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Genetic Variation and Multiple Mechanisms of Anthelmintic Resistance in *Haemonchus contortus*

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ABSTRACT

Anthelmintic treatment of livestock is an important aspect of the control of gastrointestinal parasites. Resistance to anthelmintics is common, and an understanding of resistance requires knowledge of an anthelmintic's mode(s) of action and mechanism(s) of resistance. The parasitic nematode, *Haemonchus contortus*, has developed resistance to benzimidazoles and avermectins/milbemycins. Proposed mechanisms of resistance are here supported by genetic changes observed in genes whose protein products are believed to interact with these anthelmintics. Statistically significant differences in allele frequencies were observed between untreated and ivermectin- and moxidectin-treated strains in a gene encoding a putative glutamate-gated chloride channel alpha subunit, a proposed target of avermectins/milbemycins. One allele appeared to be associated with resistance. Similar changes in allele frequencies in the same strains occurred in a gene encoding a subunit of a gamma-aminobutyric acid receptor. Significant differences in allele frequencies of a gene encoding a P-glycoprotein were found in strains of *H. contortus* treated with ivermectin and moxidectin compared to derived, untreated strains. In all treated strains, one allele appeared to be associated with resistance. Similarly, allele frequencies of this gene were significantly different between a cambendazole-treated strain and its derived, untreated strain. These results implicate glutamate-gated chloride channels and gamma-aminobutyric acid receptors in mechanisms of resistance to avermectins/milbemycins and implicate P-glycoprotein in a mechanism of resistance to avermectins/milbemycins and benzimidazoles in *H. contortus*.

ABRÉGÉ

Le traitement anthelmintique du bétail est un aspect important de la lutte contre les parasites gastrointestinaux. La résistance aux anthelmintiques est répandue et pour la comprendre on se doit de connaître le(s) mode(s) d'action des anthelmintiques et le(s) mécanisme(s) de résistance. *Haemonchus contortus*, un nématode parasitaire, a développé une résistance aux benzimidazoles et aux avermectines/milbemycines. Les mécanismes de résistance suggérés découlent de changements génétiques observés chez les gènes dont les produits protidiques réagiraient avec ces mêmes anthelmintiques. Des différences statistiques significatives ont été observées, chez les souches sensibles et traitées à l'ivermectine et moxidectine, dans la fréquence des allèles d'un gène produisant une sous-unité alpha du canal chloriné régularisé par le glutamate, une cible possible des avermectines/milbemycines. Un allèle semble associé à la résistance. Des changements similaires dans la fréquence des allèles de mêmes souches sont observés dans une sous-unité du récepteur gamma de l'acide aminobutyrique. Des différences significatives dans la fréquence des allèles d'une protèine d'un gène, la P-glycoprotéine, ont été aussi observées dans les souches d'*Haemonchus contortus* traitées à l'ivermectine et la moxidectine en comparaison aux souches non-traitées, dites sensibles. Un allèle semble associé à la résistance dans toutes les souches traitées. Parallèlement, la fréquence des allèles de ce même gène était significativement différente entre une souche traitée au cambendazole et son dérivé, la souche sensible. Ces résultats indiquent que les canaux chlorinés régularisés par le glutamate et les récepteurs gamma d'acide aminobutyrique seraient impliqués dans les mécanismes de résistance aux avermectines/milbemycines et

que la P-glycoprotéine est active dans le mécanisme de la résistance aux benzimidazoles et aux avermectines/milbemycines chez *Haemonchus contortus*.

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CONTRIBUTIONS OF AUTHORS

Analyses presented in this thesis were designed and performed by the author, under the supervision of Dr. Robin Beech, and with intellectual contributions of Dr. Roger Prichard. All experimental data presented were obtained by the author, except those presented in Figure 4 of Chapter IV, which were provided by Hao Yuan Liu. The author performed all DNA extractions for the study material except for the Fort Dodge strains of *Haemonchus contortus*, which were performed by Hao Yuan Liu, and the three BZ strains in Chapter V and Appendix A, which were performed by Dr. Robin Beech as part of an earlier study. All primers used in PCR amplifications were designed by the author except for those used for obtaining the original clone of the fragment of the glutamate-gated chloride channel subunit gene in Chapter II, which were designed by Dr. Jean-François Pouliot, the P-gp antisense primer MX-D in Chapters IV and V, which was designed by Dr. Ming Xu, and those used to amplify the fragment of the gene encoding β-tubulin isotype 1 in Appendix A, RBE31 being designed by Dr. Robin Beech and MRβ5 by Dr. Marlene Roos. All PCR reactions were performed by the author except for those of the Fort Dodge strains in Chapter IV, which were performed by Hao Yuan Liu. The DNA sequence reported in Chapter II was obtained by the author. The DNA sequence reported in Chapter III were provided by an external sequencing service from PCR products and plasmid clones prepared by the author. The choice of method for obtaining allele frequency data was that of the author. In the SSCP analyses all template preparations, labelling reactions, and polyacrylamide gel electrophoresis were performed by the author. For the RFLP experiments, all restriction enzyme digestions and

polyacrylamide gel electrophoresis were performed by the author, except for those of the Fort Dodge strains in Chapter IV, which were performed by Hao Yuan Liu. The identification of all alleles except P-gp alleles L through e in Figure 4 of Chapter IV, was made by the author. Alleles L through e were identified by Hao Yuan Liu. The determination of all allele frequencies reported in this thesis, and the Chi-square analyses of these data, were performed by the author. The preparation of this thesis, including all figures, was performed by the author.

CONTRIBUTION TO KNOWLEDGE OF THE FIELD

The material presented in this thesis provides several contributions to the study of anthelmintic resistance in *Haemonchus contortus*. Glutamate-gated chloride channels may be the principal site of action of avermectins/milbemycins, and the cloning of a fragment of a gene encoding a putative alpha subunit, the subunit with which these drugs interact, is the first report of this subunit from a parasitic nematode. The subsequent finding that genetic changes at this gene is associated with anthelmintic treatment is significant in identifying potential sites of action and mechanisms of resistance to the drugs. Likewise, the report of genetic changes associated with resistance occurring at a gene encoding a gamma-aminobutyric acid receptor subunit suggests an additional site of action for these drugs, one that was being discounted after the discovery of glutamate-gated chloride channels. These receptors may play an important role in the mechanism of action of avermectins/milbemycins, and a renewed interest in them may prove valuable. Some evidence had suggested that P-glycoprotein may also provide an important mechanism of resistance to avermectins/milbemycins, and the report here of genetic changes associated with resistance occurring in a gene encoding a P-glycoprotein provides support for an important hypothesis in the field of anthelmintic resistance. Extending this finding to include another class of anthelmintics, may be equally important. In summary, the identification of a gene in *H. contortus* that encodes a probable site of action of avermectins/milbemycins, the finding that specific alleles at three genes are apparently being selected for during the course of development of resistance to treatment with avermectins/milbemycins, and the possibility that the protein encoded by one of these genes may contribute to resistance to other anthelmintics, are

worthy contributions to the understanding of how these drugs act and how resistance to them may occur.

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INTRODUCTION

Gastrointestinal parasites of livestock cause serious losses in production worldwide. Chemical treatment of hosts with anthelmintics has become an integral part of efforts to control infection. Resistance to all the major classes of anthelmintics, though, has developed and threatens to reduce the effectiveness of chemical control. New anthelmintics are being developed, but the usefulness of existing anthelmintics could be enhanced by an understanding of how they work and how resistance to them occurs. The identification of genetic markers associated with resistance to anthelmintics would be helpful in detecting the development of resistance, and changes in control strategies could then be implemented to extend the effectiveness of anthelmintics currently in use.

Anthelmintic resistance has become a serious problem in the control of the nematode *Haemonchus contortus* in sheep. Most populations of *H. contortus* exposed to benzimidazoles have developed sufficient resistance to render treatment no longer cost effective. Resistance to the avermectin/milbemycin class of anthelmintics is not as severe a problem at present, but will become so in time if measures to slow the rate of development of resistance are not taken. The present study aims to aid the effort to understand mechanisms of resistance to benzimidazoles and avermectins/milbemycins.

The objectives of the study are 1) to identify genes that may contribute to, or be associated with, anthelmintic resistance, and 2) to identify alleles of these genes which could be used in the development of tests for detecting resistance in field populations. The strategy chosen employs the tools of population genetics. Genetically variable organisms possess multiple alleles of genes, and frequencies of these alleles will change over time. Changes can occur by random genetic drift, by hitch-hiking along with

changes occurring in neighbouring genes, by population changes, or by selection, either natural or artificial. The development of anthelmintic resistance occurs by artificial selection of alleles of genes that reduce the toxic effects of the anthelmintic being administered. The detection of changes in allele frequencies of genes suspected to be associated with resistance, then, can provide indirect evidence that these genes may contribute to resistance.

A concise review of the literature on the modes of action and mechanisms of resistance to benzimidazoles and avermectins/milbemycins is presented in Chapter I. In Chapter II, genetic evidence is presented supporting the common hypothesis that avermectins/milbemycins paralyze *H. contortus* by opening glutamate-gated chloride channels, and identifies an allele of a subunit gene that may contribute to resistance. Chapter III provides data implicating gamma-aminobutyric acid receptors as targets of avermectins/milbemycins, and identifies alleles of a subunit gene that may contribute to resistance. Chapters IV and V describe experiments that suggest P-glycoprotein contributes to a resistance mechanism to both benzimidazoles and avermectins/milbemycins. Finally, Chapter VI presents a discussion of the findings of these studies in relation to the current knowledge in the field of benzimidazole and avermectin/milbemycin resistance in *H. contortus*.

CHAPTER I

Literature Review

I.1 *Haemonchus contortus*

Haemonchus is a genus of parasitic nematodes belonging to class Secernentia, order Trichostrongyloidea, and family Trichostrongylidae. The genus *Haemonchus* was erected by Cobb (1898) to accommodate *Strongylus contortus* Rudolphi (1803). Ten species are currently recognized [1]. All species parasitize ruminants: nine species inhabit the abomasum, and one species inhabits the small intestine [2].

H. contortus parasitizes a number of African ruminants [2] and is a common parasite of domestic sheep, goats, and cattle worldwide. It infects the abomasum causing anaemia and other clinical symptoms [3] resulting in loss of production and increased mortality [4]. *H. contortus* is dioecious and has a direct life cycle, the adults passing eggs with the host's faeces. Eggs develop in the faeces where the first-stage larvae (L1) feed on bacteria. L1 larvae moult to the L2 stage, which moults but retains the L2 cuticle to give rise to L3 larvae. Infective L3 larvae develop from eggs in 4 to 6 days, when they migrate onto vegetation and are ingested by the host. Exsheathment of the L2 cuticle occurs in the rumen, the larvae migrate to the abomasum, and undergo another moult to the L4 stage. A final moult gives rise to the adult [5]. The prepatent period is about 15 days [3].

The economic impact of nematode infection can be substantial. In Australia alone, the cost from production losses and control measures for sheep in 1994 was estimated at A$222 million [6] Production losses consist mostly of reduced yield and quality of wool, followed by mortality and reduced meat production. As a means of decreasing these losses, anthelmintics have been developed to control levels of nematode infections.

I.2 Anthelmintic Resistance

Anthelmintic resistance has been defined as a reduced potency of a drug in a population of parasites where the drug was once effective [7] and is characterized by an increase in the frequency of individuals able to survive doses higher than those effective against a normal population [8]. The development of resistance to anthelmintics is not, unlike antibiotic resistance, believed to be due to spontaneous mutations which occur during treatment nor to mutations caused by the anthelmintic [9]. Rather, resistance arises by an evolutionary process of selection within a genetically diverse population of parasites. Anthelmintics disrupt vital structural or physiological functions by binding to and interfering with proteins. In a genetically diverse population, each protein species may be represented by many different alleles. Some alleles are very common, and some are rare. The structural and therefore functional variability of these alleles results in differential abilities of the anthelmintic to interfere with their function, or in differential abilities of the anthelmintic to reach its target. Individual parasites possessing alleles that can tolerate or negate the effects of the anthelmintic will have a higher probability of surviving the drug treatment. The survivors will thus be able to mate and pass their alleles to the next generation more frequently than individuals that possess alleles that confer susceptibility to the action of the anthelmintic. Over many generations, the frequency of the resistant alleles will increase in the population. The efficacy of the anthelmintic will thus decrease with continued use.

The genetics of resistance is an important factor in the speed with which resistance will develop. The initial frequency of a resistance allele in a population is very important [10]. High initial frequencies will obviously lead to resistance quickly. The

number of genes involved, which may represent multiple mechanisms of resistance, is also important. If resistance is due to a single gene, resistance will develop more quickly than if resistance is polygenic [10,11]. The mode of inheritance of resistance genes will affect the speed of resistance development. If resistance is dominant, heterozygotes will survive anthelmintic treatment, and the resistance allele will accumulate in the population faster than if resistance is recessive, where only rare homozygotes survive [11,12].

The nature of the resistance may or may not be directly related to the action of the anthelmintic and may involve more than one mechanism. Since anthelmintics act by binding to and interfering with proteins, the mechanism of resistance may involve a mutation that decreases the binding affinity of the anthelmintic for the target protein [10]. A reduction in the number of affected targets, then, could reduce the severity of the action of the anthelmintic enough to allow the parasite to survive and reproduce. If the mode of action of an anthelmintic involves a conformational change in the target protein that disrupts some vital function, resistance may be due to a mutation that alters or negates the conformational change without affecting binding affinity. Both of these mechanisms of resistance depend on mutations in the target protein that affect the mode of action of the anthelmintic at the target site.

The effects of an anthelmintic may also be diminished by reducing the concentration of drug at the target site. A transport or efflux mechanism could interfere with the amount of anthelmintic reaching its target, thereby reducing toxic effects [7]. Mutations in transport or efflux proteins could alter drug specificity or expression levels. A fourth possible mechanism of resistance could involve the chemical modification of the anthelmintic before it reaches its target [13]. Rendering an anthelmintic inactive by metabolic alteration or catabolism would reduce its effectiveness or half-life. Allelic

variants of enzymes capable of doing so might then be the targets of selection. Any one or a combination of these possible mechanisms could lead to the development of resistance to an anthelmintic through selection for alleles that enhance survival.

The common broad-spectrum anthelmintics that have been developed to control agriculturally important nematode infections belong to several chemical classes. Only the two classes that have relevance to this thesis, the benzimidazoles (BZ) and the avermectins (AVM)/milbemycins, will be discussed.

I.3 Resistance to BZ in *H. contortus*

BZ drugs were the first broad-spectrum anthelmintics to be produced. Thiabendazole (TBZ) was introduced in 1962 and was highly effective against common gastrointestinal nematodes in sheep. By 1964, however, field strains of *H. contortus* were becoming tolerant to TBZ treatment [14,15]. Other reports soon followed. BZ resistance is now prevalent around the world in a number of gastrointestinal helminths of livestock (see [16] for a brief review).

The principal mechanism of resistance to BZ is believed to involve the mode of action of the drugs. Nematodes exposed to BZ were observed to lose microtubules in intestinal cells [17]. Other studies found that BZ bound with high affinity to β-tubulin, a component of microtubules, in a wide range of organisms [18,19,20]. The loss of microtubules was greater in BZ-susceptible than in BZ-resistant nematodes [21]. The reduced effect of BZ on microtubules was associated with a decrease in high-affinity binding of BZ to β-tubulin [20,22,23,24,25,26]. The mode of action of BZ thus appeared to involve an interference with microtubule polymerization, and the mechanism of resistance appeared to involve an alteration in this interference.

Comparisons of amino acid sequences of β-tubulins revealed a candidate mutation likely to be responsible for the decrease in binding of BZ. β-tubulin is an important protein and is highly conserved among phylogenetically diverse taxa. In the fungus Aspergillus nidulans, the substitution of phenylalanine with tyrosine at position 200 of a β-tubulin confers resistance to BZ [27]. This same substitution occurs in β-tubulins of BZ-resistant isolates of several other fungi [28] and the free-living nematode

Caenorhabditis elegans [29]. Two genes encoding β-tubulins have been identified from *H. contortus* [30]. The phenylalanine to tyrosine substitution has also been found at position 200 of β-tubulin isotype 1 in BZ-resistant *H. contortus* [31]. Position 200 is near a GTP-binding domain and may therefore interfere with polymerization and stability of microtubules [32]. An alteration in the structure of the target protein, resulting in a reduced anthelmintic binding-affinity, therefore appears to be a mechanism of resistance to BZ in nematodes as well as other organisms.

Genetic analyses in *H. contortus* support a role for β-tubulin in BZ resistance. Selection for a specific allele of isotype 1 has been observed in resistant isolates as compared to sensitive isolates [9,33,34,35,36]. The role of isotype 2, however, is less clear. Beech *et al.* [36] found selection for an allele at the isotype 2 locus in resistant strains, while [34] did not. The latter group, though, found evidence that the isotype 2 locus may be deleted in highly resistant strains. The inheritance of BZ resistance in trichostrongylid nematodes appears to be controlled by more than one gene [37,38]. Whether this finding reflects the roles of multiple β-tubulin isotype loci, or the possible contribution of an unrelated gene, or both, is unknown.

A second mechanism of resistance to BZ may operate in *H. contortus*. The efflux of hydrophobic drugs from the cytoplasm and cell membrane by the membrane transport protein, P-glycoprotein (P-gp)(see Section I.5.3 below), can lead to drug resistance in human cancer cells [39,40]. This process can be impeded by reversing agents. BZ is known to act as a substrate for mammalian P-gp [41]. Beugnet *et al.* [42] have reported a decrease in the inhibition of egg-hatching by BZ when eggs of *H. contortus* are exposed

to the reversing agent, verapamil. This finding implicates the action of P-gp as a possible mechanism of resistance to BZ in *H. contortus*.

I.4 Resistance to Ivermectin (IVM)/Moxidectin (MOX) in *H. contortus*

The modes of action of and mechanisms of resistance to AVM and milbemycins are less well known than for BZ. These anthelmintics, generically called macrocyclic lactones, were developed in the 1970s and 1980s [43,44] in response to the growing problem of BZ resistance. They are derived from the fermentation products of various species of *Streptomyces*. The first of this new class of anthelmintic to become commercially available, in 1981, was IVM [45,46]. Abamectin is a natural fermentation product and is the substrate from which IVM is produced, but was released commercially after IVM [47]. The first milbemycin commercially available was MOX. AVM and milbemycins are structurally closely related. Both share a 16-membered macrocyclic unit, a benzofuran unit and a spiroketal unit. AVM possess a disaccharide substituent at C-13 that milbemycins do not [47].

Like BZ, AVM and milbemycins are broad-spectrum anthelmintics. They control a wide range of gastrointestinal helminths. Unlike BZ, they also possess insecticidal and acaricidal activity, which gave rise to the term "endectocide". In fact, milbemycins were originally developed for use in crop protection because of their activity against arthropods. The various macrocyclic lactones commercially available for controlling pests of livestock have variable activities against different species of pest [47]. Recommended doses, then, are based on the dose necessary to remove the least susceptible species for which efficacy claims are made.

Laboratory selection for IVM resistance in *H. contortus* was demonstrated by Egerton *et al.* [48]. Resistance to IVM in field strains of gastrointestinal nematodes of sheep and goats was first reported from South Africa [49,50] and Brazil [51]. Resistance

has since been reported from many regions around the world, including the United States [52], New Zealand [53,54], the United Kingdom [55], Australia [56], Malaysia [57], Kenya [58], and Denmark [59]. Although IVM is extensively used to control nematode infections in cattle, horses, and swine, resistance has been slower to develop in these animals, but reports of resistance are beginning to appear [60].

The mechanism of action of AVM and milbemycins is unknown. AVM were first observed to cause paralysis of the somatic musculature in *Ascaris* [61], *C. elegans* [61], and *H. contortus* [62]. Subsequent studies found that the pharynx of nematodes was also paralysed [63,64,65]. Attempts at elucidating the mode of action of AVM have therefore focused attention on the effects of AVM on the neuromuscular system in nematodes. Paralysis of the somatic musculature in nematodes is also caused by another anthelmintic, piperazine, which acts as an agonist at gamma-aminobutyric acid (GABA) receptors [66]. GABA receptors are inhibitory chloride channels found in neuromuscular cells of invertebrates and in neurons of the central nervous system of vertebrates. Opening of these channels leads to an influx of chloride ions and hyperpolarization of the cell. Early studies on the mechanism of action of AVM thus examined the potential interaction of AVM at GABA receptors [61, 67]. Subsequent efforts, however, shifted attention to a newly discovered family of chloride channels, the glutamate-gated chloride (GluCl) channel [68,69,70]. These channels are very similar to GABA receptors in structure and function and can cause hyperpolarization of cells when opened. Since GABA receptors and GluCl channels are potential sites of action of AVM and milbemycins, and since mechanisms of resistance to an anthelmintic may be related to the anthelmintic's mechanism of action, these structures and their possible role in IVM/MOX resistance will be discussed in more detail below.

Mechanisms of resistance to an anthelmintic, however, may not necessarily directly involve the drug's mode of action. As suggested in I.2 above, mechanisms that reduce the concentration of an anthelmintic at the site of action or that render an anthelmintic less toxic by chemical modification could also lead to resistance. Evidence that the former may play a role in IVM resistance has been reported by Xu *et al.* [71]. P-gp, which binds and removes hydrophobic cytotoxins from cell membranes and cytoplasm [40], is believed to be responsible for some cases of multidrug resistance in some human cancer cells when overexpressed. In *H. contortus*, genetic changes and increased expression of a P-gp were found in IVM-resistant relative to IVM-sensitive strains [71]. Since P-gp may contribute to IVM/MOX resistance, it will be discussed in more detail below.

To date, two potential sites of action of IVM and MOX have been suggested, GABA receptors and GluCl channels. The interaction of IVM/MOX with either or both of these sites could lead to hyperpolarization of nerve or muscle cells which in turn could cause the expulsion of *H. contortus* from a host. At least two mechanisms of resistance to IVM/MOX involving these sites of action are therefore possible. A third mechanism of action involving P-gp is also possible. The ability of P-gp's to transport chemically unrelated molecules also suggests that P-gp may contribute to resistance mechanisms of other anthelmintics, such as BZ.

Although AVM and milbemycins are structurally related and are considered by many to form a single class of anthelmintic, some controversy surrounds the contention that they have the same mode of action. Evidence for and against this contention comes from studies of the efficacy of MOX in the treatment of nematodes that have developed some level of resistance to IVM. MOX was found to be more effective than IVM against

IVM-resistant worms [72,73], suggesting the possibility of different modes of action of IVM and MOX. In contrast, MOX was found to be less efficient at removing IVM-resistant than IVM-susceptible worms, suggesting co-resistance and a common mode of action [74,75]. The effectiveness of MOX against IVM-resistant nematodes may be due to a lower level of hydrolysis of the drug [72], leading to a longer half-life. MOX also appears to be more potent than IVM [76,77], although Shoop *et al.* [47] caution that relative potencies of anthelmintics vary from species to species. Many independent lines of evidence, however, support the contention that IVM and MOX share a common mode of action (see [47]).

The genetics of AVM/milbemycin resistance has been examined in *H. contortus*. The inheritance of resistance appeared to be controlled by a single, dominant gene or gene complex [12,56]. Limited strains, however, have been examined, and treatment strategies appear to influence the number of genes that may contribute to the development of resistance [78]. The number of potential mechanisms of resistance to AVM/milbemycins in *H. contortus* may thus lead to a polygenic inheritance of resistance.

I.5 Possible Mechanisms of Resistance to IVM/MOX in *H. contortus*

I.5.1 GluCl Channels

Inhibitory hyperpolarization of cells is a common phenomenon in the animal kingdom. In vertebrates, GABA and glycine act as neurotransmitters that activate inhibitory chloride channels. In the 1970s, chloride channels activated by glutamate were discovered in invertebrates [79,80,81]. GluCl channels in insects were found to be sensitive to AVM [82,83]. Genes encoding subunits of GluCl channels were first cloned and characterized in 1994 from *C. elegans* [68]. GluCl channels belong to the family of ligand-gated ion channels that includes GABA, glycine, serotonin 5HT3, and nicotinic acetylcholine receptors. Structurally, all members of this family are very similar, each consisting of five protein molecules of varying subunits [84]. The five proteins form a ringed structure that spans the cell membrane; when in the open state, a central pore allows the passage of ions. Each subunit has a large extracellular domain to which the ligand binds [85,86,87], and four hydrophobic transmembrane domains, the second of which, M2, lines the pore [88,89,90,91]. Gating of the channel occurs when one or more ligand molecules bind to the extracellular domain of one or more subunits, causing a small rotation of this domain, which in turn causes a conformational change in the M2 domains, opening the pore [92]. The channels are postsynaptically localized and anchored in the membrane by binding with an intermediary protein to the cytoskeleton [93,94,95]. This binding appears to involve the cytoplasmic loop between M3 and M4 in one subunit type [96,97]. The clustering of receptors and ion channels at synaptic junctions by interactions with the cytoskeleton may be necessary for proper neuromuscular function

[98]. GluCl channel proteins have the same secondary structure [68], and channels are assumed to possess a similar pentameric architecture [69], as other members of the family. The cloning of GluCl genes has led to *in vitro* studies of the pharmacology and electrophysiology of channels formed by the expression of subunits.

As in insects, GluCl channels in nematodes are sensitive to AVM. Arena *et al.* [99] identified chloride currents activated by AVM in *Xenopus* oocytes injected with RNA from *C. elegans*. A later study proposed that these currents were mediated by GluCl channels [100]. Once the genes encoding channel subunits had been cloned, Cully *et al.* [68] found that homomeric channels formed by the expression of alpha subunits in *Xenopus* oocytes were gated by AVM but were insensitive to glutamate. Homomeric channels composed of only beta subunits were gated by glutamate but were insensitive to AVM. Homomeric channels of GluClβ subunits from *C. elegans* have also been found to be gated by glycine [101]. Heteromeric channels formed by the expression of both subunits were sensitive to both glutamate and AVM [68]. Low concentrations of AVM potentiated gating of heteromeric channels by subthreshold doses of glutamate. This initial study suggested the simple scenario of the GluClα subunit as the AVM receptor and the GluClβ subunit as the glutamate receptor. Subsequent studies suggest a more complex system.

A gene encoding a GluClα subunit isolated from *Drosophila* and expressed as homomeric channels in *Xenopus* oocytes is gated by both glutamate and AVM [102]. AVM does not potentiate gating by subthreshold doses of glutamate. Point mutations at a single amino acid residue in M2 of GluClα from *C. elegans* allows gating by glutamate in homomeric channels [103]. Also, when a chimeric subunit containing the extracellular

domain of GluClα and the transmembrane domains of GluClβ is expressed, the channel is gated by glutamate. These results indicate that GluClα subunits may be capable of binding glutamate, but that ligand-binding and channel gating are normally uncoupled in GluClα. Further evidence that glutamate can bind to α-subunits comes from the properties of a second GluClα subunit isolated from *C. elegans*, designated GluClα2 [69,70]. Homomeric channels of GluClα2 in *Xenopus* oocytes are gated by both glutamate and AVM. The ability of α-subunits to bind glutamate, whether or not this binding is coupled to gating in homomeric channels, may be important for channel kinetics, since more than one molecule of glutamate appears necessary for the gating of GluCl channels [68,69,70,104,105].

Few genes encoding subunits of GluCl channels other than those described above have been identified from nematodes. An alternatively spliced gene from *C. elegans* produces two different subunits, GBR-2A and GBR-2B, with the same ligand-binding site but with different transmembrane domains [106]. It is unclear whether this gene represents another GluClα, a GluClβ, or a new class of GluCl subunit. A fragment of a gene from *C. elegans* designated GluClX [104] is virtually identical in nucleotide sequence to the homologous regions of GBR-2A/GBR-2B described above and is likely the same gene. Similar fragments of GluClX genes have been isolated from *Dirofilaria immitis* and *Onchocerca volvulus* [104]. Full-length cDNA sequences are known for the *H. contortus* homologues of the GluClβ and GBR2A/GBR2B genes (A.J. Wolstenholme, unpublished data). The high degree of identity of GluCl subunits should allow the identification of more genes from other species.

Elucidation of the function of GluCl channels in nematodes will require knowledge of sites of expression. Such knowledge is limited at present. Electrophysiological studies of the pharynx have identified GluCl channels in this organ from *Ascaris* [105] and *C. elegans* [69,107]. Transformation of *C. elegans* with a GluClβ-LacZ fusion construct identified the pharynx as the only site of expression of GluClβ subunits [108]. Transformation of *C. elegans* with GluClα2 fused to green fluorescent protein also identified the pharynx as a major site of expression [69]. This study, however, revealed strong expression of GluClα2 in a few neurons of the head and two ventral cord neurons near the anus, and weaker expression in ventrally located neurons contributing to the dorsal and ventral sublateral nerve cords. The pharynx, then, appears to be the principle site of GluCl channels. The function of GluCl channels in the pharynx is to repolarize and thereby relax contracted, depolarized pharyngeal muscles [69,107].

Pharyngeal pumping is a vital behaviour in nematodes, and its disruption has serious consequences. The ability to feed is impaired, and excretion and regulation of turgor pressure may also be affected [65]. The paralysis of the pharynx by AVM/milbemycins, then, represents a likely mechanism of action of these anthelmintics, and the negation of this paralysis represents a likely mechanism of resistance.

Several studies have investigated the effects of AVM/milbemycins on pharyngeal pumping in nematodes. Inhibition of pharyngeal pumping by AVM was first reported in *Trichostrongylus colubriformis* [63] and *C. elegans* [64]. In *H. contortus*, IVM reduced pharyngeal activity in adults as measured by three different assays at picomolar concentrations [65] and in larvae at nanomolar concentrations [109]. Electrophysiological experiments in *Ascaris suum* identified hyperpolarizing effects on pharyngeal muscles

with the application of milbemycin D [105] and IVM [110]. The release of GABA on pharyngeal preparations has no effect on pharyngeal pumping [105,107] suggesting that the above effects are due to interactions of anthelmintic with GluCl channels.

Larval development assays have been developed to detect the development of resistance to anthelmintics. These assays provide indirect evidence that AVM/milbemycins have an inhibitory effect on pharyngeal pumping, on the assumption that retarded development is due to a reduction in the ingestion of nutrients [111]. Many studies have demonstrated an increased tolerance to AVM in AVM-resistant compared to AVM-susceptible isolates of various species of trichostrongylid nematodes [7,109,111,112,113,114,115,116,117]. If the inhibition of pharyngeal pumping by AVM is the sole reason for retarded development, the prevalence of reduced retardation of development upon exposure to AVM in many resistant isolates from different species indicates that interaction of AVM with GluCl channels in pharyngeal muscle could be an important contributor to the mechanisms of action of and resistance to AVM.

Another indication of the importance of GluCl channels in AVM resistance is the sensitivity of these channels to AVM. IVM concentrations of 100 pM were sufficient to inhibit pharyngeal pumping in adult *H. contortus* [65]. In contrast, IVM concentrations of 10 nM were required to inhibit motility, an increase of 100-fold. This difference may reflect different binding affinities of AVM for GluCl channels and GABA receptors. Schaeffer and Haines [118] found a similar 100-fold higher affinity of AVM binding in membrane preparations of *C. elegans* (GluCl channels) than in membrane preparations of rat brain tissues (GABA receptors). In *H. contortus* larvae, 1 nM IVM inhibits development, and >30 nM IVM inhibits motility [111]. Larvae thus appear to be less

sensitive than adults to IVM, but pharyngeal muscle is still much more sensitive than the somatic neuromuscular system.

The concentrations of IVM capable of inhibiting pharyngeal pumping are consistent with those used to measure binding affinity of IVM to membrane preparations of nematodes. A single high-affinity site of IVM binding was found in *C. elegans* [118,119] and in *H. contortus* [120]. These sites are believed to be GluCl channels. A comparison of binding characteristics in strains of *H. contortus* susceptible and resistant to IVM, however, revealed no differences between strains in either binding affinity or receptor density [120]. The authors concluded that resistance to IVM was not due to alterations in the target site. The target site here, however, can only be defined as the binding site of AVM on the GluClα subunit. Alterations elsewhere in the GluCl channel could affect channel kinetics and contribute to a mechanism of resistance to AVM/milbemycins. Paiement *et al.* [121] have recently reported an increase in glutamate binding sites in the presence of IVM in unselected, but not IVM-selected, strains of H.contortus.

GluCl channels have become a primary focus in the study of AVM/milbemycin resistance. Their presence in nematodes and arthropods, their interaction with and sensitivity to AVM/milbemycins, and their role in the vital behaviour of feeding justifies the need to explore further their potential role in a mechanism of AVM/milbemycin resistance.

I.5.2 GABA Receptors

GABA receptors in the vertebrate central nervous system are molecular targets of many drugs. Anxiety-relieving benzodiazepines (eg. valium) [122], depressant [123] and convulsant [124] barbiturates, and ethanol [125] are some of the more common drugs known to affect the function of GABA receptors. The pharmacology of GABA receptors in vertebrates, however, is extremely complex. Many types of subunit have been identified, with many subtypes of each subunit. Expression of different subunits in different cell types results in a wide array of receptors possessing unique properties.

In insects, at least two types of GABA receptors that act as chloride channels have been identified. One type is found in the brain and ganglia, and the other type is found on muscle [126]. Little is known about GABA receptors in nematodes. GABAergic neurons have been mapped in *C. elegans* [127], but the physiological and pharmacological properties of the receptors have not been elucidated. The role of GABA and its receptors in nematode locomotion, however, has been determined. Nematodes have ventral and dorsal muscles. The sinusoidal swimming movement of nematodes results from the excitation of muscles on one side of the body and the contralateral inhibition of opposing muscles on the other side of the body. This inhibition is GABAergic. If the inhibitory, GABAergic neurons are destroyed, muscles on both sides of the body contract simultaneously, and locomotion is impaired [127]. Considering the key role of GABA inhibition of the somatic musculature in nematodes and the fact that AVM/milbemycins paralyse this musculature, GABA receptors are potential suspect targets of these anthelmintics.

GABA receptors are the major neurotransmitter receptor responsible for neuromuscular inhibition in insects [128] and nematodes [129]. Various compounds are known to bind to and interfere with the functioning of GABA receptors. In insects, cyclodiene insecticides act as antagonists at GABA receptors, causing convulsions by preventing the inhibition of excitatory neuromuscular transmission [130]. Picrotoxinin and phenylpyrazoles are also antagonists of insect GABA receptors and are believed to bind to the same site as the cyclodienes [128,131]. AVM in insects act as an agonist of GABA receptors, causing ataxia and paralysis [130,132,133,134].

In nematodes, piperazine is a GABA receptor agonist on *Ascaris suum* muscle membranes [135]. Treatment of *Ascaris* with piperazine elicits a flaccid paralysis, where the worm increases in length due to the hyperpolarization of the somatic muscles, which eliminates the muscles' resting potential. Muscimol, a known agonist in vertebrates, also activates GABA receptors in *Ascaris* [67,136]. Several experimental azole derivatives [137] and arylaminopyridazines [138] act as GABA-receptor antagonists in *Ascaris*. Antagonists at GABA receptors are expected to produce a shortening of nematodes by interfering with the ability to inhibit excitatory stimuli to somatic muscles [139]. The pharmacological properties of GABA receptors differ markedly between nematodes and vertebrates [140], a property that renders these receptors ideal targets for anthelmintics. Unfortunately, pharmacological properties also vary between different species of nematodes [140], which could have negative implications for broad-spectrum status for an anthelmintic and would limit extrapolation of data collected from one species to other species.

AVM are known to interact with GABA receptors in nematodes. The current state of knowledge of this interaction, however, is quite limited and at times contradictory.

Few receptors have been identified and characterized. The large array of vertebrate GABA receptors having different properties suggests that variation may also exist in nematodes. Kass *et al.* [67] have postulated that *Ascaris* may possess at least two subtypes of receptor, as have McIntyre *et al.* [127] for *C. elegans*. In *Ascaris* somatic muscle preparations, AVM has clearly been demonstrated to act as an antagonist at GABA receptors [61,67,139,141,142], yet AVM does not cause hypercontraction in whole worms [142] as would be expected from the action of an antagonist on somatic muscle receptors. AVM does not cause a flaccid paralysis, either, characterized by a lengthening of worms, as would be expected if it acted as an agonist on somatic muscle receptors. Instead, AVM causes a paralysis that neither shortens nor lengthens worms [61,136]. Either *Ascaris* possesses different subtypes of GABA receptors having different responses to AVM, or another target for AVM is responsible for the paralysis of somatic musculature.

Few studies have attempted to resolve this problem, although some evidence suggests the presence of GABA receptors in *Ascaris* at which AVM acts as an agonist. Kass *et al.* [61,67] prepared sections of *Ascaris* where the ventral nerve cord and motoneurons innervating somatic muscle could be separately stimulated with electrodes. Stimulation of either the nerve cord or an excitatory motoneuron, which is innervated by interneurons in the nerve cord, produced a depolarizing response in muscle. The application of AVM to the ventral nerve cord irreversibly diminished the response from nerve cord stimulation, but not from excitatory motoneuron stimulation, suggesting that the mode of action of AVM involves a block of neural transmission between interneurons and excitatory motoneurons. The inhibitory effect of AVM was reversed by application of picrotoxin, a GABA receptor antagonist. The application of muscimol and piperazine,

known agonists of GABA receptors, to the ventral nerve cord mimicked the effects of AVM and were partially reversed by picrotoxin. Stimulation of inhibitory motoneurons produced a hyperpolarization of somatic muscle. The application of AVM blocked this response at the postsynaptic junction, where AVM is known to be an antagonist of GABA receptors. Picrotoxin had no effect on this response. Since excitation of inhibitory motoneurons is dependent on excitation of excitatory motoneurons, the inhibition of interneuron-excitatory motoneuron transmission described above renders the antagonistic action of AVM on GABA receptors of muscle cells inconsequential. These results are consistent with the presence of a GABA receptor in the ventral nerve cord at which AVM acts as an agonist rather than as an antagonist, and suggest that disruption of this action may be a mechanism of resistance to AVM. Inhibitory interneurons in nematodes, however, have not been identified.

An alternative explanation for the above results is the presence of non-GABA activated chloride channels at which AVM can act as an agonist. Such channels sensitive to AVM have been described from arthropod muscles [83,143,144] but not from neurons. Arena [145] has suggested that all ligand-gated chloride channels may possess a common AVM binding site. In insects and nematodes, AVM is an agonist at GluCl channels, and this effect can be blocked by picrotoxin in nematodes [68,104]. The effects of the GABA receptor agonists piperazine and muscimol on GluCl channel activity in nematodes has not been examined. GluCl channels, however, do not appear to be strongly expressed in neurons, particularly the ventral nerve cord where AVM has its effects on somatic muscle contraction in *Ascaris*. Another alternative explanation to the existence of two subtypes of GABA receptors in *Ascaris* is the possibility that AVM can bind to two different sites on the same receptor with different affinities and producing different actions. AVM does

so at GABA receptors in rat cerebellar neurons [146]. At low concentrations, AVM binds to a high-affinity site where it acts as an agonist, and at high concentrations, it binds to a low-affinity site and acts as an antagonist. Further study is clearly necessary to elucidate the role of GABA receptors in the paralyzing activity of AVM.

Although inhibitory interneurons in the ventral nerve cord of nematodes have not been found, a recent study suggests their presence. A cDNA encoding a subunit of a putative GABA receptor, HG1, has been cloned from *H. contortus* [147]. An immunocytological study found this subunit to be expressed in the ventral nerve cord and nerve ring motoneurons and interneurons of *H. contortus* [148]. This finding strengthens the possibility that different subtypes of GABA receptors with different pharmacological properties exist in nematodes. The proposed homologue of this subunit from *Ascaris*, however, was found to be expressed in the somatic muscles. Considering the variability of pharmacological properties of GABA receptors in vertebrates and from one nematode species to another [140], receptor architecture may also vary from species to species.

Since nematode locomotion depends on the proper functioning of GABA receptors, and AVM interferes with this functioning, the effect of AVM on nematode locomotion provides evidence of the potential importance of GABA receptors in the action of AVM and therefore on the mechanism of resistance. Most studies on the effects of AVM on locomotion in nematodes have involved larval motility assays. Gill *et al.* [149] found that L3 larvae of IVM-resistant isolates of *H. contortus* were less sensitive to IVM exposure than were larvae of IVM-susceptible isolates. *In vitro* inhibition of motility by various AVM analogues was also consistent with their known *in vivo* efficacies. Later studies [111,117] indicated that larval motility was less sensitive to AVM/milbemycin treatment than was larval development. Some IVM-resistant isolates,

however, did not exhibit a higher tolerance to IVM, as measured by larval motility, than their IVM-susceptible counterparts [116]. The authors interpreted this finding as evidence for multiple mechanisms of resistance operating in *H. contortus* which were dependent on the selection protocols. Multiple mechanisms of resistance to anthelmintics have been proposed to be the result of suboptimal dosing [78], a practice common in the laboratory development of anthelmintic-resistant strains of parasites.

