NOTE TO USERS

A comparison of laboratory and field resistance to macrocyclic lactones in *Haemonchus contortus*

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Abstract

Sustainable parasite control in livestock depends on anthelmintic drugs. The nematode *Haemonchus contortus*, the most important intestinal parasite of sheep and goats has developed resistance to all classes of anthelmintics including moxidectin, the most potent of the macrocyclic lactones. Pyrosequencing was used to screen *H. contortus* laboratory and field strains for single nucleotide polymorphisms (SNPs) associated with resistance in three genes, and determine their involvement in field resistance to macrocyclic lactones. Specific SNPs increased in frequency in ivermectin/moxidectin laboratory selected strains for all three genes. These did not protect a resistant field strain from a field dose of ivermectin and were not the major mechanism of resistance in the field strain. A gamma-aminobutyric acid chloride receptor SNP may be a potential marker for moxidectin resistance in the field. This study indicates results obtained from laboratory strains selected with sub-therapeutic doses of drug may not reflect the situation in the field.

Abrégé

Le développement durable de méthodes de lutte contre les parasites chez les animaux d'élevage dépend des anthelminthiques. Le nématode *Haemonchus contortus*, le plus important parasite gastro-intestinal des ovins et des caprins, a développé une résistance à toutes les familles d'anthelminthiques, incluant la moxidectine, une des lactones macrocycliques les plus puissantes. La méthode de pyroséquencage a été utilisée pour étudier au sein de souches d'*H. contortus*, provenant du milieu naturel (sauvage) ou sélectionnées en laboratoire, des SNP parmi trois gènes associés à la résistance, ceci afin de déterminer leur rôle dans le phénomène de résistance aux lactones macrocycliques .

Une augmentation de la fréquence de SNP spécifiques apparaît pour les trois gènes dans les souches résistantes à l'ivermectine et la moxidectine sélectionnées en laboratoire.

Par contre, ces mêmes SNP ne protègent pas la souche sauvage résistante contre une dose prophylactique d'ivermectine et ne correspondent pas aux mécanismes principaux de résistance dans cette souche.

Un SNP au niveau du récepteur chlorure GABA (acide gamma amino butyrique) dépendant pourrait être un marqueur de la résistance à la moxidectine en milieu naturel. Cette étude indique que les résultats obtenus avec les souches sélectionnées en laboratoire avec des doses sub-thérapeutiques de médicaments ne reflètent pas la situation en milieu naturel.

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List of Abbreviations

AVM: Avermectin

BZ: Benzimidazole

Cys-loop: Cysteine-loop

dNTPs: Deoxynucleotide triphosphates

EPG: Eggs per gram

FEC: Fecal egg count

GABA: Gamma-aminobutyric acid

GABA-A: Gamma-aminobutyric acid-gated type A

IVM: Ivermectin

LGIC: Ligand-gated ion channels

ML: Macrocyclic lactone

MOX: Moxidectin

nAChR: Nicotinic acetylcholine receptors

NSW: New South Wales

PCR: Polymerase chain reaction

P-gp: P-glycoprotein

SNP: Single nucleotide polymorphism

SSCP: Single-strand conformation polymorphism

TexR: *Haemonchus contortus* IVM resistant strain from Texas, U.S.A.

***T.* and *O. circumcincta*: *Teladorsagia circumcincta* is also called *Ostertagia circumcincta*. Both are used in this thesis in accordance to the original reference.**

Chapter I

Introduction

Worldwide agricultural production is under pressure to meet the demands of increasing population growth. The farming industry addresses this high demand by increasing productivity. However, livestock farmers suffer major production and economic losses due to parasitic infections of their animals (McLeod, 1995). One of the most important gastrointestinal parasites in sheep and goats is *Haemonchus contortus*. Infections by this parasite can have a devastating impact on the health of its host due to its blood sucking behavior. Infection results in mild to severe anemia, which can be fatal if left untreated. Current control strategies rely mainly on the use of anthelmintics and grazing management. Unfortunately, improper and frequent use of anthelmintics has accelerated the development of resistance in *H. contortus* and other trichostrongylid nematodes of livestock (Prichard, 1990).

Biological control of parasitic nematodes and vaccination strategies are currently under investigation, but are not yet available commercially (Larsen, 1999; Newton and Munn, 1999; Knox and Smith, 2001). It is therefore important to understand the mechanism of resistance to anthelmintic compounds, to understand the way these chemicals work at the molecular level to improve their use (Hennessy, 2000) and to develop tests to detect resistance in its early stages, thereby minimizing the evolution of resistance.

The avermectins (AVMs) and milbemycins, known collectively as macrocyclic lactones (MLs), are the newest class of broad spectrum anthelmintics. In 1981,

ivermectin (IVM), an AVM, was the first to be made available commercially (Chabala *et al.*, 1980; Cambell *et al.*, 1983). Moxidectin (MOX), a milbemycin, was subsequently released for commercial use (Shoop *et al.*, 1995). Unfortunately, resistance to these compounds and other MLs has already been reported in many parts of the world. IVM and other related MLs modulate glutamate and gamma-aminobutyric acid (GABA)-gated chloride ion channels, causing hyperpolarization and flaccid paralysis of the worm (Arena *et al.*, 1992; Cully *et al.*, 1994; Holden-Dye and Walker, 1990; Kass *et al.*, 1990; Boisvenue *et al.*, 1983). The mechanism of resistance to compounds of this chemical class is however still unknown and currently under investigation.

Laboratory selection with sub-therapeutic doses of MLs over multiple generations in a stable and closed environment has been used to produce ML resistant strains with a common genetic origin. It is not yet known if the result of selection under these conditions is the same as selection in a field situation. It is also important to validate results from the field for the development of new drugs, which are most often tested on laboratory strains. Comparisons between IVM/MOX laboratory selected and unselected strains show allele frequency changes in four of eight genes (Blackhall, 1999). The work presented here will look at three of these genes; a gamma-aminobutyric acid type-A (GABA-A) gated chloride channel subunit (HG1), a glutamate-gated chloride channel (GluCl) subunit (HcGluClα). These two chloride channels are closely related and both members of the ligand-gated ion channel family (LGIC). The third gene investigated in this study is β-tubulin isotype-1. HG1 is of growing importance since the resistant form shows functional differences in its activation by the drug (Feng *et al.*, 2002).

Field resistant strains were screened to determine if the alleles selected in the laboratory strains with sub-therapeutic doses were the same as those selected in the field. However selection for those resistant associated alleles was not apparent in the field strains using linked marker methods, single-strand conformation polymorphism (SSCP) for HG1, and a PCR intron-based diagnostic test for HcGluClα (W. Blackhall, personal communication; Forrester, 2002). Two possibilities could explain these observations. The first is that constant conditions in the laboratory and selection with sub-therapeutic doses of the drug impose different selection pressures on the parasite population compared to field conditions. This then leads to a different resistance mechanism selected in the laboratory. The second possibility is that mutations, for example single nucleotide polymorphisms (SNPs) conferring resistance are present on different allelic forms of the PCR product used for SSCP. While the mutations responsible for resistance may indeed increase in frequency, examining linked markers may fail to detect this.

Four amino acid substitutions differentiate the resistant HG1 GABA receptor subunit from the susceptible form (Feng *et al.*, 2002); two substitutions are found in the HcGluClα resistant allele (S. Forrester, personal communication). Evidence suggests the most important of these is a substitution found in both genes at position 169 in the cysteine loop (cys-loop). In the vertebrate GABA-A receptor a lysine to arginine point mutation at the same relative position in the cys-loop as position 169 caused a 3 fold decrease in EC_{50} for the GABA-evoked current (Amin *et al*,. 1994). The resistant form of HG1 caused a 5 fold decrease in EC_{50} for the GABA-evoked current (Feng *et al.*, 2002). This allele contains the Lys169Arg substitution and in addition three other substitutions. Selection for an allele of β-tubulin isotype-1 occurs when ML resistance is selected in the

laboratory. The only SNP known to distinguish the ML resistant allele so far is the Phe200Tyr position substitution. Although known to cause benzimidazole (BZ) resistance, which is unrelated to ML resistance, it would be useful to determine if this SNP is linked with IVM/MOX resistance.

The objectives of this research are: (i) to determine the importance of the position 169 substitution in ML resistance in both HG1 and HcGluClα in laboratory and field resistant strains of *H. contortus*, (ii) to determine the importance of the position 200 substitution in ML resistance in β-tubulin in laboratory and field resistant strains of *H. contortus*, (iii) to determine if the resistant associated SNPs identified are confined to one allelic form or found on different alleles in different geographical strains.

Resistance to all three classes of broad spectrum anthelmintics in *H. contortus* is increasing at an alarming rate. Identifying a specific SNP that could be used as a marker for IVM/MOX resistance in the field is of prime concern as is could to help slow down the development of resistance.

Chapter II

Literature Review

Gastrointestinal nematodes are a major source of economic and production loss in livestock worldwide (McLeod, 1995). This problem has led to the development of many anthelmintic drugs to control these parasitic infections. The use of these drugs has been highly successful and is the most effective method of control of livestock nematode infections worldwide.

1.0 *Haemonchus contortus*

A major parasite of sheep and goats is *Haemonchus contortus* which belongs to the superfamily Trichostrongyloidea of the order Strongylida of the class Secernentea (Cheng, 1986). *H. contortus* is dioecious with a direct life cycle. Each female worm residing in the abomasum passes 5,000 to 10,000 eggs daily *via* host feces where each egg is already in the early stages of cleavage. Subsequently (4-6 days) embryos develop into 1st stage larvae (L_1) in moist conditions. Two more larval stages (L_2 and L_3) occur with further development with L_3 remaining in its protective sheath (L_2 cuticle) feeding on stored nutrients (Blood *et al.*, 1979, Olsen, 1974). These developmental stages occur in as few as five days under optimal temperature (22°C – 26°C) and humidity (100%) but can take up to 14 days (Johnstone, 1998). Infection by the host occurs through ingestion of ensheathed L_3 (Johnstone, 1998). Once ingested, complete exsheathment will occur within 12 hours and the L_3 larva will appear on the surface of the abomasal mucosa (Olsen, 1974). After 24 hours most of the L_3's are inside the mucosa, and will molt into

L_4. These will then migrate back to the mucosal surface, undergo one last molt and become sexually mature adults (Olsen, 1974). The length of the complete cycle is approximately 14-21 days (Blood *et al.*, 1979).

Haemonchosis results from the pathology of an *Haemonchus* infection and is characterized by a hemorrhagic anemia caused by the loss of blood attributed to the blood-feeding behavior of the parasite. Infection can be acute with a sudden onset of anemia which can be fatal if left untreated. Chronic haemonchosis can also result from low level infection combined with a poor diet; the symptoms involve daily loss of small amounts of blood and subsequent weight loss (Johnstone, 1998). It is estimated that costs from production losses and control measures for sheep in Australia alone were $222 million in 1994 (McLeod, 1995) and are estimated to be $700 million by 2010 (Welsman, 2001).

2.0 Anthelmintics and their Mode of Action

There are three families of broad-spectrum anthelmintics currently available for nematode parasites of livestock: the benzimidazole (BZ) family, the levamisole and morantel family, and the avermectin (AVM) and milbemycin family known collectively as macrocyclic lactones.

2.1 The Benzimidazoles

The BZs were the first broad-spectrum anthelmintics available, with thiabendazole introduced in 1961 (Brown *et al.*, 1961). The mode of action of BZs is to bind to tubulin and inhibit its polymerization and displace the tubulin dimer microtubule equilibrium (reviewed by Prichard, 1990, 2001). This affects cellular homeostasis,

intracellular transport, subcellular organization, and cell division. BZ resistance is associated with a single nucleotide polymorphism (SNP) in the β-tubulin isotype1 gene at either position 200, where a phenylalanine is substituted by a tyrosine in *H. contortus* (Kwa *et al.*, 1994), and *O. circumcincta* (Elard *et al.*, 1996), or at position 167 where a phenylalanine is substituted by a tyrosine or a histidine (Silvestre and Cabaret, 2002). The resistant form of β-tubulin no longer binds the drug with high affinity (Lubega and Prichard, 1991). A SNP specific PCR diagnostic test is available for *H. contortus* (Kwa *et al.*, 1994) and *O. circumcincta* (Elard *et al.*, 1999).

2.2 The Levamisoles and Morantels

The levamisole/morantel class of drugs acts at nicotinic acetylcholine receptors (nAChR) as cholinergic agonists, which leads to depolarization of muscle bag membranes with an efflux of Na^+ (Coles *et al.*, 1975; Harrow and Gration, 1985). A reduced number or sensitivity of cholinergic receptors was concluded to cause resistance to levamisole/morantel in *H. contortus* (Sangster *et al.*, 1991). The precise mechanism of action and mutation(s) (SNPs) causing resistance are still unknown.

2.3 The Macrocyclic Lactones

The AVMs/milbemycins are macrocyclic lactones (MLs), the most common compounds for each class being ivermectin (IVM) and moxidectin (MOX) respectively. Both groups are closely related, and share a 16-membered macrocyclic backbone (Burg *et al.*, 1979; Takiguchi *et al.*, 1980), where the major structural difference between the two is a bisoleandrosyloxy substituent at C-13 of AVMs, which is absent in milbemycins, and there are also several different alkyl substituents at C-25 (Shoop *et al.*, 1995). Both are produced from the fermentation of actinomycetes. The AVMs are derived from the

fermentation broth of *Streptomyces avermitilis* (Campbell *et al.*, 1983), and the milbemycins are produced from the fermentation broth of *Streptomyces hygroscopicus* and *Streptomyces cyaneogriseus* (Shoop *et al.*, 1995). IVM was the first ML to be commercially available in 1981 (Chabala *et al.*, 1980), and MOX was the third macrocyclic lactone released for commercial use. Interestingly the milbemycins were discovered first in 1973 as acaricidal and insecticidal compounds for crop protection, but their nematocidal activity was not realized until the discovery of the AVMs in 1975 (Egerton *et al.*, 1979; Ostlind *et al.*, 1979; Shoop *et al.*, 1995). IVM has broad-spectrum activity against nematodes and arthropod parasites of animals. MOX however has more activity against helminth species than the balanced endectocidal activity of IVM (Shoop *et al.*, 1995). MOX has a longer *in vivo* half life than IVM, 13-15 days in host fat (Afzal *et al.*, 1994) compared to only 1-2 days for IVM (Steel, 1993). IVM has also been approved for use in humans in the treatment of onchocerciasis and lymphatic filariasis (Ottesen *et al.*, 1999; WHO, 2000).

The MLs paralyze the body wall, the pharynx and the uterine muscles of nematodes (Geary *et al*, 1993; Martin, 1996; Kass *et al.*, 1980; Boisvenue *et al.*, 1983; Holden-Dye *et al.*, 1988; Holden-Dye and Walker, 1990). They interact with high affinity to nematode glutamate-gated chloride (GluCl) channels (Shaeffer and Haines, 1989; Arena *et al.*, 1992), as well as nematode gamma-aminobutyric acid (GABA)-gated chloride channels (Holden-Dye & Walker, 1990; Kass *et al.*, 1980; Boisvenue *et al.*, 1983). However, the effect of MLs on GABA-gated channels requires a higher drug concentration (Cull-Candy & Usherwood, 1973). Geary *et al.* (1993) showed IVM inhibited feeding at concentration $\geq 10^{-10}$ M and paralyzed motility at $\geq 10^{-6}$ M.

Studies on the ML mode of action have been focused on neurotransmitter receptors. IVM binds the α-subunit of GluCl channels in *Caenorhabditis elegans* (Cully *et al.*, 1994). GluCls belong to a family of ligand-gated ion channels (LGIC) which include the GABA-gated chloride channels, glycine receptors, serotonin 5HT3 receptors, and nicotinic acetylcholine receptors (nAChR). The classical model of such receptors is that they form a pentameric channel of various subunit combinations forming a central pore (Unwin, 1989). Each subunit has four transmembrane domains, the second of which (M2) lines the pore (Hucho *et al.*, 1986; Imoto *et al.*, 1986; Unwin, 1993). GluCl channels are only found in invertebrates and this makes them ideal chemotherapeutic targets.

Binding of IVM and MOX to the channel is agonistic and leads to hyperpolarization of the cell and paralysis of the parasite. Expression of the *H. contortus* GluCl subunit HcGluClα in *Xenopus* oocytes produces a homomeric channel with a rapid and reversible response to glutamate, but a slow irreversible response to IVM/MOX (Forrester *et al.*, 2003). This irreversible binding of IVM and MOX explains its paralytic effect on nematodes by maintaining the channel open.

GABA receptors, which are also inhibitory chloride channel receptors in nematodes interact with IVM/MOX and appear to be involved in the mode of action of MLs. GABA receptors are found in both vertebrates and invertebrates. Ligand binding creates a conformational change in the protein which opens the channel causing an influx of chloride ions into the cell and leads to hyperpolarization. Most drugs used on helminth parasites target LGICs, including levamisole, morantel, pyrantel, and piperazine which causes muscular paralysis through GABA receptors (Brownlee *et al.*, 2000). Piperazine is

a GABA receptor agonist acting on somatic muscles, inducing a flaccid paralysis from hyperpolarization caused by influx of chloride ions (Martin, 1982).

For GABA channels, AVMs were originally characterized as antagonists, blocking hyperpolarization of somatic muscle cells in *Ascaris* (Holden-Dye *et al.*, 1988; Holden-Dye & Walker, 1990) although they are known agonists in vertebrates and insects. Controversy ensued as AVMs were also reported as being agonists, causing ataxia and paralysis in neuromuscular cells (Bloomquist, 1993; Clark *et al.*, 1995). Paralysis of the somatic muscles has been reported in *Ascaris suum, Caenorhabditis elegans* (Kass *et al.*, 1980) and *H. contortus* (Boisvenue *et al.*, 1983), and paralysis appears to be caused by blockage of transmission between interneurons and excitatory motorneurons (Kass *et al.*, 1980, 1984). Thus nematodes may have structurally and functionally diverse GABA receptor subunits. In a more recent study with the *H. contortus* HG1 GABA-gated channel subunit, IVM was observed to potentiate the GABA response and act as an agonist of this GABA receptor (Feng *et al.*, 2002). Also, Ros-Moreno *et al.*, (1999) showed IVM is a competitive inhibitor of [^{3}H]-GABA binding activity in *Trichinella spiralis* muscle larvae.

HG1 is a major inhibitory receptor of body muscles in *H. contortus*, being localized along the ventral nerve cord, neurons in the head region, and possibly the nerve ring (Skinner *et al.*, 1998). In *C. elegans*, six GluCl genes have been identified: avr-14, avr-15 and glc-1, -2, -3, and -4 (Cully *et al.*, 1994; Dent *et al.*, 1997, 2000; Vassilatis *et al.*, 1997; Horoszok *et al.*, 2001). In addition, avr-14 and avr-15 are alternatively spliced to make eight different subunits (Dent *et al.*, 1997; Laughton *et al.*, 1997; Vassilatis *et al.*, 1997). Most have been classified as α-subunit because they bind IVM, but glc-2 the

only β-subunit, responds to glutamate and not IVM (Cully *et al.*, 1994). Expression of glc-2 is only found in the pharynx (Laughton *et al.*, 1997), whereas, avr-14 and avr-15 are found throughout the nervous system (Dent *et al.*, 1997, 2000). The *C. elegans* GluClα subunit avr-15 is expressed in the pharynx muscles of *C. elegans* and also binds glutamate and IVM (Dent *et al.*, 1997). This correlates well with IVM paralyzing movement and pharyngeal pumping in *C. elegans*.

