increasing concentrations of glutamate, and the corresponding dose-response relationships. At a holding potential of -80mV, glutamate application elicited robust inward currents in oocytes expressing IVS and IVR GluClα3 subunits (Fig. 2A). The responses were dose-dependent, rapid in onset and completely reversible. The normalized dose-response curves for the IVS and IVR GluClα3 homomeric receptors are shown in Fig. 2B. The presence of three mutations (E114G, V235A and L246F) in the IVR GluClα3 subunit resulted in a shift of glutamate response curve of the receptor to the right. Glutamate EC_{50} values showed a significant threefold increase from 29.7 ± 4 (n = 3) in IVS GluClα3 to 96.1 ± 4.4 μM (n = 3) in IVR GluClα3 (Table 3A). Hill slopes for the two receptors were 2.4 ± 0.21 and 2.4 ± 0.45, respectively, suggesting that binding of more than one glutamate was required to gate the channel.

As with the GluClα3 subunits, the IVS GluClβ subunit also formed functional glutamate-gated homomeric channels (Figs. 2A and B). Compared to the robust responses elicited by glutamate in the GluClα3 homomeric channels, the IVS GluClβ channel responses were smaller (Fig. 2A). The mean maximum current for the GluClα3 homomeric receptor was six times that for the GluClβ homomeric receptor. The EC_{50} value for glutamate for the GluClβ receptor was 185.6 ± 24.9 μM (n = 3) and the Hill value 2.2 ± 0.02. The IVR GluClβ subunit channel did not respond to any of the glutamate concentrations tested (10nm to 1mM).

Glutamate also elicited responses in cells co-expressing IVS GluClα3 and IVS GluClβ subunits (Fig. 2C). Relative to the respective homomeric receptors, the dose-response curve of the heteromeric channel shifted to the left. The EC_{50} value was estimated to be 13.4 ± 2.5 μM (n = 3), and the Hill slope 1.9 ± 0.28. In contrast, co-expressing the IVR GluClα3 and IVR GluClβ subunits shifted the concentration-response curve to the right, relative to the IVR GluClα3 homomeric receptor, indicating a reduction in glutamate sensitivity. The EC_{50} value obtained for this channel was 171.6 ± 20.7 μM (n = 3), manifesting a significant ~ 13-fold decrease in glutamate sensitivity relative to the heteromeric IVS receptor. Uninjected and water-injected oocytes did not show any response to glutamate.

Figure 2. Activation of IVS GluClα3 and GluClβ receptors by glutamate. IVS GluClα3 and GluClβ subunits form functional glutamate-activated homomeric and heteromeric channels when expressed in *Xenopus* oocytes (A, B). **A.** Representative current traces from oocytes activated with glutamate at concentrations shown above each trace. Currents were measured using the whole-cell-two-electrode voltage clamp technique, at V_{hold} = -80 mV. The GluClβ homomeric channel is less sensitive to glutamate than the GluClα3 homomeric and GluClα3/GluClβ heteromeric channels. **B.** Normalized dose-response curves for glutamate-induced currents for oocytes expressing homomeric receptors (same oocytes as in A for IVS). EC_{50} values for IVS GluClα3 (■), IVR GluClα3 (▲) and IVS GluClβ (○) homomeric receptors = 29.7, 96.1 and 185.6 μM, respectively. IVR GluClβ (◊) receptor showed no response to glutamate. **C.** Glutamate dose-response curves for ooctyes expressing homomeric GluClα3 (same as B) and heteromeric Co GluClα3/GluClβ channels. Compared to the IVS heteromeric channel (○), the IVR heteromeric channel (◊) showed a shift to the right, with a ~ 13-fold increase in EC_{50} value (13.4 μM and 171.6 μM for the IVS and IVR heteromeric channels, respectively).

Figure 2A. REPRESENTATIVE CURRENTS

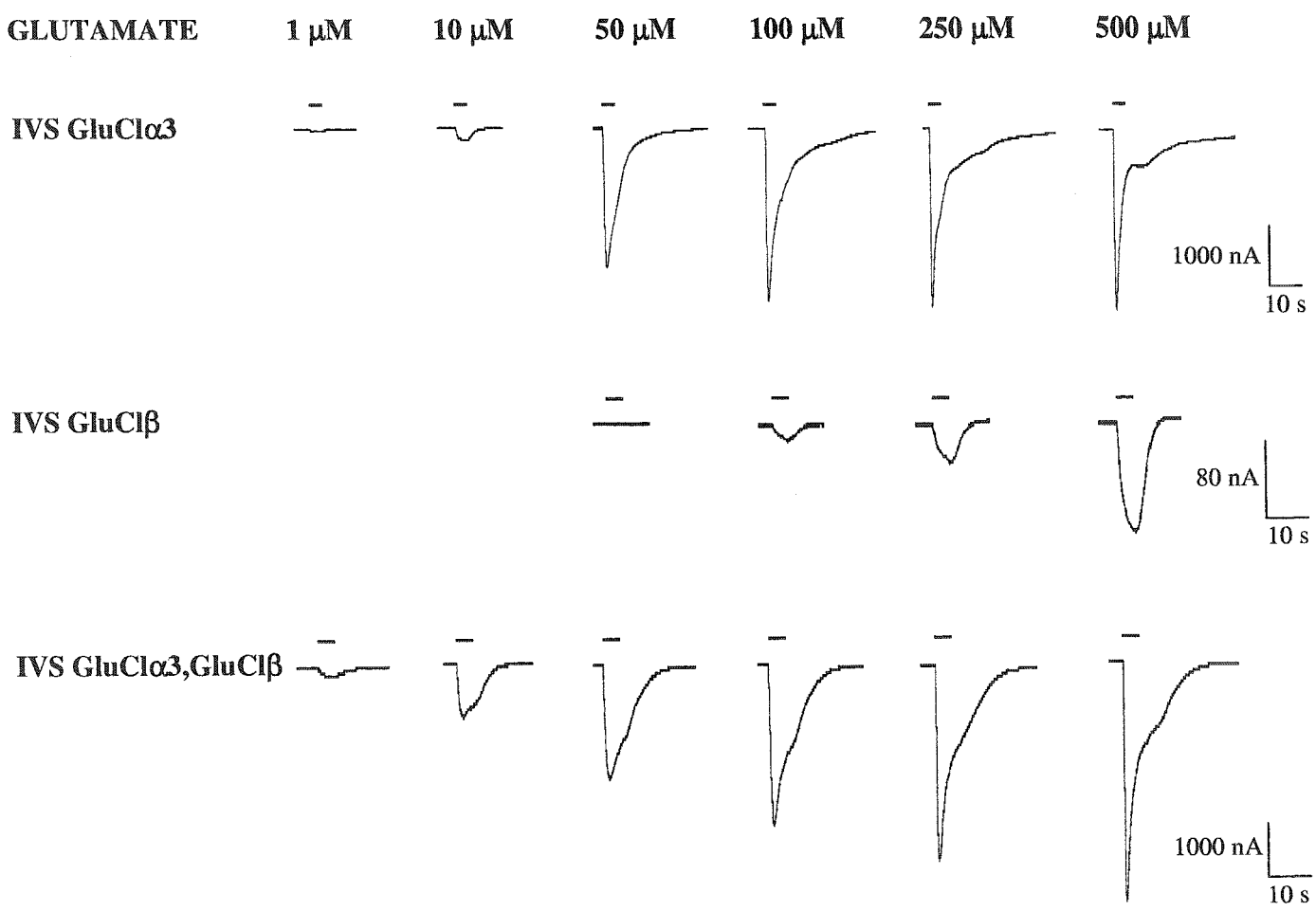


Figure 2B. Dose responses for homomeric receptors.

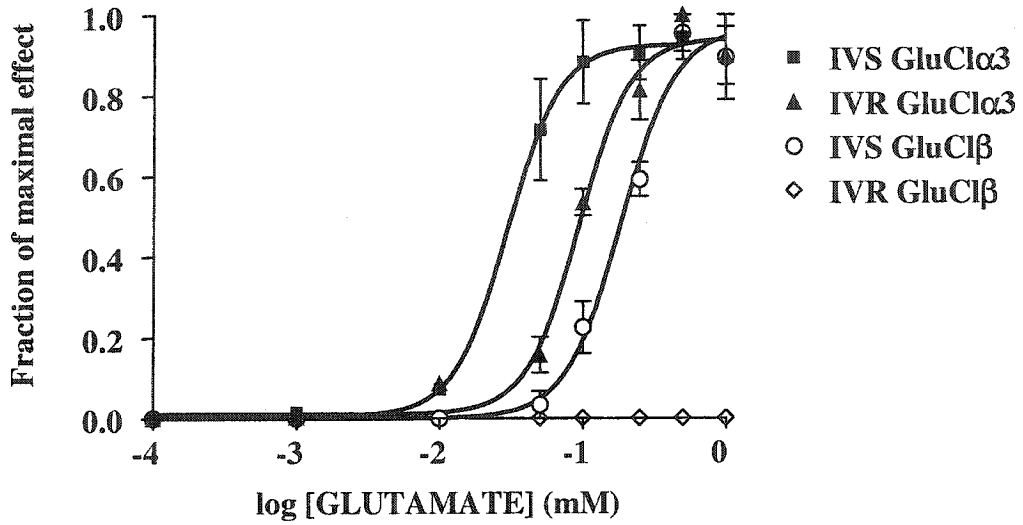
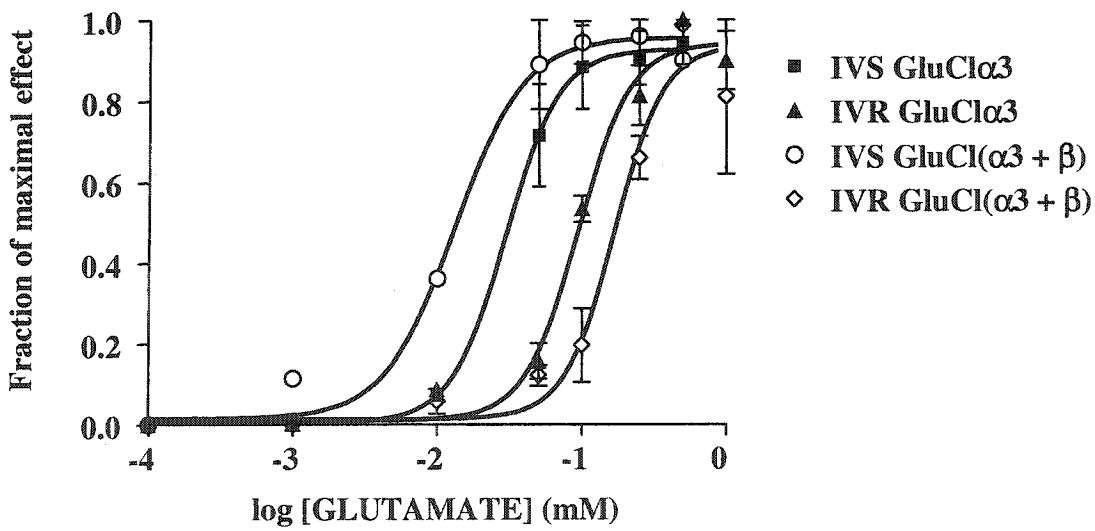


Figure 2C. Dose responses for homomeric GluClα3 and heteromeric receptors.



The current-voltage relationship for the glutamate-sensitive current from the IVS GluClα3 homomeric channels showed a slightly outwardly rectifying voltage dependence (Fig. 3). The reversal potential was -22 ± 1 mV (n = 3), close to the Nernst potential for chloride in *Xenopus* oocytes (-24 mV, with 103.6 mM extracellular chloride and assuming 40 mM intracellular chloride [Dascal 1987]).

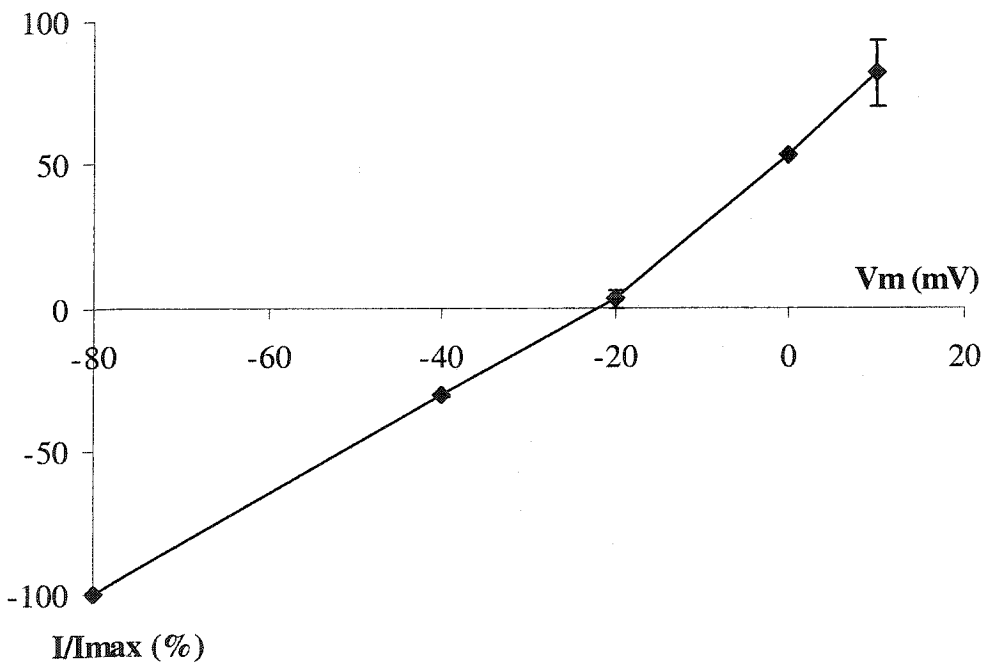


Figure 3. Current-voltage (I/V) relationship for the glutamate-gated currents of the IVS GluClα3 receptor was tested at potentials ranging from -80 mV to +10 mV. Current amplitude were normalized by assigning 100% to the glutamate-induced current recorded at -80 mV. The reversal potential was -22 ± 1 mV (n = 3 ooctyes).

The effects of ivermectin and moxidectin were also tested on the homomeric channels. Unlike the glutamate response, the responses to ivermectin and moxidectin were slow to activate and irreversible (Fig. 4A). The current did not return to baseline even after washing for several minutes with drug-free ND96. Application of 250 μM glutamate and 5 μM moxidectin during maximal response to ivermectin did not elicit additional response. No response to ivermectin or moxidectin was observed in uninjected or water-injected oocytes. Oocytes expressing IVS GluClα3 homomeric channels displayed an ivermectin dose-response relation with an EC_{50} of 0.5 ± 0.12 μM (n = 3) and a Hill slope of 2.1 ± 0.5 (Fig. 4B, Table 3B). As with the glutamate response, the ivermectin response of the IVR GluClα3 receptor was shifted to the right relative to the IVS GluClα3 receptor, with a significant increase in the ivermectin EC_{50} of about threefold (EC_{50} = 1.3 μM ± 0.11, Hill slope 2.2 ± 0.18, n = 3). In contrast, the IVS GluClα3 moxidectin dose-response curve was shifted to the left relative to the IVS GluClα3 ivermectin curve (Fig. 4B). The EC_{50} was estimated to be 0.2 ± 0.06 μM (n = 3), and the Hill slope 1.6 ± 0.36. A similar trend was seen when the IVR GluClα3 receptor dose-response curves for ivermectin and moxidectin were compared. The IVR GluClα3 moxidectin curve had an EC_{50} value similar to that of the IVS GluClα3 ivermectin curve (0.5 ± 0.05 μM, n = 3 and 0.5 ± 0.12 μM, n = 3, respectively). Relative to the IVS GluClα3 moxidectin curve, IVR GluClα3 also showed a significant decrease in moxidectin EC_{50} of ~ 2.6-fold. No ivermectin- or moxidectin-activated chloride currents were observed in oocytes expressing GluClβ homomeric channels.

Figure 4. Activation of IVS and IVR GluClα3 receptors by ivermectin (IVM) and moxidectin (MOXI) (A, B). Evidence that ivermectin and moxidectin are agonists of *C. oncophora* GluClα3 receptors. **A.** Currents induced by glutamate, ivermectin and moxidectin, applied at the concentrations indicated. While the glutamate response is rapid in activation of the membrane current and completely reversible, the ivermectin and moxidectin responses were slow in activation and irreversible. **B.** Normalized dose-response curves for ivermectin and moxidectin. The EC_{50} values for ivermectin for IVS (■) and IVR (▲) GluClα3 receptors = 0.5 and 1.3 μM respectively. The EC_{50} values for moxidectin for IVS (●) and IVR (♦) GluClα3 receptors = 0.2 and 0.5 μM respectively.

Figure 4A.