Any potential role that GABA receptors may play in AVM/milbemycin resistance, then, may depend not only on the interaction of drug and receptor, but also on the circumstances of drug treatment, which may influence the mechanism whereby parasites are inhibited from reproducing. Paralysis of somatic musculature could cause expulsion of worms from their hosts. Other mechanisms, though, could cause expulsion or other means of inhibiting reproduction.

I.5.3 P-glycoprotein

As stated above in Section I.2, mechanisms of resistance need not necessarily involve the action of an anthelmintic at its target site. A mechanism whereby anthelmintic levels are reduced at the site of action could increase a nematode's tolerance to a drug. This reduction in anthelmintic level might occur through an increase in the metabolic degradation of the anthelmintic [13] or by physically removing the anthelmintic from the target site by a mechanism of drug efflux [7]. The latter possibility has received much attention, primarily in the field of cancer treatment in humans, and involves the action of the membrane protein, P-gp.

The transport of drugs from drug resistant cancer cells has long been recognized as a possible mechanism for the failure of chemotherapy in cancer patients [150]. P-gp was then identified as the molecule responsible for this transport [151]. P-gp's belong to a large class of energy-dependent transport proteins known as ATP-binding cassette (ABC) proteins [152]. They occur in cell membranes and serve to transport various substances within and across the lipid bilayer. In cancer cells, overexpression of P-gp's leads to the phenotype of multidrug resistance (MDR), where selection with one drug can confer resistance to other, structurally unrelated drugs (for review see [39]). In addition to their role in MDR in humans, P-gp's are believed to contribute to resistances to toxic substances in a number of organisms, including nematodes.

Homologues of P-gp have been identified from some protists that cause disease. In the malaria parasite, *Plasmodium falciparum*, early experiments suggested that P-gp was responsible for resistance to antimalarials. A P-gp gene was found to be amplified and P-gp transcript levels elevated in resistant isolates relative to sensitive isolates [153].

In addition to gene amplification of P-gp in resistant isolates of *P. falciparum*, Wilson *et al.* [154] also found cross-resistance to unrelated drugs, a defining property of the MDR phenotype. Later studies, however, raised doubts about P-gp's role in resistance to antimalarials. Genetic crossing experiments found no linkage among the efflux of drugs, the MDR phenotype, and P-gp genes or the amplification of these genes [155]. In another study, a chloroquine-resistant isolate of *P. falciparum* that was subjected to further selection with chloroquine lost the previous amplification of a P-gp gene yet became 10-fold more resistant to chloroquine [156]. Also, no cross-resistance to other drugs was apparent, since the additional selection with chloroquine increased the sensitivity of the isolate to mefloquine. The role of P-gp in drug resistance in the malaria parasite remains controversial.

Evidence for a P-gp-based mechanism of resistance can be found in other protists. In *Leishmania enrietii*, a strain selected for resistance to one drug showed cross-resistance to an unrelated drug and possessed amplified copies of a P-gp homologue, all of which were derived from a single allele [157]. Cross-resistance to unrelated drugs, gene amplification, and increased gene expression have also been reported in a resistant *L. donovani* strain [158]. Wild-type strains transfected with a vector containing the P-gp gene exhibited a nearly identical multidrug resistance phenotype as did the resistant strain. Six P-gp genes have been identified in *Entamoeba histolytica* [159]. Emetine-resistant clones of these parasites show increased P-gp expression [160,161]. More than one P-gp gene may be contributing to drug resistance in this species.

Recent evidence raises the possibility that P-gp's may function in detoxification mechanisms in other organisms as well. Some insecticides have been shown to be substrates for mammalian P-gp's [162,163], and a P-gp homologue may protect the

tobacco hornworm (*Manduca sexta*) from the toxic effects of nicotine [164]. Three homologues have been identified from *Drosophila* [165,166], one of which has been implicated in conferring resistance to colchicine [165]. Plants, also, may possess P-gp homologues [167]. P-gp's share a certain degree of homology with bacterial membrane transport proteins [168], and if they share a common evolutionary origin with these proteins, P-gp's may be ubiquitous in eukaryotes.

P-gp's have been identified from nematodes. *C. elegans* possesses at least four P-gp genes [169] and possibly as many as 14 [170]. Deletion of one of these genes has been linked to increased sensitivity to chloroquine and colchicine [171]. Deletion of another P-gp gene, together with the deletion of a multidrug resistance-associated protein (MRP) gene, results in hypersensitivity to heavy metals [172]. A fragment of a P-gp homologue has been isolated from *O. volvulus* [170].

H. contortus is believed to possess at least four P-gp genes [173]. One full-length cDNA has been cloned [71], and a fragment of another gene has been isolated from genomic DNA [170]. No polymorphisms associated with resistance to BZ, IVM, levamisole, or closantel were found in the P-gp genomic fragment [170]. The other gene, designated PGP-A, however, has been linked to resistance to IVM [71].

IVM was first suspected to act as a substrate for P-gp when mice in which a P-gp gene had been disrupted were found to be sensitive to IVM [174]. Interaction of IVM with mammalian P-gp has since been confirmed [175,176]. IVM, however, was found not to bind to two P-gp's in *C. elegans* [171]. Evidence that IVM could interact with nematode P-gp, and that this interaction was a potential mechanism of resistance to IVM, came when Xu *et al.* [71] found a genetic polymorphism at a P-gp locus in genomic DNA of an IVM-resistant strain of *H. contortus*. This study also reported evidence of

overexpression of P-gp in IVM-resistant strains relative to susceptible strains and an increase in efficacy of IVM and MOX in a MOX-selected strain when coadministered with verapamil, an MDR reversing agent. The application of verapamil, however, did not affect pharyngeal pumping in larvae of IVM-resistant strains of *H. contortus* relative to sensitive strains [109]. This finding may reflect developmental or anatomical differences in P-gp expression. A role for P-gp as an efflux pump of anthelmintics thus appears to be a contributing factor in AVM/milbemycin resistance in *H. contortus*.

I.6 Summary

The development of anthelmintic resistance in nematodes is an evolutionary process whereby resistant individuals survive anthelmintic treatment and contribute their genetic material to future generations. Genetically diverse species are thus more likely to develop resistance than are invariant species. *H. contortus* is a genetically variable species and has developed resistance to the major classes of anthelmintic on the market. Different mechanisms of resistance to anthelmintics are possible. Structural alterations in a target protein, changes in metabolic processes, or mechanisms that reduce a drug's concentration at the target site can all potentially reduce a drug's effectiveness. Resistance to BZ appears to involve structural changes in β-tubulins but may also be due in part to the efflux of BZ by P-gp. Paralysis of the somatic musculature of *H. contortus* and other nematodes implicates GABA receptors as a site of action of AVM/milbemycins. Paralysis of the pharynx implicates GluCl channels as another site of action. Changes in these sites may represent mechanisms of resistance to these anthelmintics. Drug efflux by P-gp also appears to be a potential mechanism of resistance to AVM/milbemycins in *H. contortus*.

References

[1] Lichtenfels, J.R., Pilitt, P.A., Hoberg, E.P. New morphological characters for identifying individual specimens of *Haemonchus* spp. (Nematoda: Trichostrongyloidea) and a key to species in ruminants of North America. J Parasitol 1994;80:107-19.

[2] Gibbons, L.M. Revision of the genus *Haemonchus* Cobb, 1898 (Nematoda: Trichostrongylidae). Syst Parasitol 1979;1:3-24.

[3] Griffiths, H.J. A handbook of veterinary parasitology: domestic animals of North America. Minneapolis: University of Minnesota Press, 1978.

[4] Gibbs, H.C. Effects of parasites on animal and meat production. In: Gaafar, S.M.,Howard, W.E., Marsh, R.E., editors. Parasites, pests, and predators. New York: Elsevier, 1985;pp. 7-27.

[5] Read, C.P. Animal parasitism. Englewood Cliffs, NJ: Prentice-Hall, Inc., 1972; pp.112-5.

[6] McLeod, R.S. Costs of major parasites to the Australian livestock industries. Int J Parasitol 1995;25:1363-7.

[7] Sangster, N. Pharmacology of anthelmintic resistance. Parasitol 1996;113:S201-16.

[8] Prichard, R.K., Hall, C.A., Kelly, J.D., Martin, I.C.A., Donald, A.D. The problem of anthelmintic resistance in nematodes. Aust Vet J 1980;56:239-51.

[9] Roos, M.H., Boersema, J.H., Borgsteede, F.H.M., Cornelissen, J., Taylor, M., Ruitenberg, E.J. Molecular analysis of selection for benzimidazole resistance in the sheep parasite *Haemonchus contortus*. Mol Biochem Parasitol 1990;43:77-88.

[10] Anderson, T.J.C., Blouin, M.S., Beech, R.N. Population biology of parasitic nematodes: applications of genetic markers. Adv Parasitol 1998;41:219-83.

[11] Barnes, E.H., Dobson, R.J., Barger, I.A. Worm control and anthelmintic resistance: adventures with a model. Parasitol Today 1995;11:56-63.

[12] Dobson, R., Le Jambre, L., Gill, J. Management of anthelmintic resistance - inheritance of resistance and selection with persistent drugs. Int J Parasitol 1996;26:993-1000.

[13] Coles, G.C. The molecular biology of drug resistance. In: Bennet, E., Behm, C., Bryant, C., editors. Comparative biochemistry of parasitic helminths. London: Chapman and Hall, 1989;pp. 125-44.

[14] Conway, D.P. Variance in the effectiveness of thiabendazole against *Haemonchus contortus* in sheep. Am J Vet Res 1964;25:106-7.

[15] Drudge, J.H., Wyant, Z.N., Elam, G. Field studies on parasite control in sheep: comparison of thiabendazole, ruelene, and phenothiazine. Am J Vet Res 1964;25:1512-8.

[16] Prichard, R.K. Anthelmintic resistance in nematodes: extent, recent understanding and future directions for control and research. Int J Parasitol 1990;20:515-23.

[17] Borgers, M., de Nolin, S., de Brabander, M., Thienpont, D. Influence of the anthelmintic mebendazole on microtubules and intracellular organelle movement in nematode intestinal cells. Am J Vet Res 1975;36:1153-66.

[18] Davidse, L.C., Flach, W. Differential binding of methyl benzimidazole-2-yl-carbamate to fungal tubulin as a mechanism of resistance to this antimitotic agent in mutant strains of *Aspergillus nidulans*. J Cell Biol 1977;72:174-89.

[19] Hammerschlag, R.S., Sisler, H.D. Benomyl and methyl-2-benzimidazolecarbamate (MBC): biochemical, cytological and chemical aspects of toxicity in *Ustilago maydis* and *Saccharomyces cervisiae*. Pestic Biochem Physiol 1973;3:42-54.

[20] Lubega, G.W., Prichard, R.K. Specific interaction of benzimidazole anthelmintics with tubulin: high-affinity binding and benzimidazole resistance in *Haemonchus contortus*. Mol Biochem Parasitol 1990;38:221-32.

[21] Sangster, N.C., Prichard, R.K., Lacey, E. Tubulin and benzimidazole-resistance in *Trichostrongylus colubriformis* (Nematoda). J Parasitol 1985;7:645-51.

[22] Lacey, E., Prichard, R.K. Interactions of benzimidazoles (BZ) with tubulin from BZ-sensitive and BZ-resistant isolates of *Haemonchus contortus*. Mol Biochem Parasitol 1986;19:171-81.

[23] Lacey, E. The role of the cytoskeletal protein, tubulin, in the mode of action and mechanism of drug resistance to benzimidazoles. Int J Parasitol 1988;18:885-936.

[24] Lubega, G.W., Prichard, R.K. Beta-tubulin and benzimidazole resistance in the sheep nematode *Haemonchus contortus*. Mol Biochem Parasitol 1991;47:129-37.

[25] Lubega, G.W., Prichard, R.K. Interaction of benzimidazole anthelmintics with *Haemonchus contortus* tubulin: binding affinity and anthelmintic efficacy. Exp Parasitol 1991;73:203-13.

[26] Lubega, G.W., Prichard, R.K. Specific interaction of benzimidazole anthelmintics with tubulin from developing stages of thiabendazole-susceptible and -resistant *Haemonchus contortus*. Biochem Pharmacol 1991;41:93-101.

[27] Jung, M.K., Wilder, I.B., Oakley, B.R. Amino acid alterations in the benA (β-tubulin) gene of *Aspergillus nidulans* that confer benomyl resistance. Cell Motil Cytoskeleton 1992;22:170-4.

[28] Koenraadt, H., Sommerville, S.C., Jones, A.L. Characterization of mutations in the beta-tubulin gene of benomyl-resistant field strains of *Venturia inaequalis* and other pathogenic fungi. Mol Plant Pathol 1992;82:1348-54.

[29] Driscoll, M., Dean, E., Reilly, E., Bergholz, E., Chalfie, M. Genetic and molecular analysis of *Caenorhabditis elegans* β-tubulin that conveys benzimidazole sensitivity. J Cell Biol 1989;109:2993-3003.

[30] Geary, T.G., Nulf, S.G., Favreau, M.A., Tang, L., Prichard, R.K., Hatzenbuhler, N.T., Shea, M.H., Alexander, S.J., Klein, R.D. Three β-tubulin cDNAs from the parasitic nematode *Haemonchus contortus*. Mol Biochem Parasitol 1992;50:295-306.

[31] Kwa, M.S.G., Veenstra, J.G., Roos, M.H. Benzimidazole resistance in *Haemonchus contortus* is correlated with a conserved mutation at amino acid 200 in β-tubulin isotype 1. Mol Biochem Parasitol 1994;63:299-303.

[32] Farr, G.W., Sternlicht, H. Site-directed mutagenesis of the GTP-binding domain of β-tubulin. J Mol Biol 1992;227:307-21.

[33] Roos, M.H. The molecular nature of benzimidazole resistance in helminths. Parasit Today 1990;6:125-7.

[34] Kwa, M.S.G., Kooyman, F.N.J., Boersema, J.H., Roos, M.H. Effect of selection for benzimidazole resistance in *Haemonchus contortus* on β-tubulin isotype 1 and isotype 2 genes. Biochem Biophys Res Commun 1993;191:413-9.

[35] Kwa, M.S.G., Veenstra, J.G., Roos, M.H. Molecular characterisation of β-tubulin genes present in benzimidazole resistant populations of *Haemonchus contortus*. Mol Biochem Parasitol 1993;60:133-44.

[36] Beech, R.N., Prichard, R.K., Scott, M.E. Genetic variability of the β-tubulin genes in benzimidazole-susceptible and -resistant strains of *Haemonchus contortus*. Genetics 1994;138:103-10.

[37] Le Jambre, L.F., Royal, W.M., Martin, P.J. The inheritance of thiabendazole resistance in *Haemonchus contortus*. Parasitol 1979;78:107-19.

[38] Martin, P.J., McKenzie, J.A., Stone, R.A. The inheritance of thiabendazole resistance in *Trichostrongylus colubriformis*. Int J Parasitol 1988;18:703-9.

[39] Gottesman, M.M., Pastan, I. Biochemistry of multidrug resistance mediated by the multidrug tansporter. Ann Rev Biochem 1993;62:385-427.

[40] Gottesman, M.M., Hrycyna, C.A., Schoenlein, P.V., Germann, U.A., Pastan, I. Genetic analysis of the multidrug transporter. Ann Rev Gen 1995;29:607-49.

[41] Nare, B., Liu, Z., Prichard, R.K., Georges, E. Benzimidazoles, potent anti-mitotic drugs: substrates for the P-glycoprotein transporter in multidrug-resistant cells. Biochem Pharmacol 1994;48:2215-22.

[42] Beugnet, F., Gauthey, M., Kerboeuf, D. Partial *in vitro* reversal of benzimidazole resistance by the free-living stages of *Haemonchus contortus* with verapamil. Vet Rec 1997; 141:575-6.

[43] Burg, R.W., Miller, B.M., Baker, E.E., Birnbaum, J., Currie, J.A., Harman, R., Kong, V.L., Monaghan, R.L., Olson, G., Putter, I., Tunac, J.D., Wallick, H., Stapley, E.O., Oiwa, R., Omura, S. Avermectins, a new family of potent anthelmintic agents: producing organism and fermentation. Antimicrob Agents Chemother 1979;15:361-7.

[44] Takiguchi, Y., Mishima, H., Okuda, M., Terao, M. Milbemycins, a new family of macrolide antibiotics: fermentation, isolation and physico-chemical properties. J Antibiot 1980; 33:1120-7.

[45] Chabala, J.C., Mrozik, H., Tolman, R.L., Eskola, P., Lusi, A., Peterson, L.H., Woods, M.F., Fisher, M.H. Ivermectin, a new broad-spectrum antiparasitic agent. J Med Chem 1980;23:1134-6.

[46] Campbell, W.C., Fisher, M.H., Stapley, E.O., Albers-Schonberg, G., Jacob, T.A. Ivermectin: a potent new antiparasitic agent. Science 1983;221:823-8.

[47] Shoop, W.L., Mrozik, H., Fisher, M.H. Structure and activity of avermectins and milbemycins in animal health. Vet Parasitol 1995;59:139-56.

[48] Egerton, J.R., Suhayda, D., Eary, C.H. Laboratory selection of *Haemonchus contortus* for resistance to ivermectin. J Parasitol 1988;74:614-7.

[49] Carmichael, I., Visser, R., Schneider, D., Soll, M. *Haemonchus contortus* resistance to ivermectin. J S Afr Vet Assoc 1987;58:93.

[50] Van Wyk, J.A., Malan, F.S. Resistance of field strains of *Haemonchus contortus* to ivermectin, closantel, rafoxanide, and the benzimidazoles in South Africa. Vet Rec 1988;123:226-8.

[51] Echevarria, F.A.M., Trindade, G.N.P. Anthelmintic resistance by *Haemonchus contortus* to ivermectin in Brazil: a preliminary report. Vet Rec 1989;124:147-8.

[52] Craig, T.M., Miller, D.K. Resistance by *Haemonchus contortus* to ivermectin in angora goats. Vet Rec 1990;126:580.

[53] Badger, S.B., McKenna, P.B. Resistance to ivermectin in a field strain of *Ostertagia* spp. in goats. New Zeal Vet J 1990;38:72-4.

[54] Watson, T.G., Hosking, B.C. Evidence for multiple anthelmintic resistance in two nematode parasite genera on a Saanen goat dairy. New Zeal Vet J 1990;38:50-3.

[55] Jackson, F. Coop, R.L., Jackson, E., Scott, E.W., Russel, A.J.F. Multiple anthelmintic resistant nematodes in goats. Vet Rec 1992;130:210.

[56] Le Jambre, L.F. Ivermectin-resistant *Haemonchus contortus* in Australia. Aust Vet J 1993;70:357.

[57] Pandey, V.S., Sivaraj, S. Anthelmintic resistance in *Haemonchus contortus* from sheep in Malaysia. Vet Parasitol 1994; 53:67-74.

[58] Mwamachi, D.M., Audho, J.O., Thorpe, W., Baker, R.L. Evidence for multiple anthelmintic resistance in sheep and goats reared under the same management in coastal Kenya. Vet Parasitol 1995;60:303-13.

[59] Maingi, N., Bjorn, H., Thamsborg, S.M., Bogh, H.O, Nansen, P. A survey of anthelmintic resistance in nematode parasites of goats in Denmark. Vet Parasitol 1996;66:53-66.

[60] Vermunt, J.J., West, D.M., Pomroy, W.E. Multiple resistance to ivermectin and oxfendazole in *Cooperia* species of cattle in New Zealand. Vet Rec 1995;137:43-5.

[61] Kass, I.S., Wang, C.C., Walrond, J.P., Stretton, A.O.W. Avermectin B_{1a}, a paralyzing anthelmintic that affects interneurons and inhibitory motoneurons in *Ascaris*. Proc Natl Acad Sci USA 1980;77:6211-5.

[62] Boisvenue, R.J., Brandt, M.C., Galloway, R.B., Hendrix, J.C. *In vitro* activity of various anthelmintic compounds against *Haemonchus contortus* larvae. Vet Parasitol 1983;13:341-7.

[63] Bottjer, K.P., Bone, L.W. *Trichostrongylus colubriformis*: effect of anthelmintics on ingestion and oviposition. Int J Parasitol 1985;15:501-3.

[64] Avery, L., Horvitz, H.R. The effects of starvation and neuroactive drugs on feeding in *Caenorhabditis elegans*. J Exp Zool 1990;253:263-270.

[65] Geary, T.G., Sims, S.M., Thomas, E.M., Vanover, L., Davis, J.P., Winterrowd, C.A., Klein, R.D., Ho, N.F.H., Thompson, D.P. *Haemonchus contortus*: ivermectin-induced paralysis of the pharynx. Exp Parasitol 1993;77:88-96.

[66] Martin, R.J. γ-aminobutyric acid- and piperazine-activated single channel currents from *Ascaris suum* body muscle. Brit J Pharmacol 1985;84:445-61.

[67] Kass, I.S., Stretton, A.O.,and Wang, C.C. The effects of avermectin and drugs related to acetylcholine and 4-aminobutyric acid on neurotransmission in *Ascaris suum*. Mol Biochem Parasitol 1984;13:213-25.

[68] Cully, D.F., Vassilatis, D.K., Liu, K.K., Paress, P.S., Van der Ploeg, L.H.T., Schaeffer, J.M., Arena, J.P. Cloning of an avermectin-sensitive glutamate-gated chloride channel from *Caenorhabditis elegans*. Nature 1994;371:707-11.

[69] Dent, J.A., Davis, M.W., Avery, L. *avr-15* encodes a chloride channel subunit that mediates inhibitory glutamatergic neurotransmission and ivermectin sensitivity in *Caenorhabditis elegans*. EMBO J 1997;16:5867-79.

[70] Vassilatis, D.K., Arena, J.P., Plasterk, R.H.A., Wilkinson, H.A., Schaeffer, J.M., Cully, D.F., Van der Ploeg, L.H.T. Genetic and biochemical evidence for a novel avermectin-sensitive chloride channel in *Caenorhabditis elegans*. J Biol Chem 1997;272:33167-74.

[71] Xu, M., Molento, M., Blackhall, W., Ribeiro, P., Beech, R., Prichard,R. Ivermectin resistance in nematodes may be caused by alteration of P-glycoprotein homolog. Mol Biochem Parasitol 1998;91:327-35.

[72] Craig, T.M., Hatfield, T.A., Pankavich, J.A., Wang, G.T. Efficacy of moxidectin against an ivermectin-resistant strain of *Haemonchus contortus* in sheep. Vet Parasitol 1992;41:329-33.

[73] Pankavich, J.A., Berger, H., Simkins, K.L. Efficacy of moxidectin, nemadectin, and ivermectin against an ivermectin-resistant strain of *Haemonchus contortus* in sheep. Vet Rec 1992;130:241-3.

[74] Conder, G.A., Thompson, D.P., Johnson, S.S. Demonstration of co-resistance of *Haemonchus contortus* to ivermectin and moxidectin. Vet Rec 1993;132:651-2.

[75] Shoop, W.L., Haines, H.W., Michael, B.F., Eary, C.H. Mutual resistance to avermectins and milbemycins: oral activity of ivermectin and moxidectin against ivermectin-resistant and susceptible nematodes. Vet Rec 1993;133:445-7.

[76] Kieran, P.J. Moxidectin against ivermectin-resistant nematodes - a global view. Aust Vet J 1994;71:18-20.

[77] Wang, G.T., Berger, H., Simkins, K., Rock, D. The rate of resistance development by *H. contortus* to endectocides. In: Proceedings of the XV International Conference of the World Association for the Advancement of Veterinary Parasitology, Yokohama, Japan, 1995;p. 104.

[78] McKenzie, J.A. The genetics of resistance to chemotherapeutic agents. In: Anderson, N., Waller, P.J., editors. Resistance in nematodes to anthelmintic drugs. Melbourne: Australian Wool Corporation Technical Publication.1985;pp. 89-95.

[79] Cull-Candy, S.G., Usherwood, P.N.R. Two populations of L-glutamate receptors on locust muscle fibres. Nature, New Biology 1973;246:62-4.

[80] Lea, T.J., Usherwood, P.N.R. The site of action of ibotenic acid and the identification of two populations of glutamate receptors on insect muscle-fibres. Comp Gen Pharmacol 1973;4:333-50.

[81] Lea, T.J., Usherwood, P.N.R. Effect of ibotenic acid on chloride permeability of insect muscle fibres. Comp Gen Pharmacol 1973;4:351-63.

[82] Duce, I.R., Scott, R.H. Interactions of dihydroavermectin B_{1a}, GABA and ibotenic acid on locust (*Schistocerca gregaria*) muscle. Brit J Pharmacol 1985;86:431.

[83] Scott, R.H., Duce, I.R. Effects of 22,23-dihydroavermectin B_{1a} on locust (*Schistocerca gregaria*) muscles may involve several sites of action. Pestic Sci 1985;16:599-604.

[84] Unwin, N. The structure of ion channels in membranes of excitable cells. Neuron 1989;3:665-76.

[85] Devillers-Thiery, A., Galzi, J.L., Eisele, J.L., Bertrand, S., Bertrand, D., Changeux, J.P. Functional architecture of the nicotinic acetylcholine receptor: a prototype of ligand-gated ion channels. J Memb Biol 1993;36:97-112.

[86] Karlin, A., Akabas, M.H. Toward a structural basis for the function of nicotinic acetylcholine receptors and their cousins. Neuron 1995;15:1231-44.

[87] Machold, J., Utkin, Y., Kirsch, D., Kaufman, R., Tsetlin, V., Hucho, F. Photolabeling reveals the proximity of the a-neurotoxin binding site to the M2 helix of the ion channel in the nicotinic acetylcholine receptor. Proc Natl Acad Sci USA 1995;92:7282-6.

[88] Hucho, F., Oberthur, W., Lottspeich, F. The ion channel of the nicotinic acetylcholine receptor is formed by the homologous helices M II of the receptor subunits. FEBS Lett 1986;205:137-42.

[89] Imoto, K., Methfessel, C., Sakmann, B., Mishina, M., Mori, Y., Konno, T., Fukuda, K., Kurasaki, M., Bujo, H., Fujita, Y., Numa, S. Location of a delta-subunit region determining ion transport through the acetylcholine receptor. Nature 1986;324:670-4.

[90] Leonard, R.J., Labarca, C.G., Charnet, P., Davidson, N., Lester, H.A. Evidence that the M2 membrane-spanning region lines the ion channel pore of the nicotinic receptor. Science 1988;242:1578-81.

[91] Unwin, N. Nicotinic acetylcholine receptor at 9Å resolution. J Mol Biol 1993;229:1101-24.

[92] Unwin, N. Acetylcholine receptor channel imaged in the open state. Nature 1995;373:37-43.

[93] Kirsch, J., Langosch, D., Prior, P., Littauer, U.Z., Schmitt, B., Betz, H. The 93-kDa glycine receptor-associated protein binds to tubulin. J Biol Chem 1991;266:22242-5.

[94] Kirsch, J., Wolters, I., Triller, A., Betz, H. Gephyrin antisense oligonucleotides prevent glycine receptors clustering in spinal neurons. Nature 1993;366:745-8.

[95] Kirsch, J., Betz, H. The postsynaptic localization of the glycine receptor-associated protein gephyrin is regulated by the cytoskeleton. J Neurosci 1995;15:4148-56.

[96] Kirsch, J., Kuhse, J., Betz, H. Targeting of glycine receptor subunits to gephyrin-rich domains in transfected human embryonic kidney cells. Mol Cell Neurosci 1995;6:450-61.

[97] Meyer, G., Kirsch, J., Betz, H., Langosch, D. Identification of a gephyrin binding motif on the glycine receptor beta subunit. Neuron 1995;15:563-72.

[98] Gautam, M., Noakes, P.G., Mudd, J., Nichol, M., Chu, G.C., Sanes, J.R., Merlie, J.P. Failure of postsynaptic specialization to develop at neuromuscular junctions of rapsyn-defeicient mice. Nature 1995;377:232-6.

[99] Arena, J.P., Liu, K.K., Paress, P.S., Cully, D.F. Avermectin-sensitive chloride currents induced by *Caenorhabditis elegans* RNA in *Xenopus* oocytes. Mol Pharmacol 1991;40:368-74.

[100] Arena, J.P., Liu, K.K., Paress, P.S., Schaeffer, J.M., Cully, D.F. Expression of a glutamate-activated chloride current in *Xenopus* oocytes injected with *Caenorhabditis elegans* RNA: evidence for modulation by avermectin. Mol Brain Res 1992;5:339-48.

[101] Laughton, D.L., Wheeler, S.V., Lunt, G.G., Wolstenholme, A.J. The β-subunit of *Caenorhabditis elegans* avermectin receptor reponds to glycine and is encoded by chromosome 1. J Neurochem 1995;64:2354-7.

[102] Cully, D.F., Paress, P.S., Liu, K.K., Schaeffer, J.M., Arena, J.P. Identification of a *Drosophila melanogaster* glutamate-gated chloride channel sensitive to the antiparasitic agent avermectin. J Biol Chem 1996;271:20187-91.

[103] Etter, A., Cully, D.F., Schaeffer, J.M., Liu, K.K., Arena, J.P. An amino acid substitution in the pore region of a glutamate-gated chloride channel enables the coupling of ligand binding to channel gating. J Biol Chem 1996;271:16035-9.

[104] Cully, D.F., Wilkinson, H., Vassilatis, D.K., Etter, A., Arena, J.P. Molecular biology and electrophysiology of glutamate-gated chloride channels of invertebrates. Parasitol 1996;113:S191-200.

[105] Martin, R.J. An electrophysiological preparation of *Ascaris suum* pharyngeal muscle reveals a glutamate-gated chloride channel sensitive to the avermectin analogue, milbemycin D. Parasitol 1996;112:247-52.

[106] Laughton, D.L., Lunt, G.G., Wolstenholme, A.J. Alternative splicing of a *Caenorhabditis elegans* gene produces two novel inhibitory amino acid receptor subunits with identical ligand binding domains but different ion channels. Gene 1997;201:119-25.

[107] Li, H., Avery, L., Denk, W., Hess, G.P. Identification of chemical synapses in the pharynx of *Caenorhabditis elegans*. Proc Natl Acad Sci USA 1997;94:5912-6.

[108] Laughton, D.L., Lunt, G.G., Wolstenholme, A.J. Reporter gene constructs suggest that the *Caenorhabditis elegans* avermectin receptor β-subunit is expressed solely in the pharynx. J Exp Biol 1997;200:1509-14.

[109] Kotze, A.C. Effects of macrocyclic lactones on ingestion in susceptible and resistant *Haemonchus contortus* larvae. J Parasitol 1998;84:631-5.

[110] Brownlee, D.J.A., Holden-Dye, L., Walker, R.J. Actions of the anthelmintic ivermectin on the pharyngeal muscle of the parasitic nematode, *Ascaris suum*. Parasitol 1997;115:553-61.

[111] Gill, J.H., Redwin, J.M., Van Wyk, J.A., Lacey, E. Avermectin inhibition of larval development in *Haemonchus contortus* - effects of ivermectin resistance. Int J Parasitol 1995;25:463-70.

[112] Giordano, D.J., Tritschler II, J.P., Coles, G.C. Selection of ivermectin-resistant *Trichostrongylus colubriformis* in lambs. Vet Parasitol 1988;30:139-48.

[113] Lacey, E., Redwin, J.M., Gill, J.H., Demargheriti, V.M., Waller, P.J. A larval development assay for the simultaneous detection of broad spectrum anthelmintic resistance. In: Boray, J.C., Martin, P.J., Roush, R.T., editors. Resistance of parasites to antiparasitic drugs.Rahway, NJ: MSD AGVET, 1990;pp. 177-184.

[114] Taylor, M.A. A larval development test for the detection of anthelmintic resistance in nematodes of sheep. Res Vet Sci 1990;49:198-202.

[115] Hubert, J., Kerboeuf, D. A microlarval development assay for the detection of anthelmintic resistance in sheep nematodes. Vet Rec 1992;130:442-6.

[116] Gill, J.H., Kerr, C.A., Shoop, W.L., Lacey, E. Evidence of multiple mechanisms of avermectin resistance in *Haemonchus contortus* - comparison of selection protocols. Int J Parasitol 1998;28:783-9.

[117] Gill, J.H., Lacey, E. Avermectin/milbemycin resistance in trichostrongyloid nematodes. Int J Parasitol 1998;28:863-77.

[118] Schaeffer, J.M., Haines, H.W. 1989. Avermectin binding in *Caenorhabditis elegans*: a two-state model for the avermectin binding site. Biochem Pharmacol 1989;38:2329-38.

[119] Rohrer, S.P., Meinke, P.T., Hayes, E.C., Mrozik, H., Schaeffer, J.M. Photoaffinity labeling of avermectin binding sites from *Caenorhabditis elegans* and *Drosophila melanogaster*. Proc Natl Acad Sci USA 1992;89:4168-72.

[120] Rohrer, S.P., Birzin, E.T., Eary, C.H., Schaeffer, J.M., Shoop, W.L. Ivermectin binding sites in sensitive and resistant *Haemonchus contortus*. J Parasitol 1994;80:493-7.

[121] Paiement, J.P., Prichard, R.K., Ribeiro, P. *Haemonchus contortus*: characterization of a glutamate binding site in ivermectin-selected and unselected larvae and adults. Exp Parasitol 1999;(In press).

[122] Study, R.E., Barker, J.L. Diazepam and (-)-pentobarbitol: fluctuation analysis reveals different mechanisms for potentiation of γ-aminobutyric acid responses in cultured central neurons. Proc Natl Acad Sci USA 1981;78:7180-4.

[123] Trifiletti, R.R., Snowman, A.M., Snyder, S.H. Barbituate recognition site on the GABA/benzodiazepine receptor complex is distinct from the picrotoxinin/TBPS recognition site. Eur J Pharmacol 1985;106:441-7.

[124] Maksay, G., Ticku, M.K. Dissociation of [^{35}S]t-butylbicyclophosphorothionate binding differentiates convulsant and depressant drugs that modulate GABAergic transmission. J Neurochem 1985;44:480-6.

[125] Ticku, M.K., Lowrimore, P., Lehoullier, P. Ethanol enhances GABA-induced ^{36}Cl-influx in primary spinal cord cultured neurons. Brain Res Bull 1986;17:123-6.

[126] Eldefrawi, A.T., Eldefrawi, M.E. Receptors for γ-aminobutyric acid and voltage-dependent chloride channels as targets for drugs and toxicants. FASEB J 1987;1:262-71.

[127] McIntyre, S.L., Jorgensen, E., Kaplan, J., Horvitz, R. The GABAergic nervous system of *Caenorhabditis elegans*. Nature 1993;364:337-41.

[128] Matsumura, F., Ghiasuddin, S.M. Evidence for similarities between cyclodiene type insecticides and picrotoxinin in their action mechanisms. J Environ Sci Health 1983;B18:1-14.

[129] Johnson, C.D., Stretton, A.O.W. GABA-immunoreactivity in inhibitory motor neurons of the nematode *Ascaris*. J Neurosci 1987;7:223-35.

[130] Bloomquist, J.R. Toxicology, mode of action and target site-mediated resistance to insecticides acting on chloride channels. Comp Biochem Physiol C 1993;106:301-14.

[131] Bloomquist, J.R. Cyclodiene resistance at the insect GABA receptor/chloride channel complex confers broad cross resistance to convulsants and experimental phenylpyrazole insecticides. Arch Insect Biochem Physiol 1994;26:69-79.

[132] Duce, I.R., Scott, R.H. Actions of dihydroavermectin B_{1a} on insect muscle. Brit J Pharmacol 1985;85:395-401.

[133] Deng, Y., Casida, J.E. House fly head GABA-gated chloride channel: toxicologically relevant binding site for avermectins coupled to site for ethynylbicycloorthobenzoate. Pestic Biochem Physiol 1992;43:116-22.

[134] Clark, J.M., Campos, F., Scott, J.G., Bloomquist, J.R. Resistance to avermectins: extent, mechanisms, and management implications. Ann Rev Entomol 1995;40:1-30.

[135] Martin, R.J. Neuromuscular transmission in nematode parasites and antinematodal drug action. Pharmacol Ther 1993;58:13-50.

[136] Kass, I.S., Larsen, D.A., Wang, C.C., Stretton, A.O.W. *Ascaris suum*: differential effects of avermectin B_{1a} on the intact animal and neuromuscular strip preparations. Exp Parasitol 1982;54:166-74.

[137] Bascal, Z., Holden-Dye, L.,Willis, R.J., Smith, S.W.G., Walker, R.J. Novel azole derivatives are antagonists at the inhibitory GABA receptor on the somatic muscle cells of the parasitic nematode *Ascaris suum*. Parasitol 1996;112:253-9.

[138] Martin, R.J., Sitamze, J.M., Duittoz, A.H., Wermuth, C.G. Novel arylaminopyridazine-GABA receptor antagonists examined electrophysiologically in *Ascaris suum*. Eur J Pharmacol 1995;276:9-19.

[139] Martin, R.J., Pennington, A. A patch clamp study of effects of dihydroavermectin on *Ascaris* muscle. Brit J Pharmacol 1989;98:747-56.

[140] Thompson, D.P., Klein, R.D., Geary, T.G. Prospects for rational approaches to anthelmintic discovery. Parasitol 1996;113:S217-38.

[141] Holden-Dye, L., Hewitt, G.M., Wann, K.T., Krogsgaard-Larsen, P., Walker, R.J. Studies involving avermectin and the 4-aminobutyric acid (GABA) receptor of *Ascaris suum* muscle. Pestic Sci 1988;24:231-5.

[142] Holden-Dye, Walker, R.J. Avermectin and avermectin derivatives are antagonists at the 4-aminobutyric acid (GABA) receptor on the somatic muscle cells of *Ascaris*; is this the site of anthelmintic action? Parasitol 1990;101:265-271.

[143] Tanaka, K., Matsumura, F. Action of avermectin B_{1a} on the leg muscles and the nervous system of the American cockroach. Pestic Biochem Physiol 1985;24:124-35.

[144] Zufall, F., Franke, C.H., Hatt, H. The insecticide avermectin B_{1a} activates a chloride channel in crayfish muscle membrane. J Exp Biol 1989;142:191-205.

[145] Arena, J.P. Expression of *Caenorhabditis elegans* mRNA in *Xenopus* oocytes: a model system to study the mechanism of action of avermectins. Parasitol Today 1994;10:35-7.

[146] Huang, J., Casida, J.E. Avermectin B_{1a} binds to high- and low-affinity sites on the γ-aminobutyric acid-gated chloride channel of cultured cerebellar granule neurons. J Pharmacol Exp Ther 1997;281:261-6.

[147] Laughton, D.L., Amar, M., Thomas, P., Towner, P., Harris, P., Lunt, G.G., Wolstenholme, A.J. Cloning of a putative inhibitory amino acid receptor subunit from the parasitic nematode *Haemonchus contortus*. Receptors and Channels 1994;2:155-63.

[148] Skinner, T.M., Bascal, Z.A., Holden-Dye, L., Lunt, G.G., Wolstenholme, A.J. Immunocytochemical localization of a putative inhibitory amino acid receptor subunit in the parasitic nematodes *Haemonchus contortus* and *Ascaris suum*. Parasitol 1998;117:89-96.

[149] Gill, J. H., Redwin, J.M., Van Wyk, J.A., Lacey, E. Detection of resistance to ivermectin in *Haemonchus contortus*. Int J Parasitol 1991;21:771-6.

[150] Dano, K. Active outward transport of daunomycin in resistant Ehrlich ascites tumor cells. Biochim Biophys Acta 1973;323:466-83.

[151] Juliano, R.L., Ling, V. A surface glycoprotein modulating drug permeability in Chinese hamster ovary cell mutants. Biochim Biophys Acta 1976;455:152-62.

[152] Higgins, C.F. ABC transporters, from microorganisms to man. Ann Rev Cell Biol 1992;8:67-113.

[153] Foote, S.J., Thompson, J.K., Cowman, A.F., Kemp, D.J. Amplification of the multidrug resistance gene in some chloroquine-resistant isolates of *Plasmodium falciparum*. Cell 1989;57:921-30.

[154] Wilson, C.M., Serrano, A.E., Wasley, A., Bogenschutz, M.P., Shankar, A.H., Wirth, D.F. Amplification of a gene related to mammalian mdr genes in drug-resistant *Plasmodium falciparum*. Science 1989;244:1184-6.

[155] Wellems, T.E., Pantan, L.J., Gluzman, I.Y., do Rosario, V.E., Gwadz, R.W., Walker-Jonah, A., Krogstad, D.J. Chloroquine resistance not linked to mdr-like genes in a *Plasmodium falciparum* cross. Nature 1990;345:253-5.

[156] Barnes, D.A., Foote, S.J., Galatis, D., Kemp, D.J., Cowman, A.F. Selection for high-level chloroquine resistance results in deamplification of the *pfmdr1* gene and increases sensitivity to mefloquine in *Plasmodium falciparum*. EMBO J 1992;11:3067-75.