Three genes encoding four GluCl subunits have also been cloned from *H. contortus,* including two alternatively spliced subunits HcGluCla and HcGluClb (Forrester *et al.*, 1999), where the longer spliced variant HcGluCla (also called HcGluClα) binds IVM with high affinity (Forrester *et al.*, 2002) and expression of this subunit was recently localized in the pharynx of *C. elegans* (Liu *et al.*, 2004). Geary *et al.* (1993) found high affinity inhibition of pharyngeal pumping in *H. contortus*. This supports the idea that IVM paralyzes the pharynx through the HcGluClα subunit. Levitt (2004) also found the HcGluClα gene was associated with differences in adult feeding; where the susceptible, but not the resistant associated allele, was affected by the drug. No orthologous counterparts of this subunit have been identified in *C. elegans* (Forrester *et al.*, 1999). A recent study by Portillo *et al.* (2003) found the HcGluClα subunit present in *H. contortus* motor neurons commissures using immunofluorescence, and not in the pharyngeal muscles, as reported by Liu *et al.* (2004). They concluded that HcGluClα is involved in paralysis of the somatic musculature. Liu *et al.* (2004) used *C. elegans* to inject a GFP gene construct including the promoter region and a partial HcGluClα, whereas, Portillo *et al.* (2003) used specific antibodies in *H. contortus*. HcGluClβ, a β-subunit gene has also been found in *H. contortus*, this subunit is orthologous to the *C.*

elegans glc-2 (Delany *et al.*, 1998) and appears to be co-localized with HcGluClα, indicating they may coassemble to form the native GluCl (Portillo *et al.*, 2003). Finally HcGbr-2, which is orthologous to the *C. elegans* avr-14, encodes two subunits HcGluClα3A and HcGluClα3B widely distributed in the nervous system (Jagannathan *et al.*, 1999). Portillo *et al.* (2003) confirmed much of the previous staining by Jagannathan *et al.*, (1999), both HcGluClα3 subunits stained motor neuron commissures in the anterior and middle region of the worms; however they also demonstrated the probable expression of HcGluClα3B in the pharyngeal cells, which could explain the paralytic effect of IVM on feeding. There appears to be some controversy as to which GluCl is expressed where and which is responsible for the different IVM effects, but clearly GluCls are important targets of IVM and related MLs.

The concentration of IVM required to exert its effect on GABA-gated channels is much higher than the concentration needed for the effect on GluCls (Cull-Candy & Usherwood, 1973), and the concentration of IVM required to inhibit motility is 100 fold greater than the concentration required to inhibit ingestion (Geary *et al.*, 1993). It is now generally accepted that the paralyzing effect of MLs on pharyngeal muscles is *via* GluCl receptors (Arena *et al.*, 1992; Cully *et al.*, 1994; Dent *et al.*, 1997, 2000) and that its effect on the somatic musculature is *via* GABA receptors (Holden-Dye & Walker, 1990). Therefore the action of AVMs on GluCls prevents feeding, and the action of AVMs on GABA-gated channels inhibits motility. However GluCls are also found in the somatic musculature and may contribute to body muscle paralysis as well (Portillo *et al.*, 2003) although the high concentration required to inhibit motility does not support the high affinity binding of IVM to GluCl channels.

Initial reports suggested that GABA receptors on the somatic musculature were the main target of MLs; opening the chloride channels and causing a flaccid paralysis (Kass *et al.*, 1980; Turner and Schaeffer, 1889), but GABA-gated chloride channels are not the most sensitive target of this chemical group (Martin and Pennington, 1989; Martin *et al.*, 1991). Pharyngeal pumping is inhibited more potently than motility, suggesting that inhibition of ingestion may be the actual mechanism of action of IVM and other MLs (Geary *et al.*, 1993), and that the action of the drug on GluCl channels is more important. *H. contortus* viability however was not affected in these experiments at concentrations of IVM which paralyze the pharynx. It is not known if this is the case *in vivo*. Expulsion of *H. contortus* is very rapid, approximately 8-10 hours following treatment, therefore this would place more importance on the paralysis of body wall muscle, preventing the parasite to swim against the flow of digesta, and consequently flushing the parasite out. Paralysis of the pharynx and inhibition of feeding would take longer to have an effect, slowly depleting energy levels. However, as the concentration of the drug decreases within the host, the paralyzing effect on feeding will persist at lower concentration and could still contribute to worm death (Prichard, 2001). Interestingly IVM receptors in *Drosophila melanogaster* have both GABA-gated and GluCl-gated chloride channel subunits. Whether this coassembly also occurs in *H. contortus* is unknown. Portillo *et al.*, (2003) showed that HcGluClα was expressed on GABAergic motor neurons. This agrees with AVMs blocking the transmission between inhibitory motor neurons and muscles (Kass *et al.*, 1980) and implicates both GABA and GluCl channels.

3.0 Resistance

The development of anthelmintic resistance is unavoidable, unless treatment is not used or is one hundred percent effective. Improper and frequent use of the same anthelmintic can accelerate the development of resistance (Prichard, 1990). Resistance has been reported for all broad-spectrum anthelmintics (Prichard, 1994; van Wyk *et al.*, 1999). Unlike antibiotic resistance which arises by spontaneous mutation during treatment, resistance in parasites is attained by an evolutionary process of selection of resistant alleles already present in a genetically diverse population (Anderson *et al.*, 1998). Treatment with drugs eliminates susceptible individuals, leaving individuals with resistant alleles to pass them to the next generation. As the frequency of resistant genotypes increase in the population a higher drug dose is required to obtain the desired effect (Prichard, 1994). Three factors determine the rate at which resistance develops in the target population; the initial frequency of resistant alleles present in the population, the number of genes involved in resistance, and if resistance is dominant or recessive. Higher or lower doses of the drug can increase or decrease the rate of selection for resistance based on these three factors (Anderson *et al.*, 1998). For example, in a mathematical model by Smith *et al.* (1999), under-dosing promoted selection for resistance if the initial frequency of resistant alleles was low; however it impeded selection when the frequency of resistant alleles was high. Low efficacy treatment also selected for resistance rapidly in *Ostertagia* spp. (Martin, 1989).

Parasite ecology also plays an important part in the development of anthelmintic resistance. Typically, only a small proportion of the parasite population is exposed to treatment since most of the population is present as free-living stages on pasture,

otherwise known as *Refugia* (van Wyk, 2001). Under these circumstances, the response to selection pressure would be slow, as the resistant alleles are diluted in subsequent infections. If treatment occurs when the abundance of the free-living population is low due to weather conditions or when animals are moved to clean pasture following treatment, the resistant genotype will be a greater contributor to the next generation, thereby increasing the rate of resistance development substantially (Prichard, 1990; van Wyk, 2001). This is the case in the winter rainfall regions of Western Australia, where relatively few infective larvae survive over the very hot and dry summer period (Besier and Dunsmore, 1993). This contributed to IVM resistance in *Ostertagia* spp. in only four years of treatment (Swan *et al.*, 1994).

3.1 Ivermectin/Moxidectin Resistance

IVM resistance has been reported for *H. contortus* in South Africa (van Wyk and Malan, 1988), Brazil (Echevarria and Trindale, 1989), Australia (Le Jambre, 1993), and the U.S.A. (Craig and Miller, 1990). IVM resistance has also been reported for the other major trichostrongyle nematode, *Teladorsagia circumcincta* (*Ostertagia circumcincta*) in Australia (Swan *et al.*, 1994), New Zealand (Watson and Hosking, 1990: Pomroy *et al.*, 1992; Gopal *et al.*, 1999), South Africa (Reinecke *et al.*, 1991), the UK (Jackson *et al.*, 1992) and Czechoslovakia (Varady *et al.*, 1993). *Trichostrongylus colubriformis*, another important trichostrongyle nematode, was also reported to be resistant to IVM in New Zealand (Gopal *et al.*, 1999). Strains of *H. contortus* were selected in the laboratory with IVM (Egerton *et al.*, 1988) and MOX (Ranjan *et al.*, 2002). IVM resistance was also selected in *T. colubriformis* (Giordano *et al.*, 1988). MOX (the most potent ML against gastrointestinal nematodes) remains effective against most ML resistant strains; however

reports of MOX resistance are starting to emerge (Rolfe and Fitzgibbon, 1996; Veale, 2002; Love *et al.*, 2003). This is not surprising since MOX and IVM have similar modes of action, and share a common mechanism of resistance (Shoop *et al.*, 1995). In Australia the first report of MOX resistance in *H. contortus* came as a reduced period of persistent activity (Rolfe and Fitzgibbon, 1996). MOX resistance is present in both *O. circumcincta* and *T. colubriformis*, which was the first report of resistant *Trichostrongylus* spp. in Australia (Veale, 2002). MOX resistance has also been reported in *H. contortus* in Australia (Love *et al.*, 2003). Resistance is becoming very serious in some parts of the world. The situation in South Africa is so serious in some areas that some farmers have had to abandon sheep raising altogether, due to resistance to all anthelmintic groups by *H. contortus* (van Wyk *et al.*, 1999).

Resistance to the MLs appears to be inherited as a completely dominant trait (Le Jambre, 1993; Dobson *et al.*, 1996; Le Jambre *et al.*, 2000; Barnes *et al.*, 2001). This is unlike the BZs which are inherited as an incomplete dominant trait in *H. contortus* (Herlich *et al.*, 1981; Le Jambre *et al.*, 1979), and levamisole which is inherited as an autosomal recessive trait in *H. contortus* (Lacey *et al.*, 1990), and a recessive sex-linked trait in *T. colubriformis* (Martin and McKenzie, 1990). AVM resistance inherited as completely dominant could explain why resistance has developed so quickly, as a dominant trait will increase in frequency faster than a trait inherited as recessive or incompletely dominant (Dobson *et al.*, 1996). However it is unclear if resistance to MLs is actually controlled by one dominant gene across all geographical strains. In a study by Gill and Lacey (1998), different 'resistant types' were identified employing *in vitro* assays based on the larval stages of *H. contortus*, *T. colubriformis* and *O. circumcincta*,

where the structure-activity profiles were similar in susceptible isolates but different in resistant isolates. They concluded that multiple mechanisms of resistance to MLs appear to have arisen, and these mechanisms seem to differ both within and between species. It would appear ML resistance is more complicated than BZ resistance.

3.2 Ivermectin vs. Moxidectin: Identical Mechanism of Resistance?

MOX has been shown to be 99.9 and 100% effective in lambs against two IVM resistant strains (Craig *et al.*, 1992). Similar reports were also made by Pankavich *et al.* (1992), Kieran, (1994), and Echevarria *et al.* (1997). This led to the conclusion that MOX may have a different mechanism of action than IVM. This inaccurate conclusion was quickly corrected with dose titrations that clearly showed more MOX is required to kill IVM resistant worms than is needed to kill IVM susceptible ones. In the above studies, a loss of MOX sensitivity would not be apparent as the recommended dose is still effective. However, in a study by Leathwick (1995), IVM resistant strains of *Ostertagia* spp. were also resistant to MOX. Shoop *et al.* (1993) reported that *O. circumcincta* and *T. colubriformis* resistant to IVM were also resistant to MOX, and in a study by Conder *et al.* (1993) MOX was only 47.2% effective against IVM-resistant *H. contortus* at a dose which is 98% effective against IVM-susceptible *H. contortus*. In a recent study by Ranjan *et al.* (2002), selecting with both IVM & MOX separately, it was shown that co-selection for resistance to both drugs occurred. However rates of resistance development were slower for MOX than IVM. The conclusion is that MOX and IVM share a common mechanism of resistance (Shoop *et al.*, 1995).

4.0 Mechanism of Ivermectin/Moxidectin Resistance

AVMs have been reported to act at nematode GABA receptors (Martin and Pennington, 1989; Holden-Dye and Walker, 1990). AVMs have also been shown to bind to and activate glutamate-gated chloride channels in *C. elegans* (Cully *et al.*, 1994; Arena *et al.*, 1992). Therefore GABA- and GluCl-gated chloride channels make ideal targets to investigate possible mechanism(s) of resistance. However, Rohrer *et al.* (1994) found no differences IVM binding kinetics between susceptible and resistant strains of *H. contortus*. To identify genes linked with resistance, Blackhall (1999), performed single-strand conformation polymorphism (SSCP) analysis and found a link to IVM/MOX resistance in four of eight genes tested. For each of the four genes, one allele increased significantly in frequency following drug selection. These are: the gamma-aminobutyric acid (GABA) chloride channel subunit (HG1) (Blackhall *et al.*, 2003); the α-subunit of a glutamate gated chloride channel (HcGluClα) (Blackhall *et al.*, 1998[b]); β-tubulin isotype-1 (Blackhall, 1999); and P-glycoprotein (Pgp-A) (Blackhall *et al.*, 1998[a]). This evidence is associative and each gene must be explored further to confirm an involvement in resistance. For example, a gene can appear to be selected if genetic hitchhiking occurs, when a gene is physically close to another gene that is under selection pressure (Smith and Haigh, 1974; Barton, 2000).

4.1 P-glycoprotein

P-glycoprotein (P-gp) is an energy dependent transport protein belonging to a class of ATP-binding cassette (ABC) proteins. As a class they serve to transport various substances in and out of the lipid bilayer (Higgins, 1992). IVM has been shown to be a substrate of P-gp in mammalian cells (Didier and Loor, 1996; Pouliot *et al.*, 1997), and

has even been suggested as being the only transport pump of IVM (Gottesman *et al.*, 1996). P-gp has also been implicated in drug resistance since mice deficient for the P-gp homologue *mdr*1a died as a result of IVM treatment at a dose that did not affect normal mice (Schinkel *et al.*, 1994). P-gp overexpression was also found to be involved in the failure of chemotherapy in cancer treatment by transporting the drugs from cancer cells (Dano, 1973; Juliano and Ling, 1976).

In nematodes, a study by Xu *et al.* (1998) found a higher expression of Pgp-A in IVM-selected strains compared to unselected strains. In addition, the multidrug resistance reversing agent verapamil increased IVM efficacy by 13% and MOX efficacy by 26% against a MOX-selected strain. When Blackhall *et al.*, 1998[a], performed SSCP analysis on Pgp-A in *H. contortus* strains; there were significant differences between susceptible strains and IVM/MOX selected strains with selection for the same allelic form in two IVM- and one MOX-selected strain. These observations suggest P-gp may play a role in ML resistance in nematodes. Sangster *et al.* (1999) also found a link between AVM resistance and alteration of expression in a P-gp gene, where RFLP polymorphisms decreased in field resistant *H. contortus* strains. However, when Smith and Prichard (2002) investigated levels of Pgp-A mRNA in the pharynx of IVM- and MOX-selected strains of *H. contortus*, they found no significant differences relative to the unselected strain. Le Jambre *et al.* (1999) found an *H. contortus* hcpgp-1 allele increased in frequency due to ML selection following a genetic cross between *H. contortus* and *Haemonchus placei* but concluded that it is not the major gene involved in resistance but may be linked to it. The role of P-gp in ML resistance remains unclear at this time and no specific SNP(s) have yet been identified between susceptible and resistant forms.

4.2 β-tubulin

SSCP analysis comparing susceptible and resistant IVM/MOX laboratory selected *H. contortus* strains has revealed selection of one allele of β-tubulin isotype-1 (Blackhall, 1999). This was unexpected, and could be a result of genetic hitchhiking; however this it is not the only reported involvement of β-tubulin in ML resistance. An allelic form of the β-tubulin isotype-1 gene was always present in the human parasite *Oncocerca volvulus* in patients who did not respond to IVM treatment. Furthermore, three SNPs causing amino acid substitutions were identified in this allele and are linked with IVM resistance in this parasite (Eng and Prichard, submitted). Amphidial neurons are sensory neurons located in the cephalic region of nematodes as a pair of channels, the amphids, found on either side of the pharynx and in contact with the external environment. Proposing these amphids could be a critical entry point for anthelmintics, Freeman *et al.* (2003) found amphids from both laboratory and field strains resistant to MLs were shortened, structurally degenerate, and lacking microtubule structure when compared to susceptible strains. This suggests a similar mechanism for resistance as in the case of P-gp by reducing the concentration of drug at the site of action, and could explain the selection observed in β-tubulin by Blackhall (1999). The same IVM/MOX resistant strains from Fort-Dodge Laboratories (Princeton, NJ) were used in both studies. Another interesting link with amphids is GluCl, as Portillo *et al* (2003) found expression of HcGluClα3A in amphidial neurons, however it is unknown if this has any relevance to resistance and the observed structural changes in the resistant parasites.

The role of β-tubulin has been extensively studied for its involvement in BZ resistance. However, unlike BZ, IVM does not bind to or inhibit polymerization of

nematode microtubules (M. Oxberry, personal communication). The β-tubulin Phe200Tyr substitution has been well established as the key mutation in BZ resistance (Kwa *et al.*, 1994). Cloning and sequencing the β-tubulin alleles identified by Blackhall (1999) in his SSCP experiments, the same Phe200Tyr substitution involved in BZ resistance appears to be linked with the allele selected by IVM/MOX (R. Beech, personal communication). Although it seems unlikely this mutation would be involved in ML resistance as BZs and MLs are unrelated compounds, there is a circumstantial link between β-tubulin and ML resistance that should be investigated further.

4.3 GluCl Channels

Allele frequency changes were observed in two genetically distinct drug selected *H. contortus* strains in the HcGluClα subunit (Blackhall *et al.*, 1998[b]). HcGluClα allele A increased in frequency from 0.117 in the unselected MIS strain to 0.45 in the IVM selected MIR, with the two most common alleles in MIS decreasing in frequency in MIR. HcGluClα allele A also increased in frequency in the two Fort Dodge IVM and MOX selected strains, from 0.133 in the unselected PF17 to 0.283 in the IVM-selected IVF17 and 0.35 in the MOX-selected MOF17. Using the same *H. contortus* Fort Dodge strains, Paiement *et al.* (1999) found a significant increase in B_{max} for glutamate binding in adult IVF17 (4.22 pmol/mg) compared to PF17 (1.58 pmol/mg), thus suggesting glutamate binding sites may be involved in IVM resistance. This was interesting considering no difference in IVM binding kinetics was observed between susceptible and resistant strains of *H. contortus* (Rohrer *et al.*, 1994). Also, no consistent amino acid substitutions were found between IVM resistant and susceptible isolates of a *H. contortus* White River strain HcGluClα subunit cDNA (Cheeseman *et al.*, 2001).

Comparison of full length cDNA of the HcGluClα subunit alleles (Blackhall *et al.*, 1998[b]) revealed two amino acid changes in the selected allele A, the first is at position 169 within the cysteine loop, where an alanine is changed to a valine, and the second is a phenylalanine to valine change at position 353 in the large intracellular loop between transmembrane domains three and four (S. Forrester, personal communication). Interestingly an amino acid substitution in the other LGIC HG1 (GABA) subunit is found at amino acid position 169 between its susceptible and resistant alleles. Unique mutations in the intron region found in the resistance associated allele A of the HcGluClα Fort Dodge strain were also used to design a diagnostic test. However allele A was 14% less abundant in a Texas resistant strain (TexR) compared to PF20 (PF17 + 3 generations), and allele A was also not found in the White River strain (van Wyk and Malan, 1988) from South Africa (Forrester, 2002). This suggests that allele A of HcGluClα is not associated with IVM resistance in these field strains. Differences in the selection process may explain this, as selection in the laboratory strains uses sub-therapeutic doses (to kill 85-95% of the worms), and therefore allele A could be favored under those conditions but not under field conditions. There is also evidence to suggest SNPs involved in resistance may be present on different alleles in different geographical strains, as two different alleles were selected in two different laboratory selected strains of HG1 (Blackhall *et al.*, 2003). One way to confirm if the position 169 substitution SNP is implicated in field resistance would be to screen field strains for that SNP directly as opposed to a diagnostic test based on the intron.

Another GluCl gene has also been recently implicated in IVM resistance in the cattle trichostrongylid parasite *Cooperia oncophora*. Njue *et al.* (2004), reported the

GluClα3 subunit from an IVM resistant strain showed a significant three fold loss in glutamate sensitivity compared to the IVM susceptible subunit, as well as a significant decrease in IVM/MOX sensitivity. This was attributed to a single Leu256Phe substitution. This amino acid substitution was not found in two field resistant *H. contortus* strains (data not shown).