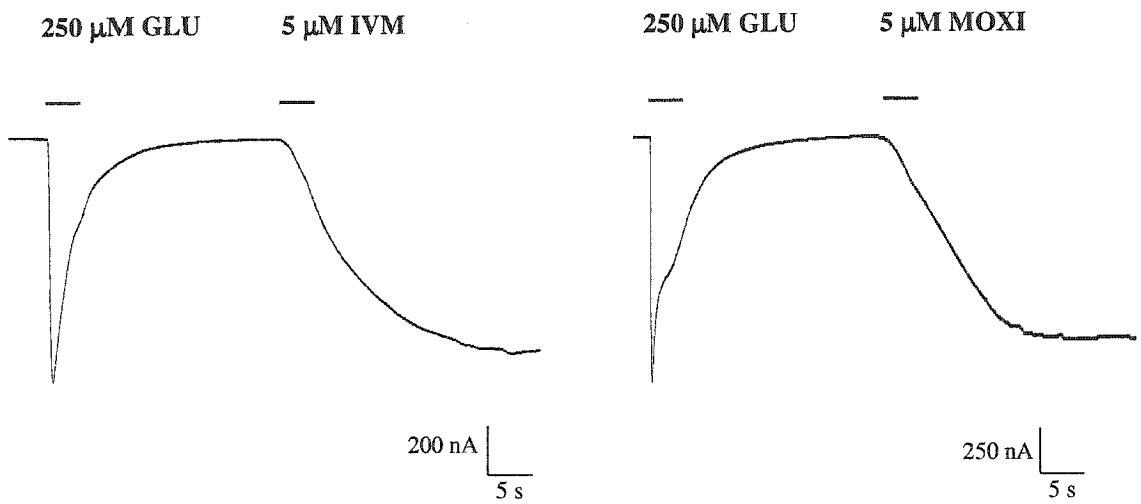
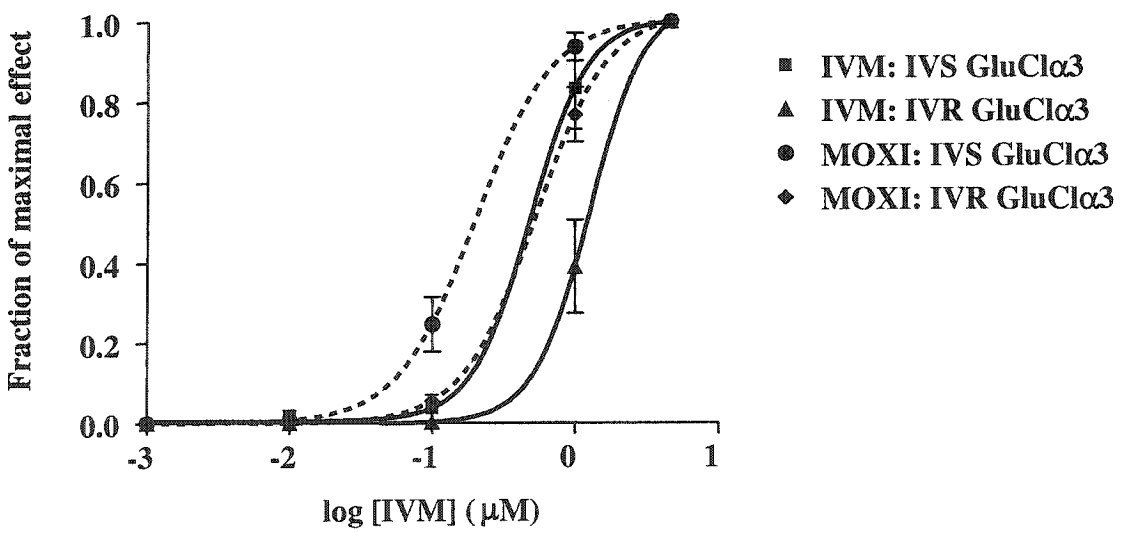


Figure 4B.



In an attempt to determine whether one, two or all three mutations in the IVR GluClα3 subunit contributed to altered response to glutamate and ivermectin, the mutants, each representing one mutation, were expressed individually in *Xenopus* oocytes. Fig. 5A compares the glutamate dose responses of the mutants with those of the IVS and IVR GluClα3 homomeric channels. Mut 1 (E114G) and Mut 2 (V235A) both showed dose response curves very similar to those of the IVS GluClα3 receptor, with EC_{50} values of 25.7 ± 5.2 (n = 2) and 27.8 ± 2.6 (n = 3) μM for Mut 1 and Mut 2, respectively. In contrast, the glutamate dose-response curve and EC_{50} value (100.6 μM ± 27, n = 2) of the Mut 3 (L256F) receptor were very similar to those of the IVR GluClα3 receptor. A similar trend was observed with the ivermectin dose-response curves (Fig. 5B). EC_{50} values for the Mut 1, Mut 2 and Mut 3 ivermectin curves were 0.6 ± 0.18, 0.4 ± 0.01 and 1.2 ± 0.11 μM, respectively.

Figure 5. Normalized dose-response curves of mutant GluClα3 receptors for glutamate and ivermectin (A, B). **A**. The EC_{50} values for glutamate for Mut 1 (E114G, ◊), Mut 2 (V235A, ○) and Mut 3 (L256F, □) GluClα3 receptors = 25.7, 27.8 and 100.6 μM, respectively. **B**. The EC_{50} values for ivermectin for Mut 1 (◊), Mut 2 (○) and Mut 3 (□) receptors = 0.6, 0.4 and 1.2 μM, respectively.

Figure 5A. Glutamate dose response curves

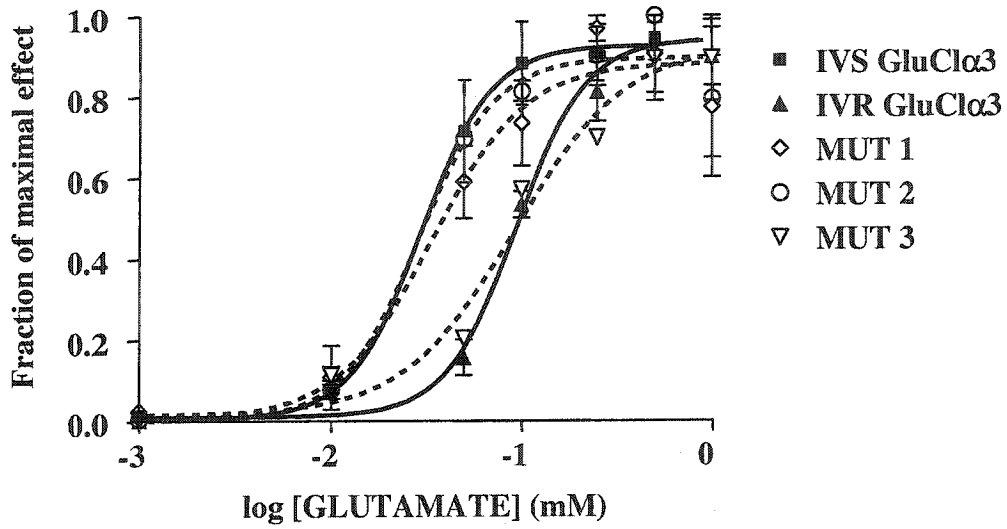
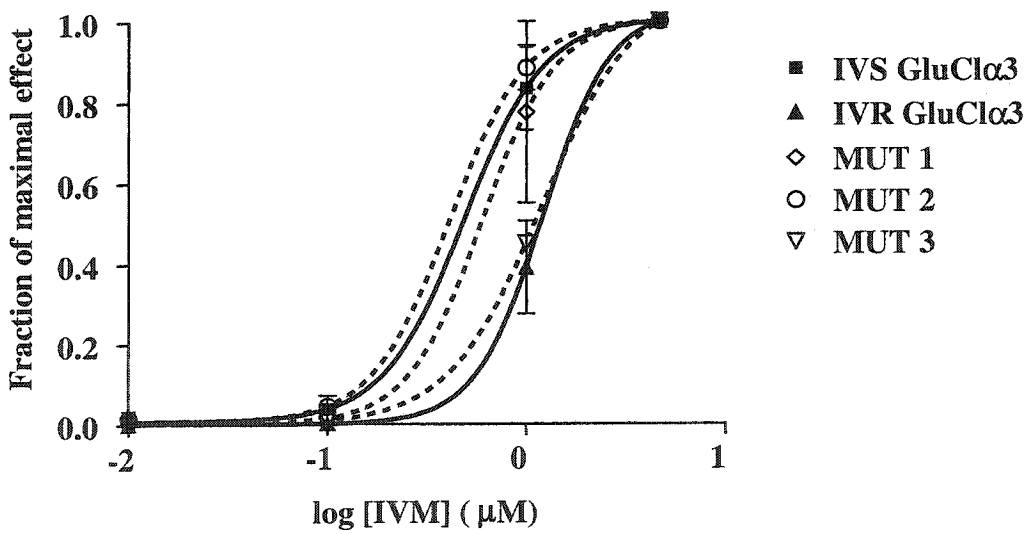


Figure 5B. Ivermectin dose response curves



The pharmacological properties of the homomeric channels were also examined. Ibotenate, a structural analogue of glutamate known to activate GluCls (Lea and Usherwood 1973; Arena et al. 1992; Cully et al. 1994), elicited responses in IVS GluClα3 and IVS GluClβ homomeric channels (Fig. 6, Table 2). For both channels, the kinetics of the glutamate and ibotenate responses were similar. While 1 mM ibotenate maximally activated the IVS GluClβ channel, the response of the IVS GluClα3 channel was 80 ± 6.1% of the response to 1 mM glutamate. Apart from ibotenate, no other agonist activated the IVS GluClα3 and IVS GluClβ homomeric channels (Table 2). The glutamate analogs D-glutamate, quisqualate, kainate and NMDA were inactive. As well, 1 mM concentrations of glycine and GABA failed to activate current.

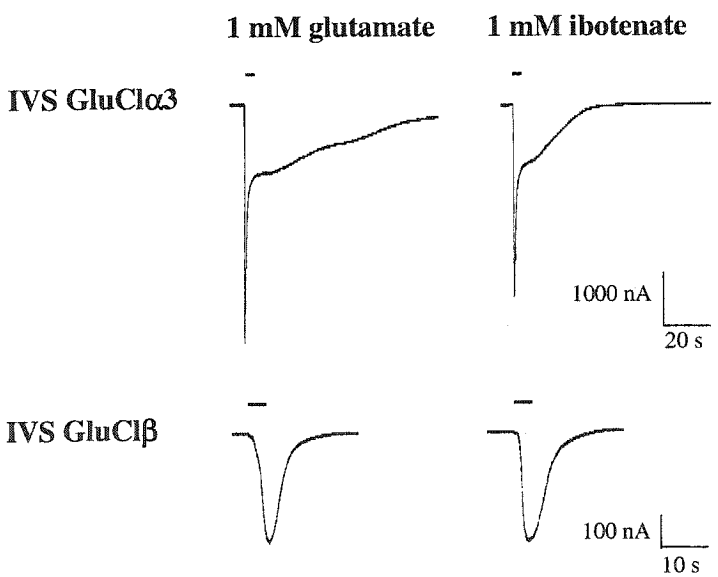


Figure 6. Ibotenate activated IVS GluClα3 and IVS GluClβ homomeric channels. Currents activated by glutamate and ibotenate at concentrations shown above each current plot. The kinetics of the glutamate and ibotenate responses are similar in both channels.

Table 2. Pharmacology of Co GluClα3 and GluClβ homomeric channels. For each ligand, at least three oocytes were tested.

AGONIST (LIGAND)	IVS GluClα3 CHANNEL RESPONSE (nA)	IVS GluClβ CHANNEL RESPONSE (nA)
250 μM Glutamate	-5936 ± 409	-311 ± 11
1 mM Ibotenate	-4785 ± 362	-310 ± 9
1 mM Aspartate	0	0
1 mM D-Glutamate	0	0
500 μM Quisqualate	0	0
500 μM Kainate	0	0
1 mM Glycine	0	0
1 mM GABA	0	0
1 mM NMDA	0	0

Table 3. Summary of effects of (A) glutamate and (B) ivermectin (IVM) and moxidectin (MOXI) on IVS, IVR and mutant GluCl receptors. EC_{50} and Hill slope values are expressed as means ± S. E. of mean. To express heteromeric channels, cRNAs for the GluClα3 and β subunits were injected in a 1: 1 ratio.

Table 3A. Glutamate responses

Receptor	EC_{50} (μM)	Hill slope	Oocytes (n)
IVS GluClα3	29.7 ± 4	2.4 ± 0.21	3
IVR GluClα3	96.1 ± 4.4	2.4 ± 0.45	3
IVS GluClβ	185.6 ± 24.9	2.2 ± 0.02	3
IVR GluClβ	-	-	3
IVS GluCl(α3 + β)	13.4 ± 2.5	1.9 ± 0.28	3
IVR GluCl(α3 + β)	171.6 ± 20.7	2.5 ± 0.28	3
MUT 1 (E114G)	25.7 ± 5.2	1.8 ± 0.21	3
MUT 2 (V235A)	27.8 ± 2.6	2.2 ± 0.27	3
MUT 3 (L256F)	100.6 ± 27	1.5 ± 0.35	3

Table 3B. IVM and MOXI responses

Receptor	EC_{50} (μM)	Hill slope	Oocytes (n)
IVM- IVS GluClα3	0.5 ± 0.12	2.1 ± 0.5	3
IVM- IVR GluClα3	1.3 ± 0.11	2.2 ± 0.18	3
MOXI- IVS GluClα3	0.2 ± 0.06	1.6 ± 0.36	3
MOXI- IVR GluClα3	0.5 ± 0.05	1.7 ± 0.22	3
IVM- MUT 1 (E114G)	0.6 ± 0.18	2.3 ± 0.3	2
IVM- MUT 2 (V235A)	0.4 ± 0.01	2.2 ± 0.33	3
IVM- MUT 3 (L256F)	1.2 ± 0.11	1.9 ± 0.16	2

4.4 Discussion

GluCls are members of the ligand gated ion channel (LGIC) superfamily which includes the cationic channels activated by acetylcholine and serotonin, as well as anionic channels activated by γ-aminobutyric acid (GABA), glycine, histamine and serotonin (Ortells and Lunt 1995; Ranganathan et al. 2000; Zheng et al. 2002). GluCls are found only in invertebrates (Cleland 1996), and are therefore ideal drug targets. Ivermectin is a widely used anthelmintic which is known to interact with GluCls (Cully et al. 1994; Arena et al. 1995; Cully et al. 1996; Dent et al. 1997; Vassilatis et al. 1997; Dent et al. 2000). This interaction is thought to mediate at least part of its anthelmintic activity, since the ability of ivermectin to activate GluCls expressed in *Xenopus* oocytes correlates well with its biological activity (Arena et al. 1995).

In this study, we describe the function of two GluCl subunits, GluClα3 and GluClβ, from *C. oncophora*. *C. oncophora* GluClα3 subunit is a homologue of *C. elegans* AVR-14B/GluClα3B (Dent et al. 2000) and *H. contortus* gbr-2B/GluClα3B (Jagannathan et al. 1999). *C. elegans* and *H. contortus GluClα3* genes are alternatively spliced to yield two subunits, GluClα3A and 3B (Jagannathan et al. 1999; Dent et al. 2000). While we did not clone a GluClα3A homologue from *C. oncophora*, it is likely that, given the close phylogenetic relationship among these three nematodes, the *C. oncophora* GluClα3 may also be alternatively spliced. Initial amplification of the full-length *C. oncophora* GluClα3 cDNA yielded two products (data not shown), and the second less abundant product, which most likely represents the GluClα3A, was not cloned. While *H. contortus* GluClα3B subunit binds ivermectin with high affinity, the 3A subunit does not (Cheeseman et al. 2001). The *C. elegans* AVR14A/GluClα3A

subunit, which is homologous to *H. contortus* GluClα3A, does not respond to glutamate or ivermectin when expressed in *Xenopus* oocytes (Dent et al. 2000). Like *C. elegans* GluClα3B, *C. oncophora* GluClα3 forms ivermectin-sensitive glutamate-gated channels when expressed in *Xenopus* oocytes. Other *C. elegans* GluCls that are activated by glutamate and ivermectin include glc-3 (Horoszok et al. 2001), GluClα2A and GluClα2B (Dent et al. 1997). As well, a GluClα subunit also exists in *C. elegans* that forms a homomeric channel gated by ivermectin but not glutamate (Cully et al. 1994). The second *C. oncophora* subunit, GluClβ, is homologous to the *C. elegans* (Cully et al. 1994) and *H. contortus* (Delany et al. 1998) GluClβ subunits. Unlike *C. elegans* AVR-14B/ GluClα3B and *C. oncophora* GluClα3 receptors, homomeric channels formed by *C. oncophora* IVS GluClβ and *C. elegans* GluClβ are activated by glutamate, but not ivermectin.

C. oncophora GluClα3 and GluClβ subunits cloned from IVS and IVR worms show amino acid differences. Mutations in both subunits were found in the N-terminal extracellular domain, which carries the ligand binding site (Etter et al. 1996). 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 modest but significant threefold decrease in sensitivity to glutamate, and significant ~ 2.5-fold reductions in sensitivity to ivermectin and moxidectin. Mutations in the IVR GluClβ subunit abolished responsiveness to glutamate. Mutations in genes encoding LGIC receptor subunits have been shown to confer drug resistance. A single A302S mutation in the *Drosophila melanogaster Rdl* gene, which encodes a GABA receptor subunit, confers resistance to cyclodiene insecticides (Ffrench-Constant et al.

1998). As well, a proline to serine mutation in the gene coding for the *D. melanogaster* GluClα subunit causes a significant reduction in sensitivity to ivermectin and nodulisporic acid (Kane et al. 2000). In *C. elegans*, mutations of *lev-1,* a gene which encodes a non-α nicotinic acetylcholine receptor (nAchR) subunit, causes a reduction in sensitivity to levamisole, even though this subunit only forms a levamisole-sensitive channel in the presence of other subunits (Fleming et al. 1997). Also in *C. elegans*, mutations in two of three GluCl genes, *avr-14*, *glc-1* or *avr-15*, confers modest or no resistance to ivermectin, while mutations in the three genes results in a 4000-fold resistance to ivermectin (Dent et al. 2000).