[157] Chow, L.M., Wong, A.K., Ullman, B., Wirth, D.F. Cloning and functional analysis of an extrachromosomally amplified multidrug resistance-like gene in *Leishmania enrietii*. Mol Biochem Parasitol 1993;60:195-208.

[158] Henderson, D.M., Sifri, C.D., Rodgers, M., Wirth, D.F., Hendrickson, N., Ullman, B. Multidrug resistance in *Leishmania donovani* is conferred by amplification of a gene homologous to the mammalian mdr1 gene. Mol Cell Biol 1992;12:2855-65.

[159] Descoteaux, S., Shen, P.S., Ayala, P., Orozco, E., Samuelson, J. P-glycoprotein genes of *Entamoeba histolytica*. Arch Med Res 1992;23:23-5.

[160] Samuelson, J., Ayala, P., Orozco, E., Wirth, D. Emetine-resistant mutants of *Entamoeba histolytica* overexpress mRNAs for multidrug resistance. Mol Biochem Parasitol 1990;38:281-90.

[161] Descoteaux, S., Ayala, P., Samuelson, J., Orozco, E. Increase in mRNA of multiple Eh pgp genes encoding P-glycoprotein homologues in emetine-resistant *Entamoeba histolytica* parasites. Gene 1995;164:179-84.

[162] Bain, L.J., LeBlanc, G.A. Interaction of structurally diverse pesticides with the human MDR1 gene product P-glycoprotein. Toxicol Appl Pharmacol 1996;141:288-98.

[163] Lanning, C.L., Fine, R.L., Sachs, C.W., Rao, U.S., Corcoran, J.J., Abou-Donia, M.B. Chlorpyrifos oxon interacts with the mammalian multidrug resistance protein, P-glycoprotein. J Toxicol Environ Health 1996;47:395-407.

[164] Murray, C.L., Quaglia, M., Arnason, J.T., Morris, C.E. A putative nicotine pump at the metabolic blood-brain barrier of the tobacco hornworm. J Neurobiol 1994;25:23-4.

[165] Wu, C.T., Budding, M., Griffin, M.S., Croop, J.M. Isolation and characterization of *Drosophila* multidrug resistance gene homologs. Mol Cell Biol 1991;11:3940-8.

[166] Gerrard, B., Stewart, C., Dean, M. Analysis of Mdr50: a *Drosophila* P-glycoprotein multidrug resistance gene homolog. Genomics 1993;17:83-8.

[167] Dudler, R., Hertig, C. Structure of an mdr-like gene from *Arabidopsis thaliana*. J Biol Chem 1992;267:5882-8.

[168] Chen, C., Chin, J.E., Ueda, K., Clark, D.P., Pastan, I., Gottesman, M.M., Roninson, I.B. Internal duplication and homology with bacterial transport proteins in the *mdr1* (P-glycoprotein) gene from multidrug-resistant human cells. Cell 1986;47:381-9.

[169] Lincke, C.R., The, I., van Groeningen, M., Borst, P. The P-glycoprotein gene family of *Caenorhabditis elegans*. Cloning and characterization of genomic and complementary DNA sequences. J Mol Biol 1992;228:701-11.

[170] Kwa, M.S.G., Okoli, M.N., Schulz-Key, H., Okongkwo, P.O., Roos, M.H. Use of P-glycoprotein probes to investigate anthelmintic resistance in *Haemonchus contortus* and comparison with *Onchocerca volvulus*. Int J Parasitol 1998;28:1235-40.

[171] Broeks, A., Janssen, H.W., Calafat, J., Plasterk, R.H.A. A P-glycoprotein protects *Caenorhabditis elegans* against natural toxins. EMBO J 1995;14:1858-66.

[172] Broeks, A., Gerrard, B., Allikmets, R., Dean, M., Plasterk, R.H.A. Homologues of the human multidrug resistance genes MRP and MDR contribute to heavy metal resistance in the soil nematode *Caenorhabditis elegans*. EMBO J 1996;15:6132-43.

[173] Sangster, N.C. P-glycoproteins in nematodes. Parasitol Today 1994;10:319-22.

[174] Schinkel, A.H., Smit, J.J., van Tellingen, O., Beijnen, J.H., Wagenaar, E., van Deemter, L., Mol, C.A., van der Valk, M.A., Robanus-Maandag, E.C., Te Riele, H.P., Berns, A.J.M., Borst, P. Disruption of the mouse *mdr1a* P-glycoprotein gene leads to a deficiency in the blood-brain barrier and to increased sensitivity to drugs. Cell 1994;77:491-502.

[175] Didier, A., Loor, F. The abamectin derivative ivermectin is a potent P-glycoprotein inhibitor. Anti-Cancer Drugs 1996;7:745-51.

[176] Pouliot, J.-F., L'Heureux, F., Liu, Z., Prichard, R.K., Georges, E. Reversal of P-glycoprotein-associated multidrug resistance by ivermectin. Biochem Pharmacol 1997;53:17-25.

CONNECTING STATEMENT I

Avermectins and milbemycins paralyse nematodes. Most recent research on the mode of action of this class of anthelmintic has focused on glutamate-gated chloride channels. Avermectins have been found to gate these channels, leading to an inhibitory hyperpolarisation of neuromuscular cells and thus to paralysis. Genes encoding subunits of glutamate-gated chloride channels, however, had been described only from the free-living nematode, *Caenorhabditis elegans*. We undertook the task of cloning from *H. contortus* a fragment of a gene encoding a channel subunit in order to acquire genetic evidence that these channels contribute to avermectin/milbemycin resistance. The results are presented in Chapter II.

CHAPTER II

***Haemonchus contortus*: Selection at a Glutamate-gated Chloride Channel Gene in Ivermectin- and Moxidectin-Selected Strains (Manuscript I)**

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ABSTRACT

Anthelmintic resistance in nematode parasites of livestock is a serious problem worldwide. Ivermectin, an avermectin, and moxidectin, a milbemycin, are potent endectocides commonly used to control these parasites. The proposed mode of action of avermectins and possibly the milbemycins involves the binding of the drug to the alpha subunit of a glutamate-gated chloride channel, which opens or potentiates gating of the channel and leads to the hyperpolarization of the target neuromuscular cell. Glutamate gates the channel by binding to the beta subunit. We have cloned a fragment of a putative alpha subunit gene from *Haemonchus contortus*. The sequence of the beta subunit is available from GenBank™. Genetic variability of this fragment was analysed by single-strand conformation polymorphism in five strains of *H. contortus*: two strains passaged without drug selection, two strains selected with ivermectin, and one strain selected with moxidectin. One allele of the putative alpha subunit gene appeared to be associated with resistance to the drugs, increasing in frequency in the three drug-selected strains relative to the unselected strains. Another allele appeared to be associated with susceptibility, decreasing in frequency in the three drug-selected strains relative to the unselected strains. A similar analysis of the beta subunit gene showed no significant differences in allele frequencies between the unselected and drug-selected strains. Our findings suggest a correlation between changes in allele frequencies of the putative alpha subunit gene and resistance to ivermectin and moxidectin.

INTRODUCTION

Anthelmintic resistance has become a serious problem in the control of gastrointestinal parasites of livestock [1]. *Haemonchus contortus*, a common trichostrongylid parasite of sheep, cattle, and goats, has developed resistance to the major classes of drugs used to control infection. Resistance to the avermectins, the newest class of anthelmintic commonly in use, has appeared in nematodes of sheep, cattle, and goats [2, 3, 4, 5, 6, 7, 8]. The avermectins are hydrophobic, macrocyclic lactones. IVM, the most widely used avermectin, is known to bind to the alpha subunit of GluCl channels in neuromuscular cells of the free-living nematode, *Caenorhabditis elegans* [9]. These chloride channels are believed to be multimeric structures similar to glycine and GABA receptors, with alpha and beta subunits having been identified from *C. elegans* [9]. Glutamate gates the channel by binding to the beta subunit. GluCl channels have been found only in invertebrates [10], rendering them ideal targets for drugs in the control of parasites of vertebrates. The binding of IVM to the alpha subunit directly opens the channel and, in low concentrations, can potentiate glutamate gating [9]. The opening of the channel leads to hyperpolarization of the cell and ultimately to a flaccid paralysis of the organism. The action of milbemycins, another class of macrocyclic lactones, may be similar to that of the avermectins [11]. In support of this conjecture, a concentration-dependent cross-resistance between the avermectins and the milbemycins has been experimentally observed [12, 13].

The development of drug resistance in eukaryotic organisms is usually due to the selection of existing alleles of a gene whose product is somehow involved in the action of the drug, rather than to the appearance of a new mutation. Individuals in a population that

possess alleles that are susceptible to the action of the drug will succumb to the drug's toxic effects. Such individuals, and therefore their alleles, will be removed from the population. Individuals that possess alleles that can negate or diminish the toxic effects of the drug will survive to reproduce and pass their alleles to future generations, thereby increasing the frequency of those alleles in the population. In time, the frequency of a "resistance" allele will increase to an extent that renders the drug treatment ineffective in the target population [1]. The more variable an organism is genetically, the greater the probability that it will possess an allele that is capable of resisting the action of a drug. *H. contortus* is such an organism [14]. Another trichostrongylid parasite of livestock, *Ostertagia ostertagi*, is also genetically diverse. The large population sizes of these trichostrongylid parasites may account for their genetic diversity [15]. Evidence for the involvement of a gene in resistance to a drug can be obtained by examining the genetic variability of the gene in individuals sensitive and resistant to the drug. Such an analysis was performed on the b-tubulin loci from benzimidazole-susceptible and -resistant strains of *H. contortus* [14]. The genetic variability of both loci were greatly reduced in resistant strains, with the apparent selection for one resistance allele. The application of population genetics promises to be a useful tool in the analysis of drug resistance in parasites and other pest species.

We have cloned a fragment of a gene that encodes a putative alpha subunit of the GluCl channel from *H. contortus*. SSCP analyses were used to compare allele frequencies of this gene in strains of *H. contortus* that were passaged without drug selection to strains that were selected with IVM or MOX. Similar analyses were performed on the gene that encodes the beta subunit.

MATERIALS AND METHODS

Two strains of *H. contortus* were supplied by Merck & Co., Inc., Whitehouse Station, NJ, USA. Both strains were derived from the same parent population. One strain (MIS) was passaged through sheep without drug treatment, and the other strain (MIR) was passaged in parallel but was challenged at each generation with IVM. After 17 generations, 10-fold more IVM was required to kill 95% of the adult population than was required for the parent population [16]. Three strains of *H. contortus* were supplied by Fort Dodge Animal Health, Princeton, NJ, USA. All three strains from Fort Dodge were derived from the same parent population. One strain (PF17) was passaged through sheep for 17 generations without drug treatment. The other two strains were passaged in parallel, but at each generation were treated with IVM (IVF17) or MOX (MOF17) at dose rates that would be 80-95% efficacious. After the twelfth generation, 9.7- and 5.3-fold more drug, respectively, was required to kill 95% of the adult population [17].

Individual male worms were collected live and incubated in phosphate-buffered saline for 2 hr at 37°C. DNA was isolated from the individual worms as described in [14]. For the initial isolation of a fragment of a GluCl channel gene, degenerate primers were designed based on conserved sequences from the alpha and beta subunit genes from *C. elegans* [9] and a putative GABA/glycine receptor gene from *H. contortus* [18]. The sense primer, 5' TGGATGCCNGAYACNTT 3', and the antisense primer, 5' AWCCARAATGAMACCCA 3', were used to amplify, by PCR, a cDNA library (Bluescript) of *H. contortus* [19]. The PCR product was ligated into the pGEM-T vector (Promega Corporation), as described by the manufacturer, and sequenced with Sequenase v2.0 (Amersham Canada Limited), as described by the manufacturer. Primers internal to

the above degenerate primers were designed and used to amplify by PCR the alpha subunit from genomic DNA of *H. contortus*. This PCR product was cloned and sequenced as above to locate the size and position of introns (data not shown). A new sense primer, 5'CCGATTATCCGCTTGATG 3', and a new antisense primer, 5' CCGTATTGGTAACTGACG 3', were designed to amplify by PCR a fragment of genomic DNA 256 bp in length to be used for SSCP analysis. PCR was performed on genomic DNA from 30 individual male worms from each of the strains. Reactions consisted of 2.5 µl 10X *Taq* buffer, 2.5 µl 2 mM dNTPs, 1 µl 25 mM $MgCl_2$, 0.5 µl 20 mM primer solutions, 0.5 unit *Taq* polymerase, approximately 1 ng DNA template, and water to a final volume of 25 µl. Amplification conditions were: 95°C for 4 min followed by 40 cycles of 95°C for 5 sec, 49°C for 30 sec, and 70 °C for 30 sec, with a final extension at 70°C for 5 min. All reactions were carried out on an MJ Research, Inc. PTC-100 Programmable Thermal Controller. The beta subunit was amplified by PCR using the sense primer, 5' TTATCAAGACAGCCAACG 3', and the antisense primer, 5' GGTAGTCGGGTTTTGTGA 3'. These primers were designed based on the sequence of the beta subunit available from GenBank™ (accession No. Y09796). The reactions were carried out as above, except with an annealing temperature of 52°C and with 2.5 µl of 25 mM $MgCl_2$.

For SSCP analysis, PCR products from individual worms were electrophoresed on a 1% agarose gel, stained with ethidium bromide, visualized under UV illumination, and excised from the gel. DNA was extracted from the agarose by placing the excised bands in the top of 0.5-10 µl filter pipette tips, placing the tips in microcentrifuge tubes, and centrifuging the tubes at 14,000 rpm for 30 sec. Two µl of the eluate were used as template in the following labelling reactions: 0.5 µl 10X *Taq* reaction buffer, 0.2 µl 25

mM $MgCl_2$ (0.5 µl for the beta subunit), 0.5 µl 50 µM dNTPs, 0.25 µl 2 µM antisense primer (both primers for the beta subunit, in separate reactions), 0.1 µl 1000 Ci/mM dATP $\alpha^{35}S$, 0.25 unit *Taq* polymerase, and water to a final volume of 5 µl. The reactions were overlaid with a drop of mineral oil and thermal-cycled as above. Six µl of stop solution (10 mM NaOH, 95% formamide, 0.05% bromophenol blue, and 0.05% xylene cyanole) were added at the end of the reactions. The reactions were heated at 80°C for 2 min, and 2.5 µl were loaded onto a 10%, 39:1 acrylamide:N,N'-methylenebisacrylamide, 1X TBE acrylamide gel and electrophoresed at 50W for 6 hr in a 6°C cold room. Gels were dried and exposed to X-ray film overnight. Alleles were identified by their differing rates of migration through the gel.

Allele frequencies for each strain were tabulated. Chi-square analyses were performed between the strains passaged without drug treatment and the corresponding selected strains. Rare alleles having frequency values lower than 5 were grouped for the analyses. A Chi-square analysis was also performed on genotype frequencies from both sets of strains to test for Hardy-Weinberg equilibrium to detect any excess of homozygotes that could indicate the presence of alleles that failed to amplify during PCR. Alleles whose counts were 5 or lower in a population were grouped together for the Chi-square analyses. The count of rare alleles in a population can vary from sample to sample, and the grouping of their counts to form a single category representing "rare alleles" lessens the statistical impact they would have in a Chi-square analysis.

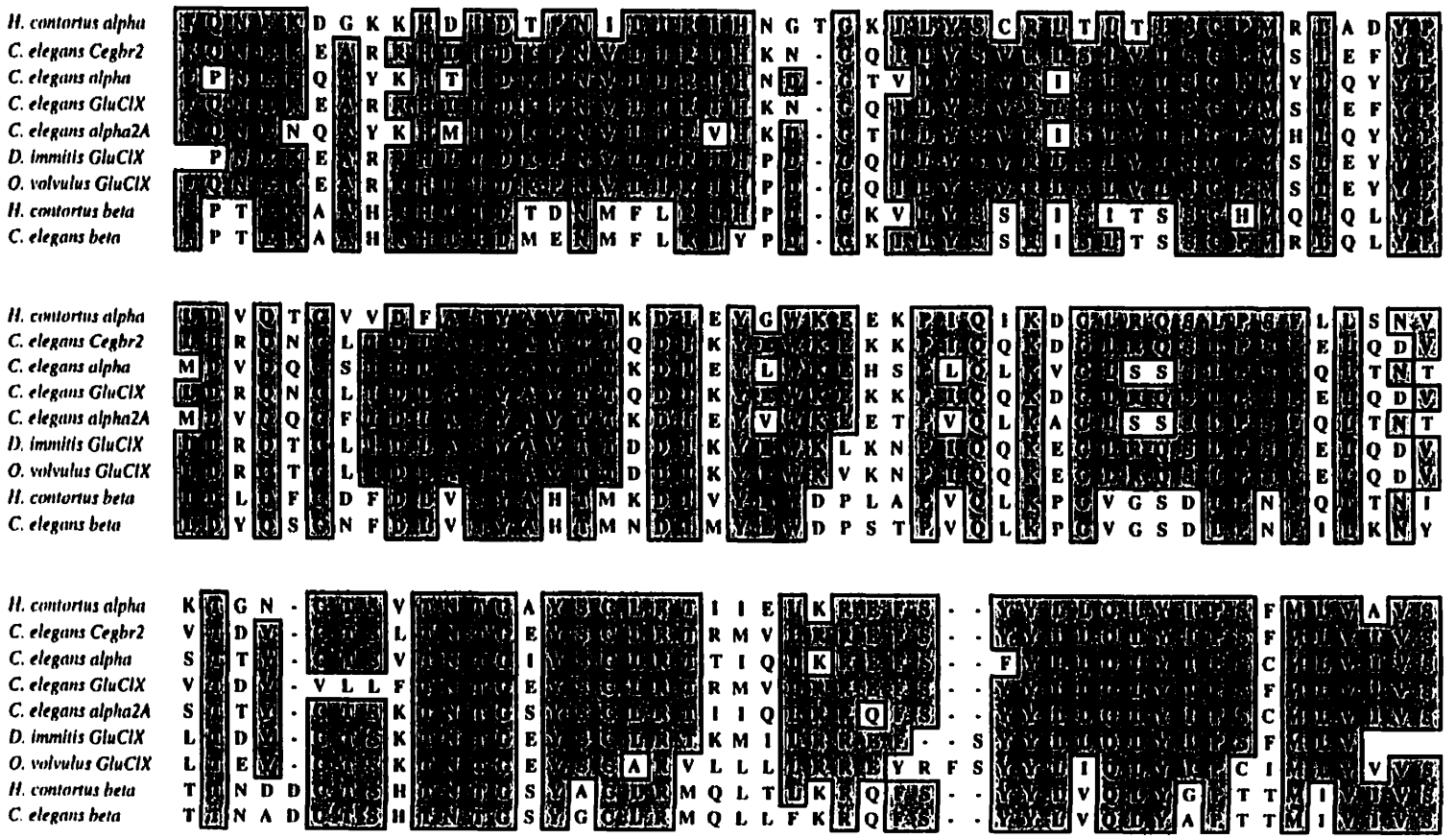
RESULTS

The predicted amino acid sequence of the putative alpha subunit cDNA fragment isolated from *H. contortus* is shown in Fig. 1 aligned with homologous sequences of the beta subunit and subunits from *C. elegans*, *Onchocerca volvulus*, and *Dirofilaria immitis*. Considerable amino acid identity or similarity existed among these sequences. The highest degree of homology of the cloned alpha subunit fragment was found with the *C. elegans* Cegbr2 sequence (71% identity), the *C. elegans* alpha and GluClX subunits (69% identity), and the *C. elegans* alpha2A subunit (68% identity).

Five alleles of the alpha subunit gene were identified from both Merck strains (Fig. 2). Two alleles, C and E, were most common in the unselected (MIS) strain, having frequencies of 0.35 and 0.383, respectively. Both of these alleles decreased in frequency in the IVM-selected strain (MIR), to 0.25 for both alleles. Allele D also decreased in frequency in MIR. Allele A, however, increased in frequency from 0.117 in MIS to 0.45 in MIR. A Chi-square analysis indicated a significant difference ($p < 0.025$) in allele frequencies between the strains. An analysis of the genotype frequencies indicated that the strains were in Hardy-Weinberg equilibrium.

Five alleles (Fig. 3) were also observed in the unselected Fort Dodge strain (PF17). Allele B, which was rare in both Merck strains, was not observed in the Fort Dodge strains. Allele C was most abundant in PF17. This allele decreased in frequency in both the IVM- and MOX-selected strains, as in the Merck IVM-selected strain. One allele, D, which was rare in PF17, was not seen in either selected strain. Allele E, which decreased in frequency in the Merck IVM-selected strain, increased slightly in frequency in both Fort Dodge drug-selected strains. The Fort Dodge strains also had an allele, F, that

Figure 1. The deduced amino acid sequences of glutamate-gated chloride channel subunits aligned in the region of the cloned fragment from *H. contortus*. Boxed residues indicate identity or similarity. Shading indicates identities. The programme MacVector was used to predict and align amino acid sequences. GenBank? accession numbers: U40573, *C. elegans* Cegbr2; U14524, *C. elegans* alpha subunit; AJ000538, *C. elegans* alpha2A subunit; AJ000537, *C. elegans* alpha2B subunit; U14525, *C. elegans* beta subunit; U59743, *C. elegans* GluClX; U59744, *D. immitis* GluClX; U59745, *O. volvulus* GluClX.


H. contortus alpha
C. elegans Cegbr2
C. elegans alpha
C. elegans GluClX
C. elegans alpha2A
D. immitis GluClX
O. volvulus GluClX
H. contortus beta
C. elegans beta

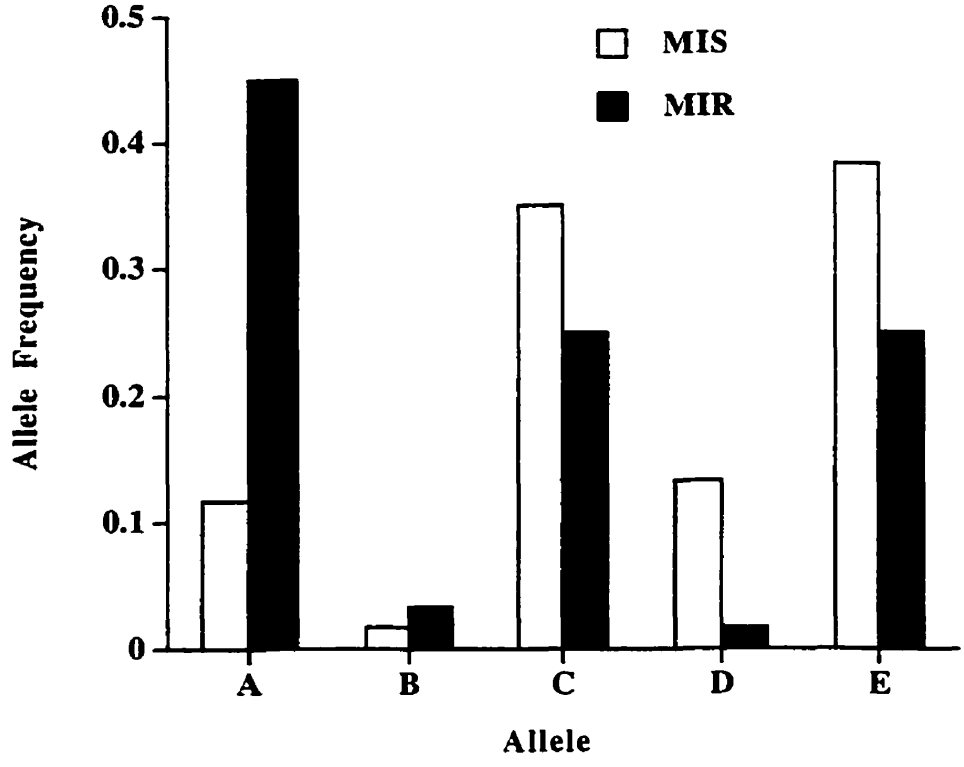


Figure 2. Allele frequencies of the putative GluCl alpha subunit gene, Merck strains of *H. contortus*.

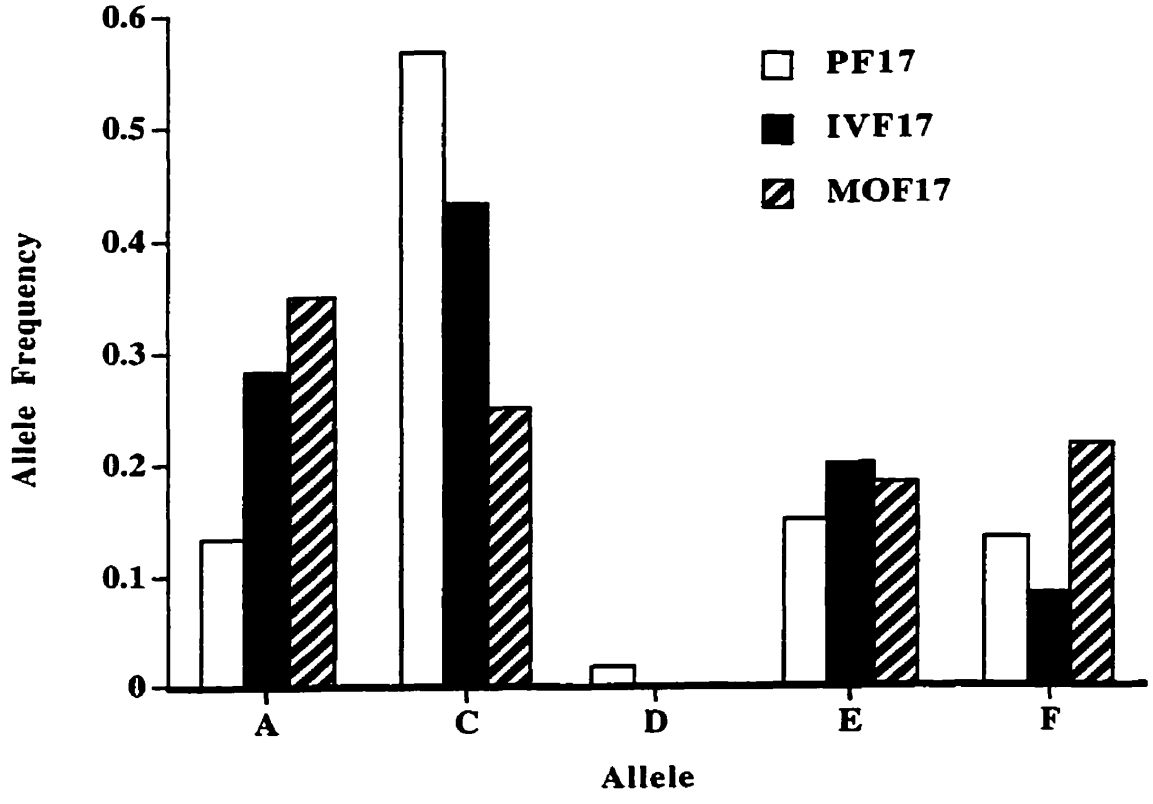


Figure 3. Allele frequencies of the putative GluCl alpha subunit gene, Fort Dodge strains of *H. contortus*.

was not found in the Merck strains. As in the Merck drug-selected strain, Allele A increased in frequency in both Fort Dodge drug-selected strains, from 0.133 in PF17 to 0.283 in the IVM-selected strain (IVF17) and to 0.35 in the MOX-selected strain (MOF17). Allele frequencies were not significantly different between PF17 and IVF17 but were significantly different ($p < 0.05$) between PF17 and MOF17. Allele frequencies, however, were not significantly different between the two drug-selected strains. An analysis of the genotype frequencies indicated that the strains were in Hardy-Weinberg equilibrium.

A total of 10 alleles of the beta subunit gene were observed in the Merck strains (Fig. 4). Little difference in allele frequencies was seen between MIS and MIR. One allele, F, dominated in both strains. Another allele, J, was not found in MIS but occurred at a frequency of 0.117 in MIR. Allele frequencies were not significantly different between MIS and MIR. An analysis of the genotype frequencies indicated that the strains were in Hardy-Weinberg equilibrium.

The Fort Dodge strains possessed fewer alleles of the beta subunit gene than the Merck strains (Fig. 5). Allele F, though, was still the most common allele. One allele, L, was moderately common in all three Fort Dodge strains but was not seen at all in the Merck strains. Allele frequencies were not significantly different between PF17 and either of the drug-selected strains or between the two drug-selected strains. An analysis of the genotype frequencies indicated that the strains were in Hardy-Weinberg equilibrium.

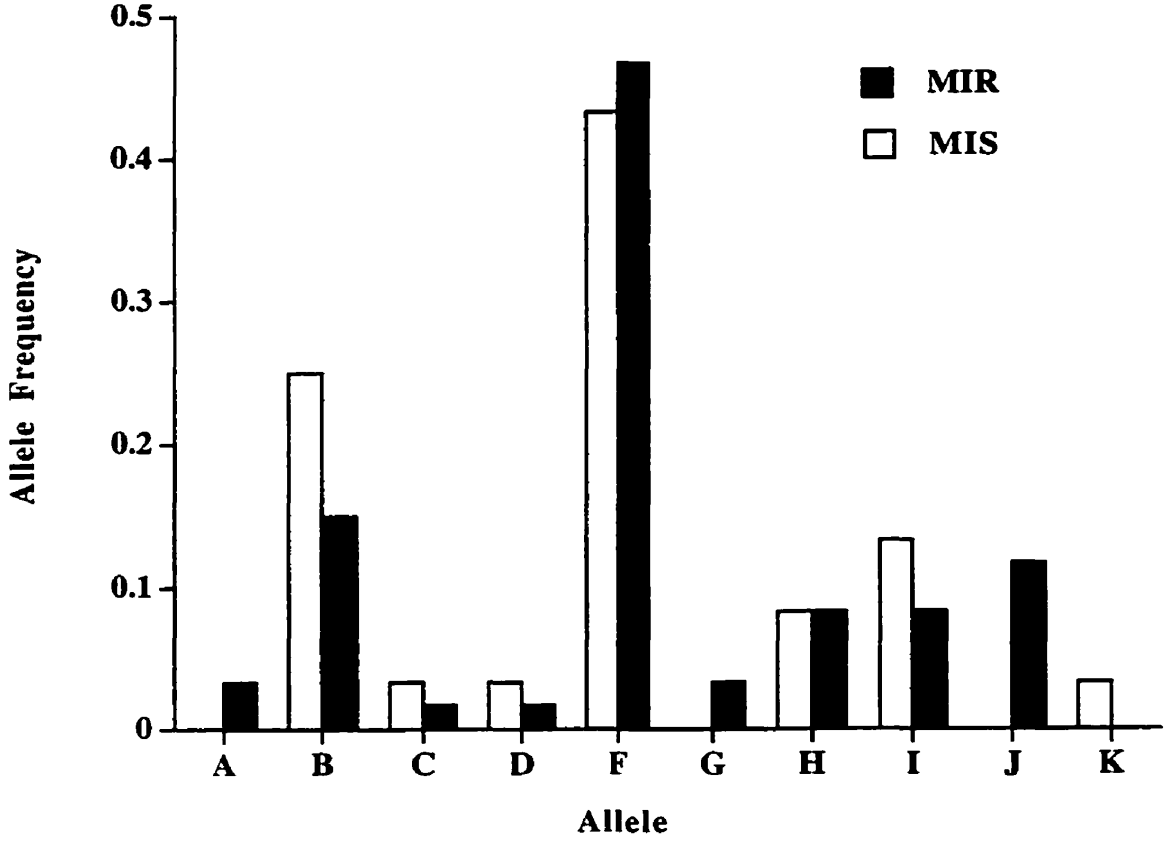


Figure 4. Allele frequencies of the GluCl beta subunit gene, Merck strains of *H. contortus*.

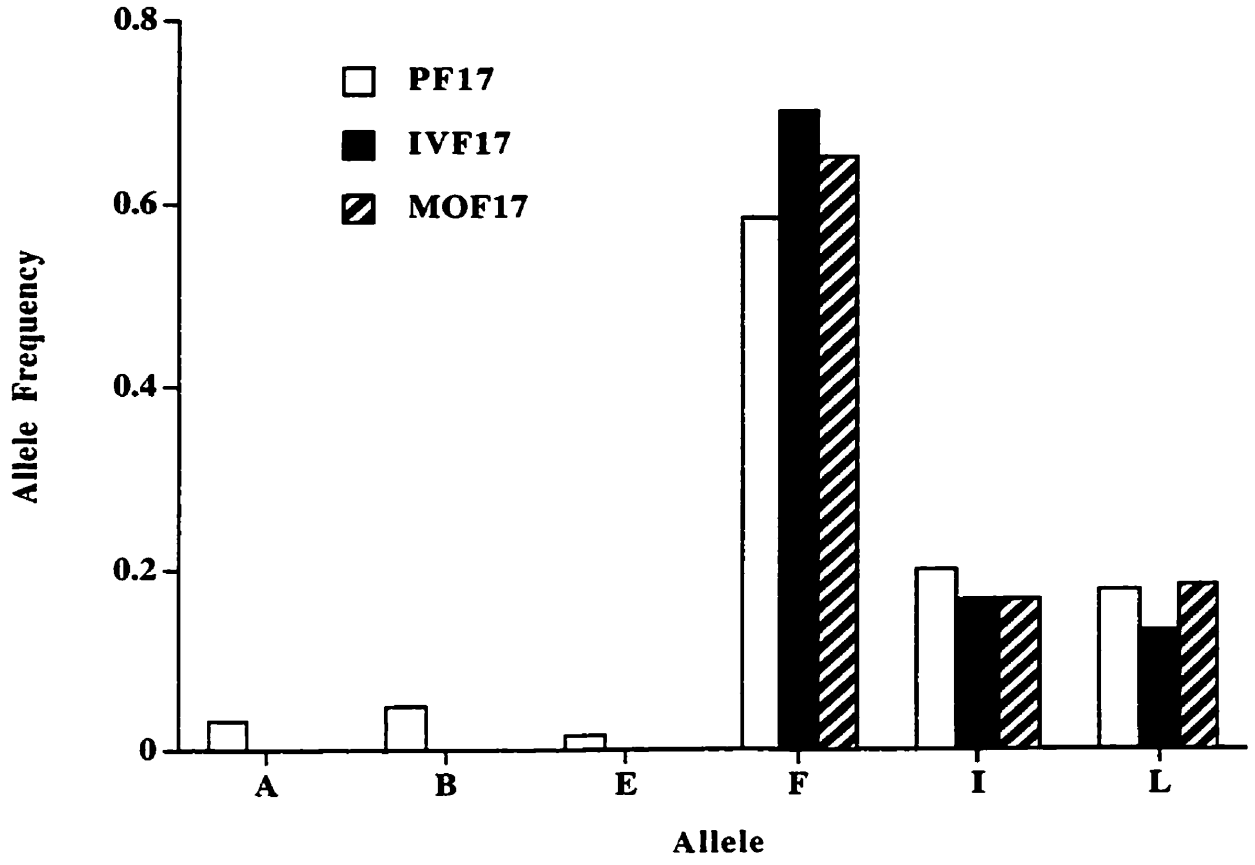


Figure 5. Allele frequencies of the GluCl beta subunit gene, Fort Dodge strains of *H. contortus*.

DISCUSSION

GluCl channels are inhibitory receptors found on neuronal and muscular tissues of insects [20], crustaceans [21], molluscs [22], and nematodes [23]. In nematodes, the channels or the genes encoding their subunits have been identified from *C. elegans* [23], *Ascaris suum* [24], *D. immitis* and *O. volvulus* [10], and *H. contortus* (Wolstenholme, unpublished data). These channels are believed to be heteromeric [9], perhaps pentameric as in the closely related glycine receptor [25]. In studies with *C. elegans*, homomeric channels formed from alpha subunits expressed in *Xenopus* oocytes are sensitive to IVM but not to glutamate, and channels formed from homomeric beta subunits are sensitive to glutamate but not to IVM [9]. A single amino acid change in the alpha subunit, however, can render homomeric channels sensitive to glutamate [26].

The possibility that an ion channel may be involved in resistance to the avermectins and milbemycins is not without precedence. The anthelmintic levamisole is known to gate a nicotinic acetylcholine receptor/cation channel in *C. elegans*, and a mutation in the alpha subunit of the channel is associated with levamisole resistance [27]. Resistance to cyclodiene insecticides is caused by a mutation in the GABA receptor/chloride channel [28]. Mutation in the voltage-sensitive sodium channel is believed to be responsible for conferring resistance to DDT and pyrethroid insecticides in the house fly [29].

In the present study, one allele of the putative alpha subunit gene consistently increased in frequency and another allele consistently decreased in frequency in the drug-selected strains relative to the unselected strains. This finding of genetic polymorphism associated with selection by IVM and MOX in the alpha subunit gene is consistent with

the hypothesis that IVM can gate channels by interacting with this subunit. If the mode of action of the avermectins and possibly the milbemycins does involve the GluCl channels, the lack of any apparent selection at the beta subunit gene is also consistent with the involvement of the alpha subunit. The nature of any mutation in the alpha subunit that may result in drug resistance, however, remains speculative. Resistance to many drugs is due to mutations that affect the ability of the drug to bind to its target. However, studies involving *H. contortus* have demonstrated that IVM binds equally well to cell-membrane preparations from IVM-sensitive and -resistant strains [16]. The binding affinity of glutamate to membrane preparations also appears to be similar in sensitive and resistant strains of *H. contortus* (J.P. Paiement, personal communication). Ion channels are believed to function by undergoing a conformational change when the ligand binds. Since IVM can gate GluCl channels directly, a mutation in the alpha subunit may affect its ability to undergo an IVM-induced conformational change, without affecting the ability of the drug to bind to the subunit. Alternatively, a mutation in the alpha subunit may affect the ability of alpha and beta subunits to properly interact with one another to form functional heteromeric channels. If this scenario were true, then this allele of the alpha subunit would in effect be a null allele, selection pressure on it would be less than on functional alleles, and it would therefore be expected to have an increased rate of nucleotide substitutions relative to the other alleles. Obtaining the nucleotide sequence of the alleles could determine the validity of this possibility. Another possibility is that a mutation in the promoter region of the alpha subunit gene may downregulate the expression of the gene, resulting in fewer heteromeric channels that are sensitive to the drugs. Further study will be necessary to determine the possible role of the alpha subunit in the mechanism of resistance.

The tissues in which GluCl channels are expressed in nematodes have not been fully identified. The channels appear to be present in pharyngeal muscle tissue in *C. elegans* [30] and *A. suum* [24]. Pharyngeal pumping was inhibited in *H. contortus* when exposed to low doses of IVM [31]. A recent study describing a second alpha subunit gene from *C. elegans* [32] indicates that GluCl channels sensitive to IVM are also expressed in a few neurons of the head and in some ventral motor neurons. Higher doses of avermectins can lead to paralysis of the body musculature, but whether this paralysis is mediated by GluCl channels or by GABA receptors, which in *C. elegans* are expressed in neurons that innervate the body musculature involved in locomotion [33], is unknown. GABA receptors in *Ascaris* are sensitive to IVM in high doses [34]. Future studies will examine genetic variation at a putative GABA receptor gene from *H. contortus* [18] to determine if allelic selection is occurring that may indicate involvement of this receptor in IVM or MOX resistance.

Multiple mechanisms may be contributing to IVM resistance in *H. contortus*. Xu *et al.* [35] have found a genetic polymorphism between IVM-sensitive and -resistant strains when using a fragment of a P-gp homologue as a probe, overexpression of P-gp mRNA in IVM-resistant strains, and the ability of a multidrug reversing agent to reverse resistance in IVM-resistant *H. contortus*. P-gp is a membrane protein that is capable of transporting aromatic, hydrophobic drugs from cell membranes and cytoplasm and is responsible for some cases of multidrug resistance in human cancer cells [36]. IVM is known to be a substrate for mammalian P-gp [37, 38]. Evidence of two different genes being involved in IVM resistance points either to multiple mechanisms of resistance to the drug or to a physical or functional linkage of the two genes or their protein products.

Our findings support the contention that resistance to IVM may be associated with

mutation of the GluCl alpha subunit gene. The apparent selection for the same GluCl allele in the Merck IVM-selected strain and in the Fort Dodge MOX-selected strain also lends support to the hypothesis that the avermectins and milbemycins share a common mode of action involving GluCl channels. Genetic polymorphism associated with resistance, however, may be due to phenomena unrelated to the mechanism of resistance. Genetic hitch-hiking or epistatic interactions may be responsible for shifts in allele frequencies of genes not directly involved in resistance. Nevertheless, the reported binding of IVM to the GluCl alpha subunit and its putative association with the drug's mode of action, and the evidence presented here of selection of the alpha subunit, suggest an involvement of the GluCl alpha subunit in a mechanism of resistance. At the very least, the allele frequency changes observed in this study may represent a marker for avermectin and milbemycin resistance in parasitic nematodes. This marker could prove useful as genetic evidence for the observed development of resistance to these drugs in field situations.

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REFERENCES

[1] Prichard, R.K. Anthelmintic resistance in nematodes: extent, recent understanding and future directions for control and research. Int J Parasitol 1990;20:515-23.

[2] Craig, T.M., Miller, D.K. Resistance by *Haemonchus contortus* to ivermectin in angora goats.Vet Rec 1990;126:580.