4.4 GABA Channels

A GABA-A subunit (HG1) was isolated and characterized in *H. contortus* (Laughton *et al.*, 1994). This HG1 subunit was shown to be under selection in IVM and MOX laboratory selected strains (Blackhall *et al.*, 2003). The HG1 E allele increased significantly in frequency from 0.150 in the unselected strain PF17 compared to 0.550 in the IVM selected IVF17 strain and 0.683 in the MOX selected MOF17. Conversely, the most common allele in the unselected PF17 strain, allele A, decreased from 0.450 to 0.333 in the IVF17 and 0.200 in MOF17. It was the M2 region of this subunit that was used in SSCP. This transmembrane region of LGICs is believed to line the pore of the pentameric channel (Hucho *et al.*, 1986; Imoto *et al.*, 1986; Unwin, 1989, 1993). A point mutation in the M2 region of Rdl, a GABA-responsive subunit in *Drosophila* confers resistance to the insecticide cyclodiene (ffrench-Constant *et al.*, 1993). Also, in nAChR receptors, amino acid changes in that region alter ion permeability (Bertrand *et al.*, 1993), and can change ion selectivity from cationic to anionic (Galzi *et al.*, 1992).

Feng *et al.* (2002) cloned and sequenced the full length HG1 E resistant allele as well as the most common susceptible allele HG1 A. Four SNPs were found in the coding region that results in amino acid substitutions in the resistant HG1 E allele; however all changes found in the M2 region were silent mutations (Blackhall *et al.*, 2003). Of the four

substitutions found in the resistant form, two are present in the extracellular cysteine loop (cys-loop) (lysine-169 to arginine, glutamine-176 to leucine), and two are present in the membrane spanning M4 region (valine-436 to isoleucine, histidine-442 to tyrosine). To determine if the four SNPs in the HG1 E alleles are involved in IVM/MOX resistance, Feng *et al.* (2002) expressed both the resistant and susceptible alleles in *Xenopus* oocytes to test the electrophysiological effects of the channel. When both alleles are co-expressed as heteromeric channels with the *C. elegans* β-like subunit GAB-1, application of IVM and 10 μM GABA potentiates the GABA-induced current with the susceptible allele HG1 A, but attenuates it with the resistant allele HG1 E. This implicates HG1 further with resistance, adding a functional effect in the presence of the drug to the selection observed by Blackhall *et al.* (2003).

Feng *et al.* (2002) speculated the first SNP in the cys-loop may be most important since a SNP at the same amino acid position was also found in the HcGluClα resistant allele A (S. Forrester, personal communication). All members of the LGIC superfamily have a 15 residue cys-loop in the N-terminal (Cockcroft *et al.*, 1990), which is also the site of neurotransmitter binding based on mutational analysis. The cys-loop was originally proposed as part of the agonist binding pocket, however studies on the vertebrate GABA-A receptor indicated this was not the case. Rather, it was suggested the cys-loop may be required for proper assembly and transport to the plasma membrane (Criado *et al.*, 1986; Sumikawa and Gehle, 1992; Amin *et al.*, 1994). The SNP at position 169 in the HG1 coding sequence is the result of a substitution from an adenine (A) to a guanine (G) converting the codon sequence from a lysine (AAG) to an arginine (AGG) at position 169. Although studies on the cys-loop indicated it did not form the binding

pocket, point mutations in the cys-loop from studies on the vertebrate GABA receptor provide some interesting information about position six in the cys-loop which happens to be the relative location of position 169 in HG1. Amin *et al.*, (1994) showed a three fold decrease in EC_{50} of the GABA-induced current with a Lysine to Arginine substitution at position six in the cys-loop in the vertebrate GABA-A receptor, and Feng *et al.* (2002) showed a five fold decrease in EC_{50} of the GABA-induced current with the same substitution at position six in the cys-loop along with three other substitutions in HG1 E. This indicates the 169 position in HG1 may be responsible for much of the changes in the GABA-induced current by this allelic form observed by Feng *et al.* (2002).

The evidence linking HG1 with IVM/MOX resistance has been obtained from laboratory selected strains selected with sub-therapeutic doses of drug. It is not known if selection under these circumstances will be similar to selection in the field. It would be useful to know if the SNPs identified in these laboratory selected strains shown to have a functional impact in the presence of the drug, are also being selected in the field and play a role in IVM/MOX resistance. The Lys169Arg substitution would be an ideal candidate to screen IVM/MOX field resistant strains.

4.5 Mechanism of Resistance in the Field

The research on IVM/MOX resistance thus far has been conducted on strains selected for resistance in the laboratory. Can this research on laboratory selected strains be applied to the field? The HG1 subunit allele HG1 E, which was selected in laboratory resistant strains, was not found in field resistant strains of *H. contortus* when screened by SSCP (W. Blackhall, personal communication). The HcGluClα resistant allele A was also screened in field resistant strains using primers based on the intron sequence, and the

allele was present at levels below that of the unselected laboratory strain (Forrester, 2002). This could indicate either a different mechanism of resistance has been selected in the laboratory, or that screening field strains using linked markers failed to detect resistant individuals. This would be the case if the SNP which confers resistance is present on different alleles in different geographically separated strains. This could occur in one of three ways; first, the SNP leading to resistance arose independently on different alleles; or second, the mutation occurred on one allele and recombination moved it to a different allele; or a third possibility is that the mutation evolved before subsequent mutations distinguished different alleles.

There is evidence that suggests more than one HG1 allele is involved in IVM/MOX resistance and the Lys169Arg substitution could be the key mutation. Allele frequency changes were determined from two parent strains for the *H. contortus* HG1 subunit. The HG1 E allele was selected in a Fort Dodge strain; however a different allele was selected in a Merk Frosst strain (HG1 L) (Blackhall *et al.*, 2003). The HG1 E allele was present in both strains but was not selected in the Merk strain. Sequence information revealed allele E from the Merck strain as being different than allele E from the Fort Dodge strain, and therefore the SSCP allele E represents a population of two alleles which co-migrate during SSCP. Only one of these may increase in frequency in the Fort Dodge strain (Blackhall *et al.*, 2003). A PCR diagnostic test revealed all worms with the HG1 E allele from the Fort Dodge strain had the Lys169Arg substitution SNP. However this SNP was rare in worms with the HG1E allele from the Merck Strain (data not shown). We hypothesize the Fort Dodge strain consist of only the 'resistant' HG1 E with the Lys169Arg substitution, and that the Merck strain HG1 E population is a mixture of

both 'resistant' and susceptible HG1 E, which could explain the lack of selection for this allele in this strain as it would be masked by the susceptible HG1E, which could have decreased in frequency. If the Lys169Arg substitution confers resistance, the strongly selected HG1 L allele in the Merck strain should also carry it; unfortunately no genetic material remains to test this hypothesis. Regardless, there is enough evidence to suggest the initial screens on the field resistant strains based on allele specific markers were not specific enough to determine if the SNPs identified under laboratory selection are also contributing to resistance in the field, and the substitutions at position 169 found in both the HG1 and HcGluClα genes in the laboratory selected strains are the best candidates to verify this.

5.0 Laboratory vs. Field Strains

The majority of the knowledge on gastrointestinal nematodes and the mechanism of resistance to anthelmintics have been obtained from laboratory reared strains. The strains used for selection usually predate the use of anthelmintics, and have been maintained under laboratory conditions ever since.

5.1 Loss of Genetic Variability in the Laboratory

There are many arguments concerning the effect of artificial breeding conditions on reduction of genetic variability. Nadler (1990) reported multiple evidence of reduction in genetic variability in *Schistosoma* and *Ascaris* populations being maintained in the laboratory for many years. Allozyme electrophoresis studies of laboratory reared and wild populations of *T. circumcincta* showed laboratory strains were similar to each other but different from the wild population (Gasnier *et al.*, 1992). A similar study identified

that field populations of *T. colubriformis* were genetically different than populations reared in the laboratory. These examples demonstrate genetic variation can change in trichostrongyle nematodes as a result of acclimation to closed laboratory conditions. A study on *T. circumcincta* by Gasnier & Cabaret (1998) observed environmental perturbation occurring during acclimation of a population in the laboratory can modify genetic variability. It is believed that reduction of genetic diversity is a result of acclimation due to the stable environment present in a laboratory setting compared to unstable conditions in the field.

5.2 Selection in the Laboratory vs. the Field

The recommended dose rate for IVM and MOX in the field is 0.2 mg/kg body weight. Laboratory selection uses sub-therapeutic doses of MOX and IVM so that adequate numbers of infective larvae (L_3) could be obtained to infect the next generation and to prevent loss of rare alleles through random genetic drift. The selection process for the Fort Dodge *H. contortus* strain was as follows: Eggs per gram (EPG) of feces were determined at each generation and the drug dose was maintained or increased to a target of 85-95% EPG reduction. For the IVM selected strain, the drug dosage increased from 0.010 mg/kg to 0.15 mg/kg after 17 generations, and for the MOX selected strain, the drug dosage increased from 0.002 mg/kg to 0.015 mg/kg after 17 generations (Ranjan *et al.*, 2002). Therefore the initial drug dose for laboratory selection was 100 fold lower than the recommended field dose for MOX, and 20 fold below that of IVM. Differences between laboratory and field selection are summarized in **Table 1**. Differences between laboratory and field selection could alter the selection pressure and the way the strain responds. This could ultimately affect the mechanism(s) of resistance selected.

Table 1: comparison between laboratory and field selection

Laboratory	Field
• Host: A few naïve hosts at each generation.	• Host: Many hosts with a resistance status to parasitic infection.
• Conditions: Fecal cultures performed under standard conditions (temperature and humidity).	• Conditions: Unstable climatic conditions in the field.
• Infection level: Intermediate sized inoculums.	• Infection level: Variable inoculums, based on the season, the climate, and grazing behavior.
• Dose: Sub-therapeutic - target 85-95% reduction	• Dose: Recommended – target >95% reduction
• Frequency: Rapid-passaging (every 4-6 weeks)	• Frequency: 2-6 times per year
• Population exposed to treatment: All	• Population exposed to treatment: Variable (*Refugia*)

5.3 Different Mechanism of Resistance in the Laboratory?

In-vitro techniques that aim to characterize the effects of AVM/milbemycin appear to have identified different mechanisms of resistance based on motility of L_1 larvae and development to the L_3 stage in *H. contortus* (Gill *et al.*, 1998). A much higher dose of AVM/milbemycin is required to inhibit development to the L_3 stage compared to inhibition of L_1 motility (Gill *et al.*, 1995). These *in vitro* techniques were performed on laboratory strains of *H. contortus* selected for resistance at sub-therapeutic concentrations of IVM as well as a field resistant isolate selected under recommended field doses of IVM (Gill *et al.*, 1998). Results show that sub-therapeutic doses of drug during selection produce a different mechanism of resistance to that produced by selection under

recommended field conditions. Interestingly, the laboratory selected strains showed no decreased sensitivity to IVM inhibition of movement in the field resistant strains. They hypothesized either that resistance was developmentally regulated in these isolates, or a mechanism that allows only a transient relief from the effects of the drug, which is not apparent in these assays due to the long period of drug exposure required. It was proposed by McKenzy (1985) that suboptimal doses of drug (LD_{95}: Kills 95% of adult population) selecting for resistance are more likely to select for polygenic resistance, whereas, field exposure of the drugs which are much higher than the LD_{95}, will select for monogenic resistance. Although there is evidence which supports a different mechanism of resistance may be selected under laboratory conditions, screening field resistant strains for the specific SNPs identified from laboratory selected strains would be the only way to confirm this.

6.0 Population Genetics: Do Inter- and Intra-Species Variations Affect Resistance?

Parasitic nematodes are a genetically diverse group. Trichostrongyle nematodes have up to ten times more mtDNA diversity than is usually observed in vertebrates (Blouin *et al.*, 1992, 1995). The highest nuclear sequence diversity based on 18s rRNA was found in nematodes when compared to other metazoan phyla (Philippe *et al.*, 1994). Beech *et al.* (1994) found extremely high nucleotide diversity of 0.094 and 0.091 in β-tubulin isotypes-1 and -2 introns respectively in *H. contortus*. In contrast, 41 *Drosophila* genes varied from 0 to 0.034 (Moriyama and Powell, 1996), a common range for other animal species. A similar study using the same techniques as Beech *et al.* (1994) only found 0.002 to 0.008 variations in five *Ascaris* introns. Large effective population size

may explain the high diversity found within trichostrongyle nematodes; however the rate of evolution may actually be higher.

It is important to know if the same mechanism of resistance will evolve in different geographical isolates and in closely related species. Most studies tend to focus on a limited number of geographical isolates. This raises questions about the interpretations of the results, and if these results can be applied to other geographical areas. In the three most important gastrointestinal trichostrongyle nematodes *H. contortus*, *T. colubriformis*, and *T. circumcincta*, resistance to BZs has been attributed to a single amino acid change at position 200 of β-tubulin isotype-1 and -2 where a phenylalanine is substituted for a tyrosine (Kwa *et al*, 1993, 1994; Grant and Mascord, 1996; Silvestre and Humbert, 2000; Silvestre and Cabaret, 2002; Elard *et al.*, 1996). Resistance has also been found associated to a single amino acid change at position 167 of β-tubulin isotype-1, where a phenylalanine is substituted for a tyrosine (Prichard *et al.*, 2000). However, this 167 substitution was not common in the field (Silvestre and Cabaret, 2002). The position 200 resistant genotype has been linked with BZ resistance in *H. contortus* from the UK, The Netherlands, South Africa, Zimbabwe, Australia and North America (Kwa *et al.*, 1993, 1994), in *T. circumcincta* from France (Elard and Humbert, 1999; Elard *et al.*, 1996) and Morocco (Leignel and Humbert, 2000), and in *T. colubriformis* from France, the UK, Guadeloupe and Zaire (Silvestre and Cabaret, 2002). It appears the resistance to BZs as developed in all three species across the world *via* the same mechanism. Will a similar outcome occur in other anthelmintic classes? In the case of levamisole the answer seems to be no. For example with levamisole, resistance

appears to be inherited as an autosomal recessive trait in *H. contortus* (Lacey *et al.*, 1990), and a recessive sex-linked trait in *T. colubriformis* (Martin and McKenzie, 1990).

Geographically distant populations in most parasites of domestic animals show little genetic differentiation, which would be consistent with high gene flow (Hartl and Clark, 1989). This is a direct result of humans moving domestic ruminants across continents, therefore eliminating the natural mating barriers. *H. contortus* and *T. circumcincta*, parasites of a domestic ruminant (Sheep) show little variation throughout different geographical areas (Blouin *et al.*, 1995; Gasnier and Cabaret, 1996), whereas *Mazamastrstongylus odocoilei*, a trichostrongylid parasite of wild deer, not subject to movement by humans, shows high variation and subdivision (Blouin *et al.*, 1995). Blouin *et al.* (1992, 1995), looked at four trichostrongylids of domestic animals including *H. contortus*, and found 95-99% of the nucleotide variations was within populations. This could explain BZ resistance selecting the same mutation on a global scale; however can we expect the same from ML resistance? Using three different larval stage assays to characterize IVM/MOX resistance, IVM field resistant *H. contortus* strains from both Australia and South Africa show consistent response patterns (Gill *et al.*, 1991, 1995; Gill and Lacey, 1993; Le Jambre *et al.*, 1995) thus indicating the mechanism of resistance to IVM in *H. contortus* may be the same in Australia and South Africa. Another study by the same group using the same *in vitro* assays looked at many different resistant isolates from *H. contortus*, *T. colubriformis*, and *O. circumcincta*. Based on the results from the three assays they identified different 'resistant types' which suggests mechanisms of resistance vary within and between different species (Gill and Lacey, 1998). Based on a larval development assay, *H. contortus* was the most sensitive to AVMs/milbemycins

followed by *T. colubriformis* and then by *O. circumcincta*. In a larval motility assay *H. contortus* and *O. circumcincta* were the most sensitive and *T. colubriformis* was the least (Gill and Lacey, 1998). These results indicate differences in the way different, though related, species respond to the same assays, and could indicate different mechanisms of resistance. IVM/MOX paralyzes both motility and pharyngeal pumping in adult nematodes at recommended field concentrations, however inhibition of pharyngeal pumping is much more sensitive (Geary *et al.*, 1993). Paralysis of motility prevents the worms from swimming against the flow of digesta and the worms get flushed out, whereas paralysis of the pharynx causes death by means of energy depletion, preventing the worm from feeding. Which of the two paralyzing effects of IVM/MOX is more critical for worm death? Following an oral treatment of 0.2 mg/kg IVM (field dose) the kinetics of expulsion were measured for *H. contortus*, *T. colubriformis* and *O. circumcincta* (Gill and Lacey, 1998). Both *H. contortus* and *T. colubriformis* and 25% of *O. circumcincta* were expelled quickly, 8-10 hours following treatment, which is similar to the anticipated rate of digesta flow (Ali and Hennessy *et al.*, 1996). The remaining *O. circumcincta* were gradually expelled after 14 hours. This suggests the effect of the drug on motility is more critical for *H. contortus* and *T. colubriformis,* and the effect on pharyngeal pumping is more critical for *O. circumcincta*. This would lead to different selection pressures as different effects of the drugs are having the lethal effect in different species, and may suggest different mechanisms of resistance would develop in different species. GluCl's have been implicated in both motility and pharyngeal pumping, and could therefore be involved in resistance with both targets of IVM/MOX. Further studies

will be needed to understand the mechanisms of resistance within and between different species.

The study presented here attempts to clarify the current situation regarding resistance attained from different selection protocols, namely the field situation compared to laboratory strains selected with sub-therapeutic concentrations of drug. SNPs have been identified in three different genes linked with IVM/MOX resistance based on allele frequency changes following drug treatment. No one has yet screened field resistant strains for these SNPs. This could provide valuable information on IVM/MOX resistance, potentially identify a genetic marker for field resistance, and answer questions about differences between laboratory and field selection.

Chapter III

Materials and Methods

1.0 Parasite Strains

1.1 Laboratory Strains

Three strains of *Haemonchus contortus* were supplied by Fort Dodge Animal Health, Princeton, New Jersey (Wang *et al.*, 1995). All strains were derived from a single parent strain obtained from the United States Department of Agriculture (USDA). PF17 was passaged through sheep for 17 generations without drug treatment. IVF17 and MOF17 were passaged in parallel; however each was treated with ivermectin (IMV) and moxidectin (MOX) respectively at each generation. Dose rates were adjusted based on fecal egg counts to be 80-95% efficacious. During 17 generations the drug dosage increased from 0.010 mg/kg to 0.15 mg/kg for IVF17 and from 0.002 mg/kg to 0.015 mg/kg for MOF17 (Ranjan *et al.*, 2002). After the seventeenth generation 15- and 7.5 fold more drugs, respectively, was required to kill 95% of the adult worms. The same strains selected further for a total of 23 generations, PF23 and IVF23 were also obtained. The dosage increased to 0.2 mg/kg for the IVF23 strain, thus 20 fold more drug was required to kill 95% of the adult worms.

1.2 Field Resistant Strains

Two field resistant *H. contortus* strains: VHR29 and BUSTA, were donated by Drs. Chick and Chambers (Veterinary Health Research PTY. LMT, University of New England, Armidale, NSW).

VHR29 originated from a farm 30 km east of Walsha in the New England region of New South Wales (NSW) Australia. Parasites were isolated in 1996 following a higher

than expected number of strongyle eggs after administration of IVM controlled release capsule (mean 360 eggs/g, range 0 to 2640 eggs/g) (Wooster *et al.*, 2001). In a pen trial by Wooster *et al.* (2001), IVM resulted in a 54% reduction and its analogue abamectin, a 74% reduction. MOX resulted in a 100% reduction; however its persistent activity was greatly reduced (Wooster *et al.*, 2001). VHR29 was exposed to MOX and IVM oral formulations, as well as IVM controlled release capsules repeatedly over a five year period.

BUSTA originated from a farm near Wallangra in northern NSW (29°14'E, 150°53'S). In a field study in 2001-2002, IVM resulted in a 0% reduction, abamectin, a 19% reduction, and MOX a 67% reduction (Love *et al.*, 2003). BUSTA was exposed to IVM oral treatment once in 1995, and MOX oral treatment was used exclusively from 1997 (5-6 treatments per year) until its failure in autumn 2001 (Love *et al.*, 2003).