To determine whether all three mutations in the IVR GluClα3 contribute to loss of glutamate and ivermectin sensitivity, each mutation was introduced singly to the IVS GluClα3 using site-directed mutagenesis. The responses of Mut 1 (E114G) and Mut 2 (V235A) receptors were similar to those of the IVS GluClα3 receptor, suggesting that the mutations at these positions had no influence on ligand binding. In contrast, Mut 3 glutamate and ivermectin dose-response curves were similar to the IVR GluClα3 receptor responses, suggesting that the L256F mutation accounted for the difference between IVS and IVR GluClα3 channels in response to these two ligands. Whether this amino acid contributes directly to ligand binding, or whether it causes conformational change that influences ligand binding, is not known, and requires further characterization. The L256F mutation does not map onto any of the six domains of the nAchR subunits that form the acetylcholine binding site (Corringer et al. 1995, 2000; Brejc et al. 2001). That this mutation is not found in critical regions may explain the modest loss of agonist sensitivity observed. In *D. melanogaster*, a single mutation in the TM2 region of the *rdl*

gene that codes for a GABA-gated channel confers high-level resistance to the insecticide dieldrin (Ffrench-Constant et al. 1993). Also in *D. melanogaster*, a single mutation in the M2-M3 linker region of DmGluClα gene confers over 20-fold resistance to the novel insecticide and acaricide, nodulisporic acid (Kane et al. 2000).

In oocytes expressing IVR GluClβ, no glutamate-activated currents were detected. This could be due to failure of the receptor to assemble on the cell surface. However, this seems unlikely, since co-expression of this subunit with the IVR GluClα3 resulted in the formation of receptors that were less sensitive to glutamate than the homomeric IVR GluClα3 receptors, suggesting co-assembly of the two subunits. It is possible that the mutations in IVR GluClβ may have altered the binding site, preventing glutamate from binding. Heteromeric channels formed by co-expressing IVS GluClβ with IVS GluClα3 (Fig. 2C) or IVR GluClα3 (data not shown) were more sensitive to glutamate than the homomeric IVR GluClα3 channels. This reflects the contribution of a functional non-mutant GluClβ subunit to the glutamate sensitivity of the heteromeric channel. It is likely that, *in vivo*, the function of the IVR GluClβ subunit may be replaced by other non-mutant subunits. In *C. elegans*, avr-14/GluClα3, which is expressed in extrapharyngeal neurons, can functionally replace avr-15 and inhibit pharyngeal pumping in *avr-15* mutants (Dent et al. 2000).

Of the two amino acid positions substituted in IVR GluClβ, the position 60 residue is highly conserved, being occupied by a Val in all GluCls, except for *D. melanogaster*. While the residue at position 100 is Arg in most GluCls, this position is more variable. An attempt to rescue the function of IVR GluClβ by mutating A60V was unsuccessful. It would appear therefore that the R100H mutation may be important in

determining the function of the GluClβ subunit. Whether the R100H mutation alone can abolish agonist sensitivity, or whether both mutations are required is not known, and requires further characterization. Interestingly, in *H. contortus* GluClβ, the residue at position 100, Arg, corresponds to the residue identified in our mutant non-functional subunit. Whether the *H. contortus* GluClβ subunit forms a functional receptor in *Xenopus* oocytes is not known.

Heteromeric channels formed after co-injection of IVS GluClα3 and GluClβ cRNA showed a higher sensitivity to glutamate than either of the homomeric channels, demonstrating the co-assembly of the two subunits. Interestingly, the IVR GluClα3/ IVR GluClβ heteromeric channels were ~13-fold less sensitive to glutamate than the IVS GluClα3/ IVS GluClβ heteromeric channels. Whether the GluClα3 and GluClβ subunits assemble to form native receptors in the nematode is not known. LGIC receptors exist in their native form as heteromeric pentamers composed of two to four different yet closely related subunit types (Unwin 1993; Bechade et al. 1994; Macdonald and Olsen 1994; Chang et al. 1996). As members of the LGIC superfamily, the native GluCls are thought to exhibit the same heteropentameric structure. However, the exact composition of native GluCl receptors is not known. In *C. elegans*, the GluClβ subunit is expressed solely in the pharynx (Laughton et al. 1997b), while AVR-14/GluClα3 is expressed in extrapharyngeal neurons (Dent et al. 2000), and it is therefore unlikely that these two subunits co-assemble to form a receptor. AVR-15/GluClα2 is expressed in the pharynx, and the native pharyngeal receptor may be composed of AVR-15 and GluClβ subunits (Dent et al. 1997; Pemberton et al. 2001). As well, other glutamate-sensitive, ivermectin-insensitive GluCl subunits may contribute to formation of the pharyngeal receptor

complex (Pemberton et al. 2001). In *H. contortus*, GluClα and β co-localize in motor neuron commisures, and most likely contribute to formation of the GluCl receptor at this site (Delany et al. 1998; Portillo et al. 2003). The splice variants of the *H. contortus gbr-2/GluClα3* gene, GluClα3A and 3B, are also expressed in motor neurons (Jagannathan et al. 1999; Portillo et al. 2003). Whether or not these two splice variants co-localize with GluClα and β subunits has not been confirmed (Portillo et al. 2003). *H. contortus* GluClα3B subunit, which is homologous to *C. oncophora* GluClα3, appears to be expressed in pharyngeal neurons (Portillo et al. 2003), and may therefore mediate ivermectin's inhibitory action on pharyngeal pumping (Geary et al. 1993). The complexity of native receptors is highlighted by a recent report that suggests co-assembly of a GluClα subunit and the GABA-gated chloride channel Rdl subunit in some receptors in *Drosophila* head membranes (Ludmerer et al. 2002).

Both IVS and IVR GluClα3 homomeric receptors were sensitive to ivermectin and moxidectin. Application of saturating concentrations of glutamate and moxidectin during maximal response to ivermectin caused no further change in membrane potential. Similarly, glutamate and ivermectin did not elicit any additional current during maximal response to moxidectin, suggesting that glutamate, ivermectin and moxidectin activate the same channel. Heteromeric channels formed by IVS GluClα3 and β subunits gave a dose-response curve to ivermectin with an EC_{50} of 0.5 μM (data not shown), suggesting that the presence of the β subunit does not affect ivermectin sensitivity. Similar results have been demonstrated with *C. elegans* GluCl subunits, where expression of *C. elegans* GluClα2 together with *C. elegans* GluClβ had no apparent effect on the ivermectin dose response curve (Vassilatis et al. 1997).

Our results show that the moxidectin dose response curves are left-shifted relative to the ivermectin dose-response curve, indicating a higher sensitivity to moxidectin than ivermectin. However, as for glutamate, ivermectin and moxidectin dose-response curves for the IVR GluClα3 homomeric channel are right-shifted relative to the respective IVS GluClα3 channel curves, with significant increases in EC_{50} values. Interestingly, the moxidectin dose-response curve of the IVR GluClα3 channel was comparable to that of the IVS GluClα3 channel response to ivermectin. These findings suggest that while loss of sensitivity to ivermectin also results in a loss of moxidectin sensitivity, the efficacy of moxidectin in IVR is still comparable to the efficacy of ivermectin in IVS. In a dose-titration study, (Ranjan et al. 2002) showed that in *H. contortus*, resistance to ivermectin results in resistance to moxidectin, and *vice-versa*, though moxidectin resistance develops more slowly. Consequently, ivermectin resistant parasites may demonstrate sensitivity to the use-level of moxidectin.

In conclusion, we have shown that *C. oncophora* GluCl subunits form functional homomeric and heteromeric receptors when expressed in *Xenopus* oocytes. Mutation in GluClα3 subunit causes a modest loss of agonist sensitivity, while mutation in the GluClβ abolishes agonist sensitivity. The IVR worms used in this study are known to be resistant to ivermectin (Coles et al. 1998). In *C. elegans*, simultaneous mutations in three GluCl genes are required for high level resistance to manifest (Dent et al. 2000). It remains to be seen what changes occur in other *C. oncophora* GluCls, as well as other ivermectin targets such as the GABA receptor, that may enhance the effect of the L256F mutation, and help explain the mechanisms of ivermectin resistance.

Acknowledgements

We thank Dr. G. Coles for supplying the parasites, and D. Eshelby and G. Bingham for technical assistance. We are grateful to Dr. A. Orth for permitting us to use the electrophysiological equipment. This study was supported by FMC Corporation, Fort Dodge Animal Health and NSERC, Canada.

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CONNECTING STATEMENT III

Apart from the GluCls, other genes have been implicated in ivermectin's mode of action and mechanism of resistance. Based on genetic variability analysis, beta-tubulin is thought to be involved in ivermectin resistance in *H. contortus*. To determine whether this gene is involved in ivermectin resistance in *C. oncophora*, we cloned two isotype cDNAs, and analyzed the genetic variability in IVS and IVR worms. However, we found no association between the two isotype genes and ivermectin resistance. Mutations in beta-tubulin are known to confer resistance to the benzimidazole class of anthelmintics. We therefore decided to analyze the two beta-tubulin isotypes for benzimidazole resistance-associated changes. The results of this analysis are presented in the following chapter.

CHAPTER 5

Cloning two full-length beta-tubulin isotype cDNAs from *Cooperia oncophora*, and screening for benzimidazole resistance-associated mutations in two isolates

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Parasitology (2003) 127: 579-588

Abstract

Two full-length beta-tubulin cDNAs, representing isotypes 1 and 2, were cloned from the cattle nematode *Cooperia oncophora*. The predicted protein sequences span 448 amino acids, and show a high degree of identity to beta-tubulins from other nematodes. While *C. oncophora* isotype 1 sequence had highest identity to *Haemonchus contortus* isotype 1 and *Teladorsagia circumcincta* sequences (95% identity), the *C. oncophora* isotype 2 sequence was most similar to *H. contortus* isotype 2 and *Trichostrongylus colubriformis* (92% identity). Alignment of the two *C. oncophora* sequences with other trichostrongylid beta-tubulins deposited in GenBank showed a clear distinction between isotype 1 and 2 beta-tubulin classes. The two classes differed at 19 amino acid positions, most notably at the carboxy terminus. These isotype-defining residues were conserved among different trichostrongylid species within a class. Analysis of fragments of both genes revealed a high degree of genetic variability in coding and non-coding regions. However, all nucleotide differences detected in the coding region were silent, as they did not result in any amino acid substitution. Analysis of two groups of worms for the codon 200 polymorphism associated with benzimidazole resistance revealed a proportion of worms in one of the groups bearing a tyrosine at this position.

5.1 Introduction

Cooperia oncophora (Strongylida: Trichostrongyloidea) is an important parasite of cattle and is prevalent in the temperate regions of the world (Parmentier *et al.* 1995). The ability of infected animals to mount an effective immune response to this parasite increases with age, and infections are usually restricted to young calves (Kloosterman, Ploeger & Frankena, 1991). Although considered a parasite of relatively low pathogenicity, infections can sometimes cause clinical disease (Vermunt, West & Pomroy, 1996; Armour *et al.* 1987). While broad-spectrum anthelmintic drugs have been effective in controlling *Cooperia* infections in cattle, their usefulness is threatened by the emergence of anthelmintic resistance. Resistance is widespread in nematode parasites of sheep, including *Haemonchus contortus* and *Teladorsagia circumcincta* (Prichard, 1994; Waller *et al.* 1996; Waller, 1997; Gopal, Pomroy & West, 1999), and while it has been slower to emerge in nematodes of cattle, there are several reports of its occurrence. Benzimidazole (BZ) resistance in species of *Cooperia* has been reported in New Zealand (Jackson *et al.* 1995, McKenna, 1996), while ivermectin resistance in this species has been reported in New Zealand, UK and Argentina (e. g., Vermunt *et al.* 1996; Coles, Stafford & MacKay, 1998; Coles, Watson & Anziani, 2001; Familton, Mason & Coles, 2001; Anziani *et al.* 2001; Fiel *et al.* 2001). Also, multiple resistance of *Cooperia* species against the avermectin and BZ classes has been reported in New Zealand (Vermunt, West & Pomroy, 1995). Since resistance is not yet widespread, the efficacy of anthelmintics against nematodes of cattle can be maintained if control strategies which limit the frequency of treatment are adopted (Coles, 2002). In addition, the development

of sensitive tests that detect resistance at the earliest stage would enable action to be taken before significant selection had occurred.

The faecal egg count reduction test (FECRT) is the most widely used method for detecting anthelmintic resistance (Taylor, Hunt & Goodyear, 2002). This test is suitable for all types of anthelmintics, and has been used to detect all cases of anthelmintic resistance in *Cooperia* species reported to date. It has several limitations, including the necessity for repeat visits to affected farms, and a lack of sensitivity (Waller, 1997). Sensitive molecular tests have been described for detecting BZ resistance in *H. contortus*, *T. circumcincta* and *Trichostrongylus colubriformis* (Kwa, Veenstra & Roos, 1994; Elard & Humbert, 1999; Silvestre & Humbert, 2000). These tests detect a mutation (phenylalanine to tyrosine) at codon 200 of the beta-tubulin isotype 1 gene, a change which is linked to BZ resistance (Kwa *et al.* 1994, 1995; Elard & Humbert, 1999). Designing a molecular test for monitoring BZ resistance in *C. oncophora* requires knowledge of the beta-tubulin sequence. Here, we describe the cloning of isotypes 1 and 2 beta-tubulin cDNAs from this parasite, which show a high degree of sequence identity to similar sequences from other trichostrongylids. The genetic variability of both isotype genes was also analyzed using the single-strand conformation polymorphism (SSCP) method. Also, the presence of the Phe-Tyr mutation was investigated in two *C. oncophora* populations, and shown to occur at a low frequency in one of the populations.

5.2 Materials and Methods

5.2.1 Parasites

Two *C. oncophora* isolates (IVS and IVR), kindly provided by Dr. Coles (University of Bristol, UK) were used in this study. The IVS isolate was maintained without anthelmintic pressure at Weybridge Experimental Station, UK, while IVR represents a field isolate originally obtained from a farm in Somerset, UK, where ivermectin resistance was reported (Coles *et al.* 1998). The two isolates were maintained by regular passage through donor (male Holstein) calves at the Macdonald Campus, McGill University Farm. The animals were housed indoors. Third-stage larvae (L3) were obtained by copro-cultures (Borgsteede & Hendriks, 1979) and kept in water at 4°C until used to infect other calves. To ensure viability, L3's were less than 3 months old when used for infection. Adult *C. oncophora* were collected live at necropsy from the small intestine, washed in RPMI medium (Sigma) at 37°C, and stored in liquid nitrogen until used for RNA and DNA extraction.

5.2.2 Cloning of the full-length beta-tubulin Isotypes 1 and 2 cDNAs of C. oncophora

Total RNA was isolated from bulk adult worms using the TRIZOL method (GibcoBRL, Burlington, ON, Canada). First strand cDNA synthesis was performed using 2 µg of the total RNA using 200 U murine Moloney leukemia virus reverse transcriptase (M-MLV, GibcoBRL) and 0.2 µg oligo $dT_{12\text{-}18}$ primer (GibcoBRL). For the initial isolation of the *C. oncophora* beta-tubulin isotype 1 (Co b1) sequence, cDNA was amplified using Advantage 2 cDNA kit (ClonTech) with degenerate primers. These primers were designed based on conserved regions of beta-tubulin isotype 1 sequences from *H.*

contortus and *T. circumcincta*. Two sets of primers were designed for a nested PCR approach. A fragment of the Co b1 cDNA was first amplified with the outer sense primer, Co b1 Deg F1 (5' GGNCCNTAYGGNCARCTNTTYCGNC 3'), and the outer antisense primer, Co b1 Deg R1 (5' CYTCNGCRTCNAGRTCNCCCATRTC 3'). This first-round reaction was then used as template for subsequent amplification using the nested primers Co b1 Deg F2 (5' GARGGNGCNCARCTNGTNGAYAAYG 3'), and Co b1 Deg R2 (5' GNGTNAGYTCNGCNACNGTNGANGC 3'). The PCR reaction conditions were: an initial denaturation at 94°C for 30 s, followed by 30 cycles of 94°C for 20 s, 50°C for 30 s, and 72°C for 1 min, and a final extension at 72°C for 5 min. The PCR product from the second (nested) reaction was examined on a 1% agarose gel (TBE) stained with 0.5 μg/ml ethidium bromide, and the fragment of the expected length (~ 550 bp) was purified using the Nucleospin Gel Extraction Kit (ClonTech). The purified PCR products were then sub-cloned into a TA cloning vector (Invitrogen) as described by the manufacturer, and then sequenced using standard M13 forward and/or reverse primers. Three independent clones were sequenced to obtain a consensus. Based on the sequenced fragment, gene-specific primers were designed for the 5' and 3' RACE (Rapid Amplification of cDNA Ends) reactions.

To identify the 5' end of the Co b1 cDNA, a trans-spliced 22-nucleotide conserved leader sequence (SL1) (Blaxter & Liu, 1996) was used. This sequence has been identified at the 5' end of a number of nematode mRNAs. The SL1 primer (5' GGTTTAATTACCCAAGTTTGAG 3') was used along with two gene-specific antisense primers Co b1 5' RACE 1 (5' CCACAACGGTGTCGGAGACCTTTGG 3') and Co b1 5' RACE 2 (5' CCCATACCGGATCCGGTACCTCCTC 3') in a semi-nested PCR reaction

using the Advantage 2 cDNA kit. Amplification conditions were as outlined above, with an annealing temperature of 54°C. The resulting PCR fragment was purified, ligated into a TA cloning vector (Invitrogen) and subsequently sequenced in both directions using vector primers.

To amplify the 3' end of Co b1 cDNA, a nested PCR approach was employed using the Marathon cDNA Amplification Kit (ClonTech). Two gene-specific sense primers were designed from the sequenced fragment (Co b1 3' RACE 1, 5' GGCTTCGTTCTCTGTTGTTCCTTCA 3' and Co b1 3' RACE 2, 5' CCAAAGGTCTCCGACACCGTTGTGG 3'), and used with the two antisense adaptor primers AP1 and AP2, respectively, as outlined by the manufacturer.