[3] Echevarria, F.A.M. ,Trindade, G.N.P. Anthelmintic resistance by *Haemonchus contortus* to ivermectin in Brazil: a preliminary report. Vet Rec 1989;124:147-8.

[4] Le Jambre, L.F. Ivermectin-resistant *Haemonchus contortus* in Australia. Aus Vet J 1993;70:357.

[5] Maingi, N., Bjorn, H., Thamsborg, S.M., Bogh, H.O., Nansen, P. A survey of anthelmintic resistance in nematode parasites of goats in Denmark. Vet Parasitol 1996;66:53-66.

[6] Van Wyk, J.A., Malan, F.S. Resistance of field strains of *Haemonchus contortus* to ivermectin, closantel, rafoxanide and the benzimidazoles in South Africa. Vet Rec 1988;123:226-8.

[7] Vermunt, J.J., West, D.M., Pomroy, W.E. Multiple resistance to ivermectin and oxfendazole in Cooperia species of cattle in New Zealand. Vet Rec 1995;137:43-5.

[8] Watson, T.G., Hosking, B.C., Leathwick, D.M., McKee, P.F. Ivermectin-moxidectin side resistance by *Ostertagia* species isolated from goats and passaged to sheep. Vet Rec 1996;138:472-3.

[9] Cully, D.F., Vassilatis, D.K., Liu, K.K., Paress, P.S., van der Ploeg, L.H.T., Schaeffer, J.M., Arena, J.P. Cloning of an avermectin-sensitive glutamate-gated chloride channel from *Caenorhabditis elegans*. Nature 1994;371:707-11.

[10] Cully, D.F., Wilkinson, H., Vassilatis,D.K., Etter, A., J.P. Arena. Molecular biology and electrophysiology of glutamate-gated chloride channels of invertebrates. Parasitol 1996;113 (supp):S191-200.

[11] Arena, J.P., Liu, K.K., Paress, P.S., Frazier, E.G., Cully, D.F., Mrozik, H., Schaeffer, J.M. The mechanism of action of avermectins in *Caenorhabditis elegans*: correlation between activation of glutamate-sensitive chloride current, membrane binding, and biological activity. J Parasitol 1995;81:286-94.

[12] Conder, G.A., Thompson, D.P., Johnson, S.S. Demonstration of co-resistance of *Haemonchus contortus* to ivermectin and moxidectin. Vet Rec 1993;132:651-2.

[13] Shoop, W.L., Haines, H.W., Michael, B.F., Eary, C.H. Mutual resistance to avermectins and milbemycins: oral activity of ivermectin and moxidectin against ivermectin-resistant and susceptible nematodes. Vet Rec 1993;133:445-7.

[14] Beech, R.N., Prichard, R.K., Scott, M.E. Genetic variability of the β-tubulin genes in benzimidazole-susceptible and -resistant strains of *Haemonchus contortus*. Genetics 1994;138:103-10.

[15] Blouin, M.S., Dame, J.B., Tarrant, C.A., Courtney, C.H. Unusual population genetics of a parasitic nematode: mtDNA variation within and among populations. Evolution 1992;46:470-6.

[16] Rohrer, S.P., Birzin, E.T., Eary, C.H., Schaeffer, J.M., Shoop, W.L. Ivermectin binding sites in sensitive and resistant *Haemonchus contortus*. J Parasitol 1994;80:493-7.

[17] Wang, G.T., Berger, K., Simkins, K., and Rock, D. The rate of resistance development by *H. contortus* to endectocides. Proceedings of the XV International Conference of the World Association for the Advancement of Veterinary Parasitology. Yokohama, Japan, 1995; p. 104.

[18] Laughton, D.L., Amar, M., Thomas, P., Towner, P., Harris, P., Lunt, G.G., Wolstenholme, A.J. Cloning of a putative inhibitory amino acid receptor subunit from the parasitic nematode *Haemonchus contortus*. Receptors & Channels 1994;2:155-63.

[19] Klein, R.D., Olson, E.R., Favreau, M.A., Winterrowd, C.A., Hatzenbuhler, N.T., Shea, M.H., Nulf, S.C., Geary, T.G. Cloning of a cDNA encoding phosphofructokinase from *Haemonchus contortus*. Mol Biochem Parasitol 1991;48:17-26.

[20] Cull-Candy, S.G., Usherwood, P.N.R. Two populations of L-glutamate receptors on locust muscle fibres. Nature, New Biol 1973;246:62-4.

[21] Cleland, T.A., Selverston, A.I. Glutamate-gated inhibitory currents of central pattern generator neurons in the lobster stomatogastric ganglion. J Neurosci 1995;15:6631-9.

[22] Ikemoto, V., Akaike, N. The glutamate-induced chloride current in *Aplysia* neurons lacks pharmacological properties seen for excitatory responses. Eur J Pharmacol 1988;150:313-8.

[23] Arena, J.P., Liu, K.K., Paress, P.S., Cully, D.F. Avermectin-sensitive chloride currents induced by *Caenorhabditis elegans* RNA in *Xenopus* oocytes. Mol Pharmacol 1991;40:368-74.

[24] Martin, R.J. An electrophysiological preparation of *Ascaris suum* pharyneal muscle reveals a glutamate-gated chloride channel sensitive to the avermectin analogue, milbemycin D. Parasitol 1996;112:247-52.

[25] Kuhse, J., Betz, H., Kirsch, J. The inhibitory glycine receptor: architecture, synaptic localization and molecular pathology of a postsynaptic ion-channel complex. Curr Opin Neurobiol 1995;5;318-23.

[26] Etter, A., Cully, D.F., Schaeffer, J.M., Liu, K.K., Arena, J.P. An amino acid substitution in the pore region of a glutamate-gated chloride channel enables the coupling

of ligand binding to channel gating. J Biol Chem 1996;271:16035-8.

[27] Lewis, J.A., Elmer, J.S., Skimming, J., McLafferty, S., Fleming, J., McGee, T. Cholinergic receptor mutants of the nematode *Caenorhabditis elegans*. J Neurosci 1987;7:3059-71.

[28] ffrench-Constant, R.H., Rocheleau, T.A., Steichen, J.C., Chalmers, A.E. A point mutation in a *Drosophila* GABA receptor confers insecticide resistance. Nature 1993;363:449-51.

[29] Knipple, D.C., Doyle, K.E., Marsella-Herrick, P.A., Soderlund, D.M. Tight genetic linkage between the kdr insecticide resistance trait and a voltage-sensitive sodium channel gene in the house fly. Proc Natl Acad Sci USA 1994;91:2483-7.

[30] Laughton, D.L., Lunt, G.G., A.J. Wolstenholme. Reporter gene constructs suggest that the *Caenorhabditis elegans* avermectin receptor β-subunit is expressed solely in the pharynx. J Exp Biol 1997;200:1509-14.

[31] Geary, T.G., Sims, S.M., Thomas, E.M., Vanover, L., Davis, J.P., Winterrowd, C.A., Klein, R.D., Ho, N.F.H., Thompson, D.P. *Haemonchus contortus*: ivermectin-induced paralysis of the pharynx. Exp Parasitol 1993;77:88-96.

[32] Dent, J.A., Davis, M.W., Avery, L. Avr-15 encodes a chloride channel subunit that mediates inhibitory glutamatergic neurotransmission and ivermectin sensitivity in

Caenorhabditis elegans. EMBO J 1997;16:5867-79.

[33] McIntire, S.L., Jorgensen, E., Kaplan, J., Horvitz, H.R. The GABAergic nervous system of *Caenorhabditis elegans*. Nature 1993;364:337-41.

[34] Kass, I.S., Wang, C.C., Walrond, J.P., Stretton, A.O.W. Avermectin $B1_a$, a paralyzing anthelmintic that affects interneurons and inhibitory motorneurons in *Ascaris*. Proc Natl Acad Sci USA 1980;77:6211-5.

[35] Xu, M., Molento, M., Blackhall, W., Ribeiro, P., Beech, R., Prichard, R. Ivermectin resistance in nematodes may be caused by alteration of P-glycoprotein homolog. Mol Biochem Parasitol 1998;91:327-35.

[36] Gottesman, M.M., Pastan, I. Biochemistry of multidrug resistance mediated by the multidrug transporter. Ann Rev Biochem 1993;62:385-427.

[37] Didier, A., Loor, F. The abamectin derivative ivermectin is a potent P-glycoprotein inhibitor. Anti-Cancer Drugs 1996;7:745-51.

[38] Pouliot, J.-F., L'Heureux, F., Liu, Z., Prichard, R.K., Georges, E. Reversal of P-glycoprotein-associated multidrug resistance by ivermectin. Biochem Pharmacol 1997;53:17-25.

CONNECTING STATEMENT II

Glutamate-gated chloride channels are currently considered to be the principal site of action of avermectins. Early studies, however, had suggested that gamma-aminobutyric acid receptors were a possible target. These receptors are also inhibitory chloride channels structurally related to glutamate-gated chloride channels. A gene encoding a subunit of gamma-aminobutyric acid receptors had been reported from *H. contortus*, so we analysed this gene to see if any evidence existed for its involvement in resistance to avermectins/milbemycins in *H. contortus*. Results of this analysis are presented in Chapter III.

CHAPTER III

Genetic Evidence that Gamma-aminobutyric Acid (GABA) Receptors are Target Sites of Avermectins/Milbemycins in *Haemonchus contortus* (Manuscript II)

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Submitted

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ABSTRACT

Gamma-aminobutyric acid (GABA) Type A receptors are inhibitory chloride channels in membranes of vertebrate and invertebrate neuromuscular cells. Gating of the channels by GABA leads to an influx of chloride ions into, and hyperpolarization of, the cell. GABA receptors are believed to form channels by the association of five protein molecules of varying subunit types, with the second transmembrane (M2) domain of each protein molecule forming a central pore through which chloride ions can pass. We have analysed by single-strand conformation polymorphism the genetic variation of a putative GABA-receptor gene, HG1, from two sets of unselected and anthelmintic-selected strains of the parasitic nematode *Haemonchus contortus*. Significant differences in allele frequencies were detected between one unselected strain and its derived ivermectin-selected strain and between the other unselected strain and its derived ivermectin- and moxidectin-selected strains. In each set of strains, one allele increased substantially in frequency in the drug-selected strains relative to their respective unselected strains. The selected allele, however, differed between the two sets of strains. Deduced amino acid sequences of the M2 domains of all alleles detected are identical. Similar analyses were performed on a phosphoenolpyruvate carboxykinase gene and a nicotinic acetylcholine receptor subunit gene. No significant differences were found in allele frequencies between the unselected and their derived anthelmintic-selected strains. These results implicate the GABA receptor as a site of action for avermectins and milbemycins, and suggest its involvement in resistance to these anthelmintics. However, the results indicate that the mechanism of resistance does not directly involve the M2 region.

INTRODUCTION

Gamma-aminobutyric acid (GABA) Type A receptors are inhibitory chloride channels found in invertebrate neuromuscular cells and in neurons of the vertebrate central nervous system. These receptors belong to the family of ligand-gated ion channels that includes the glutamate-gated chloride channels, glycine receptors, serotonin 5HT3 receptors, and nicotinic acetylcholine receptors. All members of this family are believed to possess a similar morphology: five protein molecules of various subunits surrounding a central pore [1]. Each subunit has four transmembrane domains, with the second transmembrane domain (M2) of each subunit lining the pore [2-4].

GABA receptors are known targets for pesticides. In insects, various compounds act as antagonists of GABA receptors, causing convulsions by preventing the inhibition of nerve and muscle excitation [5,6]. Avermectins (AVM), macrocyclic lactones isolated from *Streptomyces avermitilis* [7], act as agonists of GABA receptors on neuromuscular cells, causing ataxia and paralysis [5,8]. In nematodes, piperazine is a GABA-receptor agonist [9] that causes paralysis. The action of AVM on GABA receptors in nematodes, however, is unclear. AVM causes paralysis of the somatic musculature in *Ascaris* [10], *Caenorhabditis elegans* [10], and *Haemonchus contortus* [11], yet AVM has been characterized as a GABA-receptor antagonist, blocking hyperpolarization, on nematode somatic muscle [12,13]. AVM appears to cause paralysis by blocking transmission between interneurons and excitatory motorneurons [10,14], but the mechanism of this blockage is unknown.

The second transmembrane domain (M2) of ligand-gated ion channels plays an important role in the function of these channels. The mutation of amino acid residues in

M2 domains can block picrotoxinin sensitivity in glycine [15] and GABA [16] receptors, alter ion permeability in nicotinic acetylcholine receptors [17], enable coupling of ligand binding to channel gating in glutamate-gated chloride channels [18], and even change ion selectivity from cationic to anionic in nicotinic acetylcholine receptors [19]. A point mutation in M2 of a GABA receptor in *Drosophila* confers resistance to cyclodiene insecticides [20].

By examining genetic changes that occur in response to selection with ivermectin (IVM), an AVM, and moxidectin (MOX), a milbemycin, at a gene encoding a putative GABA receptor subunit, HG1, in the parasitic nematode *H. contortus*, we present evidence that GABA receptors play a role in the action of AVM and milbemycins in nematodes and are subject to selection during the development of IVM and MOX resistance. Genes encoding a phosphoenolpyruvate carboxykinase (PEPCK) and a nicotinic acetylcholine receptor (nAChR) subunit are also examined for comparison.

MATERIALS AND METHODS

Parasite strains. The strains of *H. contortus* used in this study are described in [21].

DNA isolation. DNA was extracted from individual adult males as described in [22].

DNA amplification. DNA was amplified by polymerase chain reaction (PCR) from genomic DNA of 30 individuals from each strain. Primers were designed based on the HG1 cDNA sequence reported by [23]. A sense primer (position 799 to 816), 5' GGTGATGTCATGGGTGTC 3', and an antisense primer (position 969 to 986), 5' TTGCTGCGAATACGAATC 3', amplified a fragment of genomic DNA approximately 305 base pairs in length. All reactions were performed using an MJ Research PTC-100 Programmable Thermal Controller (Watertown, MA). The reaction mixture contained 2.5 µl 10X *Taq* buffer, 2.5 µl 2 mM dNTPs, 2.5 µl 25 mM $MgCl_2$, 0.5 µl 20 µM primer solutions, 0.5 unit *Taq* polymerase, approximately 1 ng DNA template, and water to a final volume of 25 µl. Amplification conditions were: 95°C for 1 min followed by 40 cycles of 95°C for 10 sec, 53°C for 15 sec, and 70°C for 30 sec with a final extension step at 70°C for 5 min. PCR products were visualized on a 1% agarose gel containing 0.5 µg/ml ethidium bromide.

DNA amplification of the PEPCK and nAChR genes was as above but with the following differences. PEPCK primers were: sense primer, 5' CTGTCATGCACGATCCCA 3', and antisense primer, 5' CCGTAACCAGGCCAGAGG 3', based on the cDNA sequence reported by [24]. nAChR primers were: sense primer, 5' CCTCAGAGCGCGTACATT 3', and antisense primer, 5'

CAAGGCGACAAGAATGGA 3', based on the cDNA sequence reported by [25]. PEPCK PCR reactions used 2 µl 25mM $MgCl_2$ and an annealling temperature of 55 °C, and nAChR reactions used 3 µl 25 mM $MgCl_2$ and an annealling temperature of 52 °C.

Single-strand conformation polymorphism (SSCP) analysis. PCR products were excised from the agarose gel, and the DNA extracted by placing the excised bands in the top of 0.5-10 µl filter pipette tips, placing the tips in microcentrifuge tubes, and centrifuging the tubes at 14,000 rpm for 30 sec. Two µl of the eluate were used as template for the following labelling reactions: 0.5 µl 10X *Taq* buffer, 0.5 µl 50 µM dNTPs, 0.5 µl 25 mM $MgCl_2$, 0.25 µl 2 µM sense primer, 0.1 µl 1000 Ci/mmol dATP $\alpha^{35}S$, 0.1 unit *Taq* polymerase, and water to a final volume of 5 µl. The reactions were overlaid with a drop of mineral oil and thermal-cycled as above. Six µl of stop solution (10 mM NaOH, 95% formamide, 0.05% bromophenol blue, and 0.05% xylene cyanole) were added at the end of the reactions. The reactions were heated at 80°C for 2 min, and 2.5 µl were loaded onto a 16%, 99:1 acrylamide:N,N'-methylenebisacrylamide, 1X TBE acrylamide gel and electrophoresed at 50W for 6 hr in a 6°C coldroom. Gels were dried and exposed to X-ray film overnight. Alleles were identified by their differing rates of migration through the gel.

PCR products of the PEPCK and nAChR genes were processed as above, with the following differences. PEPCK labelling reactions used 0.4 µl 25 mM $MgCl_2$ and were electrophoresed on a 15%, 59:1 acrylamide:N,N'-methylenebisacrylamide gel for 4.5 hr. nAChR labelling reactions used 0.6 µl 25 mM $MgCl_2$ and were electrophoresed on a 15%, 99:1 acrylamide:N,N'-methylenebisacrylamide gel for 5.5 hr.

Allele sequence determination. Alleles of HG1 were isolated by excising representative bands from the SSCP gel. Only bands from heterozygotes were chosen for analysis. For the three most common alleles, two bands were excised, one from a Merck strain and one from a Fort Dodge strain, where applicable. One band only was excised for the other alleles. DNA was eluted from the acrylamide slices by incubating them in 25 µl TE in PCR reaction tubes at room temperature for 2 hr, heating to 100°C for 10 min, and then incubating at room temperature for another 2 hr. The alleles were reamplified using the following reaction mixtures: 10 µl 10X *Taq* buffer, 10 µl 2 mM dNTPs, 10 µl 25 mM $MgCl_2$, 2 µl 20 µM primer solutions, 2 units *Taq* polymerase, 1 µl eluted allele DNA solution, and water to a final volume of 100 µl. The amplification conditions were: 95°C for 1 min followed by 40 cycles of 95°C for 20 sec, 53°C for 30 sec, and 70°C for 1 min with a final extension step at 70°C for 5 min. PCR products were visualized on a 1% agarose gel containing 0.5 µg/ml ethidium bromide. The bands were excised from the agarose gel, and the DNA eluted using filter pipette tips as described above. DNA was further purified by ethanol precipitation and resuspended in TE. The PCR products were either sequenced directly or ligated into the pGEM-T cloning vector (Promega Corporation, Madison, WI) as described by the manufacturer . For the cloned alleles, three clones of each allele were sequenced to determine a consensus sequence. All sequencing was commercially cycle-sequenced (MOBIX, McMaster University, Hamilton, ON).

Data analysis. Allele frequencies were tabulated for each gene from all strains. Chi-square analyses were performed between the unselected and the corresponding derived drug-selected strains. Rare alleles having frequency values lower than 5 were grouped for

the analyses. To test for the presence of alleles that may have failed to amplify during PCR, Chi-square analyses were also performed on observed versus expected frequencies of homozygotes and heterozygotes. Alleles whose counts were 5 or lower in a population were grouped together for the Chi-square analyses. The count of rare alleles in a population can vary from sample to sample, and the grouping of their counts to form a single category representing "rare alleles" lessens the statistical impact they would have in a Chi-square analysis.

RESULTS

Thirteen alleles of the HG1 GABA receptor gene were identified in the Merck strains of *H. contortus* (Fig. 1). The unselected strain (MIS) possessed 11 alleles, and the IVM-selected strain (MIR) possessed 8 alleles. Except for the loss of allele H in MIR, the lower number of observed alleles in MIR may simply be due to sampling variation, as the frequencies of the alleles involved are low. Allele A was the most common allele in both strains, with frequencies of 0.317 in MIS and 0.333 in MIR. In MIS, alleles E, F, and H were also relatively common, with frequencies of 0.167, 0.133, and 0.167, respectively. The frequencies of the other alleles seen in this strain (alleles B, D, G, J, K, L, and M) were 0.067 or lower. In MIR, allele E was also relatively common, with a frequency of 0.183. Allele F decreased in frequency relative to MIS, and allele H was not observed. Allele H may be particularly sensitive to IVM treatment and may have been selected against. The most striking difference in allele frequencies between MIS and MIR was the increase in frequency of allele L, from 0.033 in MIS to 0.300 in MIR. This allele has apparently undergone strong positive selection. A Chi-square analysis indicated a significant difference in allele frequencies ($P < 0.005$) between MIS and MIR. Both strains were in Hardy-Weinberg equilibrium.

Less genetic variation was observed in the Fort Dodge strains (Fig. 2). Only 7 alleles were identified, compared to 13 in the Merck strains. Differences in the origins of the two sets of strains may account for the difference in variability at this locus. As in MIS, allele A was the most common allele observed in the unselected Fort Dodge strain (PF17) with a frequency of 0.450. Alleles D and e were also relatively common in PF17, with frequencies of 0.250 and 0.150, respectively. Alleles B, C, and F occurred at

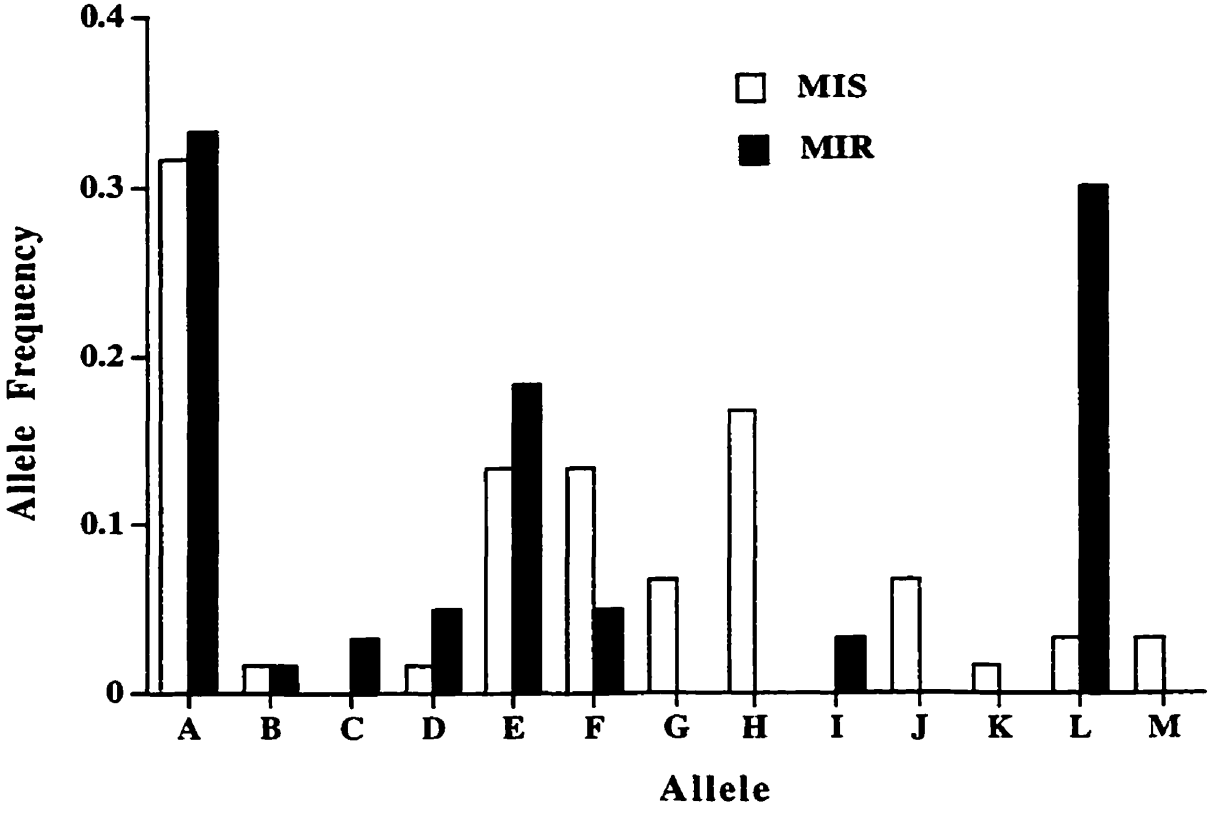

Figure 1. Allele frequencies of a GABA receptor gene from Merck unselected (MIS) and ivermectin-selected (MIR) strains of *H. contortus*.

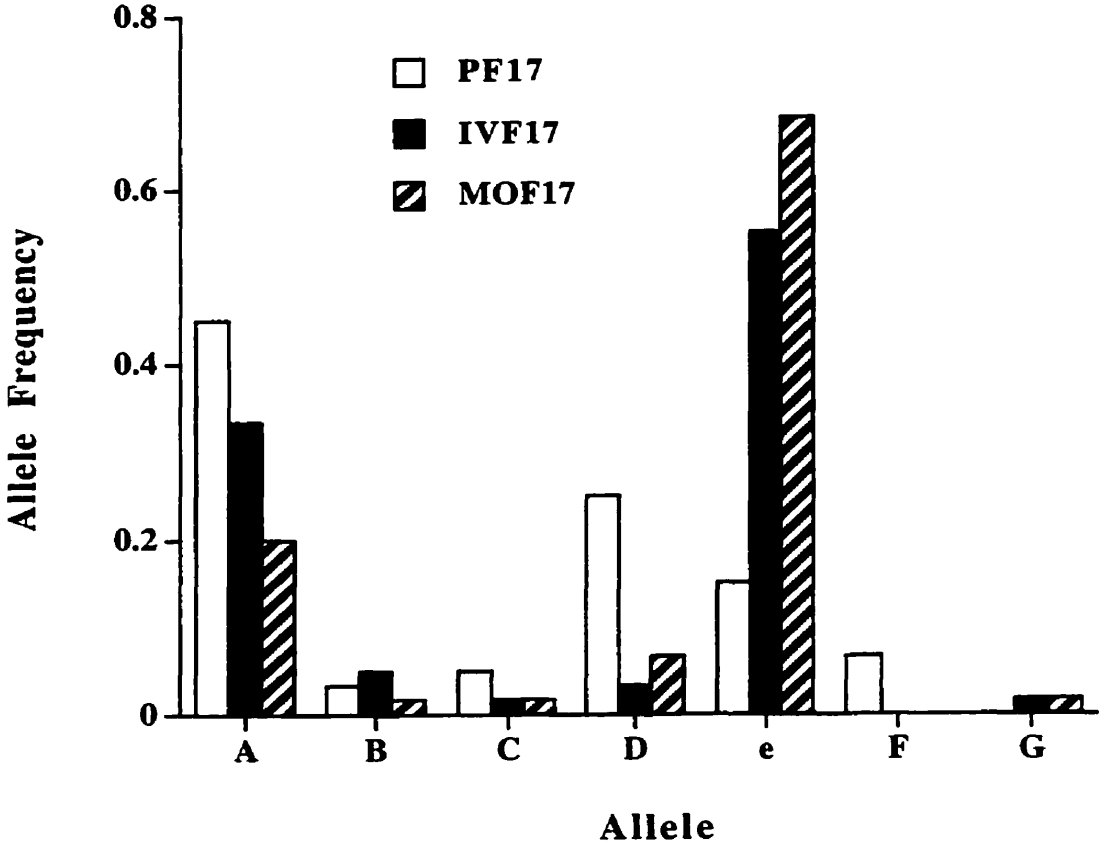

Figure 2. Allele frequencies of a GABA receptor gene from Fort Dodge unselected (PF17) and ivermectin- (IVF17) and moxidectin-selected (MOF17) strains of *H. contortus*.

frequencies of 0.067 or lower. In the drug-selected strains, allele A decreased in frequency to 0.333 in the IVM-selected strain (IVF17) and to 0.200 in the moxidectin-selected strain (MOF17). Allele D also decreased in frequency in the drug-selected strains, to 0.033 in IVF17 and to 0.067 in MOF17. Alleles B, C, D, and G occurred at frequencies of 0.067 or lower. As with the Merck strains, a large increase in frequency of one allele was the most striking difference between the unselected and drug-selected strains. The frequency of allele e rose from 0.150 in PF17 to 0.550 in IVF17 and to 0.683 in MOF17. Positive selection for allele e has apparently occurred in the drug-selected strains. The allele that underwent apparent selection in MIR, allele L, was not observed in the Fort Dodge strains. Chi-square analyses indicated a significant difference in allele frequencies between PF17 and IVF17 ($P < 0.001$) and between PF17 and MOF17 ($P < 0.0005$). Allele frequencies were not significantly different between IVF17 and MOF17. All three Fort Dodge strains were in Hardy-Weinberg equilibrium.

Nucleotide sequence data in the M2 region are shown in Fig. 3. All alleles identified have different nucleotide sequences in this region, except allele C is identical to allele E/e. All nucleotide differences in the M2 region are silent mutations: the deduced amino acid sequences of all alleles are identical to that reported for HG1. This sequence information also identified allele E from a Merck strain as being different from allele e from a Fort Dodge strain, although alleles E and e comigrated in the SSCP gels. The single SSCP band that was designated as either allele E or e represents a population of at least two alleles, only one of which may increase in frequency in the two Fort Dodge anthelmintic-selected strains. All worms from all strains possessing at least one allele E/e were re-analysed under different polyacrylamide gel conditions, but all allele E/e's continued to comigrate. The degree of differences in frequencies of allele e in IVF17 and

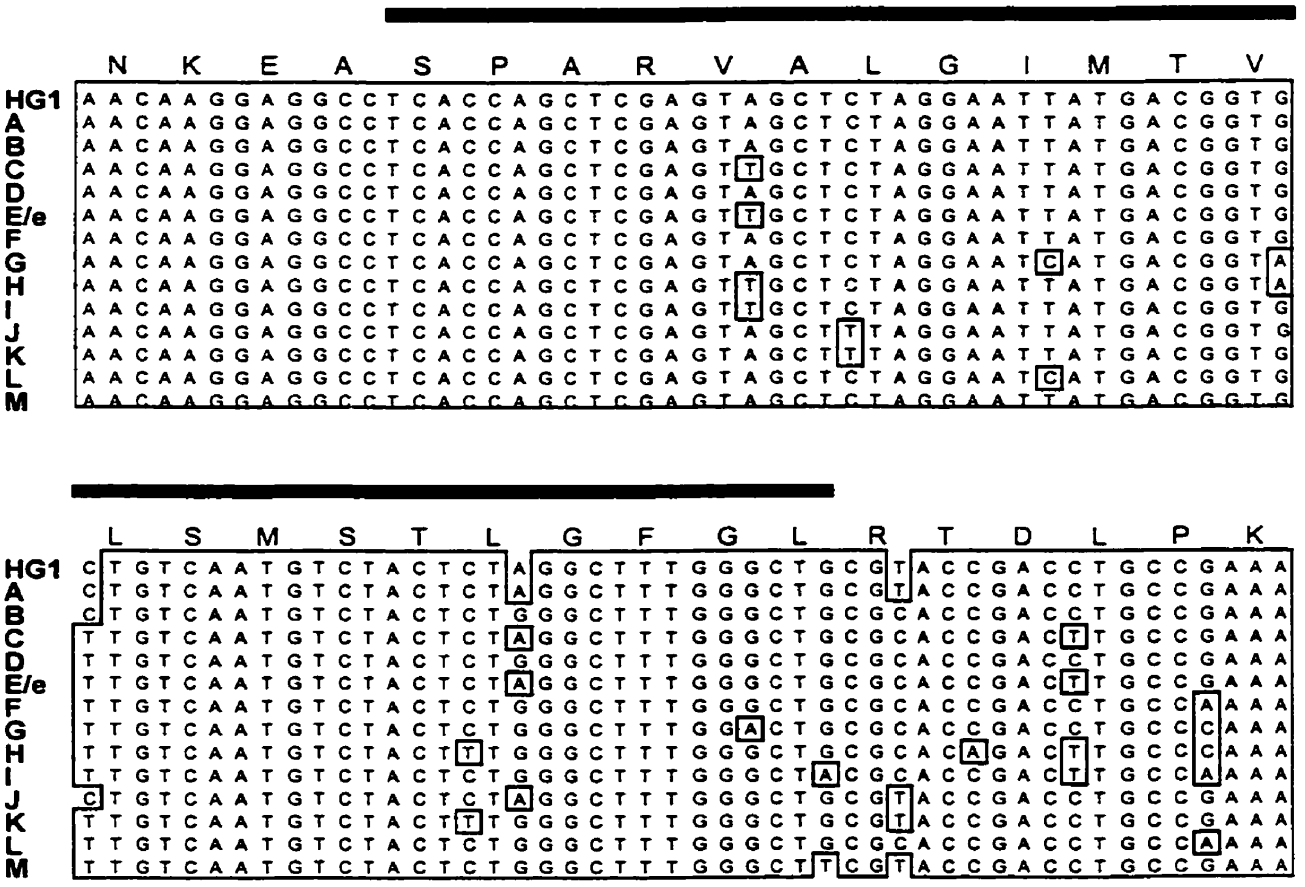

Figure 3. Nucleotide sequences of HG1 alleles in the region of M2. The deduced amino acid sequence is indicated above the nucleotide sequences. The black line indicates the M2 domain.

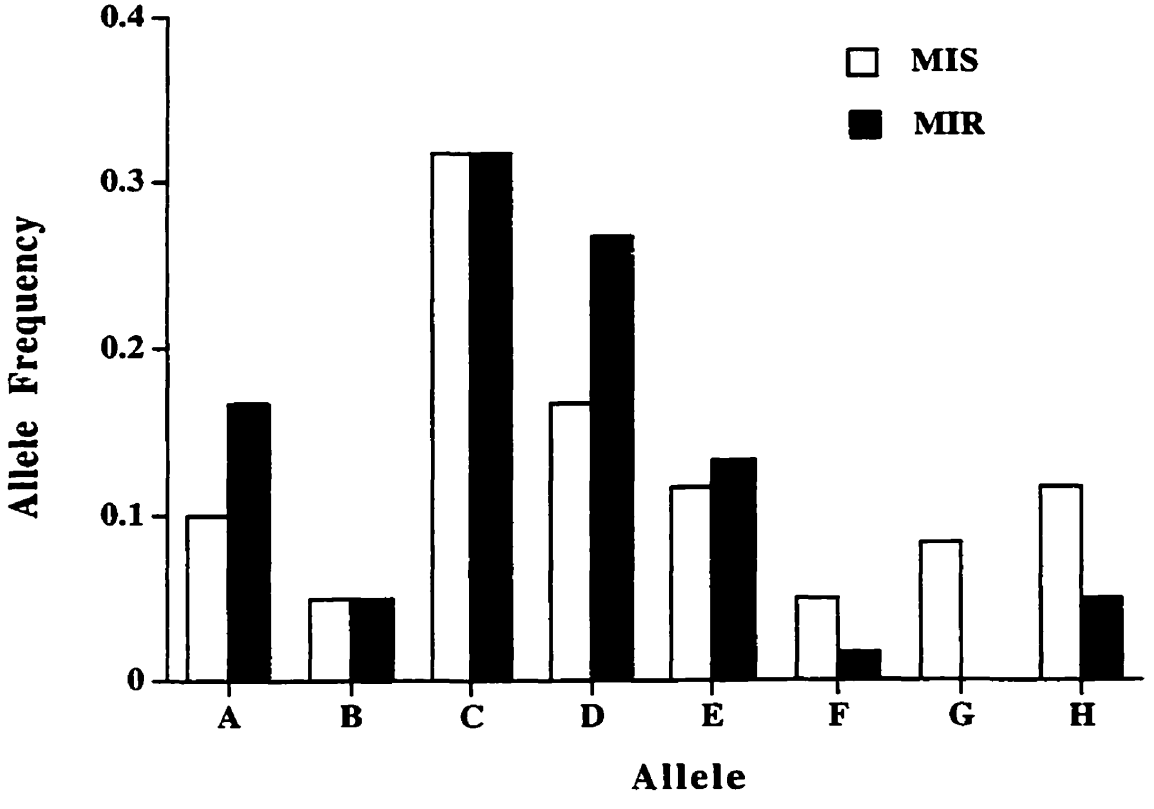

Figure 4. Allele frequencies of a PEPCK gene from Merck unselected (MIS) and ivermectin-selected (MIR) strains of *H. contortus*.

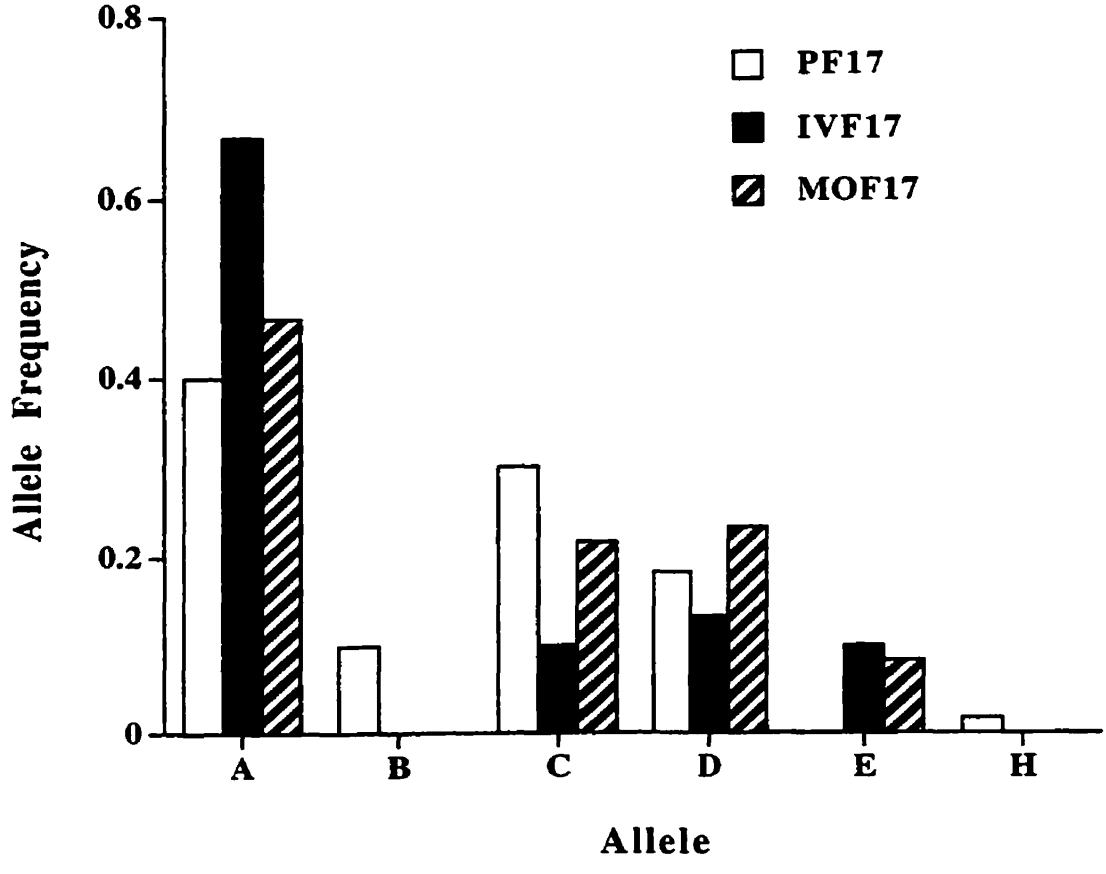

Figure 5. Allele frequencies of a PEPCK gene from Fort Dodge unselected (PF17) and ivermectin- (IVF17) and moxidectin-selected (MOF17) strains of *H. contortus*.

MOF17 relative to PF17, however, are great enough to indicate that selection is occurring.

Allele frequencies of the PEPCK gene from the Merck and Fort Dodge strains are presented in Fig. 4 and Fig. 5, respectively. Alleles A, C, and D were the most common alleles in both sets of strains. No statistically significant differences were found in allele frequencies between MIS and MIR. In the Fort Dodge strains, allele A increased in frequency from 0.400 in PF17 to 0.667 in IVF17, and allele C decreased in frequency from 0.300 in PF17 to 0.100 in IVF17. Despite these changes, a Chi-square analysis found no statistically significant differences in allele frequencies between these two strains. Similarly, no significant difference was found between PF17 and MOF17 or between IVF17 and MOF17. All strains were in Hardy-Weinberg equilibrium.

The SSCP analyses of the nAChR gene from the Merck (Fig. 6) and Fort Dodge (Fig. 7) strains also found no significant differences in allele frequencies between the unselected and derived selected strains. All strains were in Hardy-Weinberg equilibrium at this locus.