1.3 VHR29 *in vivo* Trial Protocol

Three sheep were obtained from Hugh Sutherland (Hutington, Quebec, Canada), and were approximately 3 months old. Sheep were made worm free with two consecutive anthelmintic treatments. Ten mg/kg valbazen was given initially. Parasite eggs were still found by fecal egg count (FEC) therefore 8 mg/kg levamisole was used. A FEC confirmed all sheep were worm free. The three sheep were infected with approximately 7,500 VHR29 L_3 larvae. FEC were used to monitor the status of infection, and 42 days post infection, sheep 1 was treated with 5 ml water, sheep 2 with 0.2 mg/kg ivermectin (IVM), and sheep 3 with 0.2 mg/kg moxidectin (MOX). The sheep were then killed, and the abomasa were removed immediately to obtain adult worms. The abomasum was cut open and washed thoroughly with 37°C RPMI 1640 solution (Invitrogen™ life

technologies, Burlington, ON) prepared according to manufacturer's recommendation and adjusted to pH 7.2 with NaOH or HCL to detach worms. All processing was carried out at 37°C. An equal volume of 2% agar (previously boiled and cooled to 48°C) was added and the mixture poured in baking trays containing cheesecloth. Approximately 300 ml was used per tray. The agar-cheesecloth was then placed in RPMI and incubated for 1 hour. Adult worms were collected, frozen in liquid nitrogen and stored at -80°C until DNA extraction.

2.0 DNA Isolation

Genomic DNA from individual frozen adult *H. contortus* was extracted using the QIAGEN DNeasy® Tissue Kit according to the manufacturer's protocols. Elution of DNA with EB buffer was performed in two steps for optimal results; a first elution was performed with 100 µl followed by a second 50 µl elution.

3.0 Amplification

PCR reactions were performed with a PTC-100 programmable thermocycler (MJ Research, Inc). Amplification (8 µl of PCR reaction) was examined by agarose gel electrophoresis. Samples were loaded on a 1.2% agarose gel with 0.1 µg/ml ethidium bromide and visualized with the BIO-RAD gel documentation system Gel Doc 2000™.

3.1 β-tubulin SNP

The PCR protocol and pyrosequencing assay were designed based the *H. contortus* β-tubulin gene GRU-1 (Kwa *et al.*, 1993) sequence found at the NCBI website

(http://www.ncbi.nlm.nih.gov) with the GenBank™ accession number X67489. The amplified PCR product corresponds to region 2666-2765 of the GenBank™ sequence for a 104 bp product. The Phe200Tyr amino acid substitution linked to BZ resistance results from a T/A tranversion mutation (GenBank™ X67489 position 2710). The PCR protocol for the β-tubulin T/A SNP pyrosequencing assay was as follows: the sense primer was RBE 33: 5' – ATG CTA CCC TTT CCG TC – 3', and the antisense primer was RBE34-biotin (HPLC purified, and 5' biotin labeled): 5' – biotin – TGT GAG TTT CAA AGT GCG – 3'. The PCR reaction was: 5.0 µl 10x PCR Taq reaction Buffer; 4.0 µl 2mM dNTP's; 2.0 µl 50mM $MgCl_2$; 0.4 µl primer RBE33; 0.4 µl primer RBE34-biotin; 0.2 µl platinum Taq polymerase (Invitrogen™ life technologies, Burlington, ON); 2.0 µl of DNA, and millipore water to a total volume of 50 µl. The thermo cycling conditions were as follows: 1. 2 min at 94°C; 2. 10 sec at 94°C; 3. 20 sec at 53°C; 4. 20 sec at 72°C; 5. steps 2-4 were repeated 39 times; 6. 5 min at 72°C; 7. indefinitely at 4°C.

3.2 HcGluClα SNP

The *H. contortus* HcGluClα genomic sequence is available at the NCBI website (http://www.ncbi.nlm.nih.gov) with the GenBank™ accession number AY365127, as well as the sequences for both alleles A 'resistant' and C 'susceptible', AF119791 and AF119792 respectively (Forrester *et al.*, 1999). The PCR protocol and pyrosequencing assay were designed based on these sequences. The amplified PCR product corresponds to region 5718-5902 of the GenBank™ AY365127 sequence for a 185 bp product. The Aln169Val amino acid substitution linked to ML resistance results from a C/T transition mutation (GenBank™ AY365127 position 5738). The PCR protocol for the HcGluClα C/T SNP pyrosequencing assay was as follows: the sense primer was F4275-bio (HPLC

purified, and 5' biotin labeled): 5'- biotin – GAA TTT TTC TTA CAG GTT GG – 3', and the antisense primer was R603: 5' – CGG TTT TTC CTC TTT CCA – 3'. The PCR reaction was: 5.0 µl 10x PCR Taq reaction Buffer; 5.0 µl 2mM dNTP's; 2.0 µl 50mM $MgCl_2$; 0.4 µl primer F4275-bio; 0.4 µl primer R603; 0.2 µl platinum Taq polymerase (Invitrogen™ life technologies, Burlington, ON); 2.0 µl of DNA, and millipore water to a total volume of 50 µl. The thermo cycling conditions were as follows: 1. 3 min at 94°C; 2. 15 sec at 94°C; 3. 25 sec at 53°C; 4. 20 sec at 72°C; 5. steps 2-4 were repeated 40 times; 6. 5 min at 72°C; 7. indefinitely at 4°C (Note the biotinylated primer binds the sense strand, therefore the pyrosequencing reaction is based on the reverse complimentary sequence, and a G/A SNP will be observed rather than C/T).

3.3 HG1 SNP

The *H. contortus* HG1 gene (Laughton *et al.*, 1994) sequence is found at the NCBI website (http://www.ncbi.nlm.nih.gov) with the GenBank™ accession number X73584. The amplified PCR product corresponds to region 423-540 of the GenBank™ sequence. The genomic sequence contains a 162 bp intron, and the final amplified product is 279 bp. One allelic form has a 51 bp intron deletion for a final amplified product of 228 bp. A SNP in this same allele was located which prevented optimal binding of the sequencing primer in the pyrosequencing reaction; therefore a mixture of 2 sequencing primers was used (see Chapter III, 4.0 Pyrosequencing™ Technology). The Lys169Arg amino acid substitution linked to ML resistance results from an A/G transition mutation (GenBank™ X73584 position 519). The PCR protocol for the HG1 A/G SNP pyrosequencing assay was as follows: the sense primer was F410: 5' - CTC ACC GTT CCG AAT ATC – 3', and the antisense primer was R527-bio (HPLC purified,

and 5' biotin labeled): 5'- Biotin – GAA CAT CCA TAG GGA ACT TC – 3'. The PCR reaction was: 5.0 µl 10x PCR Taq reaction Buffer; 5.0 µl 2mM dNTP's; 1.5 µl 50mM $MgCl_2$; 0.4 µl primer F410; 0.4 µl primer R527-bio; 0.2 µl platinum Taq polymerase (Invitrogen™ life technologies, Burlington, ON); 2.0 µl of DNA, and millipore water to a total volume of 50 µl. The thermo cycling conditions were as follows: 1. 3 min at 94°C; 2. 15 sec at 94°C; 3. 30 sec at 51°C; 4. 20 sec at 72°C; 5. steps 2-4 were repeated 40 times; 6. 5 min at 72°C; 7. indefinitely at 4°C.

4.0 Pyrosequencing™ technology

All pyrosequencing reactions were performed on a PSQ™96MA instrument from Biotage™ AB (previously Pyrosequencing™ AB). The sample preparation for the pyrosequencing reaction was as follows: 30 µl of the biotinylated PCR products were immobilized on streptavidin-coated Sepharose™ beads (Amersham Biosciences) with binding buffer (10mM Tris-HCL, 2M NaCl, 1mM EDTA, 0.1% Tween 20, adjusted to pH 7.6 with 1M HCL or 4M HAc) and water to a final volume of 80 µl per well. Samples were prepared in a 96 well U-bottom plate and mixed for 10 minutes on a vortex mixer. The immobilized PCR products were made single stranded by means of the Vacuum Prep Tool (Biotage™ AB). First, the immobilized template is captured on the filter probes by slowly lowering the vacuum prep tool in the 96 well U-bottom plate. Next, the filter probes are soaked and flushed in 70% ethanol for five seconds, then in the denaturing solution (0.5M NaOH) for five seconds, and finally in the washing solution (10mM Tris-Acetate adjusted to pH 7.6 with 1M HCL or 4M HAc) for five seconds. The immobilized beads are then released into the PSQ™96 Plate Low pre-filled with 0.5 µM sequencing

primer in 40 µl annealing buffer (20mM Tris-Acetate, 2 mM Mg-Acetate, adjusted to pH 7.6 with 1M HCL or 4M HAc). Sequencing primers were RBE35: 5'- AGA ACA CCG ATG AAA CA – 3' for the β-tubulin A/T SNP assay, which binds position 2692-2708 of the GenBank™ X67489 GRU-1 sequence, R4296: 5'- CTG TAC ATC AAG CGG ATA ATC G - 3' for the HcGluClα C/T SNP assay, which binds position 5739-5760 of the GenBank™ AY365127 sequence, HG1-169pyro: 5'- GAA ATG TCT CAT GTT TTT G/AA – 3' mixture for the HG1 A/G SNP assay, which binds position 499-518 of the GenBank™ X73584 sequence. The primer annealing step was performed using a PTC-100 programmable thermocycler (MJ Research, Inc) for 3 minutes at 80°C; the plate was removed and cooled at room temperature for 10 minutes prior to the sequencing reaction. All reagents for the pyrosequencing reaction were included in the SNP reagent kits obtained from Biotage™ AB, and used according to their protocol. These include the Enzyme (E) mixture (DNA polymerase, ATP-sulfurylase, luciferase, apyrase), Substrate (S) mixture (luciferin, adenosine 5' phosphosulfate), and nucleotides (dATP αS, dCTP, dGTP, dTTP) which are loaded in the PSQ™96 Reagent Cartridge. Peak heights were measured and analyzed to determine genotypes by the AQ module in the PSQ™96 Single Nucleotide position Software (Biotage™ AB).

5.0 Cloning and Sequencing

To confirm pyrosequencing results and to compare alleles of the various laboratory and field strains, PCR products were cloned into a pGEM-T Easy vector (Promega Corporation, Montreal, Quebec), as described by the manufacturer and transformed into JM109 (Promega Corporation, Montreal, Quebec) competent cells as

described by the manufacturer. Single colonies were grown overnight in a shaking incubator at 37°C and plasmid DNA isolated using the QIAGEN MiniPrep kit according to the manufacturer's protocol. The DNA was then sequenced at the McGill University Sheldon Biotechnology Center, Montreal, Quebec.

6.0 Analysis

Sequences were analyzed online at NCBI (http://www.ncbi.nlm.nih.gov) using BLASTN and BLASTX to confirm the identity of the cloned fragment. All DNA alignments were carried out using ClustalX (Thompson *et al.*, 1997), and further analysis of sequences and alignments were carried out with BioEdit (Hall, 1999). The genotype data obtained from pyrosequencing was analyzed statistically to verify if strains were in Hardy-Weinberg equilibrium and if strains were statistically different from each other in terms of allele frequencies by means of chi-square analysis.

Chapter IV

Results

1.0 VHR29 *in vivo* Trial

The field resistant VHR29 strain was passaged in sheep to collect survivors of a field dose of ivermectin (IVM) and moxidectin (MOX). The data obtained from the *in vivo* trial is presented in **Table 1**. Fecal egg counts (FEC) were used to monitor the status of infection, 42 days post infection, sheep 1 had 1045 eggs/g and was treated with 5 ml water, sheep 2 had 5260 eggs/g and was treated with 0.2 mg/kg IVM, and sheep 3 had 6272 eggs/g and was treated with 0.2 mg/kg MOX. The variability of the FEC can be attributed to host genetic factors, male vs. female ratio of established worms, random events, and human error, both in administering the larvae to the sheep, and measuring the FEC. Two weeks following treatment the FEC for sheep 1 (water) was 1220 eggs/g, sheep 2 (IVM), 264 eggs/g, and sheep 3 (MOX), 0 eggs. The FEC reduction from 5260 to 264 eggs/g was expected as IVM is still partly effective against this field resistant strain (Wooster *et al.*, 2001). No eggs or adult worms were recovered from sheep 3 following MOX treatment, therefore MOX was still 100% effective against adult *H. contortus* from this strain. This is in agreement with the pen trial by Wooster *et al.* (2001). From the water treated control sheep 1, 150 adult worms were recovered, of which 63 were male, these worms were designated strain VHR29xW. From the IVM treated sheep 2, 82 adult worms were recovered, of which 58 were males. Genotyping *H. contortus* is usually limited to male worms, as female egg DNA could potentially interfere with actual genotype. To increase sample size, we opted to genotype both male and female worms.

Genotyping results for all three SNPs in this study were not statistically different when comparing results from males and females ($P<0.05$) therefore the results from males and females were combined.

Table 1. VHR29 *in vivo* Trial

Sheep #	FEC (eggs/g) Oct 10, 03	Treatment Oct 10, 03	FEC (eggs/g) Oct 24, 03	Adults Recovered
1	1045	Water	1220	150 (63 ♂) **(VHR29xW)**
2	5260	IVM (0.2 mg/kg)	264	82 (58 ♂) **(VHR29xI)**
3	6272	MOX (0.2 mg/kg)	0	0

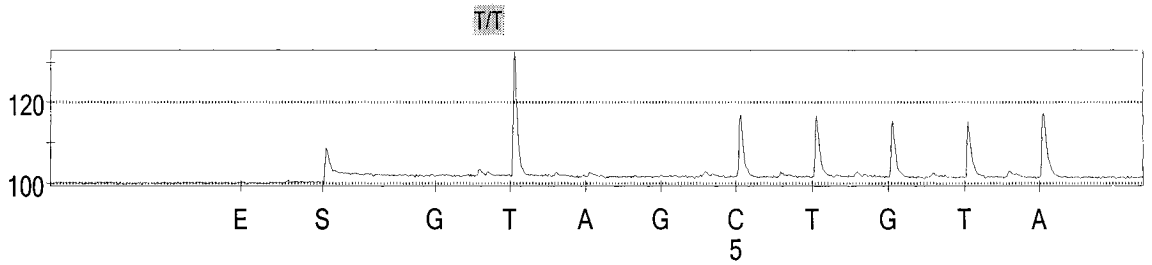
2.0 β-tubulin SNP Genotyping

The Phe200Tyr amino acid substitution involved in benzimidazole (BZ) resistance results from a T/A tranversion mutation. A pyrosequencing assay was designed to genotype this single nucleotide polymorphism (SNP). The analyzed sequence, dispensation order, and example of data obtained from the PSQ™96MA pyrosequencer are shown in **Fig. 1**. The genotype frequencies obtained for the β-tubulin position 200 SNP from laboratory strains is shown in **Fig. 2**. The results for the laboratory strains passaged for 17 generations are shown in **Fig. 2a**, and were as follows: from the unselected laboratory strain PF17, out of 40 individual *H. contortus* analyzed, 39 (0.975) were T/T, 1 was A/T (0.025) and none were A/A. From the IVM selected strain IVF17,

a. Sequence to analyze: TT/ACTGTA

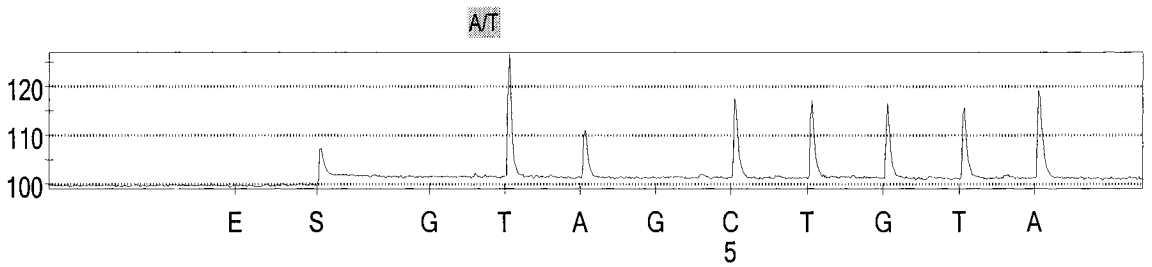
Dispensation order: GTAGCTGTA

b.



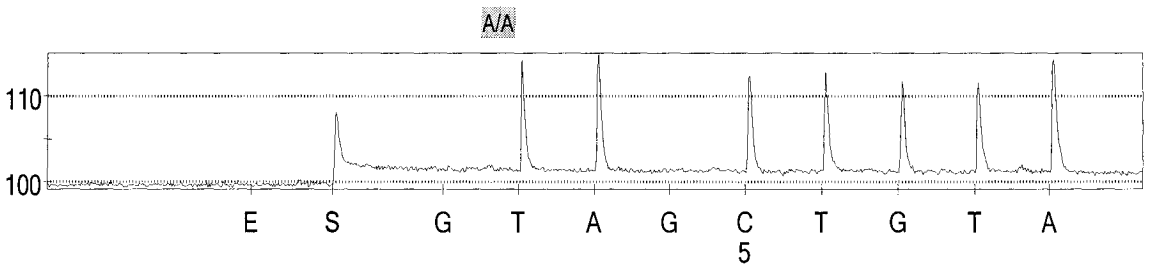
TTCTGTA
TTCTGTA

c.



TTCTGTA
TACTGTA

d.



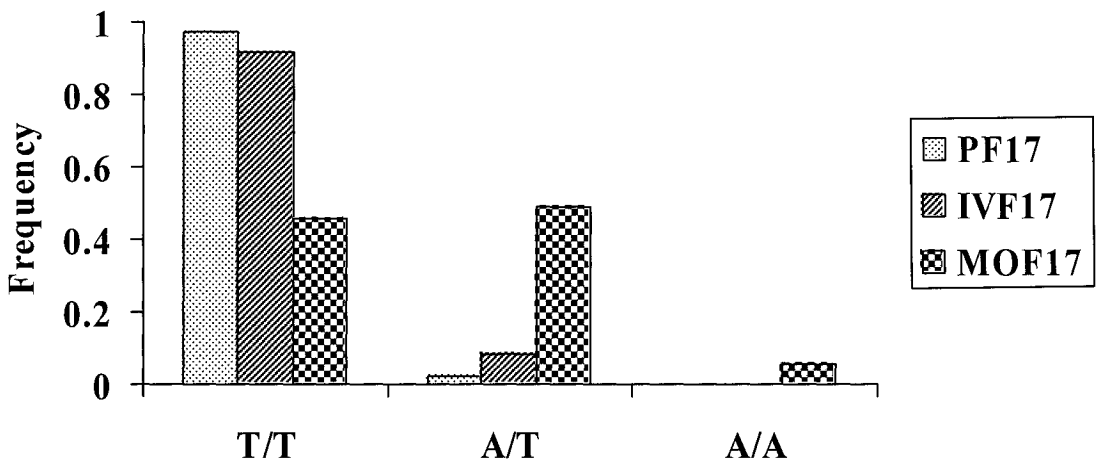
TACTGTA
TACTGTA

Figure 1. Representative pyrograms obtained from Pyrosequencing™ analysis based on the position 200 substitution of β-tubulin isotype-1. (a) The sequence to analyze following the sequencing primer RBE35, and the nucleotide dispensation order by the PSQ™96MA pyrosequencer. (b) Pyrogram for the BZ-susceptible genotype T/T. (c) Pyrogram for the heterozygote genotype A/T. (d) Pyrogram for the BZ-resistant genotype A/A. Relative light units are indicated on the y-axis. Additions of enzyme (E) and substrate (S) mixtures and incorporated nucleotides are shown on the x-axis. Interpreted sequence is shown below each pyrogram.

out of 37, 34 (0.919) were T/T, 3 (0.081) were A/T, and none were A/A. From the MOX selected strain MOF17, out of 57, 26 (0.456) were T/T, 28 (0.491) were A/T, and 3 (0.053) were A/A. Data from the further passaged PF23 and IVF23 is shown in **Fig. 2b**. From the unselected PF23 strain, out of 28, 27 (0.964) were T/T, 1 (0.036) was A/T, and none were A/A, and finally from the laboratory strains, from the IVM selected IVF23, out of 36, 23 (0.639) were T/T, 12 (0.333) were A/T, and 1 (0.028) was A/A. All strains were found to be in Hardy-Weinberg equilibrium ($P<0.05$) by means of chi-square analysis of the obtained allele frequency data. Strain PF17 and MOF17 were significantly different ($P>0.05$). PF23 and IVF23 were also significantly different, indicating positive selection for this SNP. By definition, positive selection following drug treatment should be associated with a loss of Hardy-Weinberg equilibrium. However, a single round of mating following the selection process will re-establish equilibrium, and these laboratory strains have been maintained for multiple generations following the initial drug selection trial. This explains why they are in Hardy-Weinberg equilibrium. A small increase in heterozygotes was also observed in IVF17 compared to PF17, however this was not found to be significant. The genotype frequencies for the field strains are shown in **Fig. 3**, genotypes for strains VHR29 and BUSTA obtained from freeze dried adults are in **Fig. 3a**, and were as follows: from VHR29, out of 38, none were T/T, 7 (0.184) were A/T, and 31 (0.816) were A/A, and from BUSTA, out of 98, 14 (0.143) were T/T, 59 (0.602) were A/T, and 25 (0.255) were A/A. The results from the VHR29 parasites obtained from the *in vivo* trial conducted are in **Fig. 3b**, and the results were the following: from strain VHR29xW (sheep treated with water), out of 117, none were T/T, 6 (0.051) were A/T, and 111 (0.949) were A/A, and from VHR29xI (survivors of a field dose of IVM), out of

82, none were T/T, 3 (0.037) were A/T, and 79 (0.963) were A/A. All four field strains were in Hardy-Weinberg equilibrium ($P<0.05$). Strains VHR29 and BUSTA were statistically different from each other ($P>0.05$), most likely due to the different BZ treatment histories of the two strains. Data from both males and female *H. contortus* were not significantly different ($P<0.05$), and were combined for both VHR29xW and VHR29xI.

a.



b.