C. oncophora isotype 2 (Co b2) was isolated using the same procedures as employed for isotype 1, except for the primers. The degenerate primers used for the initial isolation of a fragment of Co b2 cDNA were designed according to an alignment of the amino acid sequences of beta-tubulins from *H. contortus* (tub 12-16), *H. contortus* (tub 12-164), *H. contortus* (tub 8-9), *T. circumcincta*, *T. colubriformis* and *Caenorhabditis elegans* BEN-1. A ~ 600 bp fragment of the Co b2 cDNA was first amplified with primers Co b2 Deg F1 (5' GGNGCNGGNAAYAAYTTGGC 3'), and Co b2 Deg R1 (5' TCATRTTYTTNGCRTCRAAC 3'). This first-round reaction was then used as template for further amplification using the nested primers Co b2 Deg F2 (5' GGNCAYTAYACNGARGGNGC 3'), and Co b2 Deg R2 (5' AANGGNACCATRTTNACNGC 3'). Following cloning and sequencing of the resultant 470 bp fragment, gene-specific primers were designed for the 5' and 3' RACE reactions. The two antisense 5' RACE primers, Co b2 5' RACE 1 (5'

CCACAAGTTGGTGCACAGAAAGCGTGGC 3') and Co b2 5' RACE 2 (5' GGTTCAACTACGGTATCGGAAACCTTGG 3') were used in a semi-nested PCR with SL1 to amplify the 5' end of the Co b2 cDNA. The two 3' RACE primers, Co b2 3' RACE 1 (5' TTGACGTTGTTCGCAAGGAGGCAGAAGG 3') and Co b2 3' RACE 2 (5' CCTTCAGGGTTTCCCACTCACGCACTCG 3'), were used with the antisense adapter primers AP1 and AP2, respectively, in a nested PCR reaction.

5.2.3 Determining the presence/absence of BZ resistance mutations in IVS and IVR C. oncophora

BZ resistance in *H. contortus* and *T. circumcincta* is proposed to be mediated by a phenylalanine-to-tyrosine mutation of beta-tubulin isotype 1 at position 200 (Kwa *et al.* 1995; Elard, Comes & Humbert, 1996; Silvestre & Humbert, 2000). The same mutation at position 167 has been shown to confer BZ resistance in *H. contortus* (Prichard, 2001). To determine whether these mutations were present in the *C. oncophora* isotype 1 (Co b1) gene, genomic DNA was isolated from individual male worms (35 IVS and 33 IVR) as described by Beech, Prichard & Scott (1994). PCR products spanning positions 167 and 200 were amplified from these samples, purified using CloneTech's Nucleospin Extraction Kit, and sequenced.

5.2.4 Analyzing genetic variability using SSCP, and sequencing of alleles

To investigate the genetic variability of the gene encoding Co b1, 60 genomic DNA samples were examined by PCR-SSCP (30 IVS and 30 IVR) using the gene-specific primers Co b1 F (5' GTAACAACTGGGCAAAGGG 3') and Co b1 R (5'

TGTCAGGGTACTCCTCACG 3'). 113 samples were tested for the Co b2 gene (57 IVS and 56 IVR) using the primers Co b2 F (5' GAAATAACTGGGCGAAGGG 3') and Co b2 R (5' ATCAGGGTACTCTTCACGG 3'). The genomic DNA samples were amplified in standard PCR reactions using Taq polymerase (GibcoBRL), and the PCR products were analyzed on a 1% agarose gel to check both the size and specificity of the products.

For SSCP screening of the Co b1 samples, 2μl of each PCR product were mixed with 2 μl of loading buffer containing 95% formamide, 10 mM NaOH, 0.25% bromophenol blue, and 0.25% xylene cyanol. The samples were then denatured at 95°C for 5 min, and immediately placed on ice. 4 μl of each sample were loaded onto a 15% nondenaturing polyacrylamide gel and subjected to electrophoresis in 1XTBE for 17 hours at room temperature and 100V. For Co b2, the pcr samples were mixed with the loading dye in a product: dye ratio of 1:15. 10 μl of each sample were loaded onto a 15% polyacrylamide gel and subjected to electrophoresis for 18 hrs at 110V and room temperature. Following electrophoresis, the gels were stained with 0.5 μg/ml ethidium bromide and visualized using the Biorad Molecular Imager FX with its corresponding Quantity One (Version 4.2.1) software.

To determine the nucleotide sequence of the Co b1 and Co b2 alleles identified, at least three individual PCR samples representing each allele were selected, where possible, and sequenced using PCR primers.

5.2.5 Sequence analysis

Allele sequences were aligned using CLUSTAL W on the SDSC Biology Workbench (Version 3.2). Analysis of the beta-tubulin protein sequences was performed by first

generating a multiple sequence alignment using CLUSTAL W. Phylogenetic relationships were then determined using MEGA Version 2.1 (Kumar et al. 2001), and the statistical significance of the trees was tested by bootstrap analysis using 1000 replicates.

5.3 Results

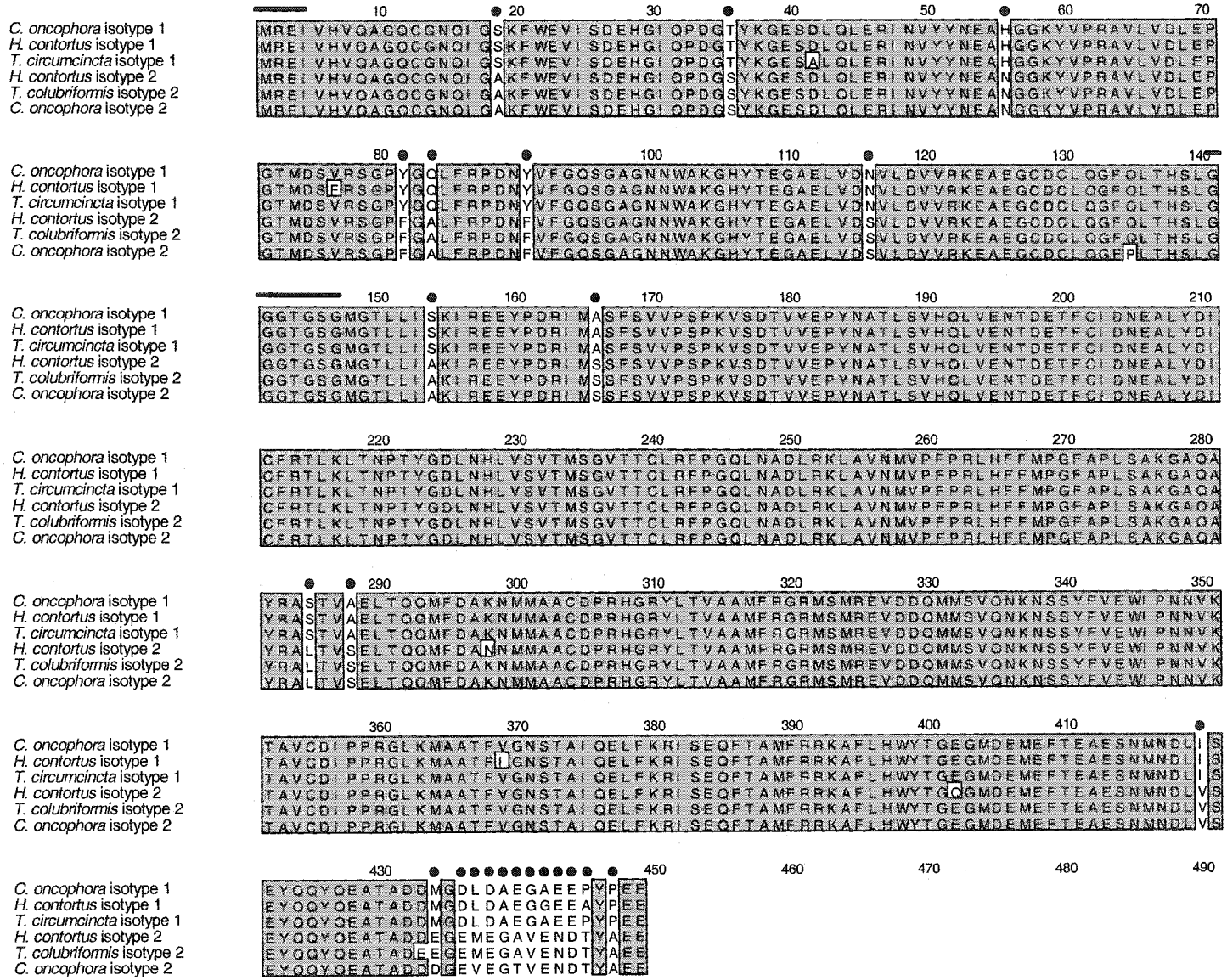
The complete nucleotide sequences of the Co b1 and Co b2 cDNAs were determined, and have been deposited in GenBank under the accession numbers AY259994 (Appendix B.3) and AY259995 (Appendix B.4), respectively. The Co b1 and Co b2 tubulin cDNAs were 1470 and 1620 nucleotides long, respectively. The translated regions of the two isotypes were both 1344 nucleotides long, and shared 80% homology. Both isotype 1 and isotype 2 sequences included the 22-nucleotide SL1 sequence at the 5' end, upstream of positions -19 and -34, respectively. The 5' and 3' untranslated regions of the Co b1 and Co b2 isotypes were highly variable, showing no significant similarity between them.

The amino acid sequences deduced from Co b1 and Co b2 cDNAs are shown in Fig. 1, aligned with sequences representing isotype 1 and isotype 2 beta-tubulins from other trichostrongylids. They were all 448 amino acids long. A characteristic feature of β tubulins is the autoregulation recognition element present at the amino terminus, which is represented by the first four amino acids Met-Arg-Glu-Ile (MREI). This conserved sequence was present in the two *C. oncophora* isotypes isolated (shown in Fig. 1). Also, residues 140-146 serve as the signature pattern for α, β and γ tubulin subunits. Co b1 and Co b2 amino acid sequences differed from each other at several positions, notably at the carboxy terminus (Fig. 1). In this region, there were 12 amino acid differences between

the two isotypes. Also, 13 other amino acid differences were identified at other positions, including 18, 35, 81, 83, and 90.

Figure 1. Alignment of the Co b1 and Co b2 sequences with other trichostrongylid beta-tubulins. The 24 amino acids unique to isotypes 1 and 2 are indicated by a filled circle. This alignment shows that the sequences in an isotype class show higher identity to each other than they do to the other isotype sequence from the same species. The autoregulation signal, MREI (Cleveland 1988), is highlighted by a black line, as is the tubulin signature sequence at position 140 – 146. Binding of GTP to this glycine-rich region stimulates microtubule assembly (Hesse, Thiefauf & Ponstingl, 1987).

Figure 1


C. oncophora isotype 1
H. contortus isotype 1
T. circumcincta isotype 1
H. contortus isotype 2
T. colubriformis isotype 2
C. oncophora isotype 2

The two predicted protein sequences were compared with other full length beta-tubulins reported in current databases (Table 1). The predicted protein sequence of Co b1 had the highest identity (95%) with the isotype 1 sequences representing *T. circumcincta* and *H. contortus*, whereas the Co b2 predicted protein was 92% identical to isotype 2 sequences of *T. colubriformis* and *H. contortus*. Other sequences which showed high identity to the Co b1 predicted protein sequence included the horse cyathostome *Cyathostomum coronatum* (94%) and *C. elegans* BEN 1 (90%); Co b2 showed 87% and 88% identity to these two sequences respectively. Co b1 showed 87% identity to beta-tubulin sequences from the filarial nematodes *Onchocerca volvulus, Dirofilaria immitis* and *Brugia pahangi*, while Co b2 showed 86% identity to these three sequences. The two *C. oncophora* sequences showed 86% identity to *Drosophila melanogaster* beta-tubulin. Comparison of the *H. contortus* and *C. oncophora* isotype 1 and isotype 2 sequences showed that the homologous isotypes from the two species had higher identity to one another than either isotype had to the other beta-tubulin isotype from the same species; *H. contortus* isotype 1 and Co b1 were 95% identical at the amino acid level, while *H. contortus* isotype 2 (β12-16 and β12-164) and Co b2 were 92% identical at the amino acid level. In contrast, *H. contortus* isotypes 1 and 2 shared 90% identity at the amino acid level, as did the Co b1 and Co b2 sequences. Phylogenetic analysis by distance-based and parsimony methods produced trees with similar topologies, and the Neighbour-Joining tree is illustrated in Fig. 2. Isotype 1 and isotype 2 predicted protein sequences of strongylids were found in distinct clusters highly supported by bootstrap analysis (Fig. 2). Co b1 grouped with isotype 1 sequences from *H. contortus*, *T. circumcincta*, *Cylicocyclus nassatus* and *C. coronatum* with a bootstrap value of 100%. Co b2 clustered with isotype

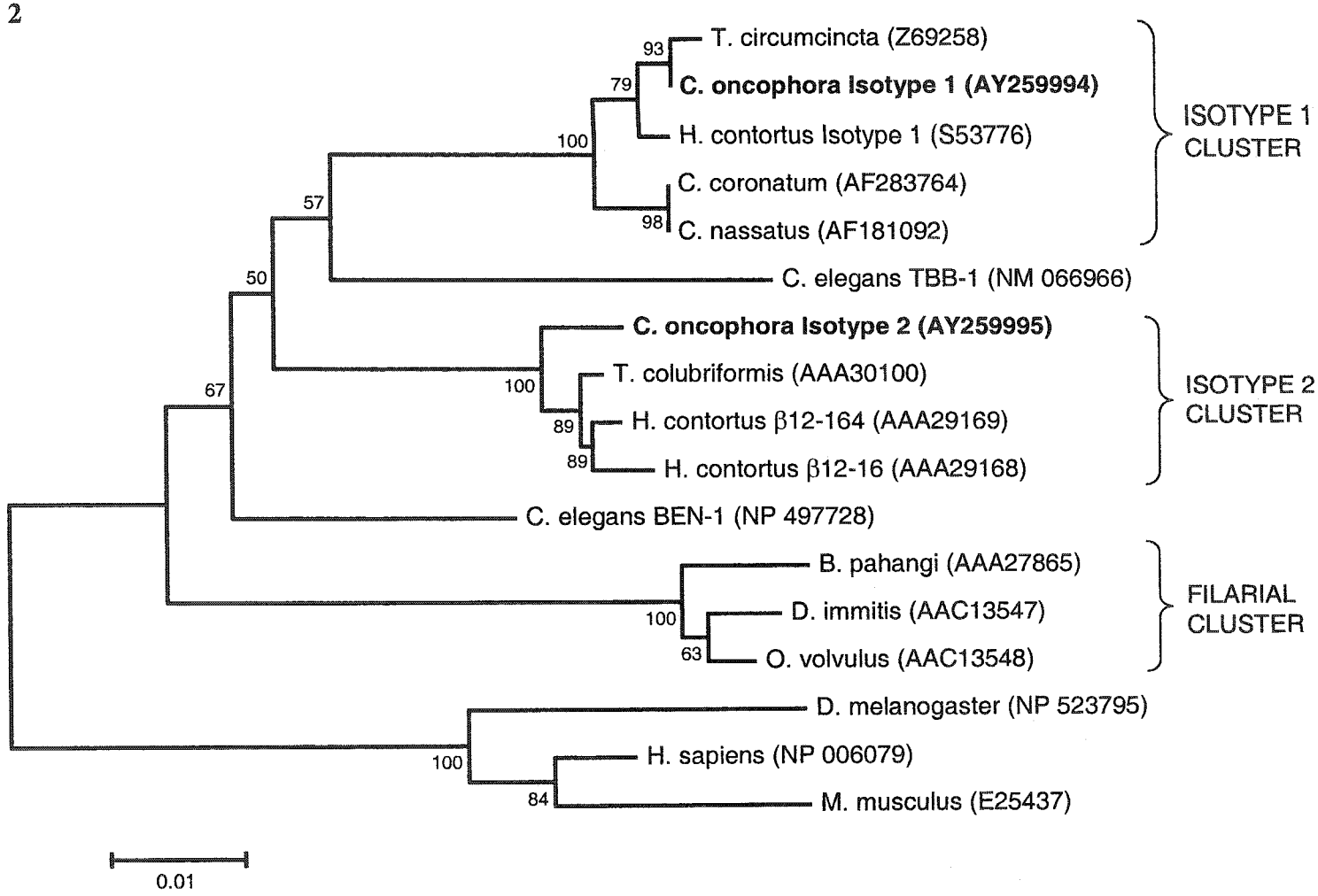
2 sequences from *H. contortus* and *T. colubriformis* (bootstrap value 100%). Sequences from the three filarial nematodes *B. pahangi*, *D. immitis* and *O. volvulus* also formed a distinct group.

Table 1. Comparison of *C. oncophora* beta-tubulin (Co b1 and Co b2) predicted proteins with other beta-tubulins for identity. The values represent pairwise percent identity in amino acid sequence. Highest identity to a different species is shown in bold type. Co b1 had highest identity to *H. contortus* and *T. circumcincta* isotype 1 sequences (95% identity). Co b2 had highest identity to *H. contortus* β12-16 and β12-164, and *T. colubriformis*, which are isotype 2 sequences.