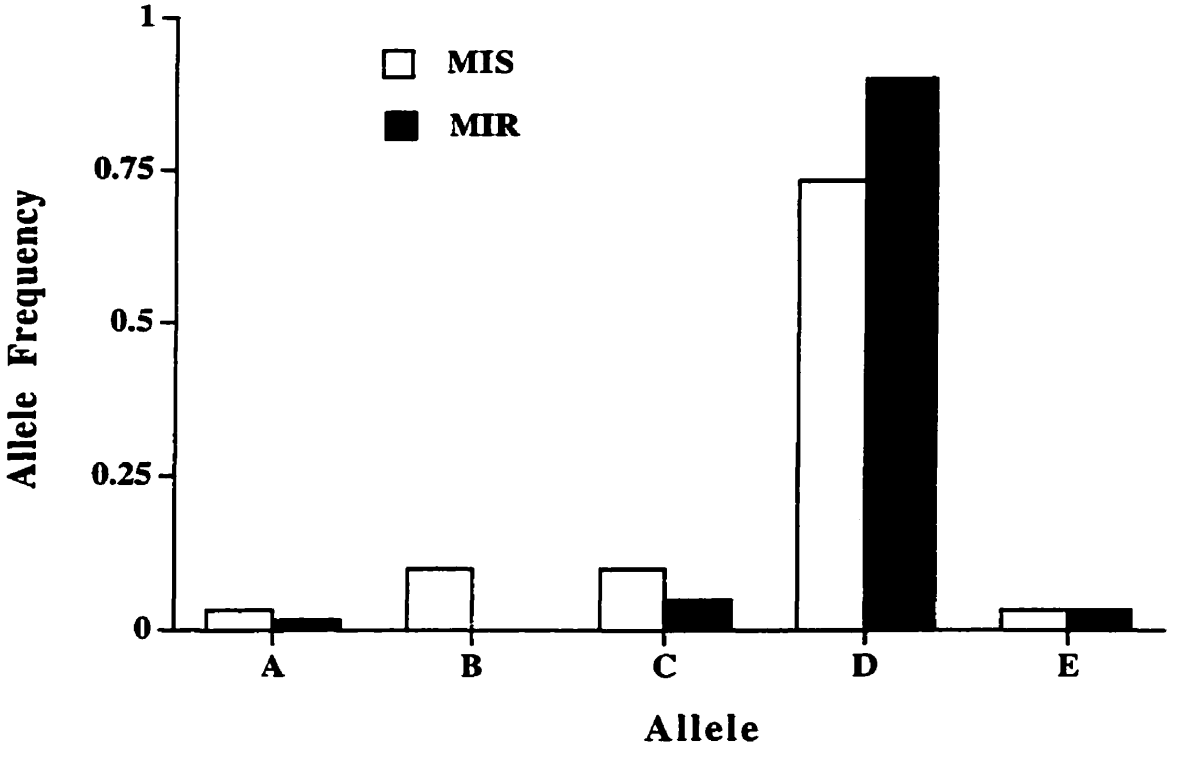

Figure 6. Allele frequencies of a nAChR gene from Merck unselected (MIS) and ivermectin-selected (MIR) strains of *H. contortus*.

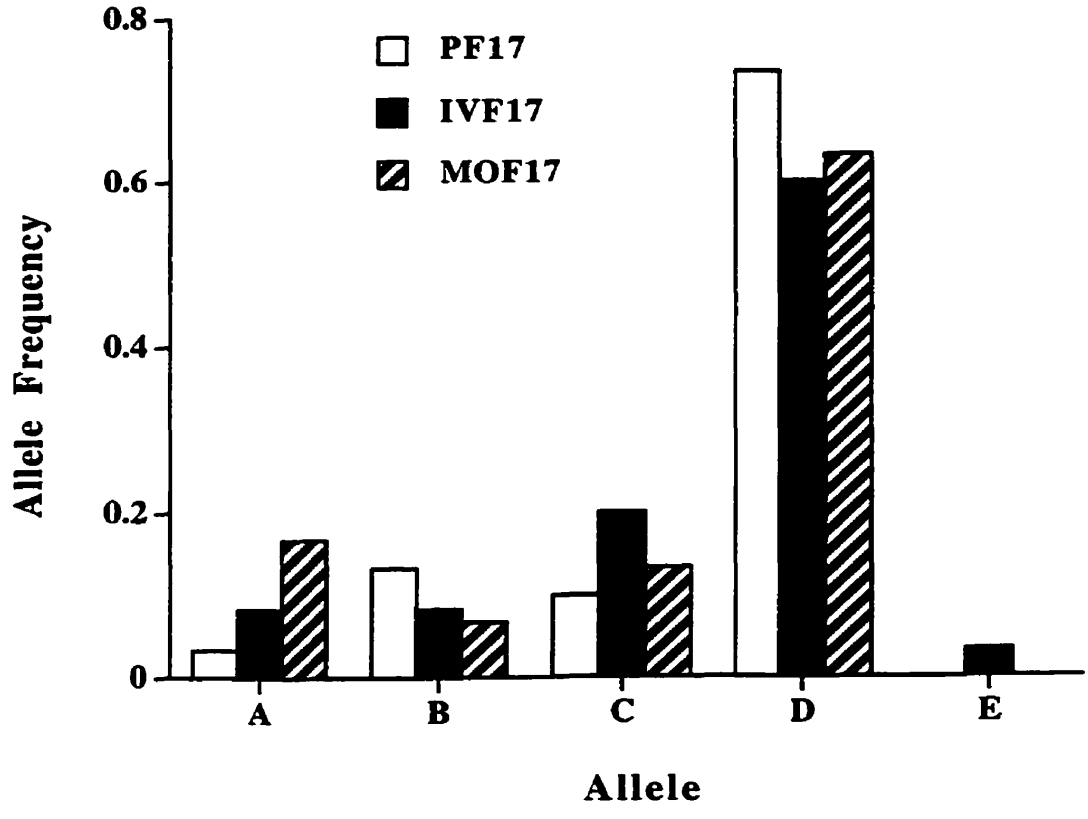

Figure 7. Allele frequencies of a nAChR gene from Fort Dodge unselected (PF17) and ivermectin- (IVF17) and moxidectin-selected (MOF17) strains of *H. contortus*.

DISCUSSION

The apparent selection for alleles associated with resistance to IVM and MOX at a gene encoding a GABA receptor subunit in *H. contortus* implicates the GABA receptor in the modes of action of these anthelmintics. The lack of significant changes in allele frequencies at the PEPCK and nAChR loci suggest that the changes seen in HG1 are due to selection occurring during anthelmintic treatment and not due to possible inbreeding or population bottlenecks. Early studies on the paralyzing action of AVM in nematodes also suggested that GABA receptors were involved [10,14], but later studies have focused attention on other chloride channels [26,27], particularly glutamate-gated chloride (GluCl) channels [28-32,21].

The *in vitro* evidence that AVM induces hyperpolarizing chloride currents by opening GluCl channels is clear. These channels are expressed in muscles of the pharynx [31,33], and *in vivo* experiments have shown that AVM inhibits pharyngeal pumping in nematodes [34-36]. Yet to date, only the GluClα2 gene in *C. elegans* and the GluClβ gene in *H. contortus* have been shown to be expressed in nonpharyngeal cells. GluClα2 is also expressed in a few neurons of the head and two ventral cord neurons near the anus, and weakly expressed in some ventral neurons that contribute to the dorsal and ventral sublateral nerve cords [32]. GluClβ in *H. contortus* is expressed in motor commissures anterior to the vulva and possibly in the nerve cord [37]. Unless the ventrally located neurons in *C. elegans* are key inhibitory neurons affecting contraction of the body musculature, or unless other GluCl subunits are expressed in tissues affecting locomotion, attributing the locomotory paralyzing effects of AVM to GluCl channels in *C. elegans* is difficult. In *H. contortus*, the presence of GluClβ subunits in motorneurons, possibly in

the nerve cord itself, suggests that paralysis of the body musculature may be mediated in part through GluCl channels.

GABAergic neurons are major components of the nematode nervous system that control locomotory functions [38]. The action of AVM at GABA receptors on somatic muscle cells, however, has clearly been demonstrated to be antagonistic rather than agonistic [10,12-14,27]. AVM, then, does not paralyze nematodes by hyperpolarizing muscle cells. Kass and co-workers [10,14] found that AVM interferes with the transmission of signal between interneurons and excitatory motorneurons in *Ascaris*, but inhibitory interneurons have not been described from nematodes, whether glutamatergic or GABAergic. Such a mechanism, though, if GABAergic, would also require a different subtype of GABA receptor from those found on somatic muscle, one where AVM is agonistic rather than antagonistic. Evidence consistent with this mechanism is provided by [14]. Muscimol and piperazine, known agonists at GABA receptors in *Ascaris*, mimic the effects of AVM on blocking interneuron-excitatory motorneuron transmission. Also, picrotoxin, a GABA receptor antagonist, reverses the effects of AVM on interneuron-excitatory motorneuron transmission but not at the neuromuscular junction. AVM does not cause a flaccid paralysis in intact *Ascaris*, nor a hypercontraction. When injected into the pseudocoelomic cavity, AVM induces paralysis without an overall lengthening or shortening of the body [39]. This observation is consistent with a neural, rather than a muscular, site of action for AVM.

Electrophysiological studies on the action of AVM in nematodes presently cannot clearly define a role for GABA receptors. Our findings, however, suggest that GABA receptors do contribute to the action of IVM and MOX. We have previously reported selection at a gene encoding a GluCl subunit [21], supporting the contribution of GluCl

channels to AVM action. The degree of selection, though, is greater at the HG1 GABA receptor gene than at the GluCl channel gene, from the same individual worms of both sets of strains. This difference rules out genetic hitch-hiking or epistasis with the GluCl gene or protein as being responsible for the allele frequency differences found at the GABA receptor gene. We hypothesize that AVM acts as an agonist at a yet unknown subtype of GABA receptor located in the ventral nerve cord of nematodes. Paralysis results from the hyperpolarization of neurons, and thus from an inhibition of excitatory signals to the muscles rather than from an inhibition of muscle cells directly. Evidence consistent with this hypothesis has recently been provided [40]. The GABA receptor subunit analysed in the present study is expressed in *H. contortus* along the ventral nerve cord and in several neurons in the head, possibly nerve ring neurons. No expression in somatic muscles was seen. In *Ascaris*, however, expression of the homologue of HG1 is seen in somatic muscles but not in the nervous system. HG1 and its homologues, then, appear to have different physiological functions in different species. Another GABA receptor subunit may perform the functions in *Ascaris* that HG1 performs in *H. contortus*. If so, AVM may act as an agonist at this subunit, causing hyperpolarization of ventral cord neurons and thus paralysis. Testing of the hypothesis of neural inhibition by AVM via GABA receptors must await the identification and physiological characterization of further subunits from *Haemonchus* and *Ascaris*.

Studies of AVM binding to membrane preparations from *C. elegans* [26] and *H. contortus* [41] have identified a single high-affinity binding site. The concentrations of AVM used in these studies are comparable to concentrations that inhibit pharyngeal pumping. The binding site is thus likely to be a GluCl channel. Concentrations of AVM required to inhibit motility in *H. contortus*, however, are 100-fold higher [36], suggesting

the presence of a low-affinity binding site, which is not likely to be a GluCl channel, but may be a GABA receptor. Binding studies using higher AVM concentrations may identify this low-affinity binding site.

The identity, at the amino acid level, of the M2 domains of all alleles indicates that this region of the protein does not directly contribute to any potential mechanism of resistance to IVM or MOX involving this HG1 GABA subunit. IVM or MOX may, of course, interact with another region of subunit proteins. The lipophilic nature of macrocyclic lactones could implicate other transmembrane domains. In the nicotinic acetylcholine receptor, mutations in M4, the transmembrane domain believed to be exposed to the lipid bilayer, can affect the gating kinetics of the receptor [42].

The present analysis has assumed that GABA receptor subunits are each encoded by separate genes, and that HG1 is one such gene. This assumption may not be true. In the free-living nematode *C. elegans*, only one gene has so far been found to encode GABA receptor subunits (B.A. Bamber and E.M. Jorgensen, personal communication). This gene contains three tandem open reading frames, none of which possess all domains typically found in subunits of ligand-gated ion channels. Subunits appear to be produced by complex splicing from among the three open reading frames. If GABA receptor subunit formation occurs by a similar process in *H. contortus*, then the selection reported here may represent selection for alleles of this large GABA receptor gene and not specifically for alleles of the HG1 subunit. A mutation that confers resistance to AVM occurring anywhere in the GABA receptor gene could, due to genetic hitch-hiking, be detected by changes in allele frequencies from analyses of any region of the gene. The expression of the HG1 subunit in the ventral nerve cord, then, may offer no clues to the location of a GABA receptor that may contribute to AVM resistance. The observation of

selection at HG1, however, still implicates GABA receptors as a site of action of AVM and MOX.

The relative importance of pharyngeal inhibition, likely to involve GluCl channels, and inhibition of motility, likely to involve GABA receptors but possibly GluCl channels as well, in the expulsion of *H. contortus* by macrocyclic lactones has yet to be determined. The examination of allele frequencies of the genes encoding the subunits of these chloride channels from field isolates resistant to macrocyclic lactones may shed some light on the cause of expulsion.

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REFERENCES

[1] Unwin, N. The structure of ion channels in membranes of excitable cells. Neuron 1989;3:665-76.

[2] Hucho, F., Oberthur, W., Lottspeich, F. The ion channel of the nicotinic acetylcholine receptor is formed by the homologous helices MII of the receptor subunits. FEBS Lett 1986;205:137-42.

[3] Imoto, K., Methfessel, C., Sakmann, B., Mishina, M., Mori, Y., Konno, T., Fukuda, K., Kurasaki, M., Bujo, H., Fujita, Y., Numa, S. Location of a delta-subunit region determining ion transport through the acetylcholine receptor channel. Nature 1986;324:670-4.

[4] Unwin, N. Nicotinic acetylcholine receptor at 9Å resolution. J Mol Biol 1993;229:1101-24.

[5] Bloomquist, J.R. Toxicology, mode of action and target site-mediated resistance to insecticides acting on chloride channels. Comp Biochem Physiol C 1993;106:301-14.

[6] Casida, J.E. Insecticide action at the GABA-gated chloride channel: recognition, progress, and prospects. Insect Biochem Physiol 1993;22:13-23.

[7] Burg, R.W., Miller, B.M., Baker, E.E., Birnbaum, J., Currie, J.A., Harman, R., Kong, V.L., Monaghan, R.L., Olson, G., Putter, I., Tunac, J.D., Wallick, H., Stapley, E.O., Oiwa, R., Omura, S. Avermectins, new family of potent anthelmintic agents: producing organism and fermentation. Antimicrob Agents Chemother 1979;15:361-7.

[8] Clark, J.M., Campos, F., Scott, J.G., Bloomquist, J.R. Resistance to avermectins: extent, mechanisms, and management implications. Ann Rev Entomol 1995;40:1-30.

[9] Martin, R.J. γ-aminobutyric acid- and piperazine-activated single channel currents from *Ascaris* suum body muscle. Br J Pharmacol 1985;84:445-61.

[10] Kass, I.S., Wang, C.C., Walrond, J.P., Stretton, A.O.W. Avermectin $B1_a$, a paralyzing anthelmintic that affects interneurons and inhibitory motoneurons in *Ascaris*. Proc Natl Acad Sci USA 1980;77:6211-5.

[11] Boisvenue, R.J., Brandt, M.C., Galloway, R.B., Hendrix, J.C. *In vitro* activity of various anthelmintic compounds against *Haemonchus contortus* larvae. Vet Parasitol 1983;13:341-7.

[12] Holden-Dye, L., Hewitt, G.M., Wann, K.T., Krogsgaard-Larsen, P., Walker, R.J. Studies involving avermectin and the 4-aminobutyric acid (GABA) receptor of *Ascaris suum* muscle. Pest Sci 1988;24:231-45.

[13] Holden-Dye, L., Walker, R.J. Avermectin and avermectin derivatives are antagonists at the 4-aminobutyric acid (GABA) receptor on the somatic muscle cells of *Ascaris*; is this the site of anthelmintic action? Parasitol 1990;101:265-71.

[14] Kass, I.S., Stretton, A.O., Wang, C.C. The effects of avermectin and drugs related to acetylcholine and 4-aminobutyric acid on neurotransmission in *Ascaris suum*. Mol Biochem Parasitol 1984;13:213-25.

[15] Pribilla, I., Takagi, T., Langosch, D., Bormann, J., Betz, H. The atypical M2 segment of the β subunit confers picrotoxinin resistance to inhibitory glycine receptor channels. EMBO J 1992;11:4305-11.

[16] Gurley, D., Amin, J., Ross, P.C., Weiss, D.S., White, G. Point mutations in the M2 region of the α, β, or γ subunit of the $GABA_A$ channel that abolish block by picrotoxin. Receptors and Channels 1995;3:13-20.

[17] Bertrand, D., Galzi, J.L., Devillers-Thiery, A., Bertrand, S., Changeux, J.P. Mutations at two distinct sites within the channel domain M2 alter calcium permeability of neuronal α7 nicotinic receptor. Proc Natl Acad Sci USA 1993;90:6971-5.

[18] Etter, A., Cully, D.F., Schaeffer, J.M., Liu, K.K., Arena, J.P. An amino acid substitution in the pore region of a glutamate-gated chloride channel enables the coupling of ligand binding to channel gating. J Biol Chem 1996;271:16035-9.

[19] Galzi, J.-L., Devillers-Thiery, A., Hussy, N., Bertrand, S., Changeux, J.-P., Bertrand, D. Mutations in the channel domain of a neuronal nicotinic receptor convert ion selectivity from cationic to anionic. Nature 1992;359:500-5.

[20] ffrench-Constant, R.H., Rocheleau, T.A., Steichen, J.C., Chalmers, A.E. A point mutation in a *Drosophila* GABA receptor confers insecticide resistance. Nature 1993;363:449-51.

[21] Blackhall, W.J., Pouliot, J.-F., Prichard, R.K., Beech, R.N. *Haemonchus contortus*: selection at a glutamate-gated chloride channel gene in ivermectin- and moxidectin-selected strains. Exp Parasitol 1998;90:42-8.

[22] Beech, R.N., Prichard, R.K., Scott, M.E. Genetic variability of the β -tubulin genes in benzimidazole-susceptible and -resistant strains of *Haemonchus contortus*. Genetics 1994;138:103-10.

[23] Laughton, D.L., Amar, M., Thomas, P., Towner, P., Harris, P., Lunt, G.G., Wolstenholme, A.J. Cloning of a putative inhibitory amino acid receptor from the parasitic nematode *Haemonchus contortus*. Receptors and Channels 1994;2:155-63.

[24] Klein, R.D., Winterrowd, C.A., Hatzenbuhler, N.T., Shea, M.H., Favreau, A., Nulf, N.C., Geary, T.G. Cloning of a cDNA encoding phosphoenolpyruvate carboxykinase from *Haemonchus contortus*. Mol Biochem Parasitol 1992;50:285-94.

[25] Hoekstra, R., Visser, A., Wiley, L.J., Weiss, A.S., Sangster, N.C., Roos, M.H. Characterization of *Haemonchus contortus* in relation to levamisole resistance. Mol Biochem Parasitol 1997;84:179-87.

[26] Schaeffer, J.M., Haines, H.W. Avermectin binding in *Caenorhabditis elegans*. A two state model for the avermectin binding site. Biochem Pharmacol 1989;38:2329-38.

[27] Martin, R.J., Pennington, A.J. A patch-clamp study of effects of dihydroavermectin on *Ascaris* muscle. Br J Pharmacol 1989;98:747-56.

[28] Arena, J.P., Liu, K.K., Paress, P.S., Schaeffer, J.M., Cully, D.F. Expression of a glutamate-activated chloride current in *Xenopus* oocytes injected with *Caenorhabditis elegans* RNA: evidence for modulation with avermectin. Mol Brain Res 1992;15:339-48.

[29] Arena, J.P., Liu, K.K., Paress, P.S., Frazier, E.G., Cully, D.F., Mrozik, H., Schaeffer, J.M. The mechanism of action of avermectins in *Caenorhabditis elegans*: correlation between activation of glutamate-sensitive chloride current, membrane binding, and biological activity. J Parasitol 1995;81:286-94.

[30] Cully, D.F., Vassilatis, D.K., Liu, K.K., Paress, P.S., Van der Ploeg, L.H.T, Schaeffer, J.M., Arena, J.P. Cloning of an avermectin-sensitive glutamate-gated chloride channel from *Caenorhabditis elegans*. Nature 1994;371:707-11.

[31] Dent, J.A., Davis, M.W., Avery, L. *avr-15* encodes a chloride channel subunit that mediates inhibitory glutamatergic neurotransmission and ivermectin sensitivity in *Caenorhabditis elegans*. EMBO J 1997;16:5867-79.

[32] Vassilatis, D.K., Arena, J.P., Plasterk, R.H.A., Wilkinson, H.A., Schaeffer, J.M., Cully, D.F., Van der Ploeg, L.H.T. Genetic and biochemical evidence for a novel avermectin-sensitive chloride channel in *Caenorhabditis elegans*. J Biol Chem 1997;272:33167-74.

[33] Laughton, D.L., Lunt, G.G., Wolstenholme, A.J. Reporter gene constructs suggest that the *Caenorhabditis elegans* avermectin receptor β-subunit is expressed solely in the pharynx. J Exp Biol 1997;200:1509-14.

[34] Bottjer, K.P., Bone, L.W. *Trichostrongylus colubriformis*: effect of anthelmintics on ingestion and oviposition. Int J Parasitol 1985;15:501-3.

[35] Avery, L., Horvitz, H.R. The effects of starvation and neuroactive drugs on feeding in *Caenorhabditis elegans*. J Exp Zool 1990;253:263-70.

[36] Geary, T.G., Sims, S.M., Thomas, E.M., Vanover, L., Davis, J.P., Winterrowd, C.A., Klein, R.D., Ho, N.F.H., Thompson, D.P. *Haemonchus contortus*: ivermectin-induced paralysis of the pharynx. Exp Parasitol 1993;77:88-96.

[37] Delaney, N.S., Laughton, D.L., Wolstenholme, A.J. Cloning and localisation of an avermectin receptor-related subunit from *Haemonchus contortus*. Mol Biochem Parasitol 1998;97:177-87.

[38] McIntire, S.L., Jorgensen, E., Kaplan, J., Horvitz, H.R. The GABAergic nervous system of *Caenorhabditis elegans*. Nature 1993;364:337-41.

[39] Kass, I.S., Larsen, D.A., Wang, C.C., Stretton, A.O.W. *Ascaris suum*: differential effects of avermectin $B1_a$ on the intact animal and neuromuscular strip preparations. Exp Parasitol 1982;54:166-74.

[40] Skinner, T.M., Bascal, Z.A., Holden-Dye, L., Lunt, G.G., Wolstenholme, A.J. Immunocytochemical localization of a putative inhibitory amino acid receptor subunit in the parasitic nematodes *Haemonchus contortus* and *Ascaris suum*. Parasitol 1998;117:89-96.

[41] Rohrer, S.P., Birzin, E.T., Eary, C.H., Schaeffer, J.M., Shoop, W.L. Ivermectin binding sites in sensitive and resistant *Haemonchus contortus*. J Parasitol 1994;80:493-7.

[42] Bouzat, C., Roccamo, A.M., Garbus, I., Barrantes, F.J. Mutations at lipid-exposed residues of the acetylcholine receptor affect its gating kinetics. Mol Pharmacol 1998;54:146-53.

CONNECTING STATEMENT III

The proposed mechanisms of resistance to AVM/milbemycins presented in Chapters II and III involve changes in the target site of the anthelmintic. Resistance, however, can arise through mechanisms acting independent of the target. Overexpression of the multidrug transporter protein, P-glycoprotein, contributes to resistance to a wide range of drugs in human cancers. The finding of overexpression of a gene encoding a P-glycoprotein in *H. contortus* led to a search for an association between genetic polymorphism in this gene and resistance to ivermectin or moxidectin. Chapter IV presents evidence of such an association.

CHAPTER IV

Selection at a P-glycoprotein Gene in Ivermectin- and Moxidectin-Selected Strains of *Haemonchus contortus*. (Manuscript III)

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ABSTRACT

Resistance to anthelmintics that are used to control parasite populations in domestic animals has become a serious problem worldwide. The development of resistance is an evolutionary process that leads to genetic changes in parasite populations in response to drug exposure. The anthelmintic ivermectin is known to bind to the human membrane transport protein, P-glycoprotein, and P-glycoprotein-deficient mice treated with ivermectin have shown signs of neurotoxicity. P-glycoprotein is believed to be involved in the multidrug resistance phenotype seen in some human cancers and for drug resistance in some protists. We have examined the genetic variation of a P-glycoprotein homologue from the nematode *Haemonchus contortus* to see if an association exists between specific alleles of this gene and survival to exposure to ivermectin or moxidectin. Two parasite strains passaged without drug treatment and three strains, subjected to anthelmintic selection and derived from the unselected strains, were examined. Allelic variation in the unselected strains showed this locus to be highly polymorphic. Chi-square analyses of allele frequencies showed significant differences between the unselected and the drug-selected derived strains. In all three drug-selected strains, an apparent selection for the same allele was observed. These findings suggest that P-glycoprotein may be involved in resistance to both ivermectin and moxidectin in *H. contortus*.

INTRODUCTION

Haemonchus contortus is a common parasitic nematode of livestock around the world and causes significant losses in production [1]. Resistance to all the broad-spectrum anthelmintic groups used to control *Haemonchus* infections has been reported. The development of resistance to anthelmintics can occur in a genetically diverse population when individuals that are able to tolerate the effects of the drugs survive and pass on their genes to the next generation [2]. Over time, the frequency of such individuals increases in the population. *H. contortus* has been shown to possess considerable genetic variability [3,4,5]. In strains resistant to benzimidazoles, the variability is greatly reduced at the loci believed to be responsible for tolerance to the drugs [4,6,7]. The reduction of variability by selection of alleles associated with resistance, whether at a locus directly involved with resistance or at a nearby locus, then, can be evidence that resistance is developing in the population.

Ivermectin (IVM) and moxidectin (MOX) are anthelmintics commonly used for the control of parasitic nematodes in domestic animals. IVM belongs to the class of drugs known as the avermectins, and MOX is a milbemycin. Both are macrocyclic lactones and share a 16-membered ring. These drugs are thought to bind to and gate, or potentiate, glutamate-gated chloride channels in *Caenorhabditis elegans* [8,9]. The opening of the channels leads to the hyperpolarization of neuromuscular cells and to paralysis. Rohrer *et al.* [10] investigated the binding of [^{3}H] IVM to membrane preparations from IVM-susceptible and -resistant strains of *H. contortus* and concluded that resistance is not due to an alteration in drug binding to the target site. Some studies suggest that MOX is effective against IVM-resistant *H. contortus* [11,12] suggesting either different modes of

action for these two drugs or differences in response to a resistance mechanism. Other studies, though, have shown experimental evidence for co-resistance between IVM and MOX and conclude that the apparent efficacy of MOX against IVM-resistant parasites is dose-dependent [13,14]. Structural similarity, the ability to interact with a common target protein, and a concentration-dependent cross-resistance suggest that the avermectins and the milbemycins may share common mechanisms of resistance in nematodes. However, factors which modulate drug concentration at the site of action may determine relative expression of resistance between different avermectins and milbemycins.

Resistance to IVM in gastrointestinal parasites of livestock in the field was first reported from South Africa [15] and Brazil [16] shortly after the introduction of this anthelmintic into the field. Since then, resistance to IVM has been reported from many countries around the world: the United States [17], New Zealand [18], United Kingdom [19], Australia [20], Malaysia [21], and Kenya [22]. This rapid increase in the incidence of resistance around the world has led to an effort to understand the development of macrocyclic lactone resistance at the genetic level.

A full-length P-glycoprotein (P-gp) cDNA from *H. contortus* has recently been cloned and sequenced [23] (GenBank accession no. AF003908). Overexpression of homologues of this gene from rodents and humans is believed to be responsible for some cases of multidrug resistance in cell cultures [24]. P-gp's are membrane proteins that bind and remove hydrophobic cytotoxins from the lipid bilayer and the cytoplasm [25]. Schinkel *et al.* [26] found that mice became sensitive to IVM when a rodent P-gp homologue, mdr1a, was disrupted. IVM has also been shown to be a substrate for P-gp in mammalian cells [27,28]. Studies on a P-gp homologue isolated from *H. contortus* suggested that this gene is overexpressed in strains resistant to IVM relative to sensitive

strains [23]. A genetic polymorphism detected by Southern blot analysis was also found associated with IVM resistance. Furthermore, the multidrug resistance reversing agent verapamil increased the efficacy of MOX against resistant *H. contortus* [23]. These findings raised the possibility that IVM may be a substrate for P-gp in nematodes and that P-gp may therefore be involved in resistance to IVM in *H. contortus*.

Four genes that encode P-gp's have been identified from *C. elegans* [29]. One of these, *pgp-1*, when deleted along with a multidrug resistance-associated protein homologue, *mrp-1*, confers hypersensitivity to heavy metals [30]. Another, *pgp-3*, when deleted, confers sensitivity to chloroquine and colchicine [31]. Since IVM is known to interact with mammalian P-gp's, and nematode P-gp's appear to play some role in resistance to cytotoxic substances, we analyzed a P-gp homologue from *H. contortus* to see if an association exists between the population genetics of this gene and exposure to either IVM or MOX.

MATERIALS AND METHODS

Parasite strains. Two strains of *H. contortus* were supplied by Merck & Co., Inc., Whitehouse Station, NJ, USA. Both strains were derived from the same parent population. One strain (MIS) was passaged through sheep without drug treatment, and the other strain (MIR) was passaged in parallel but was challenged at each generation with IVM. After 17 generations, 10-fold more IVM was required to kill 95% of the adult population than was required for the parental population [10]. Three strains of *H. contortus* were supplied by Fort Dodge Animal Health, Princeton, NJ, USA. All three strains from Fort Dodge were derived from the same parent population. One strain (PF17) was passaged through sheep for 17 generations without drug treatment. The other two strains were passaged in parallel, but at each generation were treated with IVM (IVF17) or MOX (MOF17) at dose rates that would be 80-95% efficacious. The drug dosages in the final generation were 0.15 mg/kg for IVM and 0.015 mg/kg for MOX. At the twelfth generation, the ED95 for PF12 was 0.01125 mg/kg body weight for IVM and 0.00179 mg/kg body weight for MOX [32]. The ED95 for IVF12 was 0.10874 mg IVM/kg body weight, and for MOF12 was 0.00949 mg MOX/kg body weight [32].

DNA isolation. Individual adult male worms were collected live from the abomasums of sheep and incubated in phosphate-buffered saline for 2 hr at 37°C. DNA was then extracted as described in [4] from 30 adult males from MIS and MIR, and from 40 adult males from PF17, IVF17, and MOF17.

Polymerase chain reaction (PCR) amplification. A sense primer PGP2S, 5' GAAATGACTCAAGCACAAG 3', was designed based on the cDNA sequence reported by Xu *et al.* [23]. An antisense primer MX-D, 5' AGACAAAGACATTCAGAG 3', was designed from intronic sequence. In cases when amplification was poor, an antisense primer PGP1A, 5' AGTGAACTAAGATGGGGT 3', located 50 bp downstream from MX-D, was used with PGP2S, and the product was reamplified with PGP2S and MX-D. The PCR product obtained with PGP2S and MX-D from genomic DNA is approximately 840 bp in length and amplifies a region that encodes a portion of the central cytoplasmic loop of the protein's primary structure (Fig. 1). The reaction mixture contained 5 µl 10X *Taq* buffer, 5 µl 2 mM dNTPs, 4 µl 25 mM $MgCl_2$, 1 µl 20 mM primer solutions, 1 unit *Taq* polymerase, approximately 2 ng DNA template, and water to a final volume of 50 µl. PCR was performed on an MJ Research, Inc. (Watertown, MA) PTC-100 Programmable Thermal Controller. Amplification conditions were: 95°C for 4 min followed by 40 cycles of 95°C for 15 s, 53°C for 30 s, and 70°C for 1 min and 30 s with a final extension at 70°C for 5 min. PCR products were visualized on a 1% agarose gel containing 0.5 µg/ml ethidium bromide under UV illumination.

Restriction fragment length polymorphism analysis (RFLP). PCR products were digested with the restriction enzymes *Dde*I, *Hinf*I, *Rsa*I, and *Alu*I as described by the manufacturer (Promega Corporation, Madison, WI). For each reaction, 10 µl of the PCR products were digested in a total volume of 20 µl. The reaction products were electrophoresed through a 6% nondenaturing polyacrylamide gel, stained with ethidium bromide, visualized under UV illumination, and photographed. Alleles were identified

with each enzyme when found as homozygotes or as commonly occurring fragment patterns. The genotype of each worm was determined by distinguishing unique combinations of alleles identified by the digestions with each individual enzyme. Fig. 2 shows a typical polyacrylamide gel containing PCR products amplified from individual worms and digested with *Hinf*I.

Data analysis. Chi-square analyses of allele frequencies were performed between strains passaged without drug treatment and the corresponding derived strains exposed to drugs. A Chi-square analysis was also performed on genotype frequencies from both sets of strains to test for Hardy-Weinberg equilibrium. Any excess of homozygotes could indicate the presence of alleles that failed to amplify during PCR. Alleles whose counts were 5 or lower in a population were grouped together for the Chi-square analyses. The count of rare alleles in a population can vary from sample to sample, and the grouping of their counts to form a single category representing "rare alleles" lessens the statistical impact they would have in a Chi-square analysis.

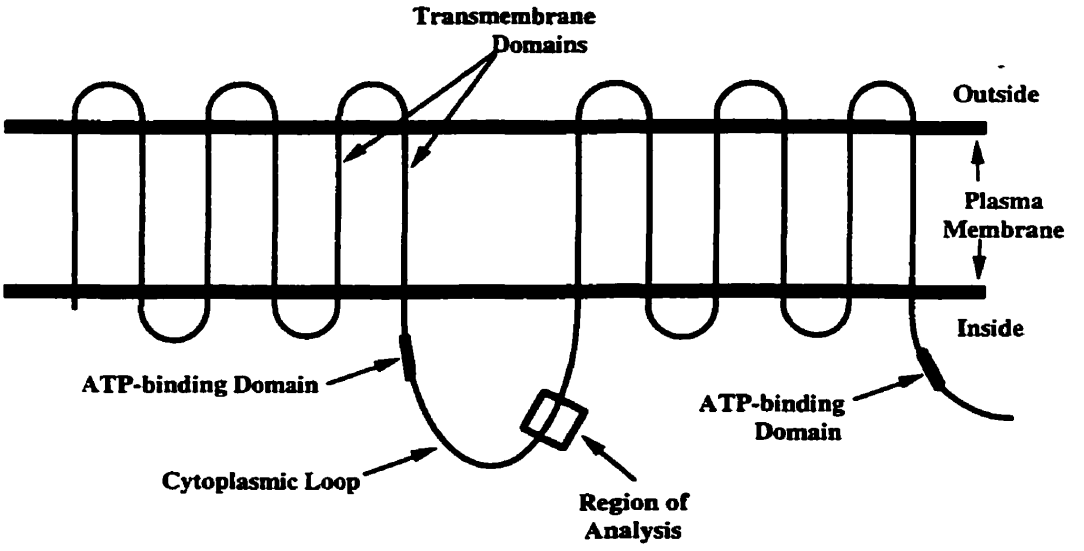

Figure 1. Schematic drawing of P-gp in cell membrane. The box indicates the approximate region of genomic DNA amplified by PCR and analysed by RFLP.

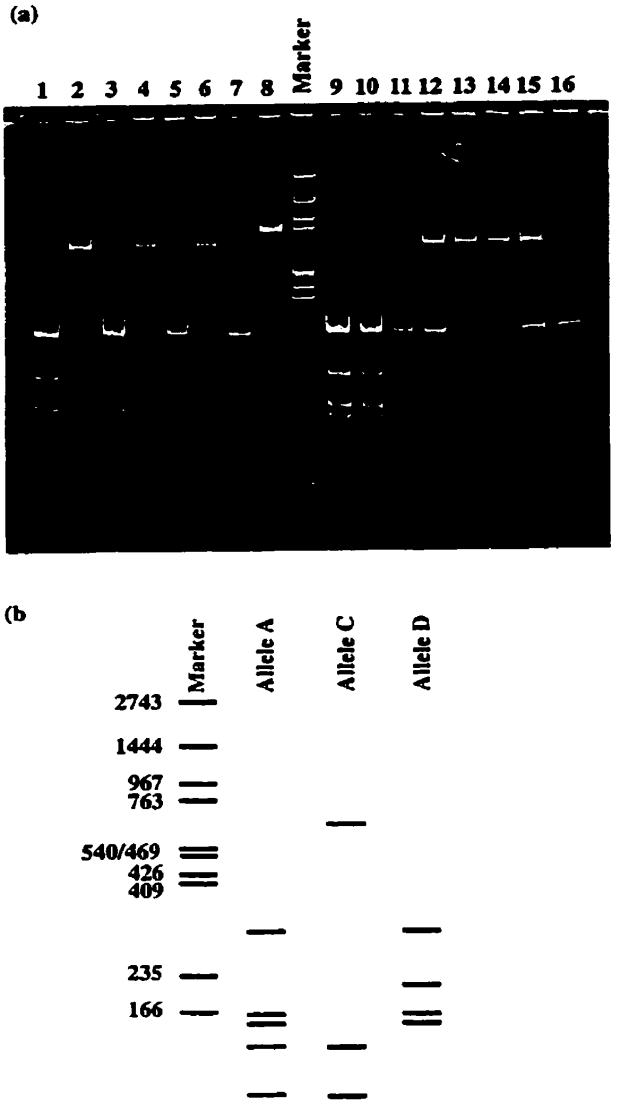

Figure 2. (a) Photograph of P-gp PCR products digested with HinfI and electrophoresed through a polyacrylamide gel. Lane 8 is undigested PCR product. Lanes 1-7 and 9-16 represent digestion products of individual worms with *HinfI* genotypes: Lane 1, AD; Lane 2, CC; Lane 3, AD; Lane 4, AC; Lane 5, DD; Lane 6, CC; Lane 7, AA; Lane 9, AD; Lane 10, AD; Lane 11, DD; Lane 12, AC; Lane 13, CC; Lane 14, CC; Lane 15, CD; Lane16, DD. **(b)** Schematic representation of DNA size marker and alleles shown in (a). Marker fragment sizes given in base pairs.

RESULTS

Merck strains. Genetic variation at this P-gp locus was examined in two sets of strains of *H. contortus*. In the Merck strains (Fig. 3), a reduction in genetic variability as measured by the number of alleles present was not apparent in the strain (MIR) passaged with drug treatment when compared to the unselected strain (MIS). However, the allele frequencies of these two strains were significantly different ($p < 0.01$). A total of 11 alleles was found, with each strain possessing seven alleles. Both strains possessed the three most common alleles (A, B, and C), but MIS possessed four unique alleles (G, H, I, and K), as did MIR (alleles D, E, F, and J). This difference may simply be due to random sampling, as the numbers involved are small in most cases. Allele D, which had a frequency of 0.15 in MIR but was not seen in MIS, may represent an allele that underwent positive selection during drug treatment. Of particular interest is the change occurring at the three most common alleles. Allele A increased in frequency from 0.267 in MIS to 0.567 in MIR, while the frequencies of alleles B and C were lower in MIR.

Another measure of genetic variability is the number of homozygotes in a population. An increase in homozygosity is generally associated with a reduction in variability, assuming the alleles are in Hardy-Weinberg equilibrium. The homozygosity of MIS was 0.267 and increased to 0.367 in MIR. This modest increase in homozygosity in the drug-selected strain together with the lack of reduction in the number of alleles in this strain suggests, at most, only a slight decrease in variability resulting from selection at this locus. A test of the genotype frequencies for Hardy-Weinberg equilibrium indicated that the strains were in equilibrium, and any difference in homozygosity was not due to the presence of one or more alleles that failed to amplify during PCR.

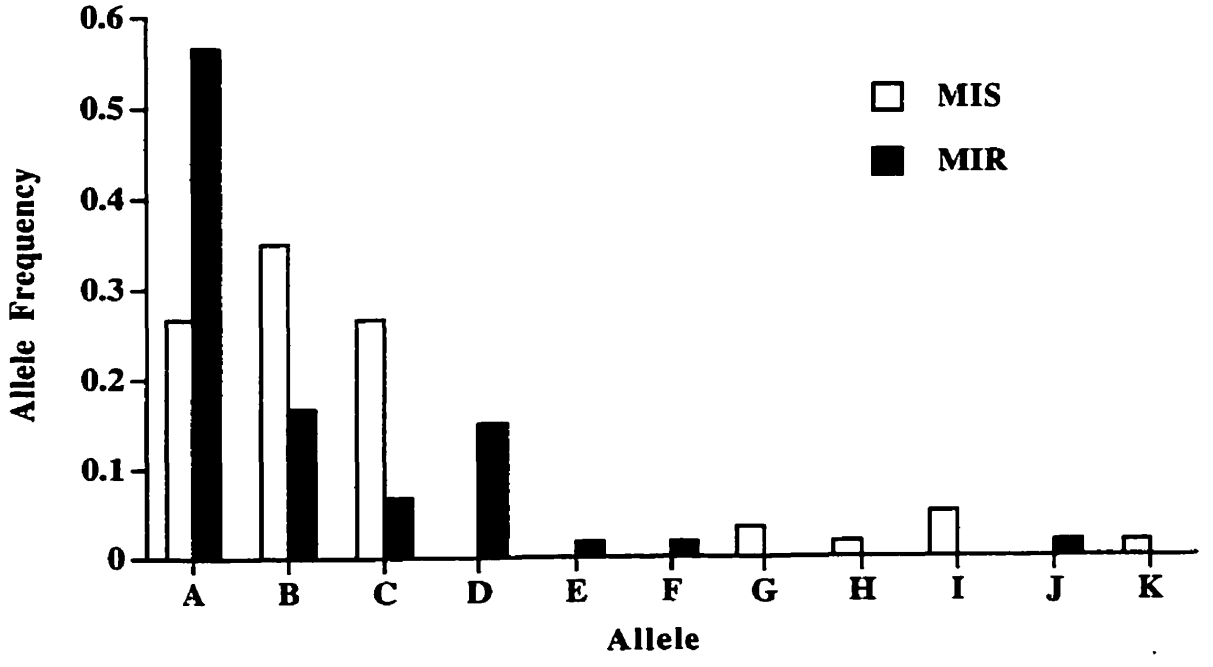

Figure 3. Allele frequencies of Merck unselected (MIS) and IVM-selected (MIR) strains of *H. contortus*.