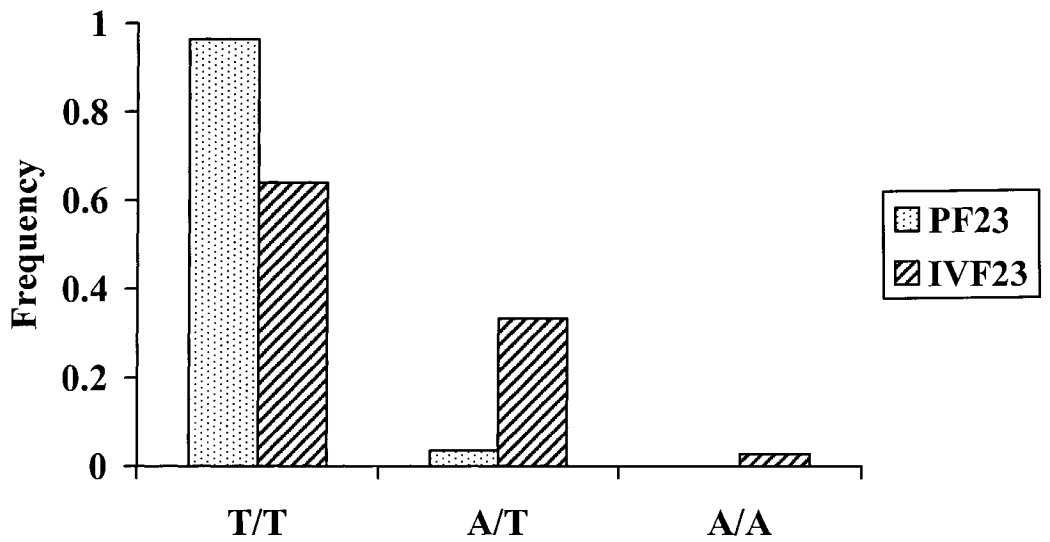
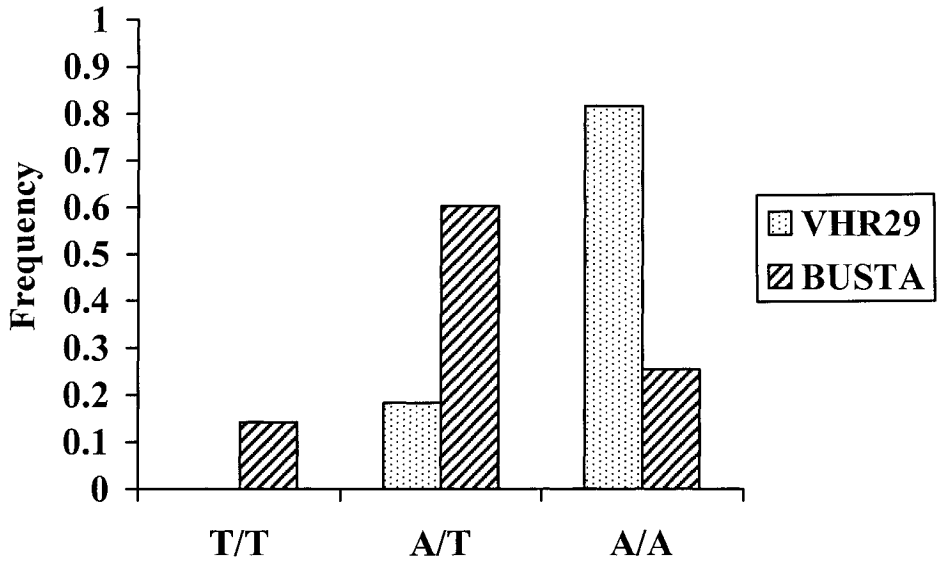


Figure 2. Genotype frequencies for the β-tubulin isotype-1 position 200 substitution SNP obtained from *H. contortus* laboratory strains. (a) From laboratory strains PF17 (unselected), IVF17 (IMV selected), and MOF17 (MOX selected). (b) From laboratory strains PF23 and IVF 23.

a.



b.

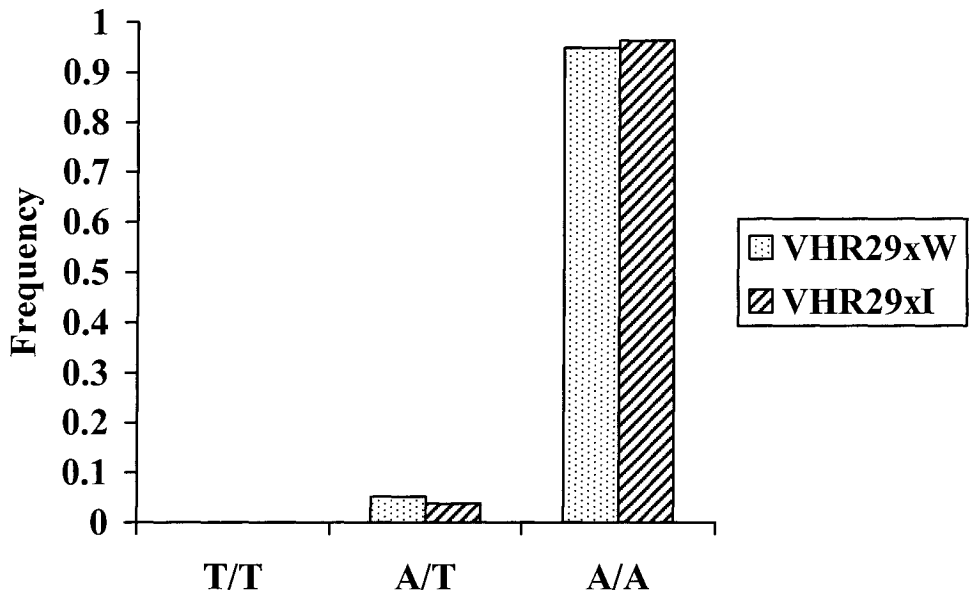


Figure 3. Genotype frequencies for the β-tubulin isotype-1 position 200 substitution SNP obtained from *H. contortus* field resistant strains. (a) From field strains VHR29, and BUSTA. (b) From the VHR29 *in vivo* trial: VHR29xW (water treated control), and VHR29xI (survivors of an IVM field dose).

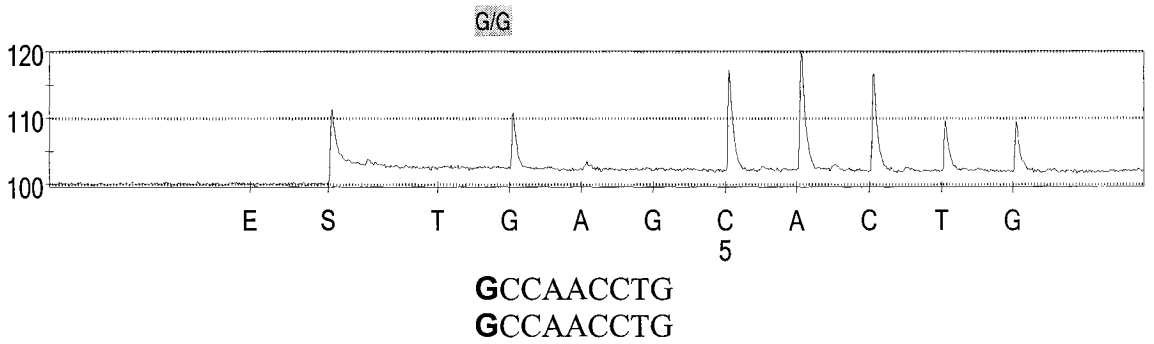
3.0 HcGluClα SNP Genotyping

The Aln169Val amino acid substitution linked to ML resistance results from a C/T transition mutation. A pyrosequencing assay was designed to genotype this SNP. The analyzed sequence, dispensation order, and example of data obtained from the PSQ™96MA pyrosequencer are shown in **Fig. 4.**

a. Sequence to analyze: G/ACCAACCTG

Dispensation order: TGAGCACTG

b.



c.

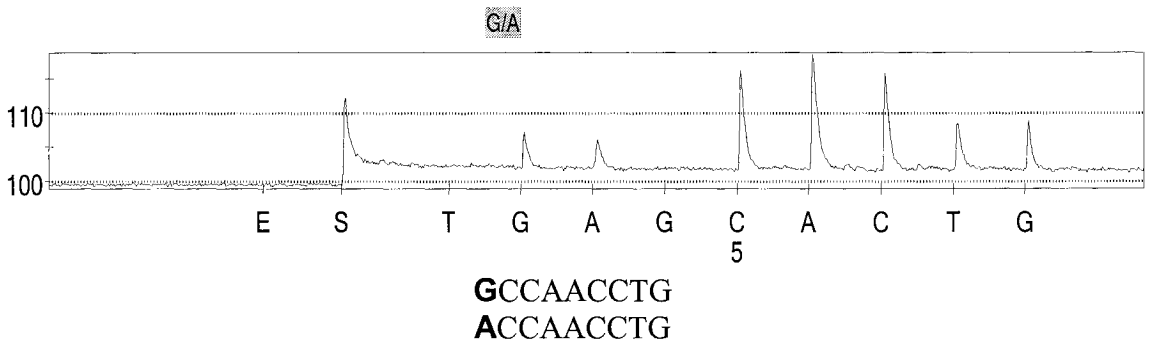


Figure 4. Representative pyrograms obtained from Pyrosequencing™ analysis based on the position 169 substitution of HcGluClα. (a) The sequence to analyze following the sequencing primer R4296, and the nucleotide dispensation order by the PSQ™96MA pyrosequencer. (b) The susceptible genotype C/C. (c) The heterozygote genotype C/T. Relative light units are indicated on the y-axis. Additions of enzyme (E) and substrate (S) mixtures and incorporated nucleotides are shown on the x-axis. Interpreted sequence is shown below each pyrogram (assay is performed on the reverse complimentary strand, and no T/T (A/A) homozygotes were observed).

The genotype frequencies obtained for the HcGluCLα pos 169 SNP from laboratory strains are shown in **Fig. 5**. For the unselected PF17 strain, out of 20 individual adults analyzed, 19 (0.950) were C/C, 1 (0.050) was C/T, and none were T/T, from the IVM selected IVF17, out of 42, 33 (0.786) were C/C, 9 (0.214) were C/T, and none were T/T, and from the MOX selected MOF17, out of 23, 17 (0.739) were C/C, 6 (0.261) were C/T. and none were T/T. All three strains were in Hardy-Weinberg equilibrium, and although an increase in the resistance associated genotype is seen, it was not significant ($P<0.05$).

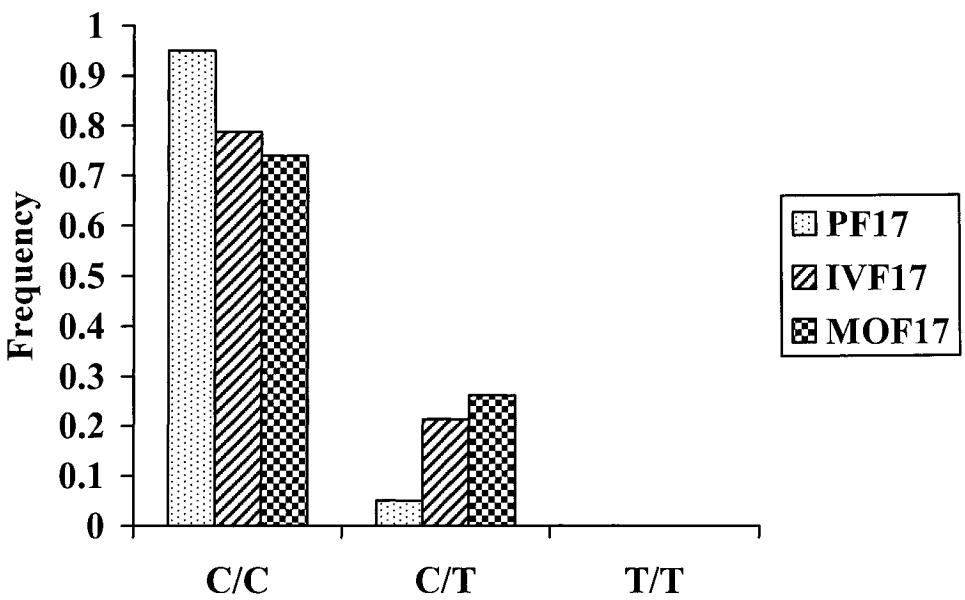
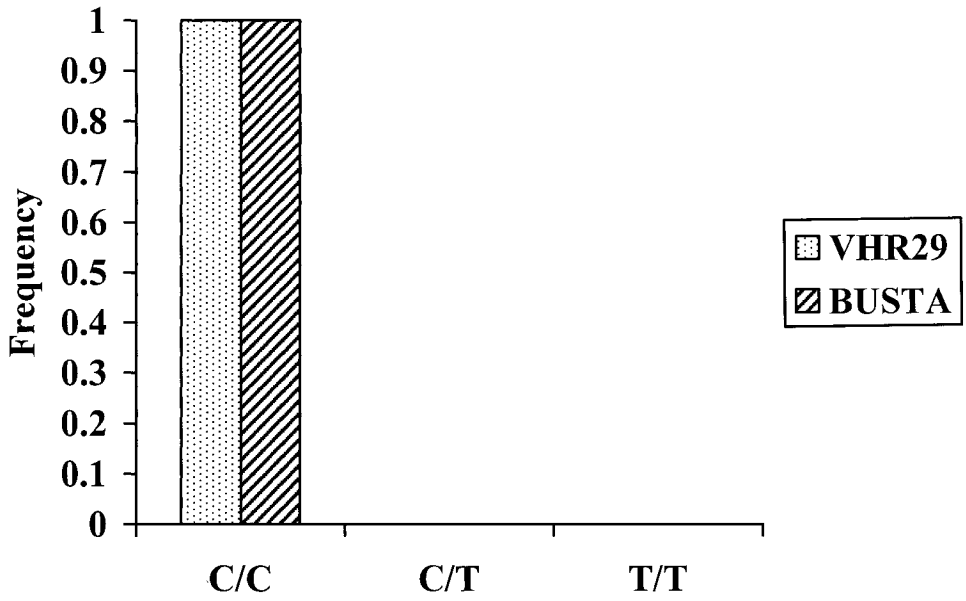


Figure 5. Genotype frequencies for the HcGluClα position 169 substitution SNP obtained from *H. contortus* laboratory strains. From strain PF17 (unselected), IVF17 (IMV selected), and MOF17 (MOX selected).

a.



b.

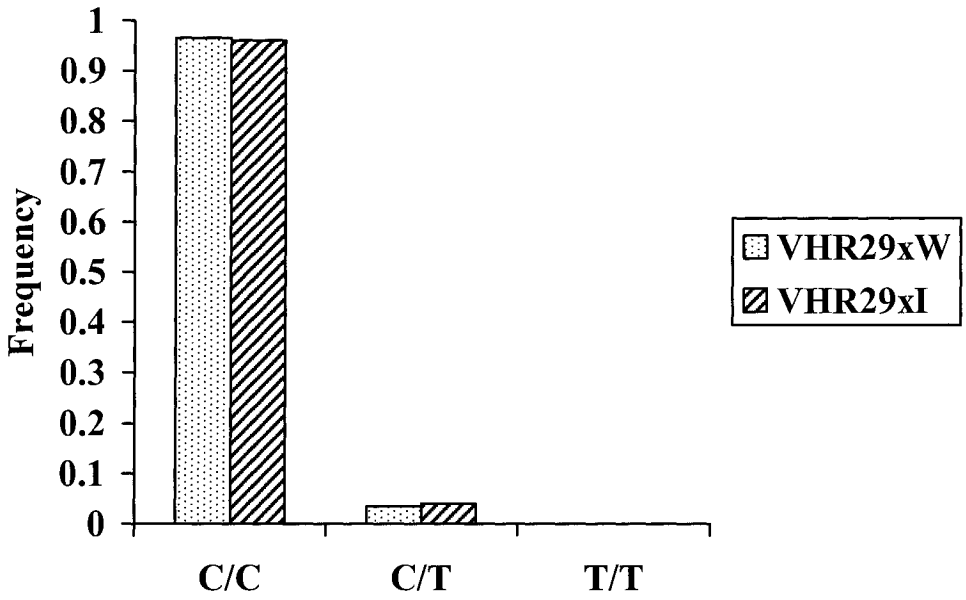


Figure 6. Genotype frequencies for the HcGluClα position 169 substitution SNP obtained from *H. contortus* field resistant strains. (a) From field strains VHR29, and BUSTA. (b) From the VHR29 *in vivo* trial: strains VHR29xW, and VHR29xI.

The genotype frequencies for the field strains are shown in **Fig. 6,** genotypes for strains VHR29 and BUSTA obtained as freeze dried adults are in **Fig. 6a**, and were as follows: from VHR29, out of 27, all individuals were C/C (1.000), and from BUSTA, out of 72, all individuals were also C/C (1.000). The results from the VHR29 parasites obtained from the *in vivo* trial experiment conducted are in **Fig. 6b**, and the results were as follows: from strain VHR29xW (sheep treated with water), out of 89, 86 (0.966) were C/C, 3 (0.034) were C/T, and none were T/T, and from VHR29xI (survivors of a field dose of IVM), out of 75, 72 (0.960) were C/C, 3 (0.040) were C/T, and none were T/T. All four strains were in Hardy-Weinberg equilibrium ($P<0.05$). Data from both males and females were not significantly different ($P<0.05$), and were combined for both VHR29xW and VHR29xI.

