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GENETIC VARIATION OF A P-GLYCOPROTEIN GENE IN UNSELECTED AND IVERMECTIN- AND MOXIDECTIN-SELECTED STRAINS OF *HAEMONCHUS CONTORTUS*

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A thesis submitted to the Faculty of Graduate Studies and Research in partial fulfillment of the requirements for the degree of Master of Science

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SUGGESTED SHORT TITLE:

Genetic variation of a Pgp gene in *Haemonchus contortus*

Abstract

Anthelmintics, antiparasitic agents, have been developed as a main weapon to control parasitic nematodes of domestic ruminants. Unfortunately, the intensive use of anthelmintics leads to the development of drug resistance in parasite populations. Anthelmintic resistance has compromised the control of nematode parasites and has become a major problem in many countries of the world. Resistance to the newest anthelmintics such as ivermectin (IVM) and related anthelmintics in *Haemonchus contortus* in sheep has been developing rapidly in recent years. The development of drug resistance is an evolutionary process that leads to genetic changes in parasite populations in response to drug exposure. However, the mechanism of ivermectin resistance in nematode parasites is unknown. P-glycoprotein (Pgp) has been well documented in mammalian cells as a membrane transporter by actively extruding a variety of structurally and functionally unrelated hydrophobic cytotoxic drugs out of the cell. This study was to determine whether there is an association between specific alleles at the Pgp locus and IVM or moxidectin (MOX) selection in *H. contortus*, by investigating the genetic variation of the Pgp homologue in unselected and IVM- and MOX- selected strains of *H. contortus*. Three laboratory strains, derived from the same unselected parent population, IVM-selected (IVF17), MOX-selected (MOF17) (passaged for 17 generations with IVM or MOX treatment respectively) and unselected (PF17) strains (passaged for 17 generations without any drug treatment) of *H. contortus* were surveyed in this study. Forty individual adult male worms were investigated for each strain in this study. Using restriction fragment length polymorphism (RFLP) analysis, we found that Pgp is highly polymorphic in *H. contortus*. Allelic variation is extremely high at this locus among three strains. The allele frequencies at this locus in IVM- and MOX-selected strains were significantly different

from the unselected strain ($p<0.0005$), but not between both selected strains ($P>0.25$). In the two selected strains, an apparent selection for the same allele was observed. These findings suggested that Pgp may contribute to IVM and MOX resistance in *H. contortus*. The alleles which associated with IVM and MOX resistance and susceptibility, or had high frequencies in the parasite population were then cloned and sequenced. The results show that the selection of allele A may be a diagnostic marker for testing the IVM and MOX resistance at an early stage. The results of this study provide further information to better understand the molecular and genetic mechanisms of IVM and MOX resistance in *H. contortus* in animals, and have practical implications for designing more effective drug administration strategies and minimizing selection for IVM and MOX resistance and for reducing economical losses caused by nematode infections.

ABRÉGÉ

Les anthelmintiques, des agents antiparasitaires, ont été développés comme outil principal à la lutte contre les nématodes parasitaires chez les ruminants. Malheureusement, l'utilisation intensive de ces produits a engendré chez les populations de parasites le développement d'une résistance à ceux-ci. Cette résistance aux anthelmintiques compromet la lutte contre les parasites et de ce fait est devenue un problème majeur dans plusieurs pays. Chez le parasite du mouton, *Haemonchus contortus*, la résistance à de nouveaux produits tel que l'ivermectin (IVM) et autres anthelmintiques de la même famille s'est développée rapidement durant les dernières années. Le développement de la résistance est un processus évolutif des populations de parasites face à l'exposition aux médicaments se traduisant par des changements génétiques. Cependant, le mécanisme de la résistance à l'IVM chez les parasites est inconnu. La P-glycoprotéine (Pgp) est bien connue dans les cellules de mammifères. Ellle agit comme transporteur au niveau de la membrane et expulse une variété de médicaments cytotoxiques hydrophobes différents de part leur structure et leur fonction. L'objectif de cette étude est de déterminer si une association existe entre des allèles spécifiques du locus Pgp et la résistance à l'IVM et au moxidectin (MOX) chez *Haemonchus contortus*. Pour ce faire, la variation génétique de l'homologue Pgp dans les lignées sensibles et résistantes à l'IVM et au MOX d'*Haemonchus contortus* a été étudiée. Créées en laboratoire, 3 lignées dérivées de la même souche parentale sensible d'*H. contortus* ont été utilisées, soit une résistante à l'IVM (IVF17), une résistante au MOX (MOF17) (17ième génération de parasite traitée à chaque fois avec IVM et MOX), et une lignée sensible (PF17) (17ième génération n'ayant jamais été exposée aux anthelmintiques). Quarante mâles *Haemonchus* ont été étudiés pour chaque lignée. Grâce à la technique de polymorphisme de taille des fragments de restriction (PTFR), il est démontré que la Pgp d'*H. contortus* est hautement polymorphe. Une grande variation des allèles est observée à ce locus parmi les 3 lignées. La fréquence des allèles à ce locus chez les lignées résistantes à l'IVM et au MOX est statistiquement différente de la lignée

sensible ($p<0.0005$). Il n'y a aucune différence entre les 2 lignées résistantes ($p>0.25$), et l'on observe une sélection apparente pour le même allèle. Ces observations suggèrent que la Pgp contribue à la résistance aux anthelmintiques, IVM et MOX chez *H. contortus*. Par la suite, les allèles associés à la résistance et à la sensibilité ainsi que les allèles ayant une grande fréquence dans la population de parasites ont été clonés et séquencés. Les résultats démontrent que l'allèle A peut être utiliser comme marqueur de diagnostique pour tester la résistance à l'IVM et au MOX à ses premiers symptômes. Les résultats de cette étude procurent des informations permettant de mieux comprendre les mécanismes moléculaires et génétiques de la résistance à l'IVM et au MOX chez *H. contortus*. Ils permettent aussi d'élaborer de nouvelles stratégies d'administration des anthelmintiques plus efficaces et de minimiser le développement de la résistance à l'IVM et au MOX et ainsi réduire les pertes économiques causées par les infestations parasitaires.

TABLE OF CONTENTS

Suggested short title.......i
Abstract.......ii
Abrégé.......iv
Table of contents.......vi
List of figures. ix
List of tables....... xi
Thesis office statement....... xii
Acknowledgments xiv
List of abbreviations xv

CHAPTER I. INTRODUCTION....... 1

1. General introduction 