Fort Dodge strains. Allele frequencies of the Fort Dodge strains are presented in Fig. 4. A total of 23 alleles was found in these 3 strains as compared to 11 alleles in the Merck strains. This difference is in part due to the increased sample size from 30 to 40 worms per strain. Only 5 alleles (A, B, O, X, and Y) were common to all Fort Dodge strains, and only 3 (alleles A, B, and K) were shared with the Merck strains. Differences in alleles between the Merck and Fort Dodge strains may reflect initial differences in the parental populations from which these strains were developed. A reduction in genetic variability in the selected strains as indicated by the number of alleles present in the population was apparent. The unselected strain (PF17) possessed 17 alleles, while the strain selected with IVM (IVF17) possessed 12 alleles, and the strain selected with MOX (MOF17) possessed only 7 alleles. As in the Merck strains, most of the alleles occurred with low frequency. Only 2 alleles change in frequency between strains to any great extent. Allele A increased in frequency from 0.063 in PF17 to 0.5 in IVF17 and to 0.438 in MOF17. This is the same allele that increased in frequency in the Merck IVM-selected strain. Allele X decreased in frequency with drug treatment, from 0.2 in PF17 to 0.063 in IVF17 and to 0.075 in MOF17. A Chi-square analysis of allele frequencies showed that both selected strains differed very significantly ($p < 0.0005$ for both) from the unselected strain. Allele frequencies did not differ significantly between the two selected strains. A significant difference ($p < 0.0005$) in allele frequencies was found between the two unselected strains used in this study, MIS and PF17. This difference is not surprising considering the independent derivation of these strains from different parent populations.

A Chi-square analysis of the observed and expected homozygote and heterozygote frequencies shows that the 2 selected strains were in Hardy-Weinberg

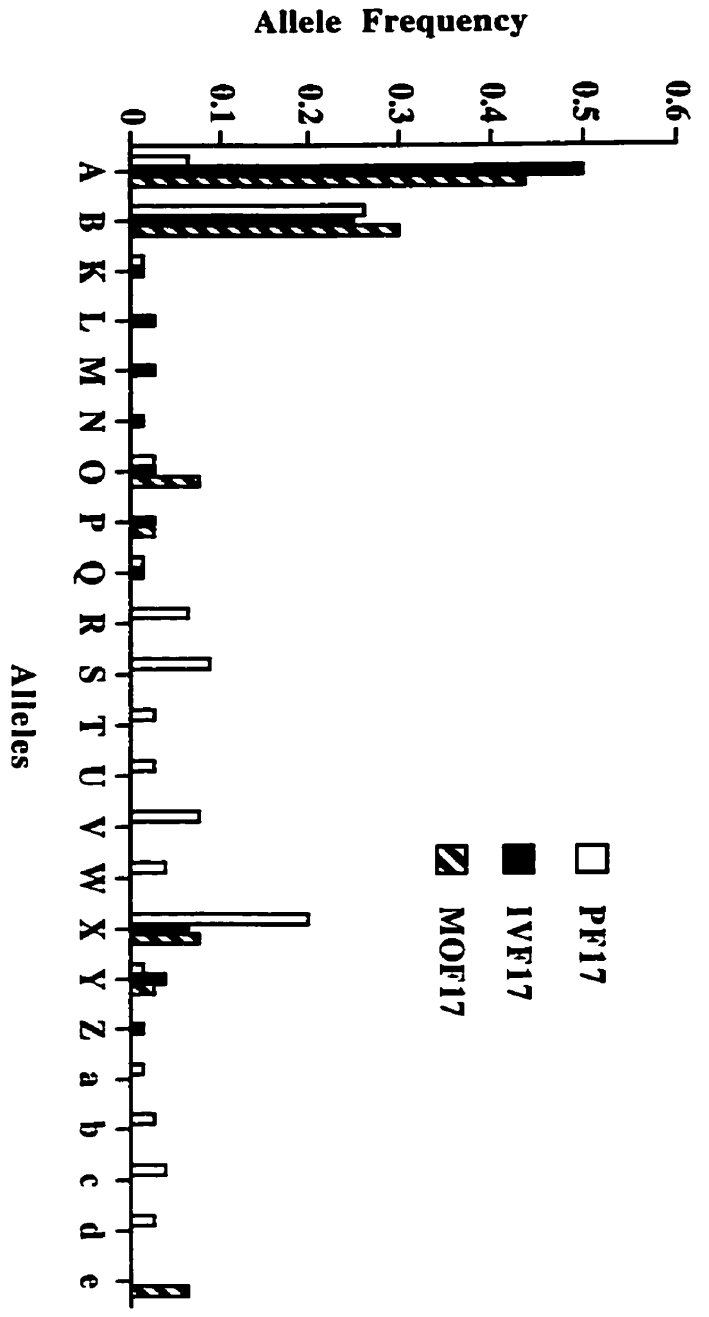

Figure 4. Allele frequencies of Fort Dodge unselected (PF17), IVM-selected (IVF17), and MOX-selected (MOF17) strains of *H. contortus*.

equilibrium but that the unselected strain was not. The excess of homozygotes in this strain may indicate the presence of one or more alleles that failed to amplify during PCR, so that the restriction digestions would appear as homozygotes when, in fact, the worms are heterozygotes. Because of this, the number of homozygotes observed cannot be used as an indicator of any change in genetic variability between strains.

DISCUSSION

The involvement of P-gp's in cases of drug resistance in mammals is well documented (see [33] for review). The case for invertebrates and protists is weaker, but some evidence implicates these proteins in the mechanisms of resistance to various toxic substances. Evidence that P-gp's may play a role in such resistance mechanisms has been reported from *C. elegans* [30,31], *Leishmania donovani* [34,35], *Entamoeba histolytica* [36], and *Plasmodium falciparum* [37,38], but later studies [39,40] have raised doubts about the importance of P-gp's in drug resistance seen in *P. falciparum*. Recent evidence raises the possibility that P-gp's may be involved in some cases of drug resistance in arthropods. An insecticide has been shown to be a substrate for rat P-gp [41], and an insect P-gp homologue is implicated in protecting the tobacco hornworm (*Manduca sexta*) from the effects of nicotine [42].

Caution must be exercised in extrapolating results obtained from laboratory strains to field situations. In the development of laboratory strains of parasites, only a subset of a parent population contributes genetic material to those strains. This may lead to a population bottleneck and a resultant loss of genetic variability. Genetic variability may also be lost through inbreeding: population sizes of passaged laboratory strains are smaller than natural populations. However, the various strains of *H. contortus* examined in this study demonstrate a high degree of polymorphism at this P-gp locus. Genetic hitch-hiking or epistatic interactions between protein products of genes may also cause shifts in allele frequencies of a gene not directly involved in any mechanism of resistance.

The finding of a genetic polymorphism associated with drug resistance at a P-gp gene in *H. contortus*, however, raises the possibility that alleles of this gene may confer

differing degrees of susceptibility to IVM and MOX. The selection for allele A in all three strains selected with drug treatment supports this possibility. The probability that the increase in frequency of allele A has occurred by some random process independently three times is low. Further support comes from a similar genetic analysis of a gene encoding the beta subunit of the glutamate-gated chloride channel. Allele frequencies at this locus, from the same individuals used in the present study, were not significantly different between unselected and drug-selected strains [43]. These data suggest that bottlenecking or inbreeding is not responsible for the observed changes in P-gp allele frequencies, but cannot rule out hitch-hiking or epistasis. Evidence for a concentration-dependent cross-resistance between the avermectins and the milbemycins [13,14] suggests a common mechanism of resistance to these two classes of drugs. Our finding of a genetic polymorphism associated with resistance to IVM and MOX in a P-gp gene in *H. contortus* is evidence for, but not proof of, P-gp involvement in the mechanism of resistance to these drugs. This polymorphism may, at the very least, serve as a marker for resistance to IVM and MOX.

P-gp may function in concentration-dependent resistance to IVM and MOX in *H. contortus* by regulating drug concentrations at the site(s) of action of these drugs. However, how P-gp may be altered in susceptible and resistant strains of this nematode is unknown. Possibilities include overexpression due to gene amplification, overexpression of a single-copy gene, an alteration in the stability of the P-gp mRNA or of the protein, or a change in substrate specificity. Higher levels of P-gp could result in higher rates of drug efflux. The work of [23] and the present study indicate that different alleles are predominantly expressed in unselected and IVM- and MOX-selected strains. No evidence for an increase in copy number was found [23]. Higher levels of P-gp mRNA, relative to

unselected strains, have been reported from the IVM-selected strains used in this study [23].

With evidence mounting for a role of P-gp's in drug resistance from a number of phylogenetically diverse organisms, this protein may play at least a contributory role in cases of resistance to various chemical control agents throughout the animal kingdom, and perhaps even the plant kingdom [44]. If so, the genetic analysis of these genes in populations will be useful in monitoring the development and extent of resistance to a variety of pesticides.

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REFERENCES

[1] Gibbs, H.C. Effects of parasites on animal and meat production. In: Gaafar, S.M., Howard, W.E., and Marsh, R.E., editors. Parasites, pests, and predators. New York, USA:Elsevier, 1985;7-27.

[2] Prichard, R.K. Anthelmintic resistance in nematodes: extent, recent understanding and future directions for control and research. Int J Parasitol 1990;20:515-23.

[3] Blouin, M.S., Dame, J.B., Tarrant, C.A., Courtney, C.H. Unusual population genetics of a parasitic nematode: mtDNA variation within and among populations. Evolution 1992;46:470-6.

[4] Beech, R.N., Prichard, R.K., Scott, M.E. Genetic variability of the β-tubulin genes in benzimidazole-susceptible and -resistant strains of *Haemonchus contortus*. Genetics 1994;138:103-10.

[5] Blouin, M.S., Yowell, C.A., Courtney, C.H., Dame, J.B. Host movement and the genetic structure of populations of parasitic nematodes. Genetics 1995;141:1007-14.

[6] Kwa, M.S.G., Kooyman, F.S.J., Boersema, J.H., Roos, M.H. Effect of selection for benzimidazole resistance in *Haemonchus contortus* on β-tubulin isotype 1 and isotype 2 genes. Biochem Biophys Res Comm 1993;191:413-19.

[7] Kwa, M.S.G., Veenstra, J.G., Roos, M.H. Molecular characterization of β-tubulin genes present in benzimidazole-resistant populations of *Haemonchus contortus*. Mol Biochem Parasitol 1993;60:133-44.

[8] Cully, D.F., Vassiliatis, D.K., Liu, K.K., Paress, P.S., Van Der Ploeg, L.H.T., Schaeffer, J.M., Arena, J.P. Cloning of an avermectin-sensitive glutamate-gated chloride channel from *Caenorhabditis elegans*. Nature 1994;371:707-11.

[9] Arena, J.P., Liu, K.K., Paress, P.S., Frazier, E.G., Cully, D.F., Mrozik, H., Schaeffer, J.M. The mechanism of action of avermectins in *Caenorhabditis elegans*: correlation between activation of glutamate-sensitive chloride current, membrane binding, and biological activity. J Parasitol 1995;81:286-94.

[10] Rohrer, S.P., Birzin, E.T., Eary, C.H., Schaeffer, J.M., Shoop, W.L. Ivermectin binding sites in sensitive and resistant *Haemonchus contortus*. J Parasitol 1994;80:493-7.

[11] Craig, T.M., Hatfield, T.A., Pankavich, J.A., Wang, G.T. Efficacy of moxidectin against an ivermectin-resistant strain of *Haemonchus contortus* in sheep. Vet Parasitol 1992;41:329-33.

[12] Pankavich, J.A., Berger, H., Simkins, K.L. Efficacy of moxidectin, nemadectin and ivermectin against an ivermectin-resistant strain of *Haemonchus contortus* in sheep. Vet Rec 1992;130:241-3.

[13] Conder, G.A., Thompson, D.P., Johnson, S.S. Demonstration of co-resistance of *Haemonchus contortus* to ivermectin and moxidectin. Vet Rec 1993;132:651-2.

[14] Shoop, W.L., Haines, H.W., Michael, B.F., Eary, C.H. Mutual resistance to avermectins and milbemycins: oral activity of ivermectin and moxidectin against ivermectin-resistant and susceptible nematodes. Vet Rec 1993;133:445-7.

[15] Van Wyk, J.A. Malan, F.S. Resistance of field strains of *Haemonchus contortus* to ivermectin, closantel, rafoxanide and the benzimidazoles in South Africa. Vet Rec 1988;123:226-8.

[16] Echevarria, F.A.M., Trindade, G.N.P. Anthelmintic resistance by *Haemonchus contortus* to ivermectin in Brazil: a preliminary report. Vet Rec 1989;124:147-8.

[17] Craig, T.M., Miller, D.K. Resistance by *Haemonchus contortus* to ivermectin in angora goats. Vet Rec 1990;126:580.

[18] Badger, S.B., McKenna, P.B. Resistance to ivermectin in a field strain of *Ostertagia* spp. in goats. New Zealand Vet J 1990;38:72-4.

[19] Jackson, F., Coop, R.L., Jackson, E., Scott, E.W., Russel, A.J.F. Multiple anthelmintic resistant nematodes in goats. Vet Rec 1992;130:210.

[20] Le Jambre, L.F. Ivermectin-resistant *Haemonchus contortus* in Australia. Aust Vet J 1993;70:357.

[21] Pandey, V.S., Sivaraj, S. Anthelmintic resistance in *Haemonchus contortus* from sheep in Malaysia. Vet Parasitol 1994;53:67-74.

[22] Mwamachi, D.M., Audho, J.O., Thorpe, W., Baker, R.L. Evidence for multiple anthelmintic resitance in sheep and goats reared under the same management in coastal Kenya. Vet Parasitol 1995;60:303-13.

[23] Xu, M., Molento, M., Blackhall, W.J., Ribeiro, P., Beech, R.N., Prichard, R.K. Ivermectin resistance in nematodes may be caused by alteration of P-glycoprotein homolog. Mol Biochem Parasitol 1998; 91:327-35.

[24] Scotto, K.W., Biedler, J.L., Melera, P.W. Amplification and expression of genes associated with multidrug resistance in mammalian cells. Science 1986;232:751-5.

[25] Gottesman, M.M., Hrycyna, C.A., Schoenlein, P.V., Germann, U.A., Pastan, I. Genetic analysis of the multidrug transporter. Ann Rev Gen 1995;29:607-49.

[26] Schinkel, A.H., Smit, J.J, Van Tellingen, O., Beijnen, J.H., Wagenaar, E., Van Deemter, L., Mol, C.A., Van der Valk, M.A., Robanus-Maandag, E.C., Te Riele, H.P., Berns, A.J.M., Borst, P. Disruption of the mouse *mdr1a* P-glycoprotein gene leads to a

deficiency in the blood-brain barrier and to increased sensitivity to drugs. Cell 1994;77:491-502.

[27] Didier, A., Loor, F. The abamectin derivative ivermectin is a potent P-glycoprotein inhibitor. Anti-Cancer Drugs 1996;7:745-51.

[28] Pouliot, J.-F., L'Heureux, F., Liu, Z., Prichard, R.K., Georges, E. Reversal of P-glycoprotein-associated multidrug resistance by ivermectin. Biochem Pharm 1997;53:17-25.

[29] Linke, C.A., The, I., Van Groenigen, M., Borst, P. The P-glycoprotein gene family of *Caenorhabditis elegans*. Cloning and characterization of genomic and complementary DNA sequences. J Mol Biol 1992;228:701-11.

[30] Broeks, A., Gerrard, B., Allikmets, R., Dean, M., Plasterk, R.H.A. Homologues of the human multidrug resistance genes MRP and MDR contribute to heavy metal resistance in the soil nematode *Caenorhabditis elegans*. EMBO J 1996;15:6132-43.

[31] Broeks, A., Janssen, H.W., Calafat, J., Plasterk, R.H.A. P-glycoprotein protects *Caenorhabditis elegans* against natural toxins. EMBO J 1995;14:1858-66.

[32] Wang, G.T., Berger, K., Simkins, K., Rock, D. The rate of resistance development by *H. contortus* to endectocides. In: Proceedings of the XV International Conference of

the World Association for the Advancement of Veterinary Parasitology. Yokohama, Japan, 1995;104.

[33] Gottesman, M.M., Pastan, I. Biochemistry of multidrug resistance mediated by the multidrug transporter. Ann Rev Biochem 1993;62:385-427.

[34] Chow, L.M., Wong, A.K., Ullman, B., Wirth, D.F. Cloning and functional analysis of an extrachromosomally amplified multidrug resistance-like gene in *Leishmania enrietii*. Mol Biochem Parasitol 1993;60:195-208.

[35] Henderson, D.M., Sifri, C.D., Rodgers, M., Wirth, D.F., Hendrickson, N., Ullman, B. Multidrug resistance in *Leishmania donovani* is conferred by amplification of a gene homologous to the mammalian mdr1 gene. Mol Cell Biol 1992;12:2855-65.

[36] Descoteaux, S., Ayala, P., Samuelson, J., Orozco, E. Increase in mRNA of multiple Eh pgp genes encoding P-glycoprotein homologues in emetine-resistant *Entamoeba histolytica* parasites. Gene 1995;164:179-84.

[37] Foote, S.J., Thompson, J.K., Cowman, A.F., Kemp, D.J. Amplification of the multidrug resistance gene in some chloroquine-resistant isolates of *P. falciparum*. Cell 1989;57:921-30.

[38] Wilson, C.M., Serrano, A.E., Wasley, A., Bogenschutz, M.P., Shankar, A.H., Wirth, D.F. Amplification of a gene related to mammalian mdr genes in drug-resistant *Plasmodium falciparum*. Science 1989;244:1184-6.

[39] Wellems, T.E., Pantan, L.J., Gluzman, I.Y., do Rosario, V.E., Gwadz, R.W., Walker-Jonah, A., Krogstad, D.J. Chloroquine resistance not linked to mdr-like genes in a *Plasmodium falciparum* cross. Nature 1990;345:253-5.

[40] Barnes, D.A., Foote, S.J., Galatis, D., Kemp, D.J., Cowman, A.F. Selection for high-level chlororquine resistance results in deamplification of the *pfmdr1* gene and increases sensitivity to mefloquine in *Plasmodium falciparum*. EMBO J 1992;11:3067-75.

[41] Lanning, C.L., Fine, R.L., Sachs, C.W., Rao, U.S., Corcoran, J.J., Abou-Donia, M.B. Chlorpyrifos oxon interacts with the mammalian multidrug resistance protein, P-glycoprotein. J Tox Environ Health 1996;47:395-407.

[42] Murray, C.L., Quaglia, M., Arnason, J.T., Morris, C.E. A putative nicotine pump at the metabolic blood-brain barrier of the tobacco hornworm. J Neurobiol 1994;25:23-4.

[43] Blackhall, W.J., Pouliot, J.-F., Prichard, R.K., Beech, R.N. *Haemonchus contortus*: selection at a glutamate-gated chloride channel subunit gene in ivermectin- and moxidectin-selected strains. Exp Parasitol 1998;(In press).

[44] Dudler, R., Hertig, C. Structure of an mdr-like gene from *Arabidopsis thaliana*. Evolutionary implications. J Biol Chem 1992;267:5882-8.

CONNECTING STATEMENT IV

In humans, P-glycoprotein can transport a wide range of chemical substrates. A report identifying benzimidazoles as substrates for human P-glycoprotein suggested the possibility that P-glycoprotein could transport benzimidazoles in *Haemonchus*. Having acquired evidence that the P-glycoprotein gene analyzed in Chapter IV may be contributing to avermectin/milbemycin resistance in *H. contortus*, we proceeded to conduct an identical analysis of this gene in strains unselected and selected with benzimidazoles. The results of this analysis are presented in Chapter V.

CHAPTER V

P-glycoprotein Selection in Strains of *Haemonchus contortus* Resistant to Benzimidazoles (Manuscript IV)

W.J. Blackhall, R.K. Prichard, and R.N. Beech

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ABSTRACT

Anthelmintic resistance in parasitic nematodes of livestock is a chronic problem in many parts of the world. Benzimidazoles are effective, broad-spectrum anthelmintics that bind to and selectively depolymerize microtubules. Resistance to the benzimidazoles, however, developed quickly and was shown to be correlated with genetic changes in genes encoding β-tubulins, subunits of microtubules. In *Haemonchus contortus*, resistance to avermectins has been correlated with genetic changes at a gene encoding a chloride channel subunit and at a gene encoding a P-glycoprotein, a cell membrane transport protein responsible for some cases of multidrug resistance in human cancer cells. Since different classes of drugs can act as substrates for P-glycoprotein in humans, we investigated the possibility that a similar phenomenon might occur in nematodes. An analysis of restriction fragment length polymorphisms of a P-glycoprotein gene from an unselected and a cambendazole-selected strain of *H. contortus* found a significant difference in allele frequencies between strains. The frequency of one allele in particular increased dramatically. The same allele was also found at a high frequency in an independently derived thiabendazole-selected strain. Here we present genetic evidence that P-glycoprotein may contribute to resistance to benzimidazoles in *H. contortus*.

INTRODUCTION

Before development of the avermectins, benzimidazoles (BZ) were the most widely used anthelmintics for controlling parasitic nematodes in livestock. Extensive use eventually led to the development of resistance to BZ. BZ acts by binding specifically and with high affinity to β-tubulin [1, 2, 3, 4, 5], thereby selectively depolymerizing microtubules [6, 7, 8]. The molecular characterization of genes encoding β-tubulin from the parasitic nematode *Haemonchus contortus* has shown that BZ resistance is correlated with genetic changes at these loci [9, 10, 11, 12, 13]. This correlation is also found in such phylogenetically diverse organisms as fungi [14, 15, 16], yeast [17], and the free-living nematode *Caenorhabditis elegans* [18]. The effect of BZ on tubulin polymerization is thus likely to be the principal mechanism of BZ resistance in parasitic nematodes.

The development of anthelmintic resistance is an evolutionary process, where individuals that can survive drug treatment pass their genes to the next generation. Over many generations, the frequency of survivors will increase in the population. How the survivors tolerate the effects of an anthelmintic can occur in different ways. Some alleles of a protein to which a drug binds may have a lower affinity for the drug [19]. Alternatively, allele differences may reduce a drug's effect on the target protein without affecting binding affinity. Reducing the concentration of an anthelmintic at its target site by a transport or efflux mechanism could also enhance survival [20]. Another possibility for enhancing survival is the differential ability to metabolically modify the anthelmintic, thereby reducing its effectiveness [21]. Whatever the means of survival, genetic changes will occur at the population level over time, and the effectiveness of anthelmintic treatment will diminish.

Recent evidence suggests that resistance to an anthelmintic may develop by multiple mechanisms, even within a single population. Avermectins are known to bind to and gate glutamate-gated chloride (GluCl) channels in *C. elegans* [22], leading to hyperpolarization of neuromuscular cells and paralysis. Allele frequency changes in a gene encoding a putative alpha subunit of the GluCl channel from ivermectin (IVM)- and moxidectin-selected strains of *H. contortus* [23] is consistent with the involvement of GluCl channels in a mechanism of resistance to these drugs. A second mechanism, however, may be contributing to IVM resistance. P-glycoprotein (P-gp), a membrane transport protein responsible for some cases of multidrug resistance in human cancer cells [24], may also contribute to IVM resistance in *H. contortus* [25, 26]. Since BZ can act as substrates for human P-gp [27], and multidrug-reversing agents can enhance the toxicity of BZ in eggs of *H. contortus* [28], P-gp may contribute to BZ resistance in adult nematodes.

The present study is an analysis of genetic variation in a P-gp gene from cambendazole (CBZ)-sensitive, CBZ-selected, and thiabendazole (TBZ)-selected strains of *H. contortus*. An analysis of a gene unlikely to be involved in BZ resistance, a putative GluCl channel alpha subunit gene, is included as a negative control for allele frequency changes in the strains used.

MATERIALS AND METHODS

Parasite strains. Three strains of *H. contortus* were used in this study. One of these strains (CBZ-sensitive) had no prior exposure to CBZ. The second strain (CBZ-selected), derived from the CBZ-sensitive strain, was treated with CBZ for 10 generations [29, 30]. After 10 generations, CBZ treatment at twice the recommended dose had an efficacy of 45% in the treated strain compared to 99.8% in the sensitive strain [30]. A third strain (TBZ-selected) was independently derived from the field on the basis of its resistance to TBZ [3]. After selection with TBZ, this strain required 4.4 times more TBZ than a sensitive strain to cause a 50% inhibition in egg hatching.

DNA isolation. The isolation of DNA used in this study has been described previously [12].

Polymerase chain reaction (PCR) amplification. A sense primer, PGP2S, 5' GAAATGACTCAAGCACAAG 3', designed based on the cDNA sequence of a P-gp gene reported from *H. contortus* [25], and and an antisense primer, MX-D, 5' AGACAAAGACATTCAGAG 3', designed from unpublished sequence data (M. Xu, personal communication), were used to obtain a genomic PCR product approximately 870 base pairs in length from 30 individuals of each strain. The reaction mixture contained 5 µl 10X *Taq* buffer, 5 µl 2 mM dNTPs, 4 µl 25 mM $MgCl_2$, 1 µl 20 mM primer solutions, 1 unit *Taq* polymerase, approximately 2 ng DNA template, and water to a final concentration of 50 µl. Amplification reactions were performed on an MJ Research, Inc. PTC-100 Programmable Thermal Controller (Watertown, MA). Amplification conditions were: 95° for 4 min followed by 40 cycles of 95° for 15 sec, 53° for 30 sec, and 70° for 1 min with a final extension step at 70° for 5 min. A fragment of a

GluCl channel subunit gene was amplified for single-strand conformation polymorphism (SSCP) analysis as described in [23]. PCR products were visualized on a 1% agarose gel containing 0.5 μg/ml ethidium bromide under UV illumination.

Restriction fragment length polymorphism analysis. PCR products were digested with the restriction enzymes *Dde*I, *Hinf*I, *Rsa*I, and *Alu*I as described by the manufacturer (Promega Corporation, Madison, WI). Ten μl of each PCR product were digested in a total volume of 20 μl. The digestion products were electrophoresed through a 6% nondenaturing polyacrylamide gel, stained with ethidium bromide, visualized under UV illumination, and photographed. Alleles were identified with each enzyme as homozygotes or as consistently occurring fragment patterns in heterozygotes.

Single-strand conformation polymorphism. PCR products of the GluCl channel subunit gene were processed and analysed as described in [23].

Data analysis. A Chi-square analysis of allele frequencies was performed between the CBZ-sensitive and CBZ-resistant strains for both genes examined. Rare alleles having frequency values lower than 5 were grouped for the analyses. No analysis was performed between the CBZ-sensitive and TBZ-resistant strains since these two strains were independently derived. Chi-square analyses were performed on all strains to test for Hardy-Weinberg equilibrium. An excess of homozygotes could indicate the presence of alleles that failed to amplify during PCR. Alleles whose counts were 5 or lower in a population were grouped together for the Chi-square analyses. The count of rare alleles in a population can vary from sample to sample, and the grouping of their counts to form a single category representing "rare alleles" lessens the statistical impact they would have in a Chi-square analysis.

RESULTS

A total of 6 alleles was found in the strains examined (Fig. 1). The CBZ-sensitive strain possessed 4 alleles, with allele 43 being the most common, having a frequency of 0.783. Allele I was the second most common allele at a frequency of 0.15. The CBZ-selected strain had a very different allele profile. The frequency of allele 43 decreased to 0.167. Allele B increased in frequency. Allele P, which was not seen in the CBZ-sensitive strain, was the most common allele, with a frequency of 0.5. A Chi-square analysis indicated a significant difference ($P < 0.0005$) in allele frequencies between the CBZ-sensitive and CBZ-selected strains. In the TBZ-selected strain, allele P also had a frequency of 0.5, with frequencies of the other alleles being comparable to those of the CBZ-selected strain. Allele frequencies were not significantly different between the CBZ-selected and TBZ-selected strains, even though these strains were independently derived. An apparent selection for allele P has occurred during the course of BZ treatment.

The SSCP analysis of the GluCl channel subunit gene identified a total of 5 alleles (Fig. 2). Allele C was the most common allele in the CBZ-sensitive and CBZ-selected strains, with frequencies of 0.683 and 0.533, respectively. Allele E was the next most common allele in these two strains. A Chi-square analysis indicated that allele frequencies of this gene were not significantly different between the sensitive and selected strains. In the TBZ-selected strain, allele E was most common, with a frequency of 0.4. The second most common allele, C, had a frequency of 0.3.

Chi-square analyses of observed versus expected homozygote and heterozygote frequencies for both genes indicated that all three strains were in Hardy-Weinberg equilibrium and that no frequent alleles failed to amplify.

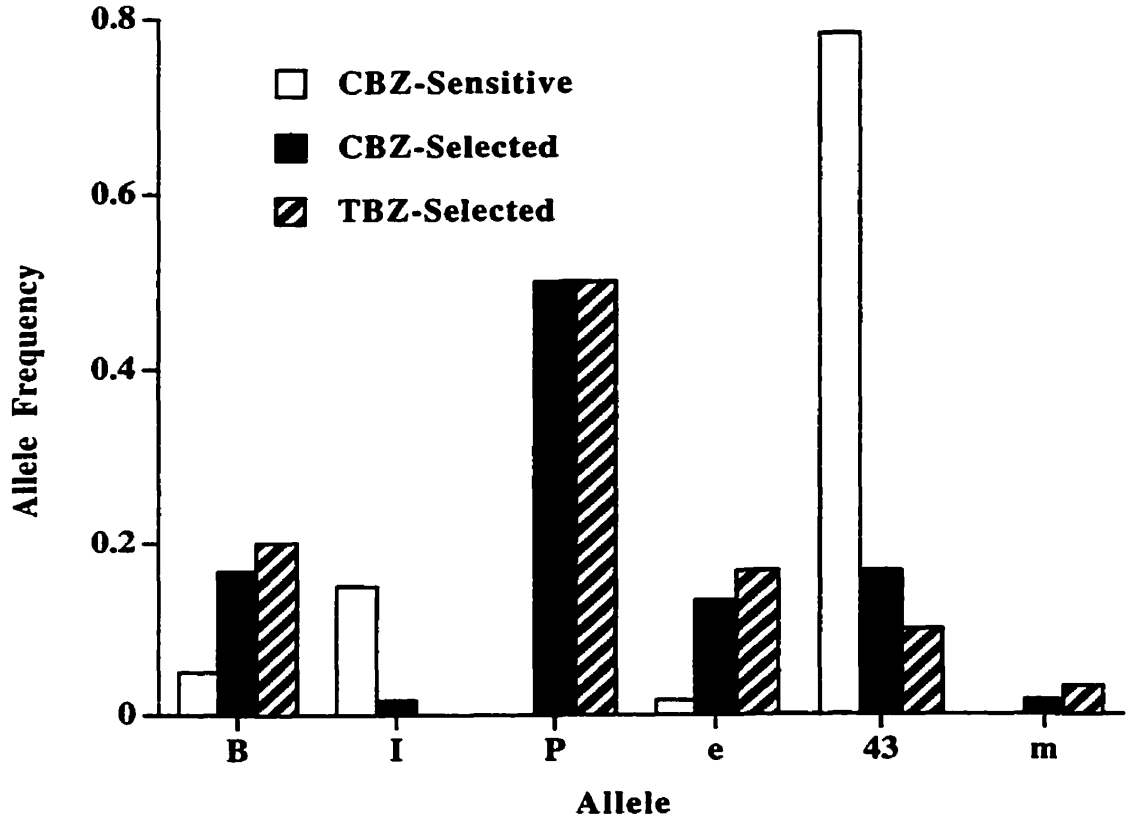

Figure 1. Allele frequencies of a P-gp gene of the CBZ-sensitive, CBZ-selected, and TBZ-selected strains of *H. contortus*.

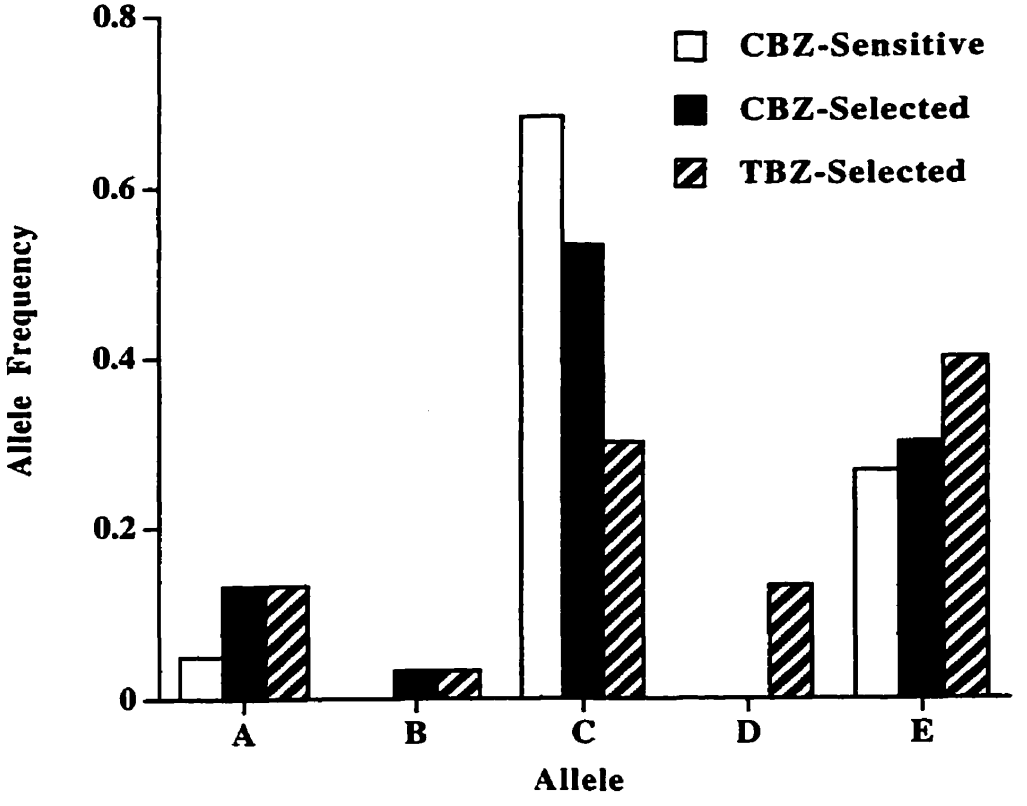

Figure 2. Allele frequencies of a GluCl channel subunit gene of the CBZ-sensitive, CBZ-selected, and TBZ-selected strains of *H. contortus*.

DISCUSSION

This study presents evidence suggesting that multiple mechanisms are contributing to BZ resistance in *H. contortus*. Previous studies on the effects of BZ drugs on microtubule polymerization and alterations in tubulin genes of resistant parasites implicate microtubule stability as a major factor in the development of BZ resistance. The changes in allele frequencies of a P-gp gene reported here most likely reflects the contribution of a second mechanism, one involving alteration of BZ concentrations at the tubulin target site.

The selection for a specific allele of P-gp, allele P, in CBZ- and TBZ-selected strains of *H. contortus* is apparent. Allele P was not seen in the sensitive strain. Changes in allele frequencies, however, may occur by genetic hitch-hiking or epistatic interactions of encoded proteins. The lack of a significant difference in allele frequencies at the GluCl subunit locus indicates that the changes at the P-gp locus are not likely due to the effects of inbreeding or population bottlenecks during the course of strain development. Allele P has been found to occur at a very low frequency at other, independently derived strains of *H. contortus* [26]. The probability that the high frequency of this allele in both the CBZ- and TBZ-selected strains is due to some stochastic process is extremely low.

How allele P of the P-gp gene may function in BZ resistance is unknown. Xu *et al.* [25] reported increased expression of the same P-gp gene in eggs of IVM-selected strains of *H. contortus* relative to unselected strains. Expression levels in adults is unknown. Overexpression of P-gp could enhance drug transport, reducing concentrations of IVM at the target site. An analysis of P-gp allele frequencies in these strains, identical to the analysis of the present study, identified selection for an allele other than allele P

[26]. This may indicate that different P-gp alleles could have differential substrate specificities.

Overexpression of P-gp is responsible for some cases of multidrug resistance in human cancer cells [24]. A defining characteristic of multidrug resistance is that selection for resistance to one drug confers resistance to other, unrelated drugs. Resistance to BZ in *H. contortus*, however, does not confer use-level resistance to IVM [31, 32, 33, 34], moxidectin [35, 36, 37, 38], levamisole [39, 40, 41, 42], closantel [34], morantel [39, 43], nor pyrantel [39]. The contribution of P-gp in BZ resistance, then, does not implicate P-gp for conferring use-level multidrug resistance in *H. contortus*. Although overexpression of P-gp has been found in IVM-selected strains of *H. contortus* [25], it is not known if these strains show any evidence of cross-resistance to other classes of anthelmintics. An alteration and/or overexpression of P-gp may contribute to anthelmintic resistance, but not in itself be sufficient for resistance to be apparent at recommended anthelmintic dose rates. These recommended dose rates are sometimes 5-50 times the ED_{95} concentrations. Thus to find evidence of a contribution of P-gp selection to cross-resistance, dose-response titrations to look for shifts in ED_{95} or ED_{50} levels would be required. Such studies have yet to be performed.

In summary, genetic selection at a P-gp locus in *H. contortus* resistant to BZ suggests that drug efflux at the cellular level might be a second mechanism contributing to the development of resistance to BZ. Combined with the report by [25] that P-gp may also cause resistance to IVM, this study leads to the speculation that P-gp, a membrane transport protein, may contribute to resistance to other anthelmintics as well.

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REFERENCES

[1] Hammerschlag, R.S., Sisler, H.D. Benomyl and methyl-2-benzimidazolecarbamate (MBC): biochemical, cytological and chemical aspects of toxicity in *Ustilago maydis* and *Saccharomyces cerivisiae*. Pestic Biochem Physiol 1973;3:42-54.

[2] Davidse, L.C., Flach, W. Differential binding of methyl benzimidazole-2-yl-carbamate to fungal tubulin as a mechanism of resistance to this anthelmintic agent in mutant strains of *Aspergillus nidulans*. J Cell Biol 1977;72:174-89.

[3] Lubega, G.W., Prichard, R.K. Specific interaction of benzimidazole anthelmintics with tubulin: high-affinity binding and benzimidazole resistance in *Haemonchus contortus*. Mol Biochem Parasitol 1990;38:221-32.

[4] Lubega, G.W., Prichard, R.K. Interaction of benzimidazole anthelmintics with *Haemonchus contortus* tubulin: binding affinity and anthelmintic efficacy. Exp Parasitol 1991;73:203-13.

[5] Lubega, G.W., Prichard, R.K. Specific interaction of benzimidazole anthelmintics with tubulin from developing stages of thiabendazole-susceptible and-resistant *Haemonchus contortus*. Biochem Pharmacol 1991;41:93-101.

[6] Borgers, M., de Nollin, S. Ultrastructural changes in *Ascaris suum* intestine after mebendazole treatment *in vivo*. J Parasitol 1975;61:110-22.

[7] Borgers, M., de Nollin, S., de Brabander, M., Thienpont, D. Influence of the anthelmintic mebendazole on microtubules and intracellular organelle movement in nematode intestinal cells. Am J Vet Res 1975;36:1153-66.

[8] Quinlan, R.A., Pogson, C.I., Gull, K. The influence of the microtubule inhibitor, methyl benzimidazole-2-yl-carbamate (MBC) on nuclear division and the cell cycle in *Saccharomyces cerevisiae*. J Cell Sci 1980;46:341-52.

[9] Roos, M.H., Boersema, J.H., Borgsteede, F.H.M., Cornelissen, J., Taylor, M., Ruitenberg, E.J. Molecular analysis of selection for benzimidazole resistance in the sheep parasite *Haemonchus contortus*. Mol Biochem Parasitol 1990;43:77-88.

[10] Kwa, M.S.G., Kooyman, F.N.J., Boersema, J.H., Roos, M.H. Effect of selection for benzimidazole resistance in *Haemonchus contortus* on β-tubulin isotype 1 and isotype 2 genes. Biochem Biophys Res Commun 1993;191:413-9.