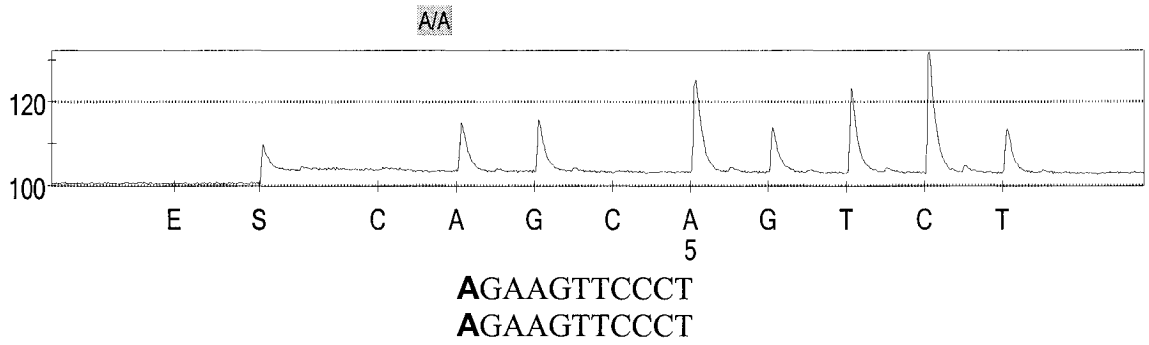
4.0 HG1 SNP Genotyping

The Lys169Arg amino acid substitution linked to ML resistance results from a A/G transition mutation. A pyrosequencing assay was designed to genotype this SNP. The analyzed sequence, dispensation order, and example of data obtained from the PSQ™96MA pyrosequencer are shown in **Fig. 7**. The genotype frequencies obtained for the laboratory strains is shown in **Fig. 8**. Data from the laboratory strains passaged for 17 generations is shown in **Fig. 8a**, and is as follows: from the unselected PF17 strain, out of 43 individual adult *H. contortus* analyzed, 28 (0.651) were A/A, 13 (0.302) were G/A, and 2 (0.047) were G/G, from the IVM selected IVF17 strain, out of 36 samples, 15 (0.417) were A/A, 19 (0.528) were G/A, and 2 (0.056) were G/G, and from the MOX

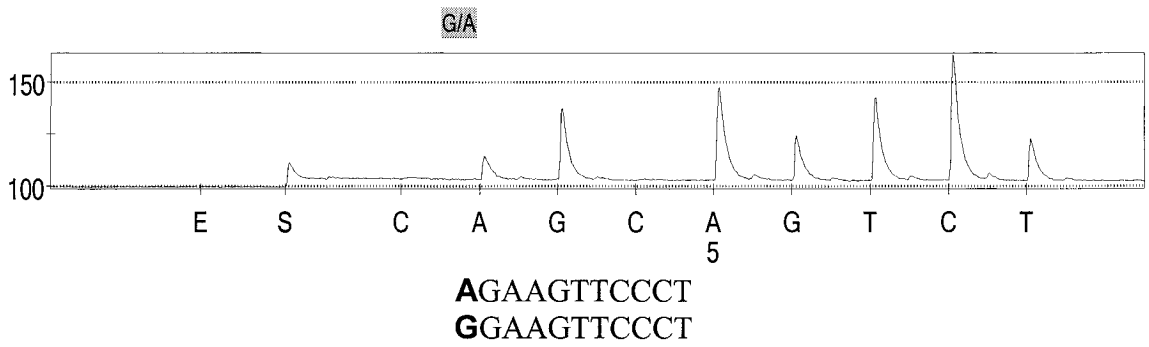
a. Sequence to analyze: G/AGAAGTTCCCT

Dispensation order: CAGCAGTCT

b.



c.



d.

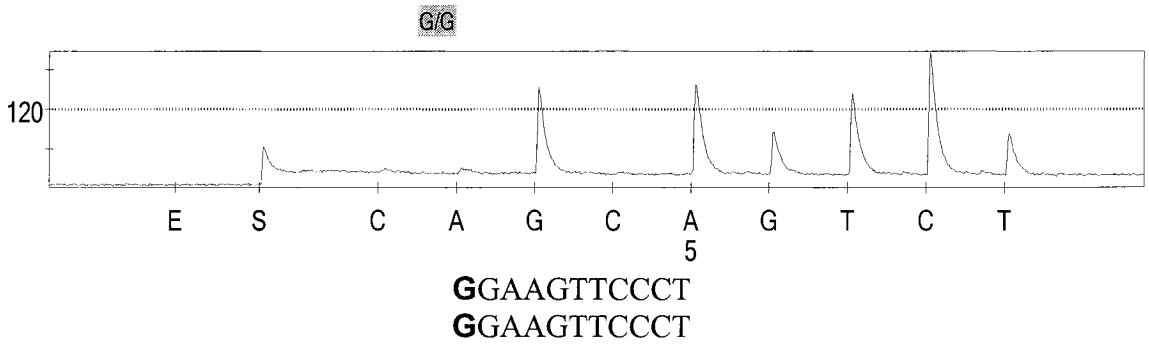
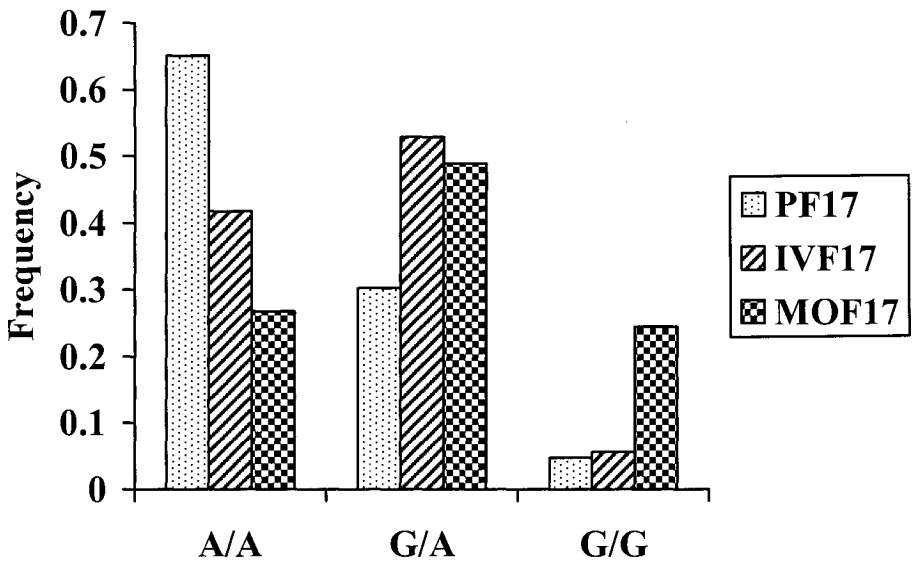


Figure 7. Representative pyrograms obtained from Pyrosequencing™ analysis based on the position 169 substitution of HG1. (a) The sequence to analyze following the sequencing primer HG1-Pyro169 and the nucleotide dispensation order by the PSQ™96MA pyrosequencer. (b) The susceptible genotype A/A. (c) The heterozygote genotype G/A. (d) The homozygote 'resistant' genotype G/G. Relative light units are indicated on the y-axis. Additions of enzyme (E) and substrate (S) mixtures and incorporated nucleotides are shown on the x-axis. Interpreted sequence is shown below each pyrogram.

selected strain MOF17, out of 45, 12 (0.267) were A/A, 22 (0.489) were G/A, and 11 (0.244) were G/G. Data from the further passaged PF23 and IVF23 strains are shown in **Fig. 8b**, and is as follows: from the unselected PF23 strain, out of 38, 30 (0.789) were A/A, 6 (0.158) were G/A, and 2 (0.053) were G/G, and from the IVM selected IVF23, out of 40, 18 (0.450) were A/A, 19 (0.475) were G/A, and 3 (0.075) were G/G. All laboratory strains were in Hardy-Weinberg equilibrium ($P<0.05$). There was an obvious increase in the resistance associated allele in the IVM selected strain IVF17 compared to the unselected PF17 although it was not statistically significant ($P<0.05$), however after 23 generations of selection with IVM, IFF23 was significantly different than PF23 ($P>0.05$), as well, the MOX selected MOF17 was statistically different from PF17 ($P>0.05$). The genotype frequencies from the field strains is shown in **Fig. 9**, genotypes for strains VHR29 and BUSTA obtained from freeze dried adults are in **Fig. 9a**, and were as follows: from VHR29, out of 38, 37 (0.974) were A/A, 1 (0.026) was G/A, and none were G/G, and from BUSTA, out of 105, 89 (0.848) were A/A, 16 (0.152) were G/A, and none were G/G. The results from the VHR29 parasites obtained from the *in vivo* trial experiment conducted are in **Fig. 9b**, and are as follows: from strain VHR29xW (sheep treated with water), out of 107, 103 (0.963) were A/A, 4 (0.037) were G/A, and none were G/G, and from VHR29xI (survivors of a field dose of IVM), out of 75, 71 (0.947) were A/A, 4 (0.053) were G/A, and none were G/G. All four strains were in Hardy-Weinberg equilibrium ($P<0.05$). Data from both males and females were not significantly different ($P<0.05$), and was combined for both VHR29xW and VHR29xI. There was an increase in heterozygotes in the more resistant BUSTA strain, however this was not statistically significant ($P<0.05$).

a.



b.

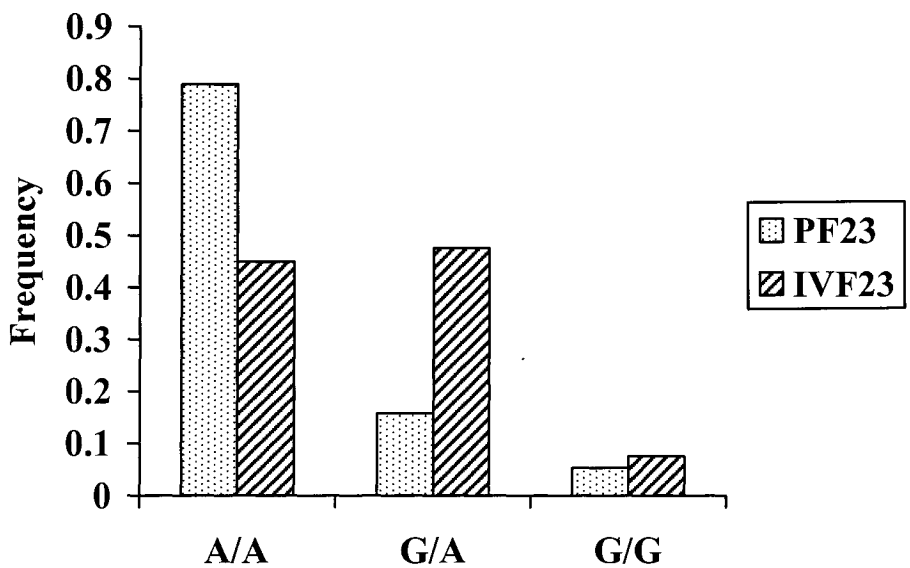
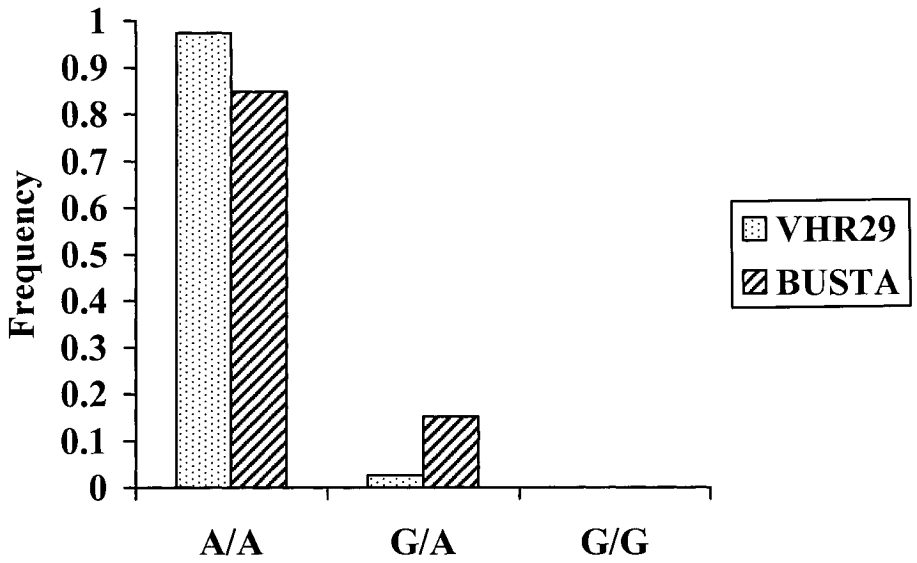


Figure 8. Genotype frequencies for the HG1 position 169 substitution SNP obtained from *H. contortus* laboratory strains. (a) From laboratory strains PF17 (unselected), IVF17 (IMV selected), and MOF17 (MOX selected). (b) From laboratory strains PF23 and IVF23.

a.



b.

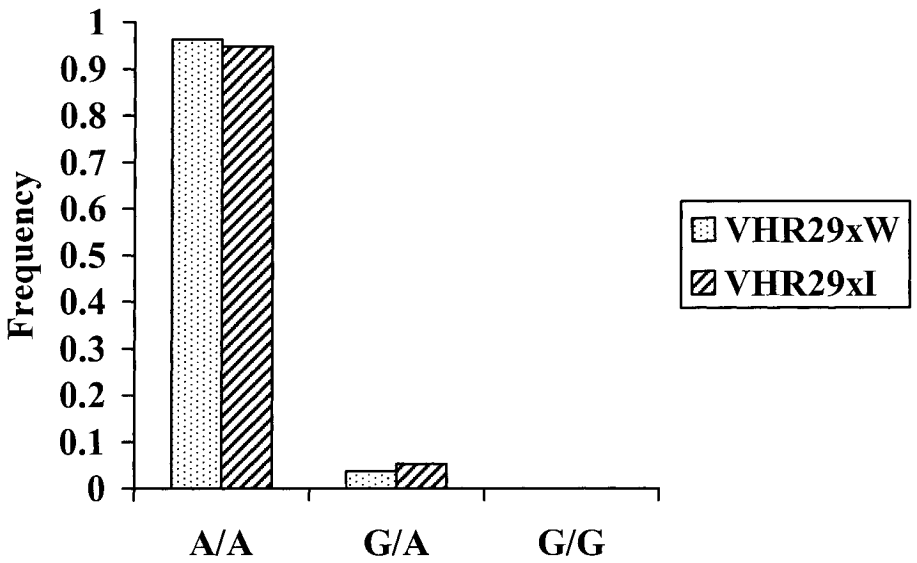


Figure 9. Genotype frequencies for the HG1 position 169 substitution SNP obtained from *H. contortus* field resistant strains. (a) From field strains VHR29, and BUSTA. (b) From the VHR29 *in vivo* trial: VHR29xW, and VHR29xI.

5.0 HG1 Sequence Alignment

The HG1E allele found to be linked with IVM/MOX resistance in laboratory selected strains by Blackhall (2003) was not found by SSCP in field selected strains (W. Blackhall, personal communication). Our hypothesis is that SNPs conferring resistance in the laboratory strains are also present in the field strains on different allelic forms; therefore a linked marker method like SSCP would fail to detect the resistant individuals. A sequence alignment was performed to compare the allelic forms of both laboratory and field resistant strains which contain the Lys169Arg associated SNP to confirm whether or not the SNP is found on different alleles in different strains, or if the Lys169Arg SNP remained conserved to one allelic form in the strains used for this study. For this alignment, the same fragment of HG1 amplified by PCR and used for pyrosequencing was sequenced and aligned. The alignment consists of the laboratory HG1E allele from the Fort Dodge strain, the alleles containing the Lys169Arg SNP from the two field resistant strains VHR29 and BUSTA, as well as cDNA from the laboratory Fort Dodge strain alleles A and E to show the location of the intron and the Lys169Arg SNP (**Fig. 10**). The sequence alignment revealed all alleles with the Lys169Arg substitution were identical in the segment aligned for all strains (**Fig. 10**). This indicates the Lys169Arg substitution is unique to allele E and does not appear to be found on different alleles on different strains as we previously hypothesized.

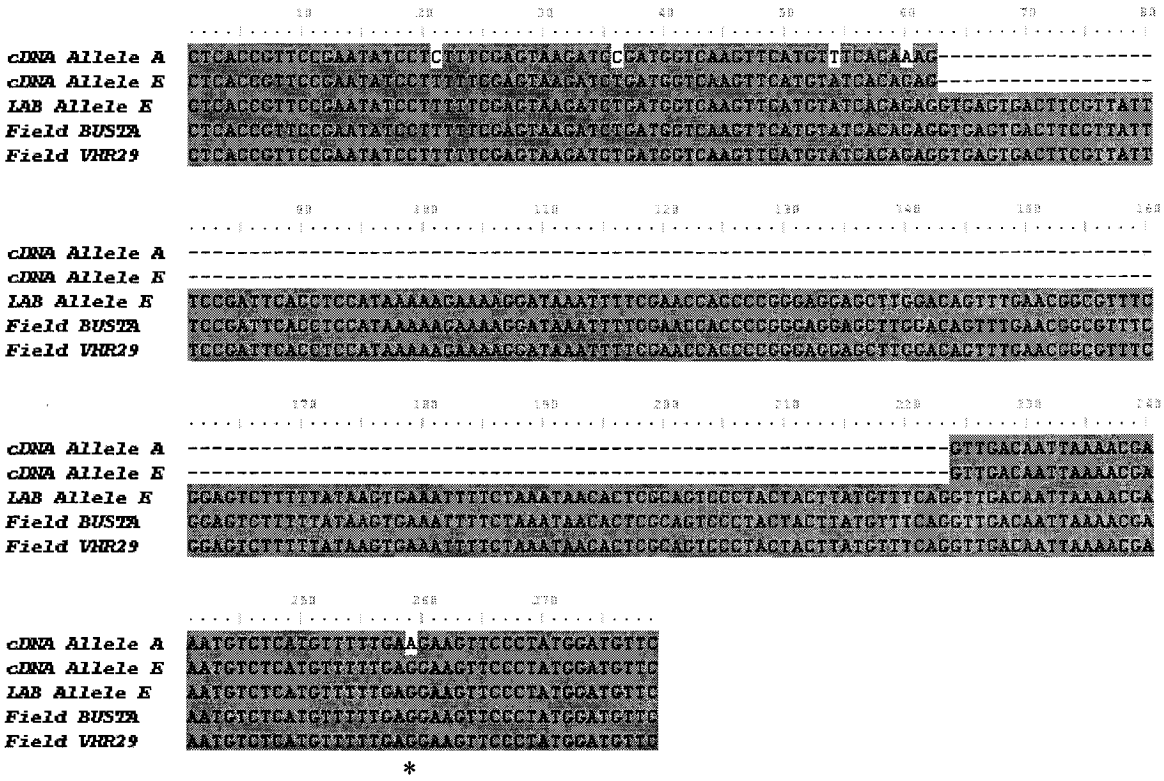


Figure 10. HG1 partial alignment of cDNA from allele E and allele A, allele E from the Fort-Dodge laboratory strain, BUSTA field strain allele with the Lys169Arg substitution SNP, and the VHR29 field strain with the Lys169Arg substitution SNP. (----) represent intron, (*) represent Lys169Arg substitution A/G SNP (multiple E alleles were sequenced from each strain, only one representative sequence from each is shown here).

Chapter V

Discussion

Controlling ivermectin (IVM) and moxidectin (MOX) resistance in gastrointestinal nematodes is becoming a serious problem worldwide. So serious in fact, that some farms in South Africa have had to abandon sheep farming altogether due to failure of all anthelmintic classes against *Haemonchus contortus* (van Wyk *et al.*, 1999). Studies based on laboratory selected *H. contortus* have identified genes linked with IVM/MOX resistance. None of these findings however have been confirmed in field resistant strains.

In this study, single nucleotide polymorphisms (SNPs) associated with resistance and identified from these *H. contortus* laboratory resistant strains were screened from various laboratory resistant and field resistant *H. contortus* strains using pyrosequencing™ technology. Genotypes were obtained for SNPs associated with IVM/MOX resistance from the following three genes: β-tubulin isotype-1; a gamma-aminobutyric acid (GABA) chloride channel subunit, HG1; and a glutamate-gated chloride channel (GluCl) subunit, HcGluClα.