	Co b1	Co b2
Co b2	90	-
H. contortus isotype 1	**95**	88
T. circumcincta isotype 1	**95**	87
C. nassatus	94	87
C. coronatum	94	87
C. elegans BEN 1	90	88
H. contortus *β*12-16	90	**92**
H. contortus *β*12-164	90	**92**
T. colubriformis	90	**92**
C. elegans TBB 1	87	86
O. volvulus	87	86
D. immitis	87	86
B. pahangi	87	86
Mus musculus	86	85
Homo sapiens	87	86
D. melanogaster	86	86

Figure 2. Neighbour-joining tree showing the relationships among beta-tubulin predicted protein sequences. GenBank accession numbers are indicated with each sequence. The two beta-tubulin sequences cloned from *C. oncophora* are in bold type. The first sequence, *C. oncophora* isotype 1, groups with isotype 1 sequences from *H. contortus* and *T. circumcincta*. The second sequence, *C. oncophora* isotype 2, is found on a distinct branch with similar sequences from *H. contortus* and *T. colubriformis*. Bootstrap values range from 50% to 100%. Sequences were aligned using CLUSTAL W on the SDSC Biology Workbench (Version 3.2), and phylogenetic analyses performed using MEGA Version 2.1 (Kumar *et al.* 2001).

Figure 2


T. circumcincta (Z69258)
C. oncophora Isotype 1 (AY259994)
H. contortus Isotype 1 (S53776)
C. coronatum (AF283764)
C. nassatus (AF181092)
C. elegans TBB-1 (NM 066966)
C. oncophora Isotype 2 (AY259995)
T. colubriformis (AAA30100)
H. contortus β12-164 (AAA29169)
H. contortus β12-16 (AAA29168)
C. elegans BEN-1 (NP 497728)
B. pahangi (AAA27865)
D. immitis (AAC13547)
O. volvulus (AAC13548)
D. melanogaster (NP 523795)
H. sapiens (NP 006079)
M. musculus (E25437)
93
79
100
57
98
50
100
67
89
89
100
63
100
84
ISOTYPE 1 CLUSTER
ISOTYPE 2 CLUSTER
FILARIAL CLUSTER
0.01

PCR fragments spanning amino acids 167 and 200 of Co b1 were amplified from individual male worms and then sequenced to determine whether the tyrosine resistance-associated mutation was present. The fragments were 310 bp in length, and 35 IVS and 33 IVR individual male worms were examined. All IVS and IVR worms were homozygous for phenylalanine (Phe/Phe) at position 167 (Table 2). At position 200, all (n=35) IVS worms were homozygous Phe/Phe. From the IVR group, eight worms were found to be heterozygous Phe/Tyr (24.2%), while one worm was homozygous Tyr/Tyr (3%). All other IVR worms (n=24) were homozygous Phe/Phe at this position (72.7%).

Table 2. Determining the genotypes of individual worms based on amino acids at positions 167 and 200 of Co b1 gene. At codon position 167, all IVS and IVR worms were homozygous for Phe. At position 200, all IVS worms were homozygous for Phe. Eight of the IVR worms were heterozygous Phe/Tyr, while one was homozygous for the BZ resistance mutation.

	GENOTYPES	**Phe/Phe (%)**	**Phe/Tyr (%)**	**Tyr/Tyr (%)**
	PHENOTYPES	**BZ-susceptible**	**BZ-susceptible**	**BZ-resistant**
CODON 167	**IVS, n=35**	35 (100)	0	0
	IVR, n=33	33 (100)	0	0
CODON 200	**IVS, n=35**	35 (100)	0	0
	IVR, n=33	24 (72.7)	8 (24.2)	1 (3)

Using SSCP analysis, 60 Co b1 and 113 Co b2 genomic DNA samples were screened for genetic variability. Figure 3 shows sequence variability of the Co b2 gene as demonstrated using SSCP. A total of nine alleles were identified by PCR-SSCP for both Co b1 and Co b2 genes (Table 3). For the Co b1 gene, all nine alleles were found in both the IVS and IVR groups. Allele A was the most abundant in both groups, and alleles F, G and H were found at lower frequencies in both groups when compared to other alleles. For the Co b2 gene, allele A was the most common in both IVS and IVR with frequencies of 0.48 and 0.46 respectively. Allele F was found only in the IVS group, and allele L was found only in IVR. These two alleles were present at low frequencies (0.03 and 0.01 respectively). Alignment of the Co b1 and Co b2 allele sequences showed nucleotide polymorphisms in the intron and exon regions (data not shown). However, all nucleotide differences in the exons were silent, as they did not result in a change in the amino acid.

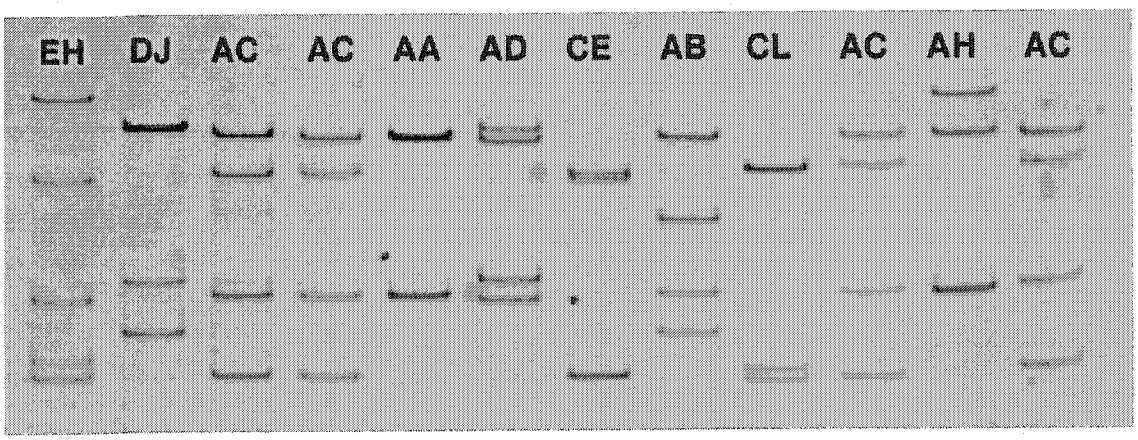


Figure 3. SSCP of the Co b2 gene. The polyacrylamide gel was stained with ethidium bromide, and the banding pattern visualized using the Biorad Molecular Imager FX. Samples in this figure were all from the IVS group. Eight of the nine alleles identified for the Co b2 gene are shown here, labeled using a letter code. Homozygotes are represented by two bands (e.g., AA) and most of the heterozygotes are represented by four bands (e.g., EH). However, where two of the bands in the heterozygote have co-migrated, three bands are seen (e.g., DJ).

Table 3. Allele frequencies for Co b1 and Co b2 genes in IVS and IVR worms. In both genes, nine alleles were identified. All nine Co b1 alleles were found in both IVS and IVR groups, while eight of the nine Co b2 alleles were found in each group.

GENE (FRAGMENT SIZE)	ALLELE	IVS	IVR
Co b1 (234 bp)		**(n = 30)**	**(n = 30)**
	A	0.37	0.4
	B	0.23	0.15
	C	0.13	0.12
	D	0.12	0.13
	E	0.05	0.1
	F	0.02	0.03
	G	0.02	0.02
	H	0.02	0.03
	J	0.05	0.02
Co b2 (252 bp)		**(n = 57)**	**(n = 56)**
	A	0.48	0.46
	B	0.01	0.04
	C	0.18	0.21
	B	0.12	0.16
	E	0.17	0.1
	F	0.03	0
	H	0.01	0.01
	J	0.01	0.01
	L	0	0.01

5.4 Discussion

Beta-tubulin sequences from three sheep trichostrongylid nematodes have been reported to date, namely *H. contortus*, *T. colubriformis* and *T. circumcincta*. However, no beta-tubulin from a cattle trichostrongylid nematode had been sequenced. Here, we report the cloning of two full-length beta-tubulin cDNAs from the cattle nematode *C. oncophora*, representing isotypes 1 and 2.

Studies by Geary *et al.* (1992) showed that there are at least two beta-tubulin isotype classes in *H. contortus*. The present results show that the same applies to *C. oncophora*, and that a high degree of sequence identity exists between similar isotypes from both nematodes. At the amino acid level, Co b1 sequence was 95% identical to the *H. contortus* isotype 1, while Co b2 was 92% identical to the two *H. contortus* isotype 2 sequences β12-16, and β12-164. This high sequence conservation extended to beta-tubulin sequences from other organisms, including the *O. volvulus*, *D. melanogaster*, and vertebrates, where the percentage identity to both Co b1 and Co b2 ranged from 85 to 87%. This high identity across species suggests that evolutionary divergence of beta-tubulins is limited by functional constraints. Phylogenetic analysis placed the Co b1 sequence with isotype 1 sequences of *H. contortus* (Geary *et al.* 1992), *T. circumcincta* (Elard *et al.* 1996), *C. nassatus* (Pape, Samson-Himmelstjerna & Schneider, 1999) and *C. coronatum* (Samson-Himmelstjerna *et al.* 2001). Co b2 was found on a distinct branch with isotype 2 sequences of *H. contortus* (β12-16 and β12-164) and *T. colubriformis*. Bootstrap analysis showed strong support for these groupings. The trichostrongylid isotypes 1 and 2 can be distinguished based on differences at their carboxy termini, as well as 12 other amino acid positions. These residues are identical among species within

a single isotype class, as seen in Fig. 1, and suggest that in trichostrongyles, at least two distinct beta-tubulin isotype classes exist. Whether these two isotypes have unique functions is not yet known. In vertebrates, seven beta-tubulin isotypes have been identified, and are distinguished based on the sequence at the isotype-defining carboxy terminus (Luduena, 1993). They differ in their cellular distribution and relative stabilities and hence, *in vivo* function (Roach *et al.* 1998; Schwarz, Liggins & Luduena, 1998). The isotype-defining carboxy terminus is thought to interact with microtubule associated proteins (MAPs) and motor proteins (Nogales, Wolf & Downing, 1998; Downing & Nogales, 1998). Isotype differences found in regions other than the carboxy terminus are proposed to determine microtubule stability (Downing & Nogales, 1998).

A Phe-Tyr mutation at position 200 of beta-tubulin isotype 1 gene is the major determinant of BZ resistance in trichostrongylid nematodes (Kwa *et al.* 1995; Elard *et al.* 1996). The same mutation at position 167 has also been reported in the absence of the position 200 mutation in resistant *H. contortus* (Prichard, 2001). To determine the prevalence of the codon 167 and 200 isotype 1 mutations in IVS and IVR worms, a fragment of the Co b1 gene spanning the two amino acid positions was amplified from randomly selected individual male worms from both groups, and sequenced. The two groups were considered independently because they originated from different locations. At position 167, all individual worms (IVS and IVR) were identified as homozygous non-mutant (Phe/Phe). The mutation at position 167 appears to be rare under field conditions in trichostrongylids of sheep, and may reflect the fitness cost associated with this mutation (Silvestre & Cabaret, 2002).

At position 200 of the Co b1 gene, all 35 IVS worms were found to be homozygous Phe/Phe at position 200 (Table 2). From the IVR group, eight of the 33 worms were identified as heterozygous Phe/Tyr at this position (24.2%). One worm was homozygous Tyr/Tyr (3%), and all other worms (n=24) were homozygous Phe/Phe (72.7%). These results are similar to those obtained by Elard & Humbert (1999) with the 'SuPRO' susceptible population of *T. circumcincta*, where 18% of the worms were heterozygous (Phe/Tyr) and 3% were homozygous resistant (Tyr/Tyr). This population was classified as being susceptible using the FECRT to estimate BZ resistance, since no eggs were found in the faeces after BZ treatment. The presence of the BZ resistance mutation in a proportion of the IVR worms suggests that this group has the potential to develop BZ resistance if selective pressure is applied, assuming BZ resistance mechanisms to be similar in *C. oncophora* and other trichostrongyles. The Phe-Tyr mutation at position 200 of isotype 1 is recessive (Elard, Suave & Humbert, 1998), and heterozygous Phe/Tyr worms are therefore eliminated along with homozygous Phe/Phe worms by using the recommended drug dose. However, underdosing seems to favour survival of heterozygous Phe/Tyr susceptible worms over homozygous Phe/Phe susceptible worms (Silvestre, Cabaret & Humbert, 2001), and benzimidazole resistance is more likely to spread rapidly when lower-than-recommended drug doses are used.

A reduction in genetic variability of isotype 1 and 2 genes has been linked to resistance in *H. contortus*, *T. colubriformis* and *T. circumcincta* (see Kwa *et al.* 1993, Beech, Prichard & Scott, 1994, Grant & Mascord, 1996, Elard & Humbert, 1999). In the most resistant worms, a loss of isotype 2 is evident (Kwa *et al.* 1993; Lubega *et al.* 1994; Roos, Kwa & Grant, 1995). SSCP analysis of short Co b1 and Co b2 gene fragments

from individual *C. oncophora* male worms showed that both genes were polymorphic- nine alleles were identified for both isotypes. Trichostrongylid nematodes show high genetic diversity (Blouin *et al.* 1992), and are therefore able to respond to selection pressure (Grant 1994). The variability of both isotypes in the IVS and IVR groups was comparable, even though the BZ resistance- associated mutation was found at very low frequency in the latter group. At very low levels of BZ resistance, there is no detectable loss of variability of isotype 1 and 2 genes (Kwa *et al.* 1993). Sequencing of the alleles revealed nucleotide variations in coding and non-coding regions, although all differences in the coding region were silent, suggesting that allelic variation is limited to positions that will not result in amino acid changes, as such changes may affect function.

To prevent the spread of resistance, early detection is essential. The FECRT, which is most widely used to monitor anthelmintic resistance in domestic animals, can only detect resistance when the proportion of resistant worms in the population is at least 25% (Martin, Anderson & Jarrett, 1989). This is much greater than the 1-2% suggested by mathematical modelling if resistance is to be managed by using a second drug before resistance develops to the first (Sangster *et al.* 2002). Sensitive molecular tests that detect very low resistance gene frequencies have been described for *H. contortus*, *T. circumcincta*, *T. colubriformis*, and small strongyles (Kwa *et al.* 1994; Elard & Humbert, 1999; Silvestre & Humbert, 2000; Samson-Himmelstjerna *et al.* 2002). Such a test would be useful for studying the prevalence of BZ resistance in *C. oncophora.*

The finding of BZ-resistance associated mutations in the IVR group, albeit at a low frequency, is important, since this group is resistant to ivermectin (Coles *et al.* 1998). Recent findings suggest that ivermectin-resistant *C. oncophora* are more pathogenic than

the susceptible parasites (Coles *et al.* 2001). We found that infecting calves with 10 000 IVR L3 larvae caused diarrhoea and poor body condition, while age-matched calves infected with the same number of IVS worms did not show any clinical signs (unpublished results). The potential for multiple anthelmintic resistance developing in an isolate which appears to be more pathogenic is of concern, and indicates the need for monitoring and early detection of resistance so as to maintain the efficacy of currently available anthelmintics. Unlike nematode parasites of sheep, BZ resistance is still considered to be rare among nematodes of cattle (Prichard 1994; Coles 2002). Importantly, knowledge of the *C. oncophora* beta-tubulin isotype 1 sequence will allow for the development of a sensitive molecular test that can be used to monitor the emergence of BZ resistance, so that measures can be taken to counter it before it becomes widespread.

Acknowledgements

This study was supported by Fort Dodge Animal Health and NSERC, Canada. We would like to thank Dr. G. Coles for supplying two isolates, and D. Eshelby and G. Bingham for technical assistance.

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CHAPTER 6

General Discussion

Gastrointestinal helminth infections play a significant role in limiting livestock productivity in many parts of the world. Control relies heavily on the use of broad-spectrum anthelmintics. Three classes are currently available for use- the benzimidazoles, which were introduced in the early 1960s, the tetrahydopyrimidines/ imidazothiazoles, introduced in the 70s, and the macrocyclic lactones (MLs), introduced in the 80s. They are classified based on their modes of action, with drugs in the same class having a common mode of action and showing cross-resistance. The effectiveness of these drugs is threatened by the development of resistance. Drug exposure selects for resistance alleles that are initially present at low frequencies in the population. Over time, and with continued selection pressure, the frequency of these resistance alleles increases, ultimately leading to treatment failure. There are currently no new drugs on the market that can be used as alternatives if existing broad-spectrum anthelmintics fail. As well, with the high cost of drug development and the demands for high returns on research investments, many animal health companies have turned to developing products for the most profitable market segments (Geary and Thompson 2003). Parasites of companion animals are considered a priority, and fewer companies are currently involved in the discovery of new antiparasitic drugs. In companion animals, anthelmintics tend to

be used against a narrower spectrum of parasites as compared to domestic ruminants. While anthelmintic resistance is a major concern in domestic ruminants, it has not been reported in companion animals. With this state of affairs, efforts should be made to preserve the shelf-life of existing anthelmintics. A key aspect of this is the ability to detect resistance at a very early stage, when the frequency of resistant individuals is low. This would allow the drug in question to be withdrawn before it looses effectiveness (Sangster 2001). This requires sensitive molecular tests that can detect low levels of resistance in populations (1-2% resistant individuals), and the development of such tests requires a good understanding of the genetic basis of anthelmintic resistance.

Studies on the molecular mechanisms of benzimidazole resistance in trichostrongylid nematodes determined that this phenotype is conferred by a phenylalanine-to-tyrosine mutation at position 200 of beta-tubulin isotype 1 (Kwa et al. 1994; Kwa et al. 1995; Elard et al. 1999). This led to the development of sensitive PCR-based tests that can detect low levels of the benzimidazole resistance-associated allele in three nematode species of sheep, including *Teladorsagia circumcincta*, *Haemonchus contortus* and *Trichostrongylus colubriformis* (Elard et al. 1999; Silvestre and Humbert 2000). No such test exists for the MLs. Such a test would be particularly useful for monitoring resistance in cattle parasites, since resistance is still not widespread. Ivermectin resistance in cattle is emerging, and all cases that have so far been reported involve *Cooperia* species (Vermunt et al. 1995; Coles et al. 1998; Anziani et al. 2001; Coles et al. 2001; Familton et al. 2001; Fiel et al. 2001). The glutamate-gated chloride channels (GluCls) are important targets for the MLs and are also thought to be involved in resistance to this class of drugs (Blackhall et al. 1998; Dent et al. 2000). This thesis

describes the genetic and functional analyses of two GluCl subunits from ivermectin-susceptible and -resistant isolates of *Cooperia oncophora*, which is the important *Cooperia* species in temperate regions. As well, the cloning of two *C. oncophora* beta-tubulin isotype cDNAs and the screening for benzimidazole resistance-associated changes is presented.