[11] Kwa, M.S.G., Veenstra, J.G., Roos, M.H. Molecular characterization of β-tubulin genes present in benzimidazole-resistant populations of *Haemonchus contortus*. Mol Biochem Parasitol 1993;60:133-44.

[12] Beech, R.N., Prichard, R.K., Scott, M.E. Genetic variability of the β-tubulin genes in benzimidazole-susceptible and -resistant strains of *Haemonchus contortus*. Genetics 1994;138:103-10.

[13] Kwa, M.S.G., Veenstra, J.G., Roos, M.H. Benzimidazole resistance in *Haemonchus contortus* is correlated with a conserved mutation at amino acid 200 in β-tubulin isotype 1. Mol Biochem Parasitol 1994;63:299-303.

[14] Sheir-Neiss, G., Lai, M.H., Morris, N.R. Identification of a gene for β-tubulin in *Aspergillus nidulans*. Cell 1978;15:639-47.

[15] Orbach, M.J., Porro, E.B., Yanofsky, C. Cloning and characterization of the gene for β-tubulin from a benomyl-resistant mutant of *Neurospora crassa* and its use as a dominant selectable marker. Mol Cell Biol 1986;6:2452-61.

[16] Foster, K.E., Burland, T.G., Gull, K. A mutant β-tubulin confers resistance to the action of benzimidazole-carbamate microtubule inhibitors both *in vivo* and *in vitro*. Eur J Biochem 1987;163:449-55.

[17] Thomas, J.H., Neff, N.F., Botstein, D. Identification and characterization of mutations in the β-tubulin gene of *Saccharomyces cerevisiae*. Genetics 1985;112:715-34.

[18] Driscoll, M., Dean, E., Reilly, E., Bergholz, E., Chalfie, M. Genetic and molecular analysis of a *Caenorhabditis elegans* β-tubulin that conveys benzimidazole sensitivity. J Cell Biol 1989;109:2993-3003.

[19] Anderson, T.J.C., Blouin, M.S., Beech, R.N. Population biology of parasitic nematodes: applications of genetic markers. Adv Parasitol 1998;41:219-83.

[20] Sangster, N. Pharmacology of anthelmintic resistance. Parasitol 1996;113:S201-16.

[21] Coles, G.C. The molecular biology of drug resistance. In: Bennet, E., Behm, C., Bryant, C., editors. Comparative Biochemistry of Parasitic Helminths. London: Chapman and Hall, 1989;125-144.

[22] Cully, D.F., Vassiliatis, D.K., Liu, K.K., Paress, P.S., Van Der Ploeg, L.H.T., Schaeffer, J.M., Arena, J.P. Cloning of an avermectin-sensitive glutamate-gated chloride channel from *Caenorhabditis elegans*. Nature 1994;371:707-11.

[23] Blackhall, W.J., Pouliot, J.-F., Prichard, R.K., Beech, R.N.*Haemonchus contortus*: selection at a glutamate-gated chloride channel gene in ivermectin- and moxidectin-selected strains. Exp Parasitol 1998;90:42-8.

[24] Gottesman, M.M., Pastan, I. Biochemistry of multidrug resistance mediated by the multidrug transporter. Ann Rev Biochem 1993;62:385-427.

[25] Xu, M., Molento, M., Blackhall, W., Ribeiro, P., Beech, R., Prichard, R. Ivermectin resistance in nematodes may be caused by alteration of P-glycoprotein homolog. Mol Biochem Parasitol 1998;91:327-35.

[26] Blackhall, W.J., Liu, H.Y., Xu, M., Prichard, R.K., Beech, R.N. Selection at a P-glycoprotein gene in ivermectin- and moxidectin-selected strains of *Haemonchus contortus*. Mol Biochem Parasitol 1998;95:193-201.

[27] Nare, B., Liu, Z., Prichard, R.K., Georges, E. Benzimidazoles, potent anti-mitotic drugs: substrates for the P-glycoprotein transporter in multidrug-resistant cells. Biochem Pharmacol 1994;48:2215-22.

[28] Beugnet, F., Gauthey, M., Kerboeuf, D. Partial in vitro reversal of benzimidazole resistance by the free-living stages of *Haemonchus contortus* with verapamil. Vet Rec 1997;141:575-6.

[29] Kates, K.C., Colglazier, M.L., Enzie, F.D. Experimental development of a cambendazole-resistant strain of *Haemonchus contortus* in sheep. J Parasitol 1973;59:169-74.

[30] Colglazier, M.L., Kates, K.C., Enzie, F.D. Cambendazole-resistant *Haemonchus contortus* strain in sheep: further experimental development. J Parasitol 1974;60:289-92.

[31] Wescott, R.B., LeaMaster, B.R. Efficacy of ivermectin against naturally acquired and experimentally induced nematode infections in sheep. Am J Vet Res 1982;43:531-3.

[32] Swan, G.E., Schroder, J., Carmichael, I.H., Louw, J.P., Harvey, R.G., Penderis, I. Efficacy of ivermectin against internal parasites of sheep. J S Afr Vet Ass 1984;55:165-9.

[33] Taylor, M.A., Hunt, K.R., Wilson, C.A., Baggott, D.G. Efficacy of ivermectin against benzimidazole-resistant nematodes of sheep. Vet Rec 1990;127:302-3.

[34] Waruiru, R.M., Weda, E.H., Otieno, R.O., Ngotho, J.W., Bogh, H.O. Comparative efficacies of closantel, ivermectin, oxfendazole, thiophanate and levamisole against thiabendazole resistant *Haemonchus contortus* in sheep. Trop An Health Prod 1996;28:216-20.

[35] Besier, R.B., Lyon, J., Kieran, P.J. The effect of moxidectin against benzimidazole- and levamisole-resistant nematodes of sheep in Western Australia. Aust Vet J 1993;70:422-3.

[36] Oosthuizen, W.T., Erasmus, J.B. Efficacy of moxidectin against a strain of *Haemonchus contortus* resistant to ivermectin, a benzimidazole and a salicylanilide. J S Afr Vet Ass 1993;64:9-12.

[37] Bauer, C., Conraths, F.J. Comparative efficacy of moxidectin and mebendazole against gastrointestinal nematodes in experimentally infected lambs. Vet Rec 1994;135:136-8.

[38] Kerboeuf, D., Hubert, J., Cardinaud, B., Blond, F. Efficacy of oral moxidectin against benzimidazole-resistant isolates of gastrointestinal nematodes in sheep. Vet Rec 1995;136:16-7.

[39] Colglazier, M.L., Kates, K.C., Enzie, F.D. Activity of levamisole, pyrantel tartrate, and rafoxanide against two thiabendazole-tolerant isolates of *Haemonchus contortus*, and two species of *Trichostrongylus*, in sheep. Proc Helm Soc Wash 1971;38:203-5.

[40] Berger, J. The resistance of a field strain of *Haemonchus contortus* to five benzimidazole anthelmintics in current use. J S Afr Vet Ass 1975;46:369-72.

[41] Kelly, J.D., Hall, C.A., Whitlock, H.V., Thompson, H.G., Campbell, N.J., Martin, I.C. The effect of route of administration on the anthelmintic efficacy of benzimidazole anthelmintics in sheep infected with strains of *Haemonchus contortus* and *Trichostrongylus colubriformis* resistant or susceptible to thiabendazole. Res Vet Sci 1977;22:161-8.

[42] Borgsteede, F.H., Duyn, S.P. Lack of reversion of a benzimidazole resistant strain of *Haemonchus contortus* after six years of levamisole usage. Res Vet Sci 1989;47:270-2.

[43] Colglazier, M.L., Kates, K.C., Enzie, F.D. Activity of cambendazole and morantel tartrate against two species of *Trichostrongylus* and two thiabendazole-resistant isolates of *Haemonchus contortus* in sheep. Proc Helm Soc Wash 1972;39:28-32.

CHAPTER VI

General Discussion

The development of anthelmintic resistance in parasitic nematodes is an evolutionary process. Genetic changes occur at the population level in genes that contribute to resistance mechanisms. The selection of alleles capable of increasing tolerance to anthelmintics leads to changes in allele frequencies of populations. These changes can be detected by standard techniques of analysis used in population genetics. Using these techniques, this thesis presents evidence that resistance to the AVM/milbemycin and BZ classes of anthelmintics is polygenic in laboratory strains of *H. contortus*. Whether resistance is polygenic or monogenic in field strains is unknown.

Despite P-gp's known function as a drug transporter in mammals and similar functions suspected in protists and invertebrates, the efflux of anthelmintics by P-gp in *H. contortus* may represent a secondary mechanism of resistance to AVM/milbemycins. As with BZ resistance, the primary mechanism of resistance is likely to involve alterations in the mode of action of these drugs. Most attention within the last five years has focused on GluCl channels as the target of AVM/milbemycins. The paralysis that results from hyperpolarization of neuromuscular cells when GluCl channels are opened is believed to cause the expulsion of worms from the host. *In vitro* electrophysiological studies clearly demonstrate AVM's ability to elicit Cl^- currents by gating GluCl channels. Selection at a gene encoding a putative GluClα subunit in IVM- and MOX-selected strains of *H. contortus* reported in Chapter II provides good support for both the contention that GluCl channels are targets for AVM/milbemycins and the possibility that alterations in these channels represent a mechanism of resistance. The same allele, allele A, increased in frequency in the independently derived Merck and Fort Dodge IVM-selected strains relative the their corresponding unselected strains. This finding suggests an allele-specific

alteration that in some way reduces or negates the ability of IVM to open GluCl channels. Selection for the same allele in the Fort Dodge MOX-selected strain is consistent with AVM and milbemycins having a similar mode of action and perhaps a similar mechanism of resistance.

The nature of the alteration in the GluClα protein leading to resistance is unknown. Amino acid residues in the M2 region that line the channel pore are known to be critical in determining channel properties in related ligand-gated ion channels. Recent experiments, however, indicate that no mutations are found in this region of allele A compared to other alleles of this gene from *H. contortus* (S.G. Forrester personal communication). Binding studies have found no difference in binding of IVM to membrane preparations of sensitive and resistant strains of *H. contortus* [1], suggesting the absence of mutations in the IVM-binding regions of GluClα subunits of resistant worms. Mutations in M4 of acetylcholine receptors can affect gating kinetics [2], but no mutations occur in this region of allele A compared to other alleles (S.G. Forrester personal communication).

Binding studies, however, have found that an IVM-selected strain of *H. contortus* has more glutamate binding sites than the corresponding unselected strain [3]. Glutamate gates GluCl channels in *C. elegans* by binding to GluClβ subunits [4], but GluClα subunits are believed to possess a cryptic glutamate binding site [5]. Allele A may thus possess a mutation that unmasks a glutamate binding site on the GluClα subunit, thereby altering the kinetics of the channel. Further study will be necessary to elucidate any possible functional difference of allele A before ascribing to it a role in a mechanism of resistance to AVM/milbemycin resistance.

Chapter III presents evidence for a third possible mechanism of resistance to AVM/milbemycins in *H. contortus*. Selection at a gene encoding a GABA receptor subunit in IVM- and MOX-selected strains relative to unselected strains implicates these receptors as a site of anthelmintic action in *H. contortus*. Paralysis as a result of hyperpolarization of neuromuscular cells is, as with GluCl channels, believed to be a potential mechanism for the action of AVM at GABA receptors. The selection observed at this locus, however, is inconsistent between the two sets of strains examined. Allele L increased dramatically in frequency in the Merck IVM-selected strain relative to the unselected strain, while allele e increased in frequency in the Fort Dodge IVM- and MOX-selected strains relative to the unselected strain. The selection of different alleles may simply be due to the absence of allele L in the Fort Dodge strains. The existence in a population of more than one allele capable of conferring resistance is not beyond reason. As with the putative GluClα subunit locus, selection at the HG1 locus and selection for the same allele in the IVM- and MOX-selected Fort Dodge strains is consistent with AVM and milbemycins having a similar mode of action.

The nature of a possible mutation in the GABA receptor that could confer resistance is unknown. The fragment analysed in Chapter III spans the M2 region, but no differences in amino acid sequences were found in any of the alleles. The structural and functional similarity between GluCl channels and GABA receptors might imply a similar interaction with AVM/milbemycins. The lack of mutations in the M2 regions of alleles associated with resistance at both loci is consistent with this hypothesis. Full-length sequencing of the alleles would determine if alleles e and L share mutations with each other or with allele A of the GluClα subunit.

The paralyzing action of AVM/milbemycins in nematodes strongly implicates GluCl channels and/or GABA receptors as principal sites of action and therefore alterations in these structures as potential primary mechanisms of resistance. Which site is more important in accounting for the effectiveness of these anthelmintics in controlling infections is a key question. The answer lies in determining the cause of expulsion of parasite from host.

Evidence points to the inhibition of pharyngeal pumping as the principal function of GluCl channels and the coordination of locomotion as the principal function of GABA receptors. *H. contortus* is expelled within 8-10 hours after oral treatment with a recommended dose of IVM (200 μg/kg)[6], which is similar to the time required for the flow of digesta through the digestive tract from the abomasum [7]. Such a rapid expulsion cannot be attributed to an inhibition of feeding but could be due to paralysis of the body musculature. Unless normal pharyngeal function is required for attachment of *H. contortus* to the walls of the abomasum, GluCl channels are unlikely to be involved in expulsion. GABA receptors, then, would appear to be the principal target of IVM resulting in expulsion. Since AVM is a known antagonist on somatic muscle cells in *Ascaris*, however, the affected receptors must be located in nerve tissue. The gene analysed in Chapter III, HG1, has been identified throughout the ventral nerve cord in *H. contortus* [8]. The action of AVM/milbemycins on GABA receptors would thus appear to be a likely candidate for expulsion of worms.

Pharyngeal pumping, however, is about 100-fold more sensitive to AVM than is motility. The question arises whether the concentration of anthelmintic in worm tissues is high enough to affect motility when administered at recommended doses. Motility in adult *H. contortus* is inhibited at IVM concentrations of 10 nM *in vitro* [9]. The

concentration of IVM in blood of sheep following administration of a recommended dose is 25 nM [10] and, because of its hydrophobicity, may attain higher concentrations in cell membranes [11]. Even though GluCl channels are more sensitive to IVM than are GABA receptors, the recommended dose appears to be high enough to interfere with locomotion. If the opening of neural GABA receptors by AVM/milbemycins causes paralysis resulting in expulsion of parasite from host, then mutations in the receptors must be considered as a possible mechanism of resistance to these anthelmintics.

The ability to reduce or negate the effects of AVM/milbemycins on locomotion could be a necessary condition for survival of drug treatment but may not be sufficient by itself. As Gill and Lacey [6] indicate, worms that avoid expulsion will still need to feed. The half-life of IVM in sheep is approximately 7 days [12], so concentrations capable of paralyzing the pharynx can persist for weeks. The ability to survive drug treatment, then, may depend on a dual mechanism of resistance involving both GABA receptors and GluCl channels. Genotyping both loci in survivors of drug treatment from a susceptible population a week or two post-treatment may be necessary to determine if this is the case.

Another important factor to consider is the genetic change selected by anthelmintic treatment that occurs in field isolates at recommended doses. The strains examined in this thesis are laboratory strains. The inbreeding and population bottlenecks inherent in maintaining laboratory strains may influence the population genetics of resistant worms. The doses of anthelmintics these strains have received are far below the recommended dose producers would use for broad-spectrum application. Suboptimal dosing may facilitate polygenic resistance [13]. Interpreting genotype data from resistant field strains, however, would be difficult without genotypic information from the strains before exposure to the anthelmintics. Also, different populations of *H. contortus* around

the world may be sufficiently diverse genetically to exhibit different selection profiles at the loci examined leading to different mechanisms of resistance in the various populations. These drawbacks to the study of laboratory strains notwithstanding, the data gathered from this thesis are valuable in identifying potential mechanisms of resistance to AVM/milbemycins in *H. contortus*.

Le Jambre and coworkers attribute AVM resistance in *H. contortus* to a single, dominant autosomal gene [14,15]. Their findings, however, are not necessarily inconsistent with the possibility of multiple mechanisms of resistance suggested by the present study. It is likely that the survival of a worm exposed to AVM depends principally on the reduction of the drug's toxic effects at the site of action, and that this reduction is principally due to the presence of an allele of the target molecule that somehow moderates the action of the drug. The main site of action of AVM may be GluCl channels or may be GABA receptors. Some worms may starve while others may be immobilised. Different isolates may react in different ways to AVM treatment depending on treatment parameters and initial allele frequencies of target molecules. One isolate may develop resistance through one mechanism and another isolate through an alternative mechanism. Each mechanism may be due to a single, dominant gene, but not necessarily the same gene. Crossing studies on a number of field isolates and laboratory strains could clarify this issue. The existence of different mechanisms dominating in different isolates and strains is suggested by the different responses to AVM exposure in larvae of various *H. contortus* isolates [6]. The presence of a third mechanism that moderates the effect of a drug's principal mode of action, as P-gp may, might not be readily apparent in crossing studies. This mechanism is not necessary for survival of the population but only enhances survival, so the presently available assays for measuring

resistance may not be sufficiently sensitive to detect a second, contributing but unnecessary, gene.

The potential of genetic differences between various strains of *H. contortus* is apparent when comparisons are made between the initial allele frequencies at the various loci examined in this study. Even though the Merck and Fort Dodge unselected strains were both developed in the eastern United States, reducing the possible influence of geographical isolation, these two strains possess their own genetic identities. Although responses to selection were generally similar in the Merck and Fort Dodge strains, the response at the GABA receptor subunit locus was quite different, perhaps reflecting differences in initial allele frequencies. The reason for selection of a very rare P-gp allele in both the CBZ- and TBZ-selected strains (Chapter V) rather than allele A as in the Merck and Fort Dodge strains (Chapter IV) remains unknown. Allele A is not a particularly rare allele in the Merck and Fort Dodge unselected strains and wouldn't be expected to be rarer than allele P appears to be. Some unknown interaction between β-tubulin isotypes 1 or 2, which have undergone strong selection in the BZ-selected strains [21], and P-gp may be occurring (see Discussion, Appendix A). If such an interaction exists, then the combines selection of BZ resistance alleles at both β-tubulin isotypes might severely constrain which P-gp allele is able to maintain this interaction. The similarity of the BZ-selected strains in their selection at the P-gp locus, then, may be due to factors other than initial allele frequencies.

In the study of resistance to BZ anthelmintics, changes in β-tubulin genes have been clearly shown to be associated with resistance in *H. contortus* [16,17,18,19,20,21]. A reduction in the interference with microtubule polymerization caused by these changes

appears to be the major mechanism of BZ resistance. The result reported in Chapter V suggests that a second mechanism, involving the efflux of anthelmintic by P-gp, is contributing to BZ resistance. The decrease in inhibition of egg-hatching by BZ by exposure to the reversing agent, verapamil, supports a role for P-gp [22].

The mechanism whereby the efflux of anthelmintic is increased in resistant strains is unknown. A higher level of P-gp in cell membranes could result in increased efflux. More P-gp could be due to an increase in copy number of a P-gp gene or to an increase in expression of a single-copy gene, as appears to be the case in resistance to IVM [23]. An increase in P-gp levels could result from an increase in transcription rate or in mRNA or protein stability. Point mutations in promoter or enhancer regions of genes can increase transcription [24], and mutations in 3' untranslated regions can influence mRNA stability [25]. Some proteins are able to regulate their own transcription through either positive or negative feedback loops, or both [26], and mutations may affect this ability.

Alternatively, an increase in efflux might result from the selection of a P-gp allele possessing a specificity for BZ greater than that possessed by the more common alleles in a population, leading to a higher efficiency of efflux. Multidrug resistance in some cancer cells is due to increased expression of P-gp [27] rather than to selection of an allele with enhanced substrate specificity. The selection of allele P in both BZ-selected strains of *H. contortus* may represent either selection for enhanced substrate specificity or selection for an allele capable of being overexpressed. A measure of P-gp expression levels from these strains would determine if overexpression is responsible for P-gp's contribution to resistance.

If selection for an allele capable of being overexpressed was causing an increase in efflux of BZ, and if this selection also occurs in field strains, then selection for BZ

resistance might lead to cross-resistance to other anthelmintics. To date, BZ and IVM are the only anthelmintics to have been shown to act as substrates for P-gp, and no cross-resistance to IVM in BZ-resistant helminths has been observed, supporting the possibility that substrate specificity is important. P-gp's contribution to anthelmintic resistance, however, may be sensitive to dose rates, and any resistance to IVM in BZ-resistant isolates due to P-gp alone may not be apparent at recommended doses of IVM. Chapter IV supports a role for P-gp in resistance to IVM in *H. contortus*, but a different allele has been selected. Allele A, not allele P, is associated with resistance to IVM. Either more than one allele is capable of being overexpressed, or alleles A and P are more specific for IVM and BZ, respectively. The IVM-selected strains of *H. contortus* used in Chapter IV have been found to overexpress this P-gp gene [23], supporting overexpression as the mechanism of resistance, but transport studies of the different alleles with the different anthelmintics would be necessary to determine if substrate specificity might also be a factor. The possibility also exists that both overexpression and efficiency of efflux of a substrate are important. Selection at a P-gp locus in strains selected with different classes of anthelmintic, however, illustrates the potential for cross-resistance, perhaps involving other classes of anthelmintics.

In Chapter IV, the selection for allele A in two independently derived sets of anthelmintic-resistant strains of *H. contortus* suggests that IVM and MOX may share similar mechanisms of resistance. If allele A is overexpressed, it appears to have similar efficiencies of efflux of IVM and MOX. The structural similarity of these anthelmintics would imply similar substrate specificities for P-gp. Whatever the mechanism of enhanced efflux, the selection for specific alleles of P-gp in anthelmintic-selected strains

of *H. contortus* reported in Chapters IV and V provides good evidence in support of a role for P-gp-mediated efflux in AVM/milbemycin resistance.

In summary, selection at three loci, genes encoding a putative GluClα subunit, a GABA receptor subunit, and a P-gp has been observed in strains of *H. contortus* selected with IVM and MOX. The selection at the putative GluCla and GABA receptor subunit loci is evidence that the proteins they encode are sites of action of AVM/milbemycins. Selection for specific alleles at all three loci is evidence that the proteins they encode contribute to mechanisms of resistance to AVM/milbemycins, and selection at the P-gp locus in the BZ strains suggests a BZ-resistance mechanism independent of tubulin. Identification of potential mechanisms of resistance will be important in the development of diagnostic tests for the presence or stage of development of resistance in the field. The evidence for a P-gp-mediated efflux of anthelmintics provides support for efforts directed at reversing the effects contributed by this mechanism of resistance.

REFERENCES

[1] Rohrer, S.P., Birzin, E.T., Eary, C.H., Schaeffer, J.M., Shoop, W.L. Ivermectin binding sites in sensitive and resistant *Haemonchus contortus*. J Parasitol 1994;80:493-7.

[2] Bouzat, C., Roccamo, A.M., Garbus, I., Barrantes, F.J. Mutations at lipid-exposed residues of the acetylcholine receptor affect its gating kinetics. Mol Pharmacol 1998;54:146-53.

[3] Paiement, J.P., Prichard, R.K., Ribeiro, P. *Haemonchus contortus*: characterization of a glutamate binding site in ivermectin-selected and unselected larvae and adults. Exp Parasitol 1999;92:32-9.

[4] Cully, D.F., Vassilatis, D.K., Liu, K.K., Paress, P.S., Van der Ploeg, L.H.T., Schaeffer, J.M., Arena, J.P. Cloning of an avermectin-sensitive glutamate-gated chloride channel from *Caenorhabditis elegans*. Nature 1994;371:707-11.

[5] Etter, A., Cully, D.F., Schaeffer, J.M., Liu, K.K., Arena, J.P. An amino acid substitution in the pore region of a glutamate-gated chloride channel enables the coupling of ligand binding to channel gating. J Biol Chem 1996;271:16035-9.

[6] Gill, J.H., Lacey, E. Avermectin/milbemycin resistance in trichostrongyloid nematodes. Int J Parasitol 1998;28:863-77.

[7] Ali, D.N., Hennessy, D.R. The effect of level of feed intake on the pharmacokinetic disposition and efficacy of ivermectin in sheep. J Vet Pharmacol Ther 1996;19:89-94.

[8] Skinner, T.M., Bascal, Z.A., Holden-Dye, L., Lunt, G.G., Wolstenholme, A.J. Immunocytochemical localization of a putative inhibitory amino acid receptor subunit in the parasitic nematodes *Haemonchus contortus* and *Ascaris suum*. Parasitol 1998;117:89-96.

[9] Geary, T.G., Sims, S.M., Thomas, E.M., Vanover, L., Davis, J.P., Winterrowd, C.A., Klein, R.D., Ho, N.F.H., Thompson, D.P. *Haemonchus contortus*: ivermectin-induced paralysis of the pharynx. Exp Parasitol 1993;77:88-96.

[10] Marriner, S.E., McKinnon, I., Bogan, J.A. Pharmacokinetics of ivermectin after oral and subcutaneous administration of sheep and horses. J Vet Pharmacol Ther 1987;10:175-9.

[11] Sangster, N. Pharmacology of anthelmintic resistance. Parasitol 1996;113:S201-16.

[12] Prichard, R.K., Steel, J.W., Lacey, E., Hennessy, D.R. Pharmacokinetics of ivermectin in sheep following intravenous, intra-abomasal or intraruminal administration. J Vet Pharmacol Ther 1985;8:88-94.

[13] McKenzie, J.A. The genetics of resistance to chemotherapeutic agents. In: Anderson, N., Waller, P.J., editors. Resistance in nematodes to anthelmintic drugs. Melbourne: Australian Wool Corporation Technical Publication, 1985;pp. 89-95.

[14] Le Jambre, L.F. Ivermectin-resistant *Haemonchus contortus* in Australia. Aust Vet J 1993;70:357.

[15] Dobson, R., Le Jambre, L., Gill, J. Management of anthelmintic resistance - inheritance of resistance and selection with persistent drugs. Int J Parasitol 1996;26:993-1000.

[16] Roos, M.H. The molecular nature of benzimidazole resistance in helminths. Parasit Today 1990;6:125-7.

[17] Roos, M.H., Boersema, J.H., Borgsteede, F.H.M., Cornelissen, J., Taylor, M., Ruitenberg, E.J. Molecular analysis of selection for benzimidazole resistance in the sheep parasite *Haemonchus contortus*. Mol Biochem Parasitol 1990;43:77-88.

[18] Kwa, M.S.G., Kooyman, F.N.J., Boersema, J.H., Roos, M.H. Effect of selection for benzimidazole resistance in *Haemonchus contortus* on β-tubulin isotype 1 and isotype 2 genes. Biochem Biophys Res Commun 1993;191:413-9.

[19] Kwa, M.S.G., Veenstra, J.G., Roos, M.H. Molecular characterisation of β-tubulin genes present in benzimidazole resistant populations of *Haemonchus contortus*. Mol Biochem Parasitol 1993;60:133-44.

[20] Kwa, M.S.G., Veenstra, J.G., Roos, M.H. Benzimidazole resistance in *Haemonchus contortus* is correlated with a conserved mutation at amino acid 200 in β-tubulin isotype 1. Mol Biochem Parasitol 1994;63:299-303.

[21] Beech, R.N., Prichard, R.K., Scott, M.E. Genetic variability of the β-tubulin genes in benzimidazole-susceptible and -resistant strains of *Haemonchus contortus*. Genetics 1994;138:103-10.

[22] Beugnet, F., Gauthey, M., Kerboeuf, D. Partial *in vitro* reversal of benzimidazole resistance by the free-living stages of *Haemonchus contortus* with verapamil. Vet Rec 1997;141:575-6.

[23] Xu, M., Molento, M., Blackhall, W., Ribeiro, P., Beech, R., Prichard, R. Ivermectin resistance in nematodes may be caused by alteration of P-glycoprotein homolog. Mol Biochem Parasitol 1998;91:327-35.

[24] Lewin, B. Genes IV. Oxford: Oxford University Press, 1990.

[25] Jacobson, A., Peltz, S.W. Interrelationships of the pathways of mRNA decay and translation in eukaryotic cells. Ann Rev Biochem 1996;693-739.

[26] Shefer-Vaida, M., Sherman, A., Ashkenazi, T., Robzyk, K., Kassir, Y. Positive and negative feedback loops affect the transcription of IME1, a positive regulator of meiosis in *Saccharomyces cervesiae*. Dev Genetics 1995;16:219-28.

[27] Gottesman, M.M., Pastan, I. Biochemistry of multidrug resistance mediated by the multidrug tansporter. Ann Rev Biochem 1993;62:385-427.

INTRODUCTION TO APPENDICES

Three appendices are included in this thesis. Appendix A presents data that was interesting but unexpected and could not easily be incorporated into a hypothesis of a mechanism of anthelmintic resistance. A hypothesis is suggested that could explain the data. Appendix B, a research note submitted for publication, does not involve a mechanism of anthelmintic resistance and so is not included as a chapter in this thesis. Appendix C lists the genotypes of the worms I have analysed. I include this material solely for the benefit of others who may want to know where they could find a particular allele for a particular gene.

APPENDIX A

Selection at a β-tubulin Gene in IVM- and MOX-Selected Strains of *Haemonchus contortus*

INTRODUCTION

β-tubulin is a component protein of microtubules, which are important structures of the cytoskeleton and mitotic spindle in eukaryotic cells. A wide range of chemicals are known to interfere with the polymerization of microtubules. Benzimidazoles (BZ), a class of anthelmintics, bind with high affinity to β-tubulin [1,2,3] leading to the loss of microtubules in nematodes [4]. In *Haemonchus contortus*, selection for a specific allele at the β-tubulin isotype 1 gene, and the resultant reduction in frequency of other alleles, has been observed in strains treated with BZ as compared to untreated strains [5,6,7,8,9]. Such changes in allele frequencies associated with anthelmintic treatment can be interpreted as genetic evidence for a gene's contribution to the mode of action of, or the mechanism of resistance to, an anthelmintic.

During the course of an analysis of genetic variability in *H. contortus*, statistically significant differences in allele frequencies at the β-tubulin isotype 1 gene were observed between strains treated with ivermectin (IVM) or moxidectin (MOX) and their derived untreated strain. These results were unexpected, since no effects of either IVM or MOX on microtubule polymerization have been reported. A similar analysis of a gene encoding a β-subunit of the glutamate-gated chloride (GluCl) channel found statistically significant differences in allele frequencies of this gene between a strain of *H. contortus* selected with cambendazole, a BZ anthelmintic, and its derived unselected strain. This result was also unexpected, since no association between BZ drugs and ion channels/receptors has been reported.

This report will present the results of these analyses and the hypothesis that GluCl channels and microtubules interact sufficiently closely to cause epistatic changes in the genes encoding the interacting proteins.

MATERIALS AND METHODS

Parasite strains. Eight strains of *H. contortus* were used in this study. The strains that were selected with IVM or MOX, and their derived unselected strains, are described in Chapter II. The strains selected with BZ and a derived unselected strain are described in Chapter V.

DNA isolation. Individual adult male worms were collected live from the abomasums of sheep and incubated in phosphate-buffered saline at 37°C for 2 hr. DNA was then extracted as described in [9].

Polymerase chain reaction (PCR) amplification. Primers were designed based on the cDNA sequence of the β-tubulin isotype 1 gene, GRU-1 [10]. RBE31, 5' AGAACACCGATGAAACGT 3', and MRβ5, 5' ACCAGACATTGTGACAGA 3', were used to obtain a genomic PCR product 190 base pairs in length from 30 individuals of each strain. The reaction mixture contained 2.5 µl 10X *Taq* buffer, 2.5 µl 2 mM dNTPs, 2.5 µl 25 mM $MgCl_2$, 0.5 µl 20 mM primer solutions, 0.5 unit *Taq* polymerase, approximately 1 ng DNA template, and water to a final concentration of 25 µl. Amplification reactions were performed on an MJ Research, Inc. PTC-100 Programmable Thermal Controller (Madison, WI). Amplification conditions were: 95° for 4 min followed by 40 cycles of 95° for 15 sec, 47° for 15 sec, and 70° for 30 sec with a final extension step at 70° for 5 min. PCR reactions for the gene encoding a β-subunit of

the GluCl channel are described in Chapter IV. PCR products were visualized on a 1% agarose gel containing 0.5 μg/ml ethidium bromide.

Single-strand conformation polymorphism (SSCP) analysis. PCR products were prepared for labelling reactions as described in Chapter IV. For the β-tubulin gene fragment, two μl of the eluate were used as template for the following labelling reactions: 0.5 μl 10X *Taq* buffer, 0.5 μl 50 μM dNTPs, 0.5 μl 25 mM $MgCl_2$, 0.25 μl 2 μM MRβ5 primer, 0.1 μl 1000 Ci/mM dATP $\alpha^{35}S$, 0.1 unit *Taq* polymerase, and water to a final volume of 5 μl. The reactions were overlaid with a drop of mineral oil and thermal-cycled as above. Six μl of stop solution (10 mM NaOH, 95% formamide, 0.05% bromophenol blue, and 0.05% xylene cyanole) were added at the end of the reactions. The reactions were heated at 80°C for 2 min, and 2.5 μl were loaded onto a 10%, 39:1 acrylamide:N,N'-methylenebisacrylamide, 1X TBE acrylamide gel and electrophoresed at 50W for 6 hr in a 6°C coldroom. Gels were dried and exposed to X-ray film overnight. SSCP analyses for the GluCl channel β-subunit gene are described in Chapter IV. Alleles were identified by their differing rates of migration through the gel.

Data analysis. Allele frequencies for each strain were tabulated. Chi-square analyses were performed between the strains passaged without drug treatment and the corresponding selected strains. Rare alleles having frequency values lower than 5 were grouped for the analyses. Alleles whose counts were 5 or lower in a population were grouped together for the Chi-square analyses. The count of rare alleles in a population can vary from sample to sample, and the grouping of their counts to form a single

category representing "rare alleles" lessens the statistical impact they would have in a Chi-square analysis.

RESULTS

Allele frequencies of the β-tubulin isotype 1 gene from the two Merck strains of *H. contortus* are presented in Fig. 1. In the unselected strain, MIS, alleles A and C were predominant, each occurring at a frequency of 0.433. Alleles B, D, E, and F were rare. In the IVM-selected strain, MIR, allele A decreased in frequency to 0.200, and allele C increased in frequency to 0.600. Allele B also increased in frequency to 0.133. Allele D was rare, and alleles E and F were not observed. Despite these differences, a Chi-square analysis indicated no significant difference between allele frequencies of MIS and MIR ($p > 0.05$). The data, however, suggest that selection may be occurring in MIR, but that the degree of selection was insufficient for statistical significance.

Allele frequencies of the β-tubulin isotype 1 gene from the three Fort Dodge strains are presented in Fig. 2. Whereas a total of six alleles were observed in the Merck strains, only three alleles were found in the Fort Dodge strains. Allele C, one of the two common alleles in the Merck strains, was by far the most common allele seen in the unselected Fort Dodge strain, PF17, with a frequency of 0.750. Alleles A and B occurred at lower frequencies, 0.150 and 0.100, respectively. In the IVM-selected strain, IVF17, allele B increased substantially in frequency, to 0.633, and allele C decreased substantially in frequency, to 0.217. Allele B also increased in frequency in the MOX-selected strain, MOF17, to 0.400. Allele C in this strain decreased substantially in frequency, to 0.317, as it did in IVF17. Allele A increased in frequency to 0.283 in MOF17. Chi-square analyses indicated that allele frequencies in both IVF17 and MOF17 were significantly different from those in PF17 ($p < 0.001$ for both). No significant difference was found between allele frequencies of IVF17 and MOF17.

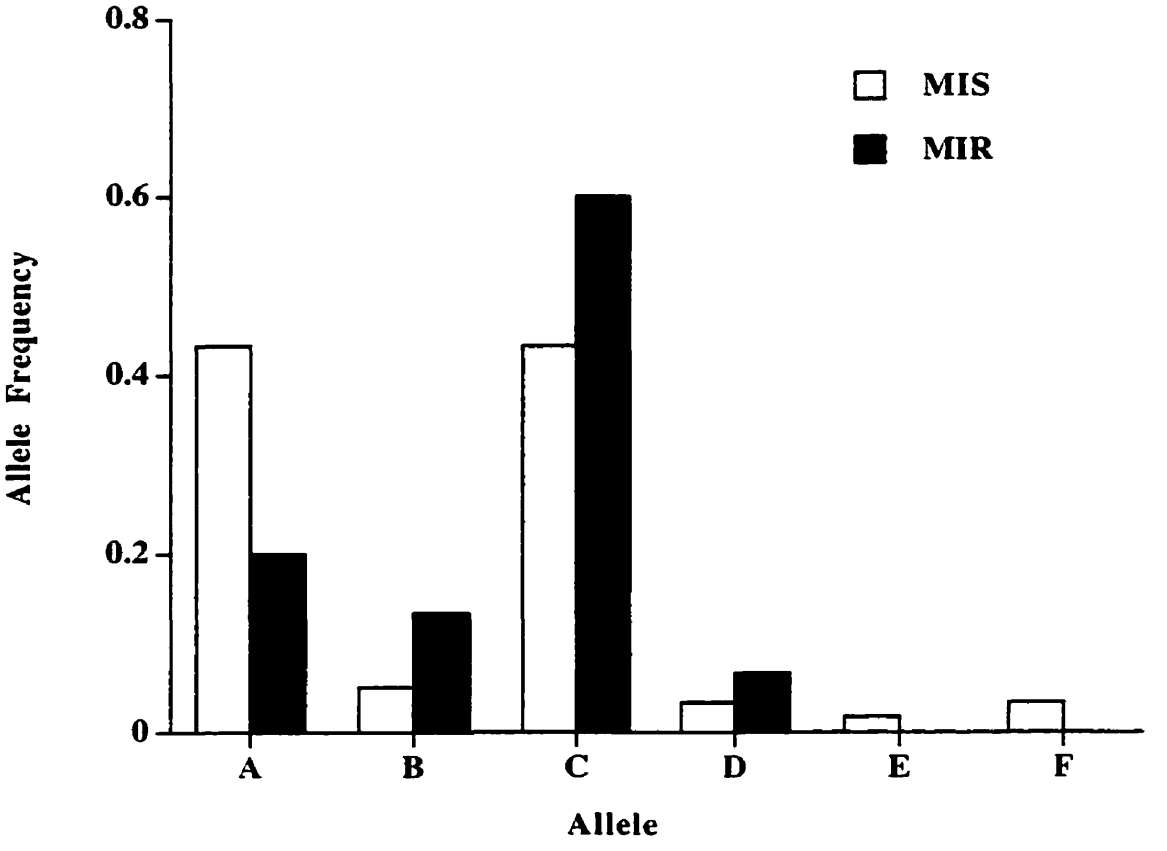

Figure 1. Allele frequencies of β-tubulin isotype 1 gene, Merck strains of *H. contortus*.

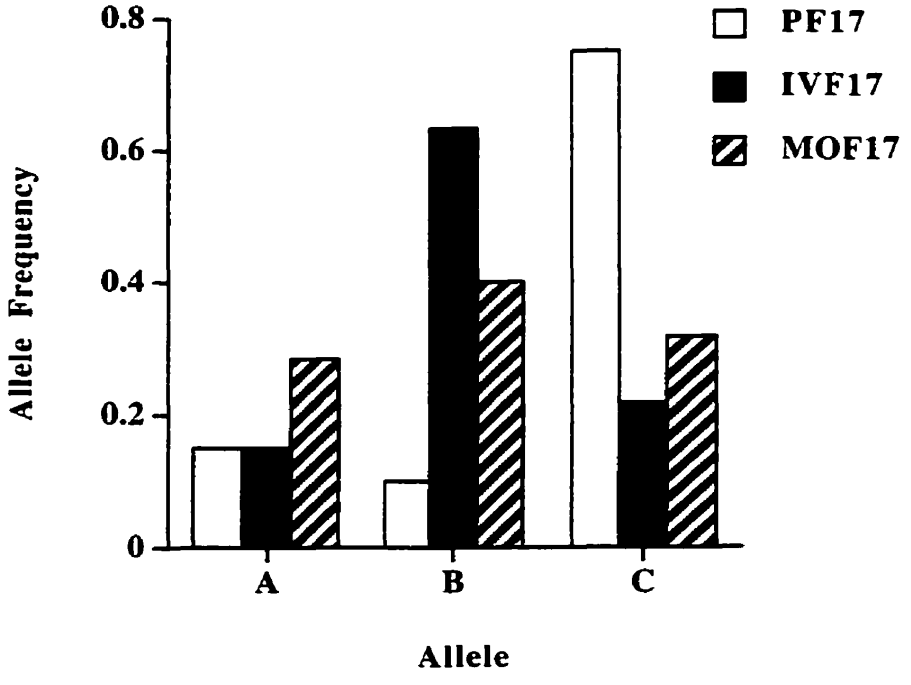

Figure 2. Allele frequencies of β-tubulin isotype 1 gene, Fort Dodge strains of *H. contortus*.