In β-tubulin isotype-1, only one SNP has been identified between the IVM/MOX laboratory resistant and susceptible alleles (R. Beech, personal communication; Blackhall, 1999). Interestingly the SNP identified is the same that produces the Phe200Tyr substitution associated with benzimidazole (BZ) resistance in *H. contortus* as well as other related species (Kwa *et al.*, 1993, 1994; Grant and Mascord, 1996; Silvestre and Humbert, 2000; Silvestre and Cabaret, 2002; Elard *et al.*, 1996). It seems unlikely

this mutation would also be involved in IVM/MOX resistance, as IVM and MOX are unrelated to the BZs, and IVM does not bind to nor inhibit polymerization of microtubules (M. Oxberry, personal communication). However, three amino acid substitutions in β-tubulin have also been linked with IVM resistance in the human parasite *Oncocerca volvulus* (Eng and Prichard, submitted), therefore we investigated the involvement of the Phe200Tyr substitution IVM/MOX resistance in *H. contortus*.

In the study reported here, significant selection was found for the Phe200Tyr substitution in the laboratory MOX selected MOF17 when compared to the unselected PF17 (**Fig. 2, p.48**). An increase in heterozygotes was also observed in the IVM selected IFV17 compared to the unselected PF17, although this increase was not statistically significant. A further six generations of selection did show significant selection by IVM. The IVM selected IVF23 was significantly different from the unselected PF23, demonstrating positive selection for the Phe200Tyr substitution by IVM in this strain. This is consistent with the observed selection by Blackhall (1999) and links this SNP with IVM/MOX resistance in these laboratory strains. It is still unknown if this SNP is directly responsible for resistance and the observed selection. It is also important to note that limited quantities of DNA samples were available from these laboratory strains, and low sample sizes limit the significance of the results. Another factor to consider is that these strains have been maintained for multiple generations following the initial drug selection trials, allowing for genetic recombination to occur. This explains why all strains were in Hardy-Weinberg equilibrium, which is not expected if positive selection has occurred. Random events and host genetic factors could also affect the genotype frequencies reported here.

BZs inhibit tubulin polymerization and alter the tubulin-microtubule equilibrium (reviewed by Prichard, 1990, 2001). This Phe200Tyr substitution inhibits the binding of BZs to β-tubulin (Lubega and Prichard, 1991). Unlike BZ, IVM does not bind to tubulin nor inhibit tubulin polymerization (M. Oxberry, personal communication). It seems unlikely the position 200 substitution would also be involved in IVM/MOX resistance. A more likely SNP may still be identified. Current work aims to sequence the full length resistant and susceptible alleles of β-tubulin isotype-1. Another explanation is that β-tubulin is indirectly linked with the resistant gene by genetic hitchhiking, where genes in close proximity to a gene under selective pressure get selected along with it (Smith and Haigh, 1974; Barton, 2000). There may be an indirect functional link between β-tubulin and resistance to a drug that targets inhibitory chloride channels. Amphids are sensory neuronal structures found on either side of the pharynx of nematodes in contact with the external environment. It as been recently argued that amphidial neurons may play a role in IVM/MOX resistance, and may be a critical entry point for the drug. Laboratory and field resistant strains have shortened and structurally degenerate amphidial neurons, but the interesting link with tubulin is that they also lack normal microtubule structure compared to susceptible strains, suggesting this may block IVM entry into the parasite, and reduce the effective concentration of drug at the target site (Freeman *et al.*, 2003; Guerrero and Freeman, 2004).

The results presented here show a significant increase in the frequency of the SNP that causes the Phe200Tyr substitution in the laboratory resistant strains (**Fig. 2 p.48**). This is consistent with the observed selection by Blackhall *et al.* (1999). Interestingly it was the frequency of heterozygotes that increased from 0.025 to 0.08 for IVF17, 0.025 to

0.491 for MOF17 (**Fig. 2a p.48**) and 0.036 to 0.333 for IVF23 (**Fig. 2b p.48**). In BZ resistance the SNP is recessive, only homozygote resistant worms are protected from the lethal effects of BZs. This indicates a different outcome from BZ selection compared to IVM/MOX selection, as it appears heterozygotes provide protection. This would be consistent with IVM/MOX resistance, which has been suggested to be inherited as completely dominant (Le Jambre, 1993; Dobson *et al.*, 1996; Le Jambre *et al.*, 2000; Barnes *et al.*, 2001). For IVM, it appears the increase was significantly higher following six more rounds of selection from 17 (IVF17) to 23 (IVF23) generations, and MOF17 was also significantly different from IVF17 ($P>0.05$). It is important to note these laboratory strains (PF17, IVF17, MOF17, PF23, and IVF23) have been passaged without drug treatment following the actual *in vivo* selection trials, thus allowing for reshuffling to occur. Therefore it is impossible to know for sure if the selection reported here is on heterozygotes or homozygotes. The passage and reshuffling history of the strains following the initial drug selection trials could also explain the unusually high difference between PF17 and IVF17 vs. PF23 and IVF23 after only six generations, although this increase in frequency from PF17 to IVF17 and finally IVF23 indicates stronger evidence for positive selection for this SNP by IVM.

In *H. contortus*, MOX is much more potent than IVM, being therapeutically effective against IVM-resistant strains (Craig *et al.*, 1992; Pankavich *et al.* 1992); Kieran, 1994; Echevarria *et al.*, 1997). Therefore the abnormally high selection seen by MOX (MOF17) compared to IVM (IVF17) (**Fig. 2a, p.48**) could indicate this mutation is more important at higher levels of resistance, giving protection against the more potent MOX,

possibly adding to another mechanism of resistance. This hypothesis is based on minimal evidence and will require further investigation.

In the two field strains screened, the Phe200Tyr substitution was very abundant, which is to be expected as these field strains were probably exposed to multiple treatments with the BZs prior to the release of IVM and MOX. VHR29xW was predominantly homozygous resistant for the Phe200Tyr substitution SNP (0.949) with no homozygous susceptible individuals (**Fig 3b p.49**). Therefore, no significant comparisons could be made from the parasites we obtained from the *in vivo* trial (**Table 1, p.44**) between the water treated VHR29xW and IVM treated VHR29xI (**Fig 3b p.49**). This indicates this mutation alone does not protect this strain from a field dose of IVM (0.2 mg/kg), as the fecal egg counts (FEC) were significantly reduced following treatment from 5260 to 264 eggs/g (**Table 1, p.44**).

The Phe200Tyr substitution and the selected allele by Blackhall (1999) does not appear important for field resistance to IVM, but this does not exclude the possibility of a minor role for β-tubulin in resistance, which is indicated by the unusually high selection by MOX compared to IVM in the laboratory strains. This could add to another mechanism(s) of resistance to give additional protection at higher doses. The role of β-tubulin in IVM/MOX resistance remains unclear at this time, but if involved, our results indicate it plays a minor role.

Both GABA- and GluCl-gated chloride channels have been well characterized as IVM/MOX target sites, although their potential role in resistance is still unclear. Both allele A of the HcGluClα subunit (Blackhall *et al.*, 1998[b]) and allele E of the HG1 subunit (Blackhall *et al.*, 2003) have been implicated in drug resistance, however these

have been identified from laboratory selected strains using sub-therapeutic doses of drugs. The question remains whether resistance selected this way is relevant to field resistance. Screens based on linked marker methods have failed to identify either of these alleles as being important in field resistance so far (W. Blackhall, personal communication; Forrester, 2002). Differences between laboratory and field selection could account for this. However, there is evidence that suggest that linked markers may be insufficient to detect the resistant individuals as other alleles may be involved in resistance (Blackhall *et al.*, 2003), suggesting SNPs involved in resistance may be present on other alleles in different strains.

In the study reported here, the SNP responsible for the 169 substitution from both HcGluClα and HG1 was screened in various laboratory and field strains using pyrosequencing™ technology. For HcGluClα, results from the laboratory strains show an increase in this SNP in both IVM (IVF17: 0.214) and MOX (MOF17: 0.261) selected strains relative to the unselected strain (PF17: 0.05) (**Fig. 5 p.51**). The increase was slightly higher in MOF17 compared to IVF17, however this increase was not statistically significant ($P<0.05$). The increase in frequency for the HcGluClα 169 SNP from PF17 to IVF17, and from PF17 to MOF17, although clearly noticeable, was not statistically significant ($P<0.05$). This is consistent with the previous allele frequency studies implicating allele A with resistance (Blackhall *et al.*, 1998[b]).

Results for the HG1 Lys169Arg substitution for the laboratory strains were similar to HcGluClα, but the selection appeared stronger in HG1 for the 169 substitution and statistically significant ($P>0.05$) for the MOX selected MOF17 (**Fig. 8a p.56**). The Lys169Arg substitution SNP increased in frequency in both drug-selected strains from

0.349 in the unselected PF17 strain to 0.583 in the IVF17 and 0.733 in MOF17 (**Fig. 8a p.56**). There was also a significant increase in frequency of the HG1 Lys169Arg substitution SNP between the unselected PF23 (0.221), and IVM selected IVF23 (0.550) (**Fig. 8b p.56**). Interestingly again, there is a greater response to MOX than IVM, even more so in HG1 than HcGluClα and this was statistically significant for HG1 ($P>0.05$). The Lys169Arg HG1 substitution may therefore be more important for MOX resistance. MOX is much more potent than IVM at the same concentration; therefore this could implicate the Lys169Arg substitution at higher levels of resistance, this will be discussed later.

A sheep infected with L_3 larvae from the VHR29 strain was treated with a field dose of IVM to eliminate susceptible individuals. Genotypes from the survivors of this treatment were compared to worms obtained from the water treated control sheep. No significant differences were found in the frequency of the HcGluClα Aln169Val substitution (**Fig. 6b p.52**) or the HG1 Lys169Arg substitution (**Fig. 9b p.57**). The SNPs from both genes were present in both groups at a frequency of 0.034 in the water treated VHR29xW, and 0.04 in the IVM treated VHR29xI for the HcGluClα Aln169Val substitution SNP (**Fig. 6b p.52**). For the HG1 Lys169Arg substitution SNP, the frequency was 0.037 in VHR29xW and 0.053 in VHR29xI (**Fig. 9b p.57**). The frequency of the SNPs was very low for both genes and did not increase significantly following a field dose of IVM. This indicates the SNPs at position 169 in either HcGluClα or HG1 were not important for field resistance to IVM in this strain. The presence of the SNP in the field indicates that selection for the SNP would have been possible if it were advantageous for survival. There has been no apparent selection for the 169 position

substitution in this field strain, demonstrating a difference between laboratory and field selection. The conditions under which strain IVF17 and MOF17 were maintained and selected seem to have produced a different mechanism of resistance to that which appears to occur in the VHR29 field strain. It is important to note that some of the data from the laboratory selected strains originated from low sample sizes. Therefore random mating events and host genetic factors become more significant. This could ultimately affect the genotype frequencies and the significance of the results presented here.

In two field resistant populations VHR29 and BUSTA, the HcGluClα Aln169Val substitution was not found (**Fig. 6a p.52**), however the HG1 Lys169Arg substitution was. The frequency of the HG1 E mutation was 0.026 for VHR29, and 0.152 for BUSTA (**Fig. 9a p.57**). IVM was still 54% effective against VHR29, but 0% effective against BUSTA in a pen trial by Wooster *et al.* (2001). MOX was 100% effective against VHR29, but only 67% effective against BUSTA (Love *et al.*, 2003). The survivors of a field dose of IVM in this study do not require the HcGluClα or HG1 169 substitutions. There remains the possibility that these SNPs could play a minor role in resistance.

Feng *et al.* (2002) showed the HG1 E allele selected by IVM and MOX in the laboratory under sub-therapeutic doses is functionally different than the susceptible HG1 A allele. The GABA-sensitive current is reduced in the presence of IVM providing relief from the effects of IVM/MOX under these conditions. Second, MOX is much more potent than IVM at the same concentration, and there was a significantly higher frequency of the HG1 E Lys169Arg substitution in the MOX selected MOF17 compared to the IVM selected IVF17 (**Fig. 8a, p.56**). Third, there was a higher frequency of the HG1 E Lys169Arg substitution in the BUSTA strain compared to VHR29 (**Fig.9a p.57**),

and BUSTA is partly resistant to MOX where VHR29 is not (Wooster *et al.*, 2001; Love *et al.*, 2003). Sequence alignment (**Fig. 10 p.59**) indicates the allele of the Lys169Arg mutation in BUSTA is identical to the laboratory selected HG1 E.

Taken together this suggests the HG1 E allele may become involved at higher levels of resistance, and more specifically in MOX resistance. The protection provided by the HG1 E allele could add to a yet unknown mechanism of IVM/MOX resistance in this field strain, providing additional protection against the more potent MOX. The Lys169Arg substitution could potentially be a genetic marker for MOX resistance in the field. MOX and IVM share a common mechanism of resistance, however there are obvious differences as MOX is still effective against IVM field resistant strains (Craig *et al.*, 1992; Pankavich *et al.* 1992; Kieran, 1994; Echevarria *et al.* 1997). It is reasonable to assume more than one gene will be involved in MOX resistance to provide the additional protection not present in these IVM-resistant strains susceptible to MOX. This hypothesis is preliminary since we are comparing only two different strains. The observed differences could be attributed to geographical differences and the higher frequency of the HG1 169 substitution in BUSTA may not be due to selection. The laboratory strain data does support stronger response to MOX than IVM, which is a consistent and links the HG1 Lys169Arg substitution with MOX resistance.

Selection in the laboratory strains used concentrations of drug far below the field recommended dose. The dose for 17 generations of IVF17 ranged from 0.002 – 0.015 mg/kg, and 0.010 – 0.15 mg/kg for MOF17 compared to the field dose of 0.2 mg/kg (Ranjan *et al.*, 2002). It is possible that a resistance mechanism that develops under these conditions can protect the parasite from low doses of drug, but not at the higher field

doses. Using sub-therapeutic doses of drug to select for resistance is a very sensitive way to identify resistance mechanisms. However, these mechanisms may each only have a small effect. As the drug concentration increases over several generations of selection, mechanisms having a minor protective effect can combine to produce a larger additive protective effect. This is not expected to occur when a sensitive population is exposed to a high field recommended dose of the drug, but rather is likely to select a single resistance locus of large effect. This was suggested in a mathematical model by McKenzie (1985). Some evidence in *H. contortus* suggests IVM resistance is caused by a single dominant trait (Le Jambre, 1993; Dobson *et al.*, 1996; Le Jambre *et al.*, 2000; Barnes *et al.*, 2001). This study has confirmed that SNPs in β-tubulin isotype-1, HcGluClα and HG1 linked with resistance in laboratory selected strains are not involved in a major mechanism of IVM resistance in the field, but an HG1 SNP may play a role in higher levels of resistance and MOX resistance.

A potential gene to play a major role in field resistance is the drug efflux pump P-gp, which could reduce the effective concentration at the target site. Pgp-A was one of the four genes selected in the laboratory strains (Blackhall *et al.*, 1998[a]). It was the only one of the four genes not investigated in this study, and it is still unknown if it is involved in field resistance. Although selection with sub-therapeutic doses do not reflect the actual situation in the field, as demonstrated in this study, it is reasonable to assume that a gene selected under strong selection from a field dose would also be selected from sub-therapeutic doses. P-gp is a leading candidate; however other genes not yet identified may also be involved in field resistance to IVM/MOX.

The purpose of this study was to determine if the alleles found in laboratory selected strains linked with resistance are important in field resistant strains. The original hypothesis was that the screening techniques previously used are not sufficiently sensitive, being based on linked markers. Mutations could be found on other alleles and using linked marker methods could fail to detect changes in the relevant mutations. A test based on the SNPs directly would be more appropriate. The results presented in this study do not agree with this hypothesis. A sequence alignment of laboratory and field alleles (**Fig. 10 p.59**) indicates that the HG1 169 substitution associated SNP identified in the laboratory is on the same allele in the field strains. Therefore using linked markers would be sufficient to detect the resistant individuals in the field. Previous screens that did not detect the resistant laboratory alleles: HcGluClα allele A and HG1 allele E are consistent with our findings. This increases the pool of field resistant strains screened for the HG1 E and HcGluClα A alleles to include Australia (VHR29 and BUSTA), South Africa (White River strain) and the U.S.A. (TexR strain). Although by screening for the SNPs directly, we confirmed the existence of the SNPs in field resistant strains, it was not in high frequency as it was in the laboratory resistant strains. It would seem that selection with sub-therapeutic doses has a different effect than selection from a field dose of the drug. Interestingly however, this data indicates a possible link between the HG1 Lys169Arg substitution and MOX resistance which could potentially be developed as a marker for MOX resistance. This would be important as MOX is still effective when IVM fails, and by detecting resistance early on, measures could be taken to extend the utility of this drug. Further evidence will be needed to confirm this link, however considering the impact, it is worth investigating.

This study presents further evidence that resistance produced from multiple generations of treatment with sub-therapeutic doses did not correspond to resistance that developed in the field. Although the SNPs in this study found in the laboratory selected strains are not the major SNPs involved in the field resistant strains investigated, they may still play a minor role in resistance. Evidence suggests different mechanism of resistance to MLs may operate within and between different species (Gill and Lacey 1998, Gill *et al.*, 1998). Genes identified from laboratory selected strains may still be involved in field resistance. It is reasonable to assume that a major gene responsible for field resistance could be identified from laboratory selected strains; however its importance will be complicated by the presence of other minor genes involved. At low drug concentration these minor genes have a more important impact. The main reason for using laboratory selected strains was to avoid the confounding effects of geographic differences between strains. The findings of this research indicate it would be advantageous to focus efforts primarily on field resistant strains to identify a major mechanism of IVM/MOX resistance.

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Appendix A

Individual Worm Genotypes

Legend:

cis:	PF17 from W. Blackhall, Institute of Parasitology, McGill, Canada, PhD Thesis, 1999 (App. C)
p:	PF17 obtained from Jeff Eng, Institute of Parasitology, McGill, Canada.
p23:	PF23 obtained from Jeff Eng, Institute of Parasitology, McGill, Canada.
cir:	IVF17 from W. Blackhall, Institute of Parasitology, McGill, Canada, PhD Thesis, 1999 (App. C)
I:	IVF17 obtained from Jeff Eng, Institute of Parasitology, McGill, Canada.
i23:	IVF23 obtained from Jeff Eng, Institute of Parasitology, McGill, Canada.
CMR:	MOF17 from W. Blackhall, Institute of Parasitology, McGill, Canada, PhD Thesis, 1999 (App. C)
moxi:	MOF17 obtained from Jeff Eng, Institute of Parasitology, McGill, Canada.
m:	MOF17 obtained from Jeff Eng, Institute of Parasitology, McGill, Canada.