In studying the mechanisms of anthelmintic resistance, differences between susceptible and resistant parasites can yield useful information on changes which may be linked to drug resistance (Sangster and Gill 1999). Two isolates, IVS (ivermectin-susceptible) and IVR (ivermectin-resistant) were used in the experiments described in this thesis. In a controlled test to determine the ivermectin susceptibility/ resistance status of the two isolates, the IVS isolate was found to be 100% susceptible to ivermectin (Chapter 2). In contrast, ivermectin was only 77.8% effective at reducing the fecal egg count in one of the calves infected with the IVR isolate. Of interest is the observation that the IVR isolate was more pathogenic than the IVS isolate. Calves infected with the IVR isolate showed clinical signs of parasitic gastroenteritis, including diarrhea, rough hair coats, and poor body condition. As well, despite having a higher worm burden, the untreated IVS-infected animal had a lower fecal egg count than the untreated IVR-infected animal. Similar results were reported by (Kelly et al. 1978), who found a benzimidazole-resistant strain of *Haemonchus contortus* to be more pathogenic than a benzimidazole-susceptible strain. However, (Barrett et al. 1998) found no difference in pathogenicity between benzimidazole-susceptible and -resistant isolates of *Teladorsagia circumcincta*. Whether there is any association between anthelmintic resistance and pathogenicity is not known and requires further study. The genes that contribute to

pathogenicity may be unrelated to anthelmintic resistance, and the two traits may occur independently of each other. Indeed, (Coles et al. 2001) have suggested that *C. oncophora* may have changed in recent years to become more pathogenic, and while this change may not be associated with anthelmintic resistance, it may represent a 'new' cattle disease, since *Cooperia* are generally considered to be parasites of low pathogenicity (Vermunt et al. 1995). Lower pathogenicity is considered an adaptation of host and parasite co-evolution. Parasites that are more pathogenic are less well adapted and are more likely to kill their hosts, and themselves in the process (Le Jambre et al. 1982).

In order to examine the role of the *C. oncophora* GluCls in ivermectin resistance, two cDNAs, encoding GluClα3 and GluClβ subunits, were cloned (Chapter 3). These subunits showed features that identified them as members of the cys-loop superfamily of receptors, including a long N-terminal extracellular domain, four transmembrane domains and the signature 15 amino-acid dicysteine loop that gives this superfamily its name. *C. oncophora* GluClα3 showed highest sequence identity to GluClα3B subunits from *H. contortus* and *C. elegans*, while *C. oncophora* GluClβ showed highest identity to similar subunits from *H. contortus* and *C. elegans*. In *C. elegans* and *H. contortus*, two GluClα3 subunits (3A and 3B) are produced by alternative gene splicing (Jagannathan et al. 1999; Dent et al. 2000). The 3A splice variant was not cloned from *C. oncophora*. It is highly likely that the *C. oncophora* GluClα3 gene is also alternatively spliced. During amplification of the full-length GluClα3, two products were amplified, and the second less-abundant product that was not cloned most likely represented the 3A splice variant, since it was similar in size (~2 kb) to the *H. contortus* GluClα3A cDNA amplification product (Jagannathan et al. 1999). However, the fact that this splice variant was not

cloned does not change the results presented in this thesis, since the genetic variability analysis examined the gene, and the same gene encodes the two products. As well, for the functional analysis, previous studies have shown that heterologously expressed 3A subunits from *H. contortus* and *C. elegans* do not respond to ivermectin (Dent et al. 2000; Cheeseman et al. 2001).

Analysis of the genetic variability of the *C. oncophora* GluClα3 and GluClβ genes revealed that there was selection at the GluClα3 gene, but not the GluClβ gene, with ivermectin resistance. These results are consistent with the observation that ivermectin binds to α-type GluCl subunits, but not the β subunit (Cully et al. 1994; Cully et al. 1996; Dent et al. 1997; Vassilatis et al. 1997; Dent et al. 2000; Horoszok et al. 2001), and suggest that the *C. oncophora* GluClα3 gene is a target for ivermectin, and may be involved in resistance to this drug. Selection for ivermectin resistance caused a significant change in allele frequency of the GluClα3 gene without causing a change in the allele frequencies of the GluClβ and actin genes, suggesting that the changes seen at the GluClα3 gene may be due to ivermectin selection and not population bottlenecking. Selection did not result in a loss of genetic variability, and whether it is maintained or lost with higher levels of selection is not known. Selection for benzimidazole resistance causes a reduction in variability of beta-tubulin isotype 1 and 2 genes (Roos et al. 1990; Kwa et al. 1993b; Kwa et al. 1993a; Beech et al. 1994; Lubega et al. 1994). That genetic variability of the *C. oncophora* GluClα3 gene was not lost may indicate the potential for further increase in the level of resistance.

The ligand binding domain lies in the N-terminal extracellular domain of cys-loop receptors, and mutations of residues that are involved in ligand binding alter channel

properties (Corringer et al. 2000). The genetic variability analysis of the *C. oncophora* GluClα3 gene was based on a short fragment in the N-terminal domain. Sequencing of all GluClα3 alleles (nine in total) showed polymorphism at the nucleotide level, and identity at the amino acid level, indicating that changes in this region do not contribute to ivermectin resistance. Cloning of full-length alleles from IVS and IVR worms revealed the presence of three non-synonymous mutations in the N-terminal extracellular domain. Two mutations were also identified in the N-terminal domain of the IVR GluClβ subunit. To determine the functional difference between the IVS and IVR subunits, they were expressed in *Xenopus laevis* oocytes, and the results are described in Chapter 4.

Except for IVR GluClβ, all receptors formed glutamate-gated homomeric channels when expressed in *Xenopus* oocytes. The IVS and IVR GluClα3 homomeric channels also responded to ivermectin and moxidectin. Responses to glutamate were rapid in activation and reversible, similar to what has previously been demonstrated in *C. elegans* and *H. contortus* (Cully et al. 1994; Dent et al. 1997; Vassilatis et al. 1997; Dent et al. 2000; Horoszok et al. 2001). In contrast, responses to ivermectin and moxidectin were slower to activate and essentially irreversible, consistent with the slow rate of dissociation observed in binding studies, and it is likely that ivermectin's neuromuscular paralysis in the worm is mediated at least in part through GluCls. Ivermectin inhibits pharyngeal pumping, and induces paralysis of somatic musculature, with pharyngeal muscle being the more sensitive site (Geary et al. 1993). In *H. contortus*, the GluClα3B subunit, which is orthologous to the *C. oncophora* GluClα3, is expressed in the pharynx, and is likely to contribute to formation of the ivermectin-sensitive receptor in this site (Portillo et al. 2003). This subunit is also expressed in motor neuron commissures, and

most likely contributes to the formation of receptors which mediate ivermectin's effects on locomotion (Jagannathan et al. 1999; Portillo et al. 2003). Given the close phylogenetic relationship between *C. oncophora* and *H. contortus*, it is likely that the *C. oncophora* GluClα3 subunit is expressed in the same sites as the *H. contortus* GluClα3B subunit.

Mutations in the *C. oncophora* GluCα3 caused modest but significant reductions in glutamate, ivermectin and moxidectin sensitivity. One of these mutations, L256F, accounted for the difference in response between the IVS and IVR GluClα3 subunits. A similar mutation (L-F) in voltage-gated sodium channels has been found to confer pyrethroid resistance in insects (Liu et al. 2002). This mutation is associated with low-level resistance, and additional sodium channel mutations, which by themselves do not confer resistance, enhance the effect of the L-F mutation, resulting in higher levels of pyrethroid resistance. Whether the L256 mutation in *C. oncophora* GluClα3 is modified by other mutations, or whether it enhances the effects of another, more important, mutation, is not known. As well, the prevalence of the L256F mutation in ivermectin-resistant *C. oncophora* populations remains to be seen. It is also possible that mutations in other GluCls, as well as other genes, contribute independently to ivermectin resistance, with high-level resistance involving changes in several of these.

The IVS and IVR GluClα3 homomeric channels were more sensitive to moxidectin than ivermectin, though selection for ivermectin resistance also caused a reduction in sensitivity to moxidectin. These drugs both act on GluCls, and appear to have a similar mechanism of resistance. Based on our results, moxidectin may be useful

against ivermectin-resistant worms, though how useful will depend on how far the ivermectin dose-response curve has shifted to the right before moxidectin is introduced.

Studies in *H. contortus* have suggested a role for beta-tubulin in ivermectin resistance (Blackhall, PhD Thesis, 1999). Ivermectin is not known to bind to beta-tubulin, and Blackhall (PhD Thesis, 1999) proposed that changes seen at the beta-tubulin gene may have been due to a functional linkage between beta-tubulin and ivermectin targets. Vertebrate cys-loop receptors have been extensively studied, and microtubules are known to anchor receptors at synaptic junctions by interacting with clustering proteins, thereby facilitating efficient neurotransmission (Kneussel and Betz 2000). Two beta-tubulin isotype cDNAs were therefore cloned from *C. oncophora* for genetic variability analysis. The sequences showed a high degree of identity to beta-tubulins of other trichostrongylid species. Genetic variability analysis of the two *C. oncophora* beta-tubulin isotype genes revealed no association between beta-tubulin and ivermectin resistance, suggesting that beta-tubulin does not play a role in ivermectin resistance in this nematode. The association between beta-tubulin and benzimidazole resistance is well established, and the *C. oncophora* beta-tubulin sequences were therefore examined for benzimidazole resistance-associated changes (Chapter 5). There was no reduction in genetic variability of both beta-tubulin isotype 1 and 2 genes, suggesting little or no selection for benzimidazole resistance. A proportion of the IVR worms were found to carry the benzimidazole resistance allele. Benzimidazole treatment will most likely impose selective pressure, leading to an increase in frequency of these alleles. That this group (IVR) is also resistant to ivermectin suggests that the use of benzimidazoles will most likely lead to multiple (benzimidazole and ivermectin) resistance. Knowledge of

the *C. oncophora* beta-tubulin isotype 1 sequence will allow for the development of a sensitive molecular test that can be used to monitor benzimidazole resistance allele frequency in the field.

In summary, this thesis demonstrates that GluCl subunits of *C. oncophora* are targets for the MLs. Genetic variability analysis provides indirect evidence that the *C. oncophora* GluClα3 gene is involved in ivermectin resistance. Functional analysis demonstrates that a L-F mutation of the GluClα3 subunit results in loss of sensitivity to the MLs. Analysis of beta-tubulin demonstrates the presence of a benzimidazole resistance associated similar to that observed in trichostrongylids of sheep. Understanding the mechanisms of drug resistance will allow for the development of molecular tools for studying the development of resistance in worm populations. Such tests would also be useful for examining the factors which contribute to the slower rate of resistance development in cattle nematodes.

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INTRODUCTION TO APPENDICES

Four appendices are included in this thesis. The manuscript presented in Appendix A shows the cloning of a GABAβ subunit from *C. oncophora*. Because this subunit has not been implicated in ivermectin resistance, it is not included as a chapter in the thesis. However, the GABAβ subunit is necessary for the formation of functional GABA receptors, and because of its relevance in functional expression studies, it is included here.

In Appendix B, data not included in the Chapter 3 manuscript is presented.

In Appendix C, the GenBank flatfiles of all the full-length sequences cloned in the course of this study are presented. Except for GABA-β, we analyzed the genetic variability of all these sequences.

Appendix D contains the Ethics Certificate (Animal Use Protocol), as required by the University for studies involving animal subjects.

APPENDIX A

Cloning a GABA-gated chloride channel cDNA from *Cooperia oncophora*

Annete I. Njue, Xiao-peng Feng and Roger K. Prichard

A.1 Introduction

GABA is an important inhibitory neurotransmitter in vertebrate and invertebrate nervous systems (McIntire et al. 1993). It mediates neuronal inhibition by binding to $GABA_A$ receptors and activating channels which are selective for chloride ions. The increased permeability to chloride ions results in membrane hyperpolarization. $GABA_A$ receptors have been identified as targets of important neuroactive drugs, including the older cyclodiene insecticides, as well as the macrocyclic lactone endectocides (Kass et al. 1980; Matsumura and Ghiasuddin 1983; Gant et al. 1987). While these two drug classes target the same receptor, they act by different mechanisms- the cyclodienes by blocking the channel, and the macrocyclic lactones by opening it (Clark et al. 1995). Like the cyclodienes, picrotoxin acts as a GABA channel blocker, and insect strains that are resistant to cyclodienes show cross-resistance to picrotoxin (Matsumura and Ghiasuddin 1983).

Macrocyclic lactone anthelmintics are widely used to treat helminth infections in domestic animals. Unfortunately, their usefulness is threatened by the development of resistance. Genetic variability studies indicate that the $GABA_A$ receptor gene HG1 may be involved in ivermectin resistance in *Haemonchus contortus*, a nematode parasite of sheep (Blackhall et al. 2003). Using *Xenopus laevis* oocytes as an expression system, Feng et al. (2002) demonstrated that mutations in the HG1 subunit affect receptor function. The GABAβ subunit is an important functional component of the GABA receptor (Sieghart et al. 1999). In their study, Feng et al. (2002) co-expressed the wild-type and mutant HG1 subunits with a GABAβ subunit, since the HG1 subunits did not form functional homomeric receptors. Here, we describe the cloning of a GABAβ

subunit from *C. oncophora*, which will be useful in heterologous expression studies involving the GABA receptors of this parasitic nematode.

A.2 Materials and Methods

A.2.1 Cloning of the full-length C. oncophora GABAβ cDNA

Isolation of total RNA from *C. oncophora* and first strand cDNA synthesis were carried out as previously described (Chapters 3 and 5). For the initial isolation of the *C. oncophora* GABAβ sequence, cDNA was amplified using Advantage 2 cDNA kit (ClonTech) with degenerate primers. These primers were designed based on an alignment of GABAβ predicted protein sequences from *Caenorhabditis elegans*, *Drosophila melanogaster*, and *Lymnaea stagnalis*. Two sets of primers were designed for a nested PCR approach. A fragment of the GABAβ cDNA was first amplified with the outer sense primer, Deg F1 (5' TAYGAYATHCGNCTNCGNCC 3'), and the outer antisense primer, Deg R1 (5' TARTTNACNGCNGCRTAYTC 3'). This first-round reaction was then used as template for subsequent amplification using the nested primers Deg F2 (5' ATHTGGGTNCCNGAYTT 3'), and Deg R2 (5' TTNACSTANGADATNCGNGG 3'). The PCR reaction conditions were: an initial denaturation at 94°C for 30 s, followed by 30 cycles of 94°C for 20 s, 50°C for 30 s, and 72°C for 1 min, and a final extension at 72°C for 5 min. The PCR product from the second (nested) reaction was examined on a 1% agarose gel (TBE) stained with 0.5 μg/ml ethidium bromide, and the fragment of the expected length (~ 570 bp) was purified using the Nucleospin Gel Extraction Kit (ClonTech). The purified PCR products were then sub-cloned into a TA cloning vector (Invitrogen) as described by the manufacturer,

and then sequenced using standard M13 forward and/or reverse primers. Three independent clones were sequenced to obtain a consensus. Based on the sequenced fragment, gene-specific primers were designed for the 5' and 3' RACE (Rapid Amplification of cDNA Ends) reactions.

To identify the 5' end of the *C. oncophora* GABAβ cDNA, The SL2 primer (5' GGTTTTAACCCAGTTACTCAAG 3') was used along with two gene-specific antisense primers 5' RACE 1 (5' TCGGACGATTCCACAGCATAAGTAC 3') and 5' RACE 2 (5' AGGTTCATCGAGCAGCTAAGCGTTG 3') in a semi-nested PCR reaction using the Advantage 2 cDNA kit. Amplification conditions were as outlined above, with an annealing temperature of 54°C. The resulting PCR fragment was purified, ligated into a TA cloning vector (Invitrogen) and subsequently sequenced in both directions using vector primers.

To amplify the 3' end of GABAβ cDNA, a nested PCR approach was employed using the Marathon cDNA Amplification Kit (ClonTech). Two gene-specific sense primers were designed from the sequenced fragment (3' RACE 1, 5' GATCGTTATGCTCTCATGGGTTCTCG 3' and 3' RACE 2, 5' CACGAGGCAACCAGTGCCCGTGTTG 3'), and used with the two antisense adaptor primers AP1 and AP2, respectively, as outlined by the manufacturer.

A.3 Results

The *C. oncophora* GABAβ sequence has been deposited in GenBank under the accession number AY372759. The 1815 bp sequence encodes a protein of 543 amino acids (Figure 1). The protein sequence has structural features that are characteristic of inhibitory cys-

loop receptor subunits, including a pair of cysteine residues in the N-terminal domain, and four hydrophobic transmembrane (TM) domains. The spliced leader sequence, SL2, is found at the N-terminus 44 bp upstream of the initiation codon, and a predicted signal peptide cleavage site occurs between residues 29 and 30 (Figure 1). Potential N-linked glycosylation sites are present in the N-terminal domain (residues 48, 54 and 60). Consensus sites for cAMP-dependent phosphorylation (residue 430) and protein kinase C phosphorylation (residues 374, 427, 472, 475 and 481) occur within the long intracytoplasmic loop between TMs 3 and 4. In Figure 2, the *C. oncophora* GABAβ predicted protein sequence is shown aligned with GABAβ sequences from *H. contortus* (Feng, unpublished) and *C. elegans* (Feng et al, 2002). At the amino acid level, the *C. oncophora* showed 89 % identity to the *H. contortus* sequence, and 69 % identity to the *C. elegans* sequence. The *C. oncophora* sequence is also closely related to GABAβ sequences from *D. melanogaster*, *Anopheles gambiae* and *Lymaea stagnalis*, and shows lower levels of sequence identity to vertebrate GABAβ subunits (Table 1).

Figure 1. Sequence of the *C. oncophora* GABAβ cDNA. The predicted amino acid sequence is shown above the nucleotide sequence. The transmembrane domains are overlined, and the signal peptide cleavage site is indicated by ↓. The two cysteine residues are indicated by ●, and the possible N-linked glycosylation sites by ▼. Potential phosphorylation sites for cAMP-dependent protein kinase (▽) and protein kinase C (★) are also indicated.

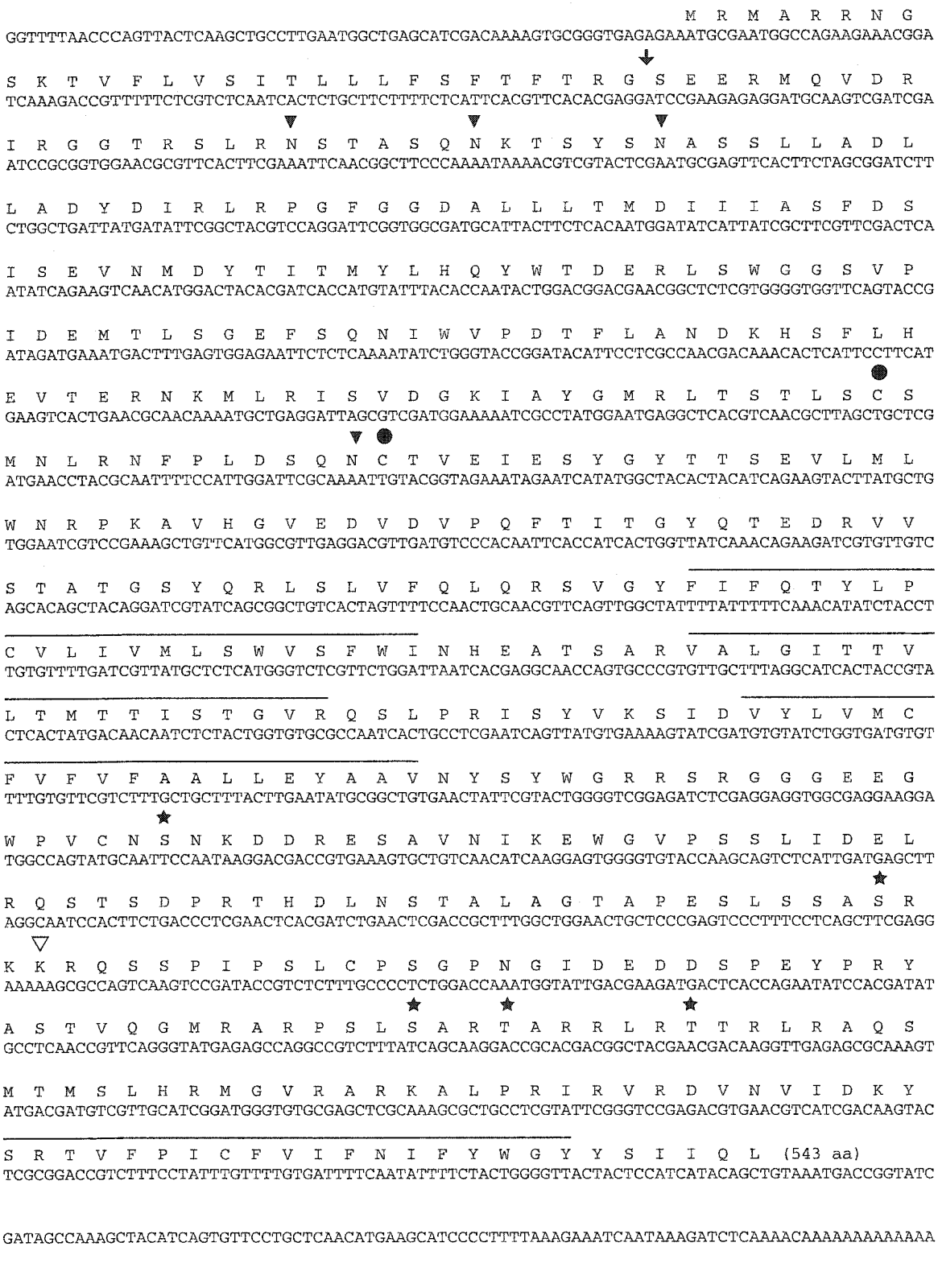
M R M A R R N G
GGTTTTAACCCAGTTACTCAAGCTGCCTTGAATGGCTGAGCATCGACAAAAGTGCGGGTGAGAGAAATGCGAATGGCCAGAAGAAACGGA
S K T V F L V S I T L L L F S F T F T R G S E E R M Q V D R
TCAAAGACCGTTTTTCTCGTCTCAATCACTCTGCTTCTTTTCTCATTCACGTTCACACGAGGATCCGAAGAGAGGATGCAAGTCGATCGA
I R G G T R S L R N S T A S Q N K T S Y S N A S S L L A D L
ATCCGCGGTGGAACGCGTTCACTTCGAAATTCAACGGCTTCCCAAAATAAAACGTCGTACTCGAATGCGAGTTCACTTCTAGCGGATCTT
L A D Y D I R L R P G F G G D A L L L T M D I I I A S F D S
CTGGCTGATTATGATATTCGGCTACGTCCAGGATTCGGTGGCGATGCATTACTTCTCACAATGGATATCATTATCGCTTCGTTCGACTCA
I S E V N M D Y T I T M Y L H Q Y W T D E R L S W G G S V P
ATATCAGAAGTCAACATGGACTACACGATCACCATGTATTTACACCAATACTGGACGGACGAACGGCTCTCGTGGGGTGGTTCAGTACCG
I D E M T L S G E F S Q N I W V P D T F L A N D K H S F L H
ATAGATGAAATGACTTTGAGTGGAGAATTCTCTCAAAATATCTGGGTACCGGATACATTCCTCGCCAACGACAAACACTCATTCCTTCAT
E V T E R N K M L R I S V D G K I A Y G M R L T S T L S C S
GAAGTCACTGAACGCAACAAAATGCTGAGGATTAGCGTCGATGGAAAAATCGCCTATGGAATGAGGCTCACGTCAACGCTTAGCTGCTCG
M N L R N F P L D S Q N C T V E I E S Y G Y T T S E V L M L
ATGAACCTACGCAATTTTCCATTGGATTCGCAAAATTGTACGGTAGAAATAGAATCATATGGCTACACTACATCAGAAGTACTTATGCTG
W N R P K A V H G V E D V D V P Q F T I T G Y Q T E D R V V
TGGAATCGTCCGAAAGCTGTTCATGGCGTTGAGGACGTTGATGTCCCACAATTCACCATCACTGGTTATCAAACAGAAGATCGTGTTGTC
S T A T G S Y Q R L S L V F Q L Q R S V G Y F I F Q T Y L P
AGCACAGCTACAGGATCGTATCAGCGGCTGTCACTAGTTTTCCAACTGCAACGTTCAGTTGGCTATTTTATTTTTCAAACATATCTACCT
C V L I V M L S W V S F W I N H E A T S A R V A L G I T T V
TGTGTTTTGATCGTTATGCTCTCATGGGTCTCGTTCTGGATTAATCACGAGGCAACCAGTGCCCGTGTTGCTTTAGGCATCACTACCGTA
L T M T T I S T G V R Q S L P R I S Y V K S I D V Y L V M C
CTCACTATGACAACAATCTCTACTGGTGTGCGCCAATCACTGCCTCGAATCAGTTATGTGAAAAGTATCGATGTGTATCTGGTGATGTGT
F V F V F A A L L E Y A A V N Y S Y W G R R S R G G G E E G
TTTGTGTTCGTCTTTGCTGCTTTACTTGAATATGCGGCTGTGAACTATTCGTACTGGGGTCGGAGATCTCGAGGAGGTGGCGAGGAAGGA
W P V C N S N K D D R E S A V N I K E W G V P S S L I D E L
TGGCCAGTATGCAATTCCAATAAGGACGACCGTGAAAGTGCTGTCAACATCAAGGAGTGGGGTGTACCAAGCAGTCTCATTGATGAGCTT
R Q S T S D P R T H D L N S T A L A G T A P E S L S S A S R
AGGCAATCCACTTCTGACCCTCGAACTCACGATCTGAACTCGACCGCTTTGGCTGGAACTGCTCCCGAGTCCCTTTCCTCAGCTTCGAGG
K K R Q S S P I P S L C P S G P N G I D E D D S P E Y P R Y
AAAAAGCGCCAGTCAAGTCCGATACCGTCTCTTTGCCCCTCTGGACCAAATGGTATTGACGAAGATGACTCACCAGAATATCCACGATAT
A S T V Q G M R A R P S L S A R T A R R L R T T R L R A Q S
GCCTCAACCGTTCAGGGTATGAGAGCCAGGCCGTCTTTATCAGCAAGGACCGCACGACGGCTACGAACGACAAGGTTGAGAGCGCAAAGT
M T M S L H R M G V R A R K A L P R I R V R D V N V I D K Y
ATGACGATGTCGTTGCATCGGATGGGTGTGCGAGCTCGCAAAGCGCTGCCTCGTATTCGGGTCCGAGACGTGAACGTCATCGACAAGTAC
S R T V F P I C F V I F N I F Y W G Y Y S I I Q L (543 aa)
TCGCGGACCGTCTTTCCTATTTGTTTTGTGATTTTCAATATTTTCTACTGGGGTTACTACTCCATCATACAGCTGTAAATGACCGGTATC
GATAGCCAAAGCTACATCAGTGTTCCTGCTCAACATGAAGCATCCCCTTTTAAAGAAATCAATAAAGATCTCAAAACAAAAAAAAAAAAA
AAAAAAAAAAAAAAA (1815 nt)

Figure 2. Alignment of GABAβ predicted amino acid sequences from *C. oncophora*, *H. contortus* and *C. elegans*. The grey shading represents amino acid identity. Two cysteine residues are found in the N-terminal extracellular domain (indicated by ●). The four transmembrane domains (TM1-4) are also shown.

Figure 2

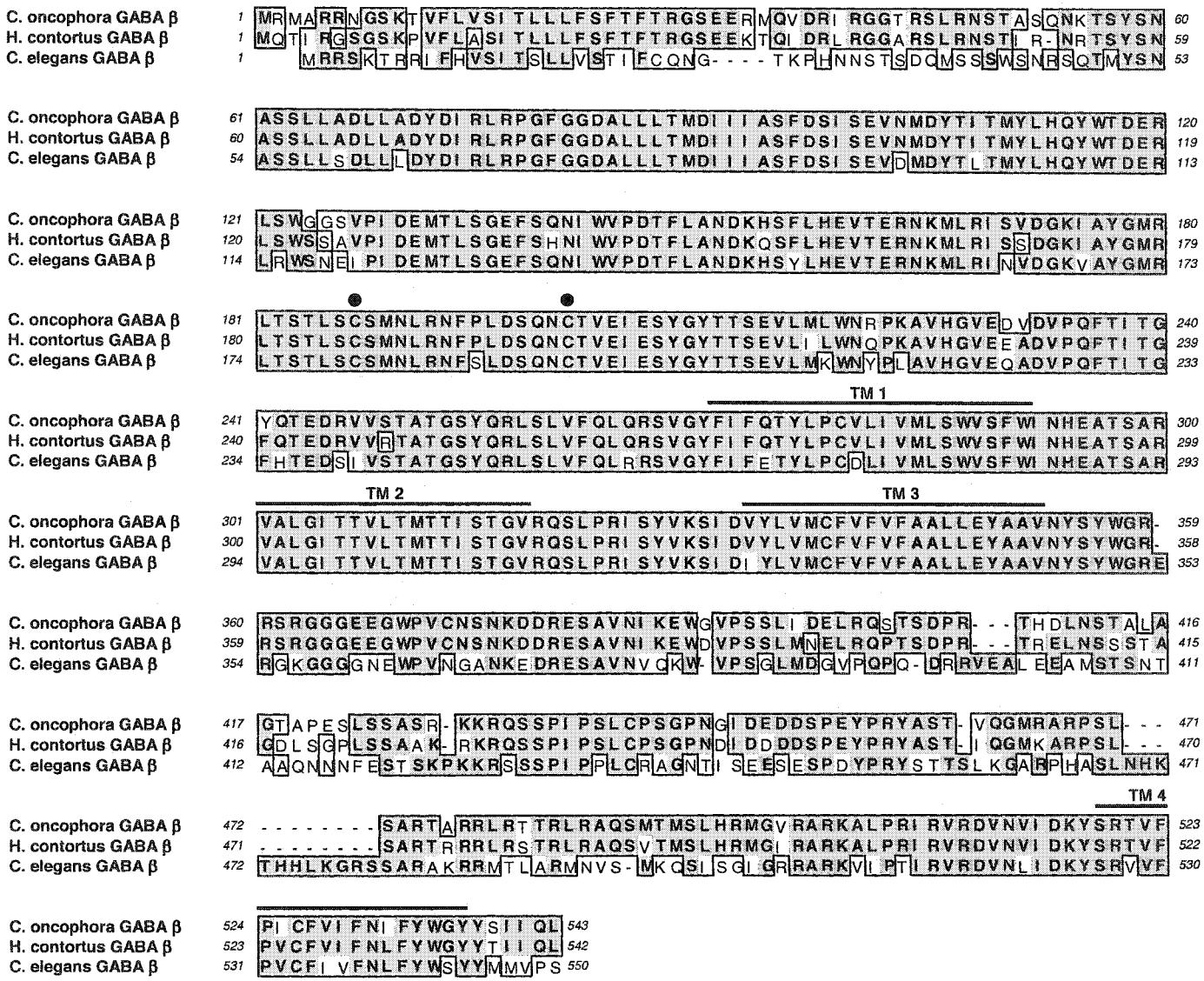
C. oncophora GABA β
H. contortus GABA β
C. elegans GABA β
TM 1
TM 2
TM 3
TM 4

Table 1. Percentage amino-acid identity of *C. oncophora* GABAβ with other cloned and predicted GABA receptor subunits.

SEQUENCE	% IDENTITY
H. contortus GABAβ	89
C. elegans GABAβ	69
D. melanogaster GABAβ	66
An. gambiae GABAβ	64
L. stagnalis GABAβ	63
D. melanogaster Rdl	44
Human GABAβ	40
Mouse GABAβ	39
Chicken GABAβ	38

A.4 Discussion

GABA receptors are members of the ligand gated ion channel superfamily, which includes nicotinic acetylcholine receptors, 5-hydroxytryptamine$_3$ (5 HT-3) serotonin receptors and glycine receptors (Vassilatis et al. 1997). Receptors in this family share structural similarities. They are pentameric in structure, and are made up of distinct, but homologous subunits (Smith and Olsen 1995). Each subunit in the receptor complex consists of a large N-terminal extracellular domain, four transmembrane (TM) domains, a

large intracellular loop between TM's 3 and 4, and an extracellular carboxy terminal. Eight subunit types (α, β γ, δ, ε, π, ρ, θ) have been identified in mammals, and various combinations of these subunits form receptors which have distinct pharmacological properties and are differentially distributed within the nervous system (Macdonald and Olsen 1994; Barnard et al. 1998; Whiting 1999). Fewer GABA receptor subunit types have been cloned from invertebrates. Three GABA receptor subunits have been cloned in *D. melanogaster* (Hosie et al. 1997). These GABA receptors are widely distributed in the insect nervous system. Three *C. elegans* genes have been identified which encode GABA receptor subunits (Bamber et al. 1999), while two subunit types have been identified in the parasitic nematode *H. contortus* (Laughton et al. 1994). Here, we report the cloning of a GABAβ receptor from *C. oncophora* which shows high sequence identity to GABAβ subunits from *H. contortus* and *C. elegans*. The GABAβ subunit is an important functional component of the $GABA_A$ receptor. While there are reports of non-β subunits (α and γ) forming functional homomeric and heteromeric channels, the β subunit is required for robust receptor expression (Sigel et al. 1990).

In *C. elegans*, GABAergic neurons control somatic musculature (McIntire et al. 1993). Avermectin causes paralysis of somatic musculature in *Ascaris suum* and *C. elegans*, and this effect is mediated at least in part through its interaction with GABA receptors (Kass et al. 1980). This effect may also be mediated partly through avermectin's interaction with glutamate-gated chloride channel (GluCl) receptors, since GluCl subunits are expressed in motor neurons (Delany et al. 1998; Jagannathan et al. 1999; Portillo et al. 2003). Genetic variability and electrophysiological studies have demonstrated the involvement of the *H. contortus* GABA HG1 gene in ivermectin

resistance (Feng et al. 2002; Blackhall et al. 2003). Attempts to clone the HG1 homolog from *C. oncophora* were unsuccessful. When expressed in *Xenopus* oocytes, the *H. contortus* HG1 subunit failed to form functional homomeric receptors, and a GABAβ subunit was necessary for the formation of functional GABA-gated and ivermectin-sensitive receptors (Feng et al. 2002). Connor et al. (1998) have shown that in *Xenopus* oocytes expressing non-β subunits, the subunits are not localized to the cell membrane, and a GABAβ subunit is needed for proper targeting of non-β subunits to the cell surface.

The *C. oncophora* GABAβ subunit was expressed in *Xenopus* oocytes. Eggs expressing the homomeric receptors did not respond to 1 mM GABA, 1 mM glycine, 1 mM glutamate, 5 μM ivermectin or 5 μM moxidectin (data not shown). Similar results were described for *C. elegans* GABAβ homomeric receptors (Feng et al. 2002). Some invertebrate GABA receptor subunits are able to form functional homomeric receptors when expressed in a heterologous system. Bamber et al. (1999) showed that *C. elegans* UNC-49B and UNC-49C GABA receptor subunits formed homomeric GABA receptors when expressed in *Xenopus* oocytes. The *D. melanogaster* Rdl GABA receptor subunits also formed homomeric GABA receptors in *Xenopus* oocytes (Ffrench-Constant et al. 1993). Whether the *C. oncophora* GABAβ homomeric receptor is actually correctly targeted to the cell membrane requires further investigation.

In conclusion, we have cloned a GABAβ subunit from *C. oncophora*, which shows a high degree of similarity to GABAβ subunits from *H. contortus* and *C. elegans*. This subunit may be necessary for the formation of functional heteromeric receptors, and will therefore be useful for future electrophysiological experiments.

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APPENDIX B

SSCP analysis of intracytoplasmic loop region of Co *GluClα3* and *GluClβ* genes

To determine the role of the Co *GluClα3* and *GluClβ* genes in ivermectin resistance, fragments of the two genes were examined using SSCP. Two regions of both genes were analyzed:

1. A fragment of the extracellular N-terminal domain between the two dicysteine loops (results presented in Chapter 3), and

2. A fragment of the intracytoplasmic loop between transmembrane domains 3 and 4. This was done to determine whether analysis of different regions of the gene would yield similar results. The results of this analysis are presented in this section. Analysis of the intracytoplasmic loop region of the *GluClα3* gene showed significant differences in allele frequencies between IVS and IVR worms, similar to what was seen with analysis of the N-terminal region (Chapter 3). With the *GluClβ* gene, unique alleles were identified at low frequency in both IVS and IVR groups following analysis of the intracytoplasmic loop region. Sequencing of Co *GluClα3* and *GluClβ* alleles revealed no amino acid changes, and the association of Co *GluClα3* with ivermectin resistance suggests that mutations in regions other than those analyzed here may be involved in ivermectin resistance. Indeed, sequencing of the full-length GluClα3 cDNAs from IVS and IVR worms identified amino acid changes in the N-terminal extracellular domain, in regions

other than those analyzed using SSCP. These changes were found to affect agonist sensitivity (Chapter 4). With the *GluClβ* gene, the finding of unique alleles in IVS and IVR worms at the intracytoplasmic loop region may explain the mutant IVR GluClβ allele reported in Chapter 4, which, when expressed in *Xenopus* oocytes, failed to form a functional channel.

B.1 Analysis of the Co *GluClα3* gene

50 IVS and 53 IVR individual male worms were analyzed, and the different alleles identified by their different banding patterns (Figure 1). A total of 9 different alleles were identified (Figure 2). Allele A was the most common allele in the IVS group, with a frequency of 0.45. The frequency of this allele in the IVR group was 0.3. Allele C was the most common allele in the IVR group, with a frequency of 0.38. The frequency of this allele in the IVS group was 0.25. Chi-square analysis revealed a significant difference in allele frequencies between the two groups ($p < 0.001$), suggesting that the *GluClα3* gene may be involved in ivermectin resistance or linked to another gene which is involved in ivermectin resistance.

Figure 1. SSCP patterns of three individual worms at the Co *GluClα3* gene (intracytoplasmic loop region). The gel shows seven of the nine alleles identified at the region examined.

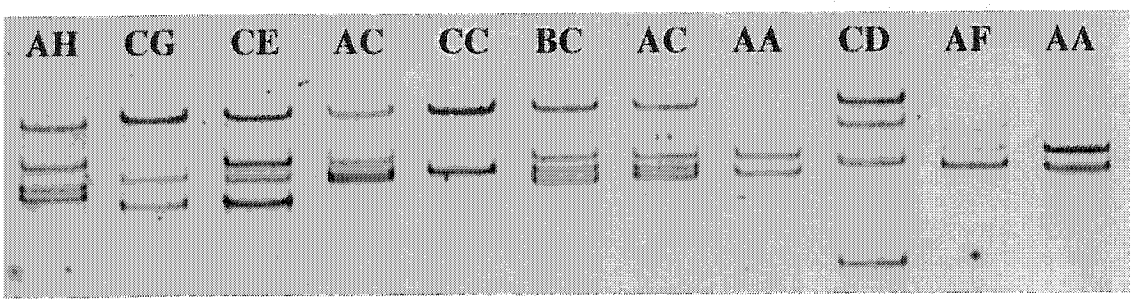
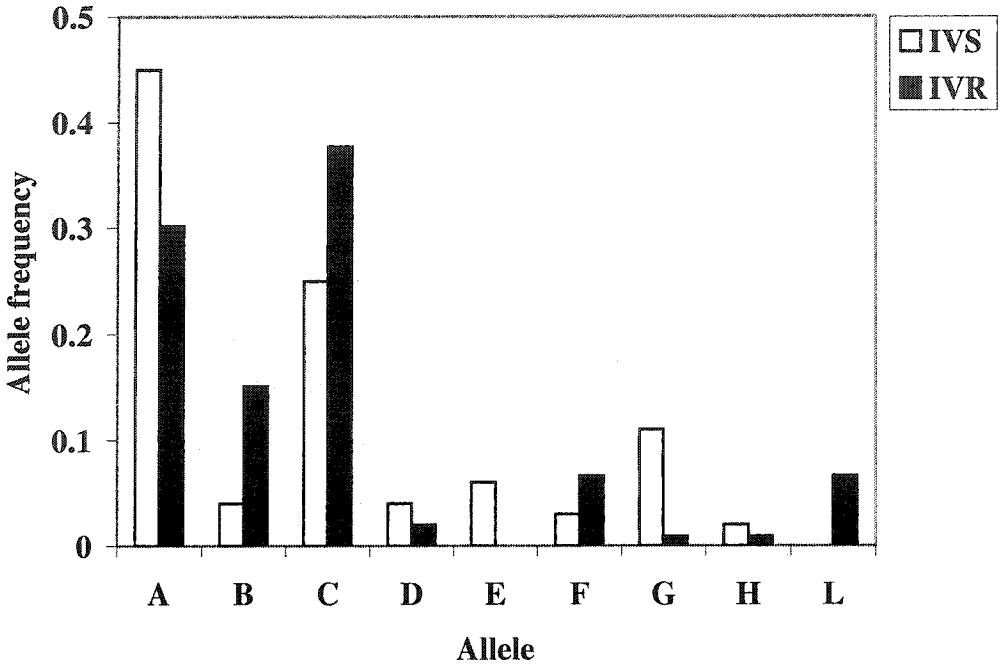
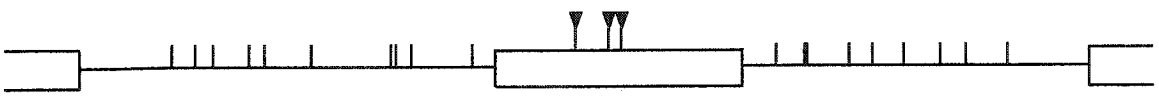


Figure 2. Co *GluClα3* allele frequencies at second region analyzed.



All nine *GluCl*α*3* alleles were sequenced. A total of 22 positions were found to be polymorphic (Figure 3). Three of these were in the exon. All three were silent mutations, as they did not result in amino acid change.

Figure 3. Positions of polymorphic sites of the Co GluCla3 gene analyzed. 22 positions were found to be polymorphic, three of which were in the exon (marked by arrows). All were silent mutations.



B.2 Analysis of the Co *GluClβ* gene

58 IVS and 58 IVR individual male worms were examined. Three alleles were detected by SSCP (Figures 4 and 5). Allele A was the most common in both groups, being found at frequencies of 0.98 and 0.97 in the IVS and IVR groups, respectively. Allele B was found only in two IVS worms, and was only present in the heterozygous state. Allele C was found only in three IVR worms, and was also only present in the heterozygous state.

Figure 4. SSCP patterns of three individual worms at the Co *GluClβ* gene.

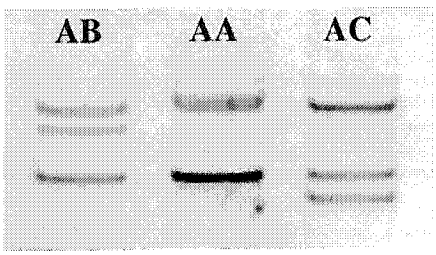
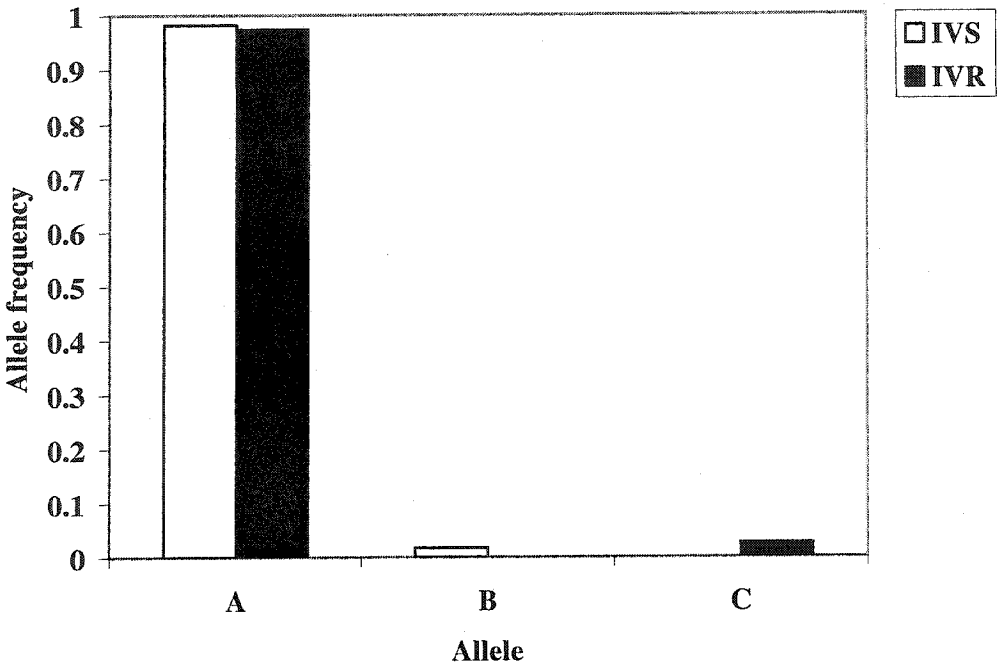


Figure 5. Co *GluClβ* allele frequencies at the second region analyzed.



The three Co *GluClβ* alleles, A, B and C, were sequenced. Unlike the Co *GluClα3* region, which spanned two introns, the same region of the Co *GluClβ* gene spanned only one intron. This suggests that the position of introns in the Co *GluClα3* and *GluClβ* genes is not conserved. Alignment of the three Co *GluClβ* alleles revealed three polymorphic sites, two of which were in the intron (Figure 6). One silent exonic mutation was also identified.

Figure 6. Positions of polymorphic sites of the Co *GluClβ* gene analyzed. 3 positions were found to be polymorphic, one of which was in the exon (marked by an arrow).

APPENDIX C

GenBank Flatfiles of Full-length *C. oncophora* cDNA Sequences Cloned

C.1 *C. oncophora* GluClα3 subunit

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ACCESSION AY372756
VERSION AY372756
KEYWORDS .
SOURCE Cooperia oncophora
ORGANISM Cooperia oncophora
Eukaryota; Metazoa; Nematoda; Chromadorea; Rhabditida; Strongylida; Trichostrongyloidea; Cooperiidae; Cooperia.
REFERENCE 1 (bases 1 to 1818)
AUTHORS Njue,A.I. and Prichard,R.K.
TITLE Genetic variability of glutamate-gated chloride channel genes in ivermectin-susceptible and -resistant strains of Cooperia oncophora
JOURNAL Unpublished
REFERENCE 2 (bases 1 to 1818)
AUTHORS Njue,A.I. and Prichard,R.K.
TITLE Direct Submission
JOURNAL Submitted (22-AUG-2003) Institute of Parasitology, McGill University, 21 111 Lakeshore Road, Ste. Anne de Bellevue, QC H9X 3V9, Canada
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C.2 *C. oncophora* GluClβ subunit

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REFERENCE   1 (bases 1 to 1480)
AUTHORS     Njue,A.I. and Prichard,R.K.
TITLE       Genetic variability of glutamate-gated chloride channel
            genes in ivermectin-susceptible and -resistant strains of
            Cooperia oncophora
JOURNAL     Unpublished
REFERENCE   2 (bases 1 to 1480)
AUTHORS     Njue,A.I. and Prichard,R.K.
TITLE       Direct Submission
JOURNAL     Submitted (22-AUG-2003) Institute of Parasitology, McGill
            University, 21 111 Lakeshore Road, Ste. Anne de Bellevue,
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C.3 *C.oncophora* beta-tubulin isotype 1

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AUTHORS     Njue,A.I. and Prichard,R.K.
TITLE       Cloning two full-length beta-tubulin isotype cDNAs from
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JOURNAL     Parasitology 127 (2003) In press
REFERENCE   2 (bases 1 to 1470)
AUTHORS     Njue,A.I. and Prichard,R.K.
TITLE       Direct Submission
JOURNAL     Submitted (21-MAR-2003) Institute of Parasitology, McGill
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1321 aagccaccgc tgacgatatg ggcgatctcg atgcagaagg cgcagaagag ccgtaccccg
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C.4 *C.oncophora* beta-tubulin isotype 2

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LOCUS       AY259995     1620     bp     mRNA     linear INV 09-OCT-2003
DEFINITION  Cooperia oncophora beta-tubulin isotype 2 mRNA, complete cds
ACCESSION   AY259995
VERSION     AY259995 GI:37594693
KEYWORDS    .
SOURCE      Cooperia oncophora
ORGANISM    Cooperia oncophora
            Eukaryota; Metazoa; Nematoda; Chromadorea; Rhabditida;
            Strongylida; Trichostrongyloidea; Cooperiidae; Cooperia.
REFERENCE   1 (bases 1 to 1620)
AUTHORS     Njue,A.I. and Prichard,R.K.
TITLE       Cloning two full-length beta-tubulin isotype cDNAs from
            Cooperia oncophora, and screening for benzimidazole
            resistance-associated mutations in two isolates
JOURNAL     Parasitology 127 (2003) In press
REFERENCE   2 (bases 1 to 1620)
AUTHORS     Njue,A.I. and Prichard,R.K.
TITLE       Direct Submission
JOURNAL     Submitted (21-MAR-2003) Institute of Parasitology, McGill
            University, 21,111 Lakeshore Road, Ste-Anne-de-Bellevue,
            QC H9X 3V9, Canada
FEATURES     Location/Qualifiers
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C.5 *C. oncophora* actin

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LOCUS       AY372758     1455 bp     mRNA     linear     INV 20-NOV-2003
DEFINITION  Cooperia oncophora actin mRNA, complete cds.
ACCESSION   AY372758
VERSION     AY372758
KEYWORDS    .
SOURCE      Cooperia oncophora
ORGANISM    Cooperia oncophora
            Eukaryota; Metazoa; Nematoda; Chromadorea; Rhabditida;
            Strongylida; Trichostrongyloidea; Cooperiidae; Cooperia.
REFERENCE   1 (bases 1 to 1455)
AUTHORS     Njue,A.I. and Prichard,R.K.
TITLE       Genetic variability of glutamate-gated chloride channel
            genes in ivermectin-susceptible and -resistant strains of
            Cooperia oncophora
JOURNAL     Unpublished
REFERENCE   2 (bases 1 to 1455)
AUTHORS     Njue,A.I. and Prichard,R.K.
TITLE       Direct Submission
JOURNAL     Submitted (22-AUG-2003) Institute of Parasitology, McGill
            University, 21 111 Lakeshore Road, Ste. Anne de Bellevue,
            QC H9X 3V9, Canada
FEATURES     Location/Qualifiers
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C.6 *C.oncophora* GABAβ subunit

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LOCUS       AY372759     1815 bp     mRNA     linear     INV 20-NOV-2003
DEFINITION  Cooperia oncophora GABA-beta subunit mRNA, complete cds.
ACCESSION   AY372759
VERSION     AY372759
KEYWORDS    .
SOURCE      Cooperia oncophora
ORGANISM    Cooperia oncophora
            Eukaryota; Metazoa; Nematoda; Chromadorea; Rhabditida;
            Strongylida; Trichostrongyloidea; Cooperiidae; Cooperia.
REFERENCE   1 (bases 1 to 1815)
AUTHORS     Njue,A.I. and Prichard,R.K.
TITLE       Sequence of GABA-beta subunit cDNA from Cooperia oncophora
JOURNAL     Unpublished
REFERENCE   2 (bases 1 to 1815)
AUTHORS     Njue,A.I. and Prichard,R.K.
TITLE       Direct Submission
JOURNAL     Submitted (22-AUG-2003) Institute of Parasitology, McGill
            University, 21 111 Lakeshore Road, Ste. Anne de Bellevue,
            QC H9X 3V9, Canada
FEATURES     Location/Qualifiers
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APPENDIX D

Ethics Certificate (Animal Use Protocol)