Allele frequencies of the GluCl channel β-subunit gene in the Merck and Fort Dodge strains, as described in Chapter II (see Figs. 4 and 5), indicated that selection at this locus was not occurring during IVM or MOX treatment. Allele frequencies of this locus from the cambendazole (CBZ)-unselected, CBZ-selected, and thiabendazole (TBZ)-selected strains are shown in Fig. 3. Of the total of twelve alleles found in the Merck and Fort Dodge strains, eight were also found in the BZ strains. Alleles G, H, J, and K were found only in the Merck or Fort Dodge strains, and alleles M, N, and O were found only in the BZ strains, at low frequencies. Allele F was the most common allele in all the Merck and Fort Dodge strains. In the CBZ-unselected strain, allele F was not common, perhaps reflecting differences in the origins of the CBZ strains from the Merck and Fort Dodge strains. Allele F, however, increased in frequency from 0.083 in the CBZ-unselected strain to 0.283 in the CBZ-selected strain. Allele I also increased in frequency in the CBZ-selected strain. Allele B was the most common allele in both strains. The CBZ-selected strain appears to possess reduced variability relative to the unselected strain, having only five alleles compared to nine in the unselected strain. Some of the missing alleles, such as L, N, and O, occurred at low frequency in the unselected strain, and their disappearance in the selected strain may be an artifact of sample size. Alleles C and E, however, occurred at sufficiently high frequencies in the unselected strain, 0.233 and 0.117, respectively, that their disappearance in the selected strain may represent a negative selection pressure against them during anthelmintic treatment. A Chi-square analysis indicates that allele frequencies of the GluCl channel β-subunit gene are significantly different between these two strains ($p < 0.025$).

The TBZ-selected strain was not derived from the CBZ-unselected strain, so no direct comparison of allele frequencies, or inferences of the effect of TBZ treatment on

GluCl channel β-subunit allele frequencies, can be made. Alleles B and F were the most common alleles in the TBZ-selected strain, with frequencies of 0.383 and 0.319, respectively (Fig. 3). Nine other alleles were present in this strain, all with frequencies of 0.067 or less.

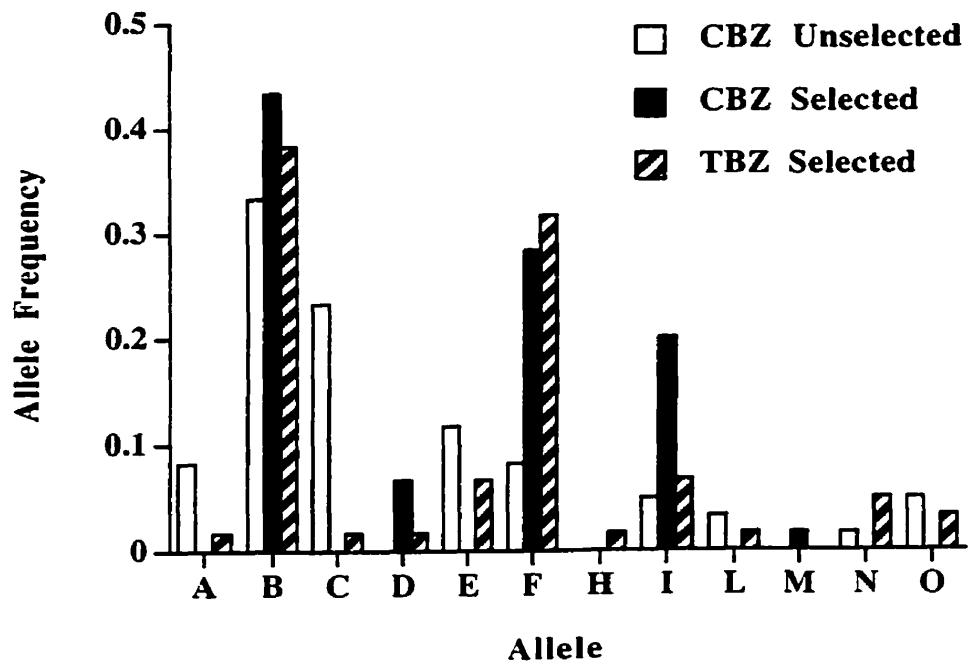

Figure 3. Allele frequencies of GluCl beta subunit gene, CBZ-unselected, CBZ-selected, and TBZ-selected strains of *H. contortus*.

DISCUSSION

The observation of apparent selection at the β-tubulin isotype 1 locus, GRU-1, in IVM- and MOX-selected strains of *H. contortus* is difficult to resolve given the known or suspected modes of action of these anthelmintics. The simplest explanation would be to infer that this gene is closely linked to one of the genes where selection due to anthelmintic treatment is apparent, and that selection for specific alleles at this other gene is causing changes in allele frequencies at the β-tubulin gene by the process of genetic hitch-hiking. Apparent selection occurs at a P-glycoprotein (P-gp) gene in the Merck and Fort Dodge drug-treated strains (Chapter IV), with selection being stronger in the Fort Dodge strains than in the Merck strain. At the β-tubulin locus, selection was also stronger in the Fort Dodge strains, supporting the hypothesis of hitch-hiking with the P-gp gene. However, the selection for a very rare P-gp allele, allele P, in the CBZ- and TBZ-selected strains (Chapter V) does not support a hitch-hiking hypothesis. Treatment with BZ anthelmintics in these strains led to a very strong selection for one BZ-resistant allele [9]. This resistance allele was a common allele in the unselected strain, having a frequency of 0.458 [9]. If the P-gp and β-tubulin genes are closely linked, and the P-gp allele P is rare, then the increase in the frequency of the BZ-resistance allele should have caused an increase in other, more common, P-gp alleles, not just allele P. Such increases were not observed (see Fig. 1, Chapter V). The possibility exists that the BZ-resistance allele identified in the CBZ-unselected strain as having a frequency of 0.458 is actually a pool of at least two alleles, with the true BZ-resistance allele occurring at a low frequency. A large increase in frequency of this rare β-tubulin allele during BZ treatment, then, could conceivably cause an increase in frequency of the rare P-gp allele P through hitch-hiking.

Such a scenario, though, would require the co-occurrence of two rare alleles in neighbouring genes, a scenario that is not impossible, but unlikely assuming mutation to be random. Although genetic hitch-hiking of β-tubulin alleles with P-gp alleles cannot be ruled out as the cause of the observed changes in β-tubulin allele frequencies in the IVM- and MOX-selected strains, evidence supporting this hypothesis is lacking at present.

Evidence also does not support the possible hitch-hiking of β-tubulin alleles with those of the putative GluCl channel alpha subunit gene. Allele frequencies of this gene were not significantly different between the CBZ-unselected and -selected strains (see Fig. 2, Chapter V), where strong selection had occurred at the β-tubulin gene. Allele frequencies of the gamma-aminobutyric acid (GABA) receptor gene in the BZ strains were not determined, so the possibility remains that β-tubulin alleles are hitch-hiking along with GABA-receptor alleles.

Changes in allele frequencies of the β-tubulin isotype 1 gene during treatment with IVM or MOX could occur if these anthelmintics interacted with tubulin. IVM and MOX are macrocyclic lactones (MCL), and other MCL are known to inhibit microtubule polymerization. Rhizoxin, an anti-mitotic antibiotic, inhibits tubulin polymerization [11]. This inhibition is believed to involve interaction with β-tubulin [12]. Spongistatin 1 also inhibits microtubule assembly and binds to a site believed to be on a β-tubulin subunit [13]. In an *in vitro* assay, however, IVM did not inhibit microtubule polymerization until applied at a concentration of 100 μM (M. Oxberry personal communication), which is about 3 orders of magnitude higher than blood concentrations of IVM following administration of a recommended dose to sheep. IVM and MOX, then, are unlikely to interact sufficiently with β-tubulin to cause the observed changes in allele frequencies.

As discussed above, changes in the frequency of β-tubulin alleles in the IVM- and MOX-selected strains do not appear to be due to a physical linkage to other genes whose allele frequencies may change as a result of anthelmintic treatment. The changes, however, may be due to a functional linkage between the protein products of these other genes and the β-tubulin isotype 1 gene. If microtubules interact with P-gp, GluCl channels, or GABA receptors, and if alleles of these structures interact differentially with β-tubulin alleles, then a change in one might provide selection pressure for a change in the other.

P-gp is a membrane-bound protein, and evidence suggests that, at least in some human cell types, it is anchored and localized in the membrane by interactions with the cytoskeleton, of which microtubules are a part [14,15]. Selection of P-gp alleles during anthelmintic treatment might cause selection for appropriately adapted β-tubulin alleles in order to maintain the integrity of the microtubule-P-gp interaction.

Since IVM and MOX paralyse nematodes apparently by opening ligand-gated ion channels, the proper functioning of these channels is key to a nematode's survival. Some neurotransmitter receptors are found extrasynaptically, but most are localized to synaptic junctions where neurotransmitter concentrations are highest. The clustering of receptors at synaptic junctions has been most extensively studied in the vertebrate glycine receptor. Glycine receptors are, like GluCl channels and GABA receptors, inhibitory chloride channels. They belong to the same family of ligand-gated ion channels as, and are structurally and functionally similar to, GluCl channels and GABA receptors. In vertebrates, glycine receptors are anchored in cell membranes at synaptic junctions by binding to a protein, gephyrin, which in turn binds to tubulin [16]. Gephyrin may also be necessary for the proper functioning of the receptor: agonist and antagonist binding

affinities are altered in homomeric alpha 2 glycine receptors when gephyrin is coexpressed with the alpha 2 subunit [17]. Vertebrate GABA Type A, acetylcholine, and glutamate receptors are also known to be associated with clustering proteins [18]. The proper anchoring of receptors in the cell membrane, then, seems vital for normal neurotransmitter signalling.

It is not known if receptor clustering in invertebrates involves an intermediary protein like gephyrin, or whether one or more receptor subunits are able to bind tubulin directly. Whatever the mechanism of receptor clustering may be in *H. contortus*, a vital association between receptor and tubulin may exist and may be responsible for the observed changes in β-tubulin allele frequencies in strains selected with IVM and MOX. In these strains, allele frequencies of the putative GluCl channel alpha subunit are altered, but those of the beta subunit are not (Chapter II). Perhaps changes in allele frequencies of the GluCl channel alpha subunit cause epistatic changes in β-tubulin allele frequencies. This effect, however, was not seen in the BZ strains. Strong selection at the β-tubulin locus did not cause a change in allele frequencies at the GluCl channel alpha subunit (Chapter V). Changes in allele frequencies, though, were observed in the GluCl channel beta subunit (Fig. 3).

Alterations in allele frequencies of the β-tubulin gene in IVM- and MOX-selected strains and of the GluCl channel beta subunit in a CBZ-selected strain may be due to epistatic interactions between GluCl channel subunits and β-tubulin. If such epistatic interactions do exist, they may be complex and may depend on many factors, such as specific inter-allele compatibilities, interactions of anthelmintic with target protein that may affect protein conformations, interactions between GluCl channel subunits in the presence or absence of IVM or MOX, whether one or both GluCl channel subunits

anchor the channel, or whether IVM or MOX may directly interfere with the anchoring mechanism. Further study will be necessary to confirm or refute this hypothesis of epistatic interaction.

REFERENCES

[1] Davidse, L.C., Flach, W. Differential binding of methyl benzimidazole-2-yl-carbamate to fungal tubulin as a mechanism of resistance to this antimitotic agent in mutant strains of *Aspergillus nidulans*. J Cell Biol 1977;72:174-89.

[2] Hammerschlag, R.S., Sisler, H.D. Benomyl and methyl-2-benzimidazolecarbamate (MBC): biochemical, cytological and chemical aspects of toxicity in *Ustilago maydis* and *Saccharomyces cervisiae*. Pestic Biochem Physiol 1973;3:42-54.

[3] Lubega, G.W., Prichard, R.K. Specific interaction of benzimidazole anthelmintics with tubulin: high-affinity binding and benzimidazole resistance in *Haemonchus contortus*. Mol Biochem Parasitol 1990;38:221-32.

[4] Borgers, M., de Nolin, S., de Brabander, M., Thienpont, D. Influence of the anthelmintic mebendazole on microtubules and intracellular organelle movement in nematode intestinal cells. Am J Vet Res 1975;36:1153-66.

[5] Roos, M.H. The molecular nature of benzimidazole resistance in helminths. Parasitol Today 1990;6:125-7.

[6] Roos, M.H., Boersema, J.H., Borgsteede, F.H.M., Cornelissen, J., Taylor, M., Ruitenberg, E.J. Molecular analysis of selection for benzimidazole resistance in the sheep parasite *Haemonchus contortus*. Mol Biochem Parasitol 1990;43:77-88.

[7] Kwa, M.S.G., Kooyman, F.N.J., Boersema, J.H., Roos, M.H. Effect of selection for benzimidazole resistance in *Haemonchus contortus* on β-tubulin isotype 1 and isotype 2 genes. Biochem Biophys Res Commun 1993;191:413-9.

[8] Kwa, M.S.G., Veenstra, J.G., Roos, M.H. Molecular characterisation of β-tubulin genes present in benzimidazole resistant populations of *Haemonchus contortus*. Mol Biochem Parasitol 1993;60:133-44.

[9] Beech, R.N., Prichard, R.K., Scott, M.E. Genetic variability of the β-tubulin genes in benzimidazole-susceptible and -resistant strains of *Haemonchus contortus*. Genetics 1994;138:103-10.

[10] Geary, T.G., Nulf, S.C., Favreau, M.A., Tang, L., Prichard, R.K., Hatzenbuhler, N.T., Shea, M.H., Alexander, S.J., Klein, R.D. Three β-tubulin cDNAs from the parasitic nematode *Haemonchus contortus*. Mol Biochem Parasitol 1992;50:295-306.

[11] Takahashi, M., Iwasaki, S., Kobayashi, H., Okuda, S., Murai, T., Sato, Y., Haraguchi-Hiraoka, T., Nagano, H. Studies on macrocyclic lactone antibiotics. XI. Anti-mitotic and anti-tubulin activity of new antitumor antibiotics, rhizoxin and its homologues. J Antibiot 1987;40:66-72.

[12] Sullivan, A.S., Prasad, V., Roach, M.C., Takahashi, M., Iwasaki, S., Luduena, R.F. Interaction of rhizoxin with bovine brain tubulin. Cancer Res 1990;50:4277-80.

[13] Bai, R., Cichacz, Z.A., Herald, C.L., Pettit, G.R., Hamel, E. Spongistatin 1, a highly cytotoxic, sponge-derived, marine natural product that inhibits mitosis, microtubule assembly, and the binding of vinblastine to tubulin. Mol Pharmacol 1993;44:757-66.

[14] Thiebaut, F., Tsuruo, T., Hamada, H., Gottesman, M.M., Pastan, I., Willingham, M.C. Cellular localization of the multidrug-resistance gene product P-glycoprotein in normal human tissues. Proc Natl Acad Sci USA 1987;84:7735-8.

[15] Tatsuta, T., Naito, M., Oh-hara, T., Sugawara, I., Tsuruo, T. Functional involvement of P-glycoprottein in blood-brain barrier. J Biol Chem 1992;267:20383-91.

[16] Kirsch, J., Langosch, D., Prior, P., Littauer, U.Z., Schmitt, B., Betz, H. The 93-kDa glycine receptor-associated protein binds to tubulin. J Biol Chem 1991;266:22242-5.

[17] Takagi, T., Pribilla, I., Kirsch, J., Betz, H. Coexpression of the receptor-associated protein gephyrin changes the ligand binding affinities of alpha 2 glycine receptors. FEBS Lett 1992;303:178-80.

[18] Colledge, M., Froehner, S.C. To muster a cluster: anchoring neurotransmitter receptors at synapses. Proc Natl Acad Sci USA 1998;95:3341-3.

APPENDIX B

Genetic Variability of a Phosphofructokinase Gene in *Haemonchus contortus*

William J. Blackhall, Roger K. Prichard, and Robin N. Beech

Submitted

(03/23/99)

The development of anthelmintic resistance to all the major classes of chemicals used to control helminthoses in livestock and, potentially, humans underscores the need to continue the search for new, effective drugs. Random screening of natural or synthetic chemicals, or screening of compounds structurally related to known drugs, has produced many effective anthelmintics [1]. Another approach to drug discovery, however, is the rational design of compounds that can interfere with the functioning of new, known targets. These targets must be sufficiently vital to parasite survival. They must also be biochemically and pharmacologically distinct between parasite and host to prevent undue damage to the host.

Glycolysis is believed to be an important means of energy metabolism in gastrointestinal nematodes [2]. This property renders enzymes of the glycolytic pathway potential targets for the design of new anthelmintics. Phosphofructokinase (PFK) is a key regulatory enzyme in glycolysis that phosphorylates fructose 6-phosphate to yield fructose 1,6-diphosphate. Nematode PFK appears to be both biochemically and pharmacologically distinct from mammalian PFK [3] and so may represent a suitable target for drug design. Other factors, however, need to be considered to warrant the considerable time and expense necessary in developing new pharmaceuticals. One such factor is the genetic variability of the target. Many of the trichostrongylid nematodes of agriculturally important animals possess considerable genetic variability [4, 5, 6, 7, 8]. If a parasite population possesses many allelic variants of the target protein of an anthelmintic, the probability is high that one or more of these alleles will be able to tolerate the action of the drug, and resistance will develop in time as a result of selection for the resistant allele(s) in the population [5,7]. Conversely, if the target protein is

relatively invariant, tolerant alleles may be very rare or absent, in which case, resistance would be very slow or unable to develop [9].

We have undertaken an analysis of the genetic variability of a gene encoding a PFK from the common trichostrongylid nematode *Haemonchus contortus* in an effort to assess the suitability of this enzyme as a target for the design of new anthelmintics. The strains of *H. contortus* used in this study are described in [7]. DNA was extracted from individual adult males as described in [5]. DNA was amplified by polymerase chain reaction (PCR) from genomic DNA of 30 individuals from each strain. Primers were designed based on the PFK cDNA sequence reported by [3]. A sense primer, 5' CAGTCGTTCGTATGGGTA 3' (positions 408 to 425 in [3]), and an antisense primer, 5' CTTCATTCGGCCTTCTCG 3' (positions 584 to 601), amplified a fragment of genomic DNA approximately 300 base pairs in length. All reactions were performed using an MJ Research PTC-100 Programmable Thermal Controller (Watertown, MA). The reaction mixture contained 2.5 µl 10X *Taq* buffer, 2.5 µl 2 mM dNTPs, 2.0 µl 25 mM $MgCl_2$, 0.5 µl 20 µM primer solutions, 0.5 unit *Taq* polymerase, approximately 1 ng DNA template, and water to a final volume of 25 µl. Amplification conditions were: 95°C for 1 min followed by 40 cycles of 95°C for 10 sec, 53°C for 15 sec, and 70°C for 30 sec with a final extension step at 70°C for 5 min. PCR products were visualized on a 1% agarose gel containing 0.5 µg/ml ethidium bromide. PCR products were excised from the agarose gel, and the DNA extracted by placing the excised bands in the top of 0.5-10 µl filter pipette tips, placing the tips in microcentrifuge tubes, and centrifuging the tubes at 14,000 rpm for 30 sec.

The genetic variability of the PCR products was analysed by the technique of single-strand conformation polymorphism (SSCP). Two µl of the eluate were used as

template for the following labelling reaction: 0.5 µl 10X *Taq* buffer, 0.5 µl 50 µM dNTPs, 0.4 µl 25 mM $MgCl_2$, 0.25 µl 2 µM antisense primer, 0.1 µl 1000 Ci/mM dATP $\alpha^{35}S$, 0.1 unit *Taq* polymerase, and water to a final volume of 5 µl. The PCR mixtures were overlaid with a drop of mineral oil and thermal-cycled as above. Six µl of stop solution (10 mM NaOH, 95% formamide, 0.05% bromophenol blue, and 0.05% xylene cyanole) were added at the end of the reactions. The reaction mixtures were heated at 80°C for 2 min, and 2.5 µl were loaded onto a 20%, 99:1 acrylamide:N,N'-methylenebisacrylamide, 1X TBE acrylamide gel and electrophoresed at 50W for 4 hr in a 6°C coldroom. Gels were dried and exposed to X-ray film overnight. Alleles were identified by their differing rates of migration through the gel.

Only two alleles of the PFK gene were identified by SSCP analysis (Table 1) in the five strains of *H. contortus*. Allele B predominated in all five strains. The independent derivation of the Merck and the Fort Dodge strains may indicate that this pattern of alleles is typical of the species. Of 90 worms from the Fort Dodge strains, only one possessed an allele A. All worms from all strains appeared as homozygotes, possessing just a single allele. *H. contortus* has an X0 sex chromosome system, so that males have only a single sex chromosome. In light of the low frequency of allele A, particularly in the Fort Dodge strains, this PFK gene is thus likely to reside on the X chromosome. The lack of variability seen at this locus, then, may in part be due to the reduced opportunity for recombination, as well as the necessity of conservation common in functionally important proteins.

The sensitivity of the SSCP technique is not absolute, so the existence of other alleles undetected here is a real possibility. Compared with other *H. contortus* genes,

Allele	Strain				
	MIS	MIR	PF17	IVF17	MOF17
A	0.100	0.200	0.033	0.000	0.000
B	0.900	0.800	0.967	1.000	1.000

Table 1. Allele frequencies of a PFK gene from Merck unselected (MIS) and ivermectin-selected (MIR) strains, and Fort Dodge unselected (PF17), ivermectin-selected (IVF17), and moxidectin-selected (MOF17) strains of *H. contortus*.

however, PFK appears to be relatively invariant. Similar analyses, of comparable fragment length and sample size, identified 6 alleles of a gene encoding a putative alpha subunit of a glutamate-gated chloride channel and 12 alleles of a gene encoding the beta subunit from the same strains of *H. contortus* used here [7]. Other SSCP analyses of these strains have found 13 alleles of a putative gamma-aminobutyric acid (GABA) receptor subunit gene, 8 alleles of a phosphoenolpyruvate carboxykinase gene, and 5 alleles of a nicotinic acetylcholine receptor gene (Chapter III). RFLP analysis of P-glycoprotein polymorphism in the same strains of *H. contortus* revealed at least 31 alleles [8]. PFK is a highly regulated, rate-limiting enzyme in glycolysis [10]. As such it has multiple receptor/functional sites. Functional sites in proteins tend to be more highly conserved , and this may have restricted the selection for polymorphism in the *H. contortus* PFK in comparison with other *H. contortus* genes investigated for polymorphism. The fragment used in the present study is upstream of the highly conserved proposed active site of the PFK protein and is in a region that is moderately conserved. The fragment, however, is approximately 1/3 intron and so should possess sufficient variability for the detection of alleles within the limits of resolution of the SSCP technique.

The isolation of a PFK gene from *H. contortus* [3] was the first step toward the potential development of a new class of anthelmintics targeting this enzyme in nematodes. The cloning and expression of this gene in a molecular screening system that could assay PFK activity when exposed to various compounds would require a larger investment. The lack of variability in the *H. contortus* PFK suggests a low probability or rate of development of resistance involving a mechanism dependent on target modifications should an anthelmintic targeting PFK be discovered. Investment in a PFK anthelmintic screen should not be easily compromised by rapid selection for resistance.

REFERENCES

[1] Thompson, D.P., Klein, R.D., Geary, T.G. Prospects for rational approaches to anthelmintic discovery. Parasitol 1996;113:S217-38.

[2] Ward, P.V.F. Aspects of helminth metabolism. Parasitol 1982;84:177-94.

[3] Klein, R.D., Olson, E.R., Favreau, M.A., Winterrowd, C.A., Hatzenbuhler, N.T., Shea, M.H., Nulf, S.C., Geary, T.G. Cloning of a cDNA encoding phosphofructokinase from *Haemonchus contortus*. Mol Biochem Parasitol 1991;48:17-26.

[4] Dame, J.B., Blouin, M.S., Courtney, C.H. Genetic structure of populations of *Ostertagia ostertagi*. Vet Parasitol 1993;46:55-62.

[5] Beech, R.N., Prichard, R.K., Scott, M.E. Genetic variability of the β-tubulin genes in benzimidazole-susceptible and -resistant strains of *Haemonchus contortus*. Genetics 1994;138:103-10.

[6] Grant, W.N., Whitington, G.E. Extensive DNA polymorphism within and between two strains of *Trichostrongylus colubriformis*. Int J Parasitol 1994;24:719-25.

[7] Blackhall, W.J., Prichard, R.K., Beech, R.N. *Haemonchus contortus*: selection at a glutamate-gated chloride channel gene in ivermectin- and moxidectin-selected strains. Exp Parasitol 1998;90:42-8.

[8] Blackhall, W.J., Liu, H.Y., Xu, M., Prichard, R.K., Beech, R.N. Selection at a P-glycoprotein gene in ivermectin- and moxidectin-selected strains of *Haemonchus contortus*. Mol Biochem Parasitol 1998;95:193-201.

[9] Anderson,T.J.C., Blouin, M.S., Beech, R.N. Population biology of parasitic nematodes: applications of genetic markers. Adv Parasitol 1998;41:219-83.

[10] Uyeda, K. Phosphofructokinase. Adv Enzymol Mol Biol 1979;48:193-244.

APPENDIX C

Worm Genotypes

Worm	P-gp	GluClα	GluClβ	GABA	β-tub	PFK	PEPCK	AchR
PF17-1	--	CC	FF	eF	BB	BB	BD	DD
PF17-2	--	CC	FF	AD	AC	BB	AC	BC
PF17-3	--	CE	II	AD	CC	AA	CC	BD
PF17-4	--	CF	FL	AF	CC	BB	BC	DD
PF17-5	--	AC	FI	Ce	CC	BB	CC	DD
PF17-6	--	CF	EF	De	AC	BB	AH	DD
PF17-7	--	AE	FI	De	CC	BB	CD	BD
PF17-8	--	CC	FF	AA	CC	BB	AC	DD
PF17-9	--	CF	AL	AD	CC	BB	AC	BC
PF17-10	--	CF	FF	ee	CC	BB	CC	BD
PF17-11	--	AE	FI	DD	CC	BB	CC	DD
PF17-12	--	CF	AL	BC	AC	BB	AD	DD
PF17-13	--	AC	FF	AA	CC	BB	AC	DD
PF17-14	--	CC	FL	AD	BB	BB	AB	AB
PF17-16	--	CE	FF	DF	AC	BB	AD	CD
PF17-17	--	CE	FL	AF	AC	BB	AA	BC
PF17-18	--	CE	FL	AA	CC	BB	AA	DD
PF17-20	--	CC	FI	AD	CC	BB	AD	DD
PF17-22	--	CC	FF	Ae	CC	BB	AB	DD
PF17-23	--	CF	FI	AD	AA	BB	AA	DD
PF17-24	--	AF	FI	AB	CC	BB	CD	DD
PF17-25	--	CF	BL	ee	AC	BB	AA	DD
PF17-26	--	CC	BF	AA	CC	BB	AA	CD

Worm	P-gp	GluClα	GluClβ	GABA	β-tub	PFK	PEPCK	AchR
PF17-27	--	CC	BF	AD	AC	BB	CD	DD
PF17-28	--	CE	FI	AA	CC	BB	AA	DD
PF17-30	--	CC	FF	AD	CC	BB	CC	DD
PF17-31	--	CC	FI	DD	CC	BB	BD	AB
PF17-32	--	AA	FF	AA	CC	BB	AD	CD
PF17-34	--	AD	II	AC	BB	BB	BD	DD
PF17-35	--	EE	FF	AA	CC	BB	AD	BD
IVF17-1	AQ	AE	IL	Ae	AA	BB	AD	AD
IVF17-2	LL	AE	IL	Ae	BB	BB	AA	BC
IVF17-3	MM	CE	FF	Ce	BB	BB	AD	AC
IVF17-4	AA	CE	IL	AA	BB	BB	AA	CC
IVF17-5	AB	AF	FF	ee	AC	BB	AD	CD
IVF17-6	XX	AC	FF	ee	CC	BB	EE	AD
IVF17-7	AA	CC	FL	AB	BB	BB	AD	DD
IVF17-8	BN	CE	FF	AA	AC	BB	DE	DD
IVF17-9	AB	CE	FF	ee	BB	BB	AD	DD
IVF17-10	AA	CE	FF	Ae	BB	BB	AE	DD
IVF17-11	AA	AE	FI	ee	CC	BB	AA	CD
IVF17-12	BB	CE	FL	Ae	BB	BB	AA	BD
IVF17-13	AA	AE	FF	Ae	BB	BB	AE	BD
IVF17-14	AA	CC	FL	Ae	CC	BB	AD	DD
IVF17-15	BB	CC	FI	Ae	AA	BB	AC	DD
IVF17-16	AA	AF	FI	Ae	BB	BB	AA	BD

Worm	P-gp	GluClα	GluClβ	GABA	β-tub	PFK	PEPCK	AchR
IVF17-17	BB	CC	FF	ee	CC	BB	AE	CE
IVF17-18	KZ	AA	FI	Ae	BB	BB	AA	BD
IVF17-19	AA	CC	FF	Be	BB	BB	AA	CD
IVF17-20	AB	AC	FL	eG	BB	BB	AA	DD
IVF17-21	AB	CE	II	AB	BB	BB	AA	CD
IVF17-22	BX	AC	FF	Ae	AC	BB	AC	CD
IVF17-23	AA	AC	FF	De	BB	BB	AA	DD
IVF17-24	BB	AA	IL	Ae	CC	BB	AA	CE
IVF17-25	AA	AC	FF	Ae	BB	BB	AC	DD
IVF17-26	AA	CF	FF	ee	AA	BB	AC	CD
IVF17-27	AO	AE	FF	ee	BB	BB	AA	DD
IVF17-28	AA	FF	FF	Ae	BB	BB	AD	AD
IVF17-29	AB	CC	FF	Ae	BB	BB	AC	AD
IVF17-30	AA	AC	FF	De	BB	BB	AC	DD
MOF17-1	AA	CC	FL	Be	AC	BB	AD	AC
MOF17-2	AA	AA	FF	Ae	AC	BB	AD	AD
MOF17-3	BX	AA	FF	De	CC	BB	AE	DD
MOF17-4	BX	AE	FF	Ae	AC	BB	DE	DD
MOF17-6	AB	AE	II	AA	AC	BB	AC	DD
MOF17-8	Ae	AE	FI	ee	AA	BB	AD	DD
MOF17-10	Be	CC	LL	Ae	AA	BB	AD	DD
MOF17-11	AB	CC	IL	ee	BB	BB	AC	DD
MOF17-12	AB	AA	FF	Ae	AC	BB	AD	CD

Worm	P-gp	GluClα	GluClβ	GABA	β-tub	PFK	PEPCK	AchR
MOF17-13	AA	FF	FI	De	BB	BB	AA	DD
MOF17-14	AA	AC	FF	ee	AA	BB	CC	BD
MOF17-15	Be	CC	FL	ee	AA	BB	AA	AB
MOF17-17	XX	AC	FF	ee	CC	BB	CE	CD
MOF17-18	AA	CC	IL	ee	BB	BB	AC	AD
MOF17-19	AB	AF	FF	ee	BB	BB	AD	AD
MOF17-20	Be	AE	FI	De	AC	BB	AD	CD
MOF17-22	AB	FF	LL	Ae	BB	BB	AA	AD
MOF17-23	AO	AF	FF	De	BB	BB	AD	AD
MOF17-24	BP	EE	IL	ee	AC	BB	AD	DD
MOF17-27	AB	AF	FL	ee	AC	BB	CC	AD
MOF17-28	BO	AC	FF	ee	CC	BB	AE	DD
MOF17-29	AO	AE	FF	eG	BB	BB	AC	DD
MOF17-30	AO	CF	FF	Ae	BB	BB	AC	DD
MOF17-31	--	EF	FF	Ae	AC	BB	CD	AD
MOF17-32	BB	FF	FF	Ce	CC	BB	AD	CD
MOF17-34	BY	AE	FF	Ae	BB	BB	AC	CD
MOF17-36	BB	EE	FL	ee	CC	BB	AD	BD
MOF17-37	AA	AC	FF	Ae	BB	BB	AC	CC
MOF17-39	AO	AA	FI	ee	BB	BB	AE	BD
MOF17-40	AB	FF	FI	Ae	BB	BB	AD	AD
MIS-2	AB	EE	BF	FH	AC	BB	GH	BC
MIS-3	CG	CE	FF	AH	CC	AA	CC	DD

Worm	P-gp	GluClα	GluClβ	GABA	β-tub	PFK	PEPCK	AchR
MIS-5	AB	CE	BF	HH	CE	BB	AH	BC
MIS-6	AC	CE	FK	EE	AC	BB	BH	DD
MIS-7	BB	CE	FF	AF	CF	BB	CG	BC
MIS-8	CC	CE	II	EE	CC	BB	CC	DD
MIS-9	AA	CD	FI	DH	AC	BB	EG	CD
MIS-11	AB	CD	FI	FG	CF	BB	AE	DE
MIS-12	AB	CE	FI	AJ	AA	BB	AC	AD
MIS-14	AB	CE	BF	BH	AC	BB	AE	DD
MIS-15	AC	AE	FF	LM	AC	BB	CE	DD
MIS-17	CC	CE	BF	AE	AA	AA	BD	DD
MIS-18	CG	DE	II	AH	CC	BB	CH	DD
MIS-19	BC	CE	FF	AG	AA	BB	BH	DD
MIS-20	BB	AD	BI	AA	AA	BB	DD	BB
MIS-21	BC	AA	HH	AJ	CC	BB	CD	DD
MIS-22	AB	AE	FH	AE	AA	BB	DE	DD
MIS-24	AB	AC	FF	AG	AA	BB	CE	DD
MIS-25	BC	CE	FF	EE	CC	BB	CD	BD
MIS-26	BB	DE	BF	JJ	AC	BB	CD	DD
MIS-27	AB	CE	HK	AH	CC	BB	DH	DD
MIS-28	AH	BD	BF	AK	AD	BB	AD	DD
MIS-29	CK	CD	BB	FF	CD	BB	CG	DD
MIS-31	AB	AE	FH	AA	AA	BB	AH	DE
MIS-33	CC	CE	BB	AG	AC	AA	CC	DD

Worm	P-gp	GluClα	GluClβ	GABA	β-tub	PFK	PEPCK	AchR
MIS-34	BB	CD	DD	AM	AA	BB	CC	DD
MIS-35	CI	CE	BB	FH	BC	BB	CF	DD
MIS-36	AI	EE	CC	FF	BC	BB	DF	AD
MIS-38	AI	CE	BB	AH	AB	BB	EF	DD
MIS-40	BC	CC	FF	AL	AC	BB	CG	CC
MIR-1	AA	CE	HI	AD	BC	AA	DE	DE
MIR-2	AA	AE	BF	DD	AD	BB	AB	DD
MIR-4	BD	CE	FF	FI	AC	BB	AC	DD
MIR-5	AC	AA	FF	LL	CC	BB	CD	DD
MIR-6	AD	CE	FI	EL	CD	BB	BC	DD
MIR-8	AC	AD	FF	AF	CC	BB	DD	DD
MIR-9	AB	AA	FH	AL	CC	BB	EH	CD
MIR-10	AA	EE	FH	LL	BB	BB	CD	CD
MIR-12	AJ	AA	BH	AL	CC	BB	CE	DD
MIR-13	AD	AC	FH	FL	CC	BB	DE	DD
MIR-19	AD	CE	FF	AA	AC	BB	AC	DD
MIR-21	AB	AA	FG	AE	CC	BB	EE	DD
MIR-22	AD	AA	FI	EL	CC	BB	CE	DD
MIR-23	DE	AE	BF	AL	CC	AA	CC	DD
MIR-24	AF	AB	BF	EE	AC	BB	AC	DD
MIR-25	AA	AA	FJ	EL	BC	BB	CD	DD
MIR-27	AB	AE	AB	IL	AC	BB	AH	DD
MIR-28	BD	CE	BF	LL	CC	AA	CD	DD

Worm	P-gp	GluClα	GluClβ	GABA	β-tub	PFK	PEPCK	AchR
MIR-29	BB	AC	DF	AA	AC	BB	AD	DD
MIR-30	AC	AB	BB	EL	AC	AA	AC	AD
MIR-31	AB	AC	FI	AB	AB	BB	AD	DD
MIR-32	AA	CC	AJ	AA	AB	BB	AD	DD
MIR-33	AA	AC	JJ	AA	BB	BB	DD	DD
MIR-34	AD	AE	FF	CC	AC	BB	AC	CD
MIR-35	AA	AC	FF	EL	CC	AA	DE	DD
MIR-36	AA	CE	CJ	AA	AC	BB	CD	DD
MIR-37	AC	AE	JJ	AL	CD	AA	BC	DD
MIR-38	BB	CE	FG	AL	CC	BB	CH	DE
MIR-39	AD	AE	BF	AE	CD	BB	CF	DD
MIR-40	43 43	AC	FI	EE	AC	BB	CD	DD
CBZS-1	43 43	CE	CC					
CBZS-2	43 43	AE	BC					
CBZS-6	43 43	CE	BB					
CBZS-7	43 43	CC	BF					
CBZS-11	43 43	EE	BC					
CBZS-12	II	CE	BE					
CBZS-14	43 43	CE	BC					
CBZS-17	43 43	CE	AA					
CBZS-19	43 43	CC	BE					
CBZS-23	43 43	CC	CC					
CBZS-24	43 43	CC	BI					

Worm	P-gp	GluClα	GluClβ	GABA	β-tub	PFK	PEPCK	AchR
CBZS-25	43 43	CC	BI					
CBZS-28	43 e	CE	AA					
CBZS-31	43 43	CE	CF					
CBZS-32	II	CC	BC					
CBZS-34	43 43	CC	BF					
CBZS-35	43 43	AC	BC					
CBZS-36	43 43	CC	EE					
CBZS-37	43 43	CC	CI					
CBZS-38	43 43	EE	CO					
CBZS-39	43 43	AC	BB					
CBZS-40	43 43	CE	BB					
CBZS-42	BI	CE	CC					
CBZS-43	43 43	CC	FO					
CBZS-44	BI	CC	EL					
CBZS-45	43 I	CE	EL					
CBZS-47	43 B	CC	BB					
CBZS-48	43 43	CC	AB					
CBZS-50	43 43	CC	FN					
CBZS-51	II	CE	EG					
CBZC-6	43 43	BC	DI					
CBZC-7	Be	CE	FF					
CBZC-8	43 43	CC	BM					
CBZC-9	PP	EE	BF					

Worm	P-gp	GluClα	GluClβ	GABA	β-tub	PFK	PEPCK	AchR
CBZC-10	BB	AE	BB					
CBZC-13	PP	CE	BF					
CBZC-14	PP	CE	BI					
CBZC-19	Be	CC	BF					
CBZC-23	PP	CC	II					
CBZC-24	PP	AC	BF					
CBZC-25	43 e	CE	BF					
CBZC-26	PP	CE	II					
CBZC-27	PP	AA	BF					
CBZC-28	43 e	BE	FI					
CBZC-29	PP	CC	BD					
CBZC-31	PP	CE	BF					
CBZC-32	PP	AC	BB					
CBZC-33	PP	CC	BB					
CBZC-34	PP	EE	BD					
CBZC-35	43 m	CE	BD					
CBZC-36	43 e	CC	FF					
CBZC-37	PP	EE	FI					
CBZC-38	Be	AC	BF					
CBZC-39	BB	EE	BI					
CBZC-40	BB	AC	BI					
CBZC-41	Be	CC	BF					
CBZC-42	PP	CC	BI					

Worm	P-gp	GluClα	GluClβ	GABA	β-tub	PFK	PEPCK	AchR
CBZC-43	43 I	CC	BB					
CBZC-44	43 e	AE	BF					
CBZC-45	PP	CC	FI					
TBZT-1	PP	EE	BO					
TBZT-3	PP	CE	FF					
TBZT-5	PP	DE	FL					
TBZT-6	PP	EE	BF					
TBZT-7	Pm	BE	EE					
TBZT-8	43 e	AC	BF					
TBZT-9	PP	CC	BB					
TBZT-10	Be	CC	CH					
TBZT-12	BB	CE	AB					
TBZT-13	43 43	CE	DF					
TBZT-14	43 e	CE	EO					
TBZT-15	PP	AA	BF					
TBZT-16	PP	DE	FN					
TBZT-18	Be	CC	EI					
TBZT-19	PP	AE	BF					
TBZT-20	PP	CE	BF					
TBZT-21	Be	AE	BF					
TBZT-22	PP	CE	BF					
TBZT-23	BB	EE	BF					
TBZT-24	43 e	CD	BF					

Worm	P-gp	GluClα	GluClβ	GABA	β-tub	PFK	PEPCK	AchR
TBZT-26	43 e	CE	BF					
TBZT-27	PP	BE	BN					
TBZT-28	PP	AD	IN					
TBZT-30	Pm	AD	BB					
TBZT-31	PP	CD	BF					
TBZT-34	Be	EE	FF					
TBZT-35	Be	CE	BB					
TBZT-37	BB	EE	BI					
TBZT-39	Be	AC	BB					
TBZT-40	PP	DD	FI					