	HG1	HcGluClα	β-tubulin
PF17			
cis1	G/A	G/G	A/T
cis5	G/A	G/G	T/T
cis7	G/A	G/G	T/T
cis9	G/A		T/T
cis12	G/A		T/T
cis14	A/A	G/G	A/A
cis21	G/G	G/G	T/T
cis23	A/A		T/T
cis27	A/A	G/G	T/T
cis29	A/A	G/G	T/T
cis30	A/A	G/G	T/T
cis33	A/A	G/G	T/T
cis34	A/A	G/A	A/A
cis43	A/A	G/G	T/T
cis44	A/A	G/G	T/T
cis45	A/A	G/G	T/T
cis46	G/A	G/G	T/T
cis47	A/A	G/G	T/T
cis48	A/A	G/G	T/T
cis49	G/A	G/G	T/T
cis50	G/A	G/G	T/T
cis51	A/A	G/G	T/T
cis52	A/A		T/T
p1	A/A		T/T
p2	G/A		T/T
p3	G/A		T/T
p4	A/A		T/T
p5	A/A		T/T

	HG1	HcGluClα	β-tubulin
p6	A/A		T/T
p7	G/A		
p8	A/A		T/T
p9	A/A		T/T
p10	A/A		T/T
p11	G/A		T/T
p12	A/A		T/T
p13	G/G		T/T
p14	A/A		A/T
p15	G/A		T/T
p16	A/A		T/T
p17	A/A		T/T
p18	A/A		T/T
p19	G/G		T/T
p20	A/A		T/T
p21	A/A		T/T
p22	A/A		T/T
p23	G/A		
p24	G/A		T/T
p25	A/A		
p26	A/A		T/T
p27	G/A		T/T
p28	A/A		T/T
p29	A/A		T/T
p30	G/A		T/T
p31	A/A		T/T
p32	A/A		T/T
p33	A/A		T/T
p34	G/A		T/T
p35	G/A		T/T
p36	A/A		T/T
p37	G/A		T/T
p38	A/A		T/T
p39	A/A		T/T
p40	A/A		T/T
p41	A/A		T/T
p42	A/A		T/T
p43	G/A		T/T
PF23			
p23-1	A/A		T/T

	HG1	HcGluClα	β-tubulin
p23-2	G/G		T/T
p23-3	A/A		T/T
p23-4	A/A		T/T
p23-5	A/A		
p23-6	A/A		
p23-7	G/A		T/T
p23-8	G/A		T/T
p23-9	A/A		T/T
p23-10	A/A		T/T
p23-11	A/A		A/T
p23-12	A/A		T/T
p23-13	G/G		T/T
p23-14	A/A		T/T
p23-15	G/A		T/T
p23-16	A/A		T/T
p23-17	A/A		
p23-18	A/A		T/T
p23-19	A/A		T/T
p23-20	A/A		T/T
p23-21	A/A		
p23-22	A/A		T/T
p23-23	G/A		T/T
p23-24	A/A		T/T
p23-25	A/A		T/T
p23-26	G/A		T/T
p23-27	A/A		T/T
p23-28	A/A		T/T
p23-29	G/A		
p23-30	A/A		
p23-31	A/A		
p23-32	A/A		
p23-33	A/A		T/T
p23-34	A/A		
p23-35	A/A		
p23-36	A/A		T/T
p23-37	A/A		T/T
p23-38	A/A		T/T
IVF17			
cir1	G/A	G/G	T/T
cir2	G/G	G/G	T/T

	HG1	HcGluClα	β-tubulin
cir3		G/G	A/T
cir6			T/T
cir9	G/A	G/G	A/A
cir10	G/A	G/G	A/T
cir12	G/A	G/G	A/T
cir14	G/A	G/G	T/T
cir15	G/A	G/G	T/T
cir17	A/A	G/G	T/T
cir18	G/A	G/G	A/T
cir25	G/A	G/G	A/T
cir26		G/A	
cir28	G/A		A/T
cir29	G/A	G/G	A/T
cir30	G/A	G/A	A/T
cir34	A/A	G/A	A/T
cir35	G/G		A/A
cir35-2	A/A	G/A	A/T
cir36	G/A	G/G	T/T
cir37	A/A	G/A	A/T
cir37-2	G/A	G/G	A/T
cir38	A/A	G/G	A/T
cir39	G/G	G/G	T/T
cir39-2	A/A	G/G	A/T
cir41	G/G	G/A	A/T
cir47	G/A	G/A	A/T
cir65	G/A	G/G	T/T
cir70	A/A	G/G	A/T
cir71	G/A	G/G	A/T
cir72	A/A	G/G	T/T
cir73	G/A	G/G	A/T
cir74	G/A	G/G	A/T
cir75	G/G	G/G	A/T
cir76	G/G	G/G	T/T
cir77	G/A	G/G	T/T
cir78	G/A		A/T
cir79	G/G	G/G	A/T
cir80	G/G	G/G	T/T
cir88	G/A	G/A	A/T
cir90	G/G	G/G	A/T
cir91	G/A	G/G	T/T
cir92	G/A	G/A	T/T

	HG1	HcGluClα	β-tubulin
cir94	G/A	G/G	A/T
cir96	G/A	G/G	A/T
cir97	A/A		A/T
cir99	G/G	G/G	A/T
cir100	G/A		T/T
cir101	G/G		T/T
cir104	G/A		A/T
cir105	A/A		A/T
cir106	G/A		A/T
cir107	G/G		T/T
I1	A/A		T/T
I2	G/A		T/T
I3	G/A		T/T
I4	G/G		
I5	A/A		T/T
I6	G/A		
I7	G/A		T/T
I8	G/A		A/T
I9	G/A		T/T
I10	A/A		T/T
I11	G/A		T/T
I12	G/A		A/T
I13	A/A		T/T
I14	G/A		T/T
I15	G/A		A/T
I16	A/A		T/T
I17	G/A		T/T
I18	A/A		T/T
I19	G/A		T/T
I20	G/A		T/T
I21	A/A		T/T
I22	A/A		T/T
I23	G/A		T/T
I24	A/A		T/T
I25	G/A		T/T
I26	G/A		T/T
I27	G/A		T/T
I28	A/A		T/T
I29			T/T
I30			
I31	G/A		T/T

	HG1	HcGluClα	β-tubulin
I32	A/A		T/T
I33	A/A		T/T
I34	A/A		T/T
I35	A/A		T/T
I36	G/G		T/T
I37	A/A		T/T
I38	G/A		T/T
I39			T/T
I40			T/T
IVF23			
i23-1	A/A		A/T
i23-2	A/A		T/T
i23-3	A/A		
i23-4	A/A		
i23-5	A/A		
i23-6	G/A		A/T
i23-7	A/A		A/T
i23-8	G/A		T/T
i23-9	G/A		T/T
i23-10	A/A		
i23-11	G/A		T/T
i23-12	A/A		A/T
i23-13	G/A		A/T
i23-14	G/G		A/T
i23-15	G/A		T/T
i23-16	G/A		A/T
i23-17	A/A		T/T
i23-18	A/A		T/T
i23-19	A/A		T/T
i23-20	G/A		T/T
i23-21	G/A		T/T
i23-22	G/A		A/T
i23-23	A/A		T/T
i23-24	G/A		T/T
i23-25	G/A		T/T
i23-26	G/A		A/T
i23-27	G/A		T/T
i23-28	A/A		T/T
i23-29	A/A		T/T
i23-30	G/A		A/T

	HG1	HcGluClα	β-tubulin
i23-31	A/A		T/T
i23-32	G/A		T/T
i23-33	A/A		T/T
i23-34	A/A		T/T
i23-35	G/A		A/T
i23-36	A/A		T/T
i23-37	G/G		T/T
i23-38	G/G		A/A
i23-39	G/A		T/T
i23-40	G/A		A/T
MOF17			
CMR2	G/A		T/T
CMR4	G/A	G/G	T/T
CMR5	A/A	G/G	A/T
CMR7	G/A	G/A	A/T
CMR8	G/G	G/G	T/T
CMR10	G/A	G/A	A/T
CMR11	G/G	G/G	A/T
CMR12	G/A	G/G	T/T
CMR16	G/A		A/A
CMR17			A/T
CMR18	G/A	G/A	A/T
CMR20	G/A	G/G	T/T
CMR22	G/A	G/G	A/A
CMR23	A/A	G/A	A/T
CMR27	G/G	G/G	T/T
CMR28	A/A	G/G	T/T
CMR34	G/A	G/G	A/T
CMR37	G/A	G/G	T/T
CMR39	G/G	G/A	A/T
CMR40	G/A		A/T
CMR42	G/A	G/G	T/T
CMR43	G/A	G/G	A/T
CMR44	G/G	G/G	T/T
CMR47	G/A	G/A	A/T
CMR50	G/A	G/G	A/T
CMR53	G/G	G/G	T/T
CMR57	G/G	G/G	A/A
moxi1	G/A		A/T
moxi2	G/G		T/T

	HG1	HcGluClα	β-tubulin
moxi3	G/A		T/T
moxi4	A/A		A/T
m1	G/A		T/T
m2	A/A		T/T
m3	G/A		A/T
m4	G/G		A/T
m5	G/A		A/T
m6	G/G		A/T
m7	A/A		A/T
m8	G/A		A/T
m9	G/A		A/T
m10	G/A		T/T
m11	A/A		T/T
m12	G/A		T/T
m13	A/A		A/T
m14			T/T
m15	G/G		A/T
m16	A/A		A/T
m17	G/A		T/T
m18	G/A		
m19	G/A		T/T
m20	G/A		T/T
m21	G/G		T/T
m22	A/A		
m23	G/A		T/T
m24	G/G		A/T
m25	G/A		T/T
m26	G/A		A/T
m27	A/A		A/T
m28	G/A		T/T
m29			
m30	A/A		
m31	G/G		
m32			
m33	G/G		
m34	G/A		
m35			
m36			
m37	A/A		
m38	A/A		
m39			

	HG1	HcGluClα	β-tubulin
m40	G/A		
m41	G/A		
m42	A/A		
m43	G/G		
m44	G/A		
m45	G/A		
m46	G/G		
m47	G/G		
VHR29			
vhr1	A/A	G/G	A/A
vhr2	A/A	G/G	A/A
vhr3	A/A	G/G	A/A
vhr4	A/A		A/A
vhr5	A/A	G/G	A/A
vhr6	A/A		A/A
vhr7	A/A	G/G	A/T
vhr8	A/A		A/A
vhr9	A/A	G/G	A/A
vhr10	A/A	G/G	A/T
vhr11	A/A	G/G	A/A
vhr12	A/A	G/G	A/A
vhr13	A/A	G/G	A/A
vhr14	A/A		A/A
vhr15	A/A		A/T
vhr16	A/A	G/G	A/A
vhr17	A/A		A/A
vhr18	A/A	G/G	A/T
vhr19	A/A		A/A
vhr20	A/A	G/G	A/A
vhr21	A/A		A/A
vhr22	A/A	G/G	A/T
vhr23	A/A	G/G	A/A
vhr24	A/A	G/G	A/A
vhr25	A/A	G/G	A/A
vhr26	A/A	G/G	A/A
vhr27	A/A	G/G	A/A
vhr28	A/A	G/G	A/A
vhr29	A/A	G/G	A/T
vhr30	G/A	G/G	A/A
vhr31	A/A		A/A

	HG1	HcGluClα	β-tubulin
vhr32	A/A	G/G	A/A
vhr33	A/A		A/A
vhr34	A/A	G/G	A/A
vhr35	A/A		A/A
vhr36	A/A	G/G	A/A
vhr37	A/A	G/G	A/A
vhr38	A/A	G/G	A/T
VHR29xW			
VHR29xW-1		G/G	A/A
VHR29xW-2	A/A	G/G	A/A
VHR29xW-3	G/A	G/G	A/A
VHR29xW-4		G/G	A/A
VHR29xW-5	A/A	G/G	A/A
VHR29xW-6	A/A	G/A	A/A
VHR29xW-7	A/A		A/A
VHR29xW-8	A/A	G/G	A/A
VHR29xW-9	A/A	G/G	A/A
VHR29xW-10		G/G	A/A
VHR29xW-11	A/A	G/G	A/A
VHR29xW-12	A/A	G/G	A/A
VHR29xW-13		G/G	A/A
VHR29xW-14	A/A		A/A
VHR29xW-15	A/A	G/G	A/A
VHR29xW-16	A/A		A/A
VHR29xW-17	A/A	G/G	A/A
VHR29xW-18	A/A	G/G	A/A
VHR29xW-19	A/A		A/A
VHR29xW-20	A/A	G/G	A/A
VHR29xW-21	A/A	G/G	A/T
VHR29xW-22	A/A		A/A
VHR29xW-23		G/G	A/A
VHR29xW-24	A/A	G/A	A/A
VHR29xW-25	A/A		A/A
VHR29xW-26	A/A	G/G	A/A
VHR29xW-27	A/A	G/G	A/A
VHR29xW-28	A/A	G/G	A/A
VHR29xW-29	A/A		A/A
VHR29xW-30		G/G	A/A
VHR29xW-31	A/A		A/A
VHR29xW-32	A/A	G/A	A/A

	HG1	HcGluClα	β-tubulin
VHR29xW-33	A/A	G/G	A/A
VHR29xW-34			A/A
VHR29xW-35	A/A	G/G	A/A
VHR29xW-36		G/G	A/A
VHR29xW-37	A/A		A/A
VHR29xW-38	A/A	G/G	A/A
VHR29xW-39	A/A	G/G	A/A
VHR29xW-40	A/A	G/G	A/A
VHR29xW-41	G/A	G/G	A/A
VHR29xW-42	A/A	G/G	A/A
VHR29xW-43	A/A	G/G	A/A
VHR29xW-44	A/A	G/G	A/A
VHR29xW-45	A/A		A/A
VHR29xW-46	G/A	G/G	A/A
VHR29xW-47		G/G	A/A
VHR29xW-48	A/A	G/G	A/A
VHR29xW-49	A/A		A/A
VHR29xW-50	A/A		A/A
VHR29xW-51	A/A	G/G	A/A
VHR29xW-52	A/A	G/G	A/A
VHR29xW-53	A/A		A/A
VHR29xW-54		G/G	A/A
VHR29xW-55	A/A	G/G	A/A
VHR29xW-56	A/A	G/G	A/A
VHR29xW-57	A/A		A/A
VHR29xW-58	A/A	G/G	A/A
VHR29xW-59	A/A		A/T
VHR29xW-60	A/A		A/A
VHR29xW-61	A/A	G/G	A/A
VHR29xW-62	G/A	G/G	A/A
VHR29xW-63	A/A		A/A
VHR29xW-64	A/A		A/A
VHR29xW-65	A/A	G/G	A/A
VHR29xW-66	A/A	G/G	A/A
VHR29xW-67	A/A		A/A
VHR29xW-68	A/A	G/G	A/A
VHR29xW-69	A/A	G/G	A/A
VHR29xW-70	A/A		A/A
VHR29xW-71	A/A	G/G	A/T
VHR29xW-72	A/A	G/G	A/A
VHR29xW-73	A/A	G/G	A/A

	HG1	HcGluClα	β-tubulin
VHR29xW-74	A/A	G/G	A/A
VHR29xW-75	A/A	G/G	A/A
VHR29xW-76	A/A		A/A
VHR29xW-77	A/A	G/G	A/A
VHR29xW-78	A/A		A/T
VHR29xW-79	A/A	G/G	A/A
VHR29xW-80	A/A	G/G	A/A
VHR29xW-81	A/A	G/G	A/A
VHR29xW-82	A/A	G/G	A/A
VHR29xW-83	A/A	G/G	A/A
VHR29xW-84	A/A	G/G	A/A
VHR29xW-85	A/A	G/G	A/A
VHR29xW-86	A/A	G/G	A/A
VHR29xW-87	A/A	G/G	A/A
VHR29xW-88	A/A	G/G	A/A
VHR29xW-89	A/A		A/A
VHR29xW-90	A/A	G/G	A/A
VHR29xW-91	A/A	G/G	A/A
VHR29xW-92	A/A		A/A
VHR29xW-93	A/A	G/G	A/A
VHR29xW-94	A/A		A/A
VHR29xW-95	A/A	G/G	A/A
VHR29xW-96	A/A	G/G	A/A
VHR29xW-97	A/A	G/G	A/A
VHR29xW-98	A/A	G/G	A/A
VHR29xW-99	A/A		A/T
VHR29xW-100	A/A	G/G	A/A
VHR29xW-101	A/A		A/A
VHR29xW-102	A/A	G/G	A/A
VHR29xW-103	A/A	G/G	A/A
VHR29xW-104	A/A	G/G	A/A
VHR29xW-105	A/A	G/G	A/A
VHR29xW-106	A/A	G/G	A/A
VHR29xW-107	A/A	G/G	A/A
VHR29xW-108	A/A	G/G	A/A
VHR29xW-109	A/A	G/G	A/A
VHR29xW-110	A/A	G/G	A/A
VHR29xW-111	A/A	G/G	A/A
VHR29xW-112	A/A	G/G	A/A
VHR29xW-113	A/A	G/G	A/A
VHR29xW-114	A/A	G/G	A/A

	HG1	HcGluClα	β-tubulin
VHR29xW-115	A/A	G/G	A/T
VHR29xW-116	A/A	G/G	A/A
VHR29xW-117	A/A	G/G	A/A
VHR29xI			
VHR29xI-1	A/A	G/G	A/A
VHR29xI-2	A/A	G/G	A/A
VHR29xI-3	A/A	G/G	A/A
VHR29xI-4	A/A	G/G	A/A
VHR29xI-5	A/A	G/G	A/A
VHR29xI-6	A/A	G/G	A/A
VHR29xI-7	A/A	G/G	A/A
VHR29xI-8	A/A	G/G	A/A
VHR29xI-9	A/A	G/G	A/A
VHR29xI-10	A/A	G/G	A/A
VHR29xI-11	A/A	G/G	A/A
VHR29xI-12	A/A	G/G	A/A
VHR29xI-13	A/A	G/G	A/A
VHR29xI-14	A/A	G/G	A/A
VHR29xI-15	A/A		A/A
VHR29xI-16	A/A	G/G	A/A
VHR29xI-17	A/A		A/A
VHR29xI-18	A/A	G/G	A/A
VHR29xI-19	A/A	G/G	A/A
VHR29xI-20	A/A	G/G	A/A
VHR29xI-21	G/A	G/G	A/A
VHR29xI-22	A/A	G/G	A/A
VHR29xI-23	A/A	G/G	A/A
VHR29xI-24	A/A	G/G	A/A
VHR29xI-25		G/G	A/A
VHR29xI-26	A/A	G/G	A/A
VHR29xI-27	A/A	G/G	A/A
VHR29xI-28	A/A	G/G	A/A
VHR29xI-29	A/A	G/G	A/A
VHR29xI-30	A/A	G/G	A/A
VHR29xI-31	A/A	G/G	A/A
VHR29xI-32	A/A	G/G	A/A
VHR29xI-33		G/G	A/A
VHR29xI-34	A/A		A/A
VHR29xI-35	A/A	G/G	A/A
VHR29xI-36	A/A	G/G	A/A

	HG1	HcGluClα	β-tubulin
VHR29xI-37	A/A	G/G	A/A
VHR29xI-38		G/G	A/A
VHR29xI-39	A/A	G/G	A/A
VHR29xI-40			A/A
VHR29xI-41		G/G	A/A
VHR29xI-42	A/A	G/G	A/A
VHR29xI-43	A/A	G/G	A/A
VHR29xI-44	A/A	G/G	A/A
VHR29xI-45	A/A	G/G	A/A
VHR29xI-46	A/A	G/G	A/A
VHR29xI-47	A/A	G/G	A/A
VHR29xI-48	A/A		A/A
VHR29xI-49	A/A	G/G	A/A
VHR29xI-50	G/A	G/G	A/A
VHR29xI-51	A/A		A/A
VHR29xI-52		G/G	A/A
VHR29xI-53	A/A	G/A	A/A
VHR29xI-54	A/A	G/G	A/A
VHR29xI-55	A/A	G/G	A/T
VHR29xI-56	A/A		A/A
VHR29xI-57	A/A	G/G	A/A
VHR29xI-58	A/A	G/G	A/A
VHR29xI-59	A/A	G/A	A/A
VHR29xI-60	A/A	G/G	A/A
VHR29xI-61	A/A	G/G	A/A
VHR29xI-62	A/A	G/G	A/A
VHR29xI-63	A/A	G/G	A/A
VHR29xI-64	A/A	G/G	A/A
VHR29xI-65	A/A	G/G	A/A
VHR29xI-66	A/A	G/G	A/A
VHR29xI-67	A/A	G/G	A/A
VHR29xI-68	A/A	G/G	A/A
VHR29xI-69	A/A	G/G	A/T
VHR29xI-70	A/A	G/G	A/A
VHR29xI-71	G/A	G/G	A/T
VHR29xI-72	A/A	G/G	A/A
VHR29xI-73	A/A	G/G	A/A
VHR29xI-74	A/A	G/G	A/A
VHR29xI-75	A/A	G/G	A/A
VHR29xI-76	A/A	G/A	A/A
VHR29xI-77	A/A	G/G	A/A

	HG1	HcGluClα	β-tubulin
VHR29xI-78	A/A	G/G	A/A
VHR29xI-79	A/A	G/G	A/A
VHR29xI-80	A/A	G/G	A/A
VHR29xI-81	G/A	G/G	A/A
VHR29xI-82	A/A	G/G	A/A

Appendix B

Environmental Safety Documentation

Animal Use Protocol: Sheep

Biohazard: *Haemonchus contortus*

Biohazard: *e-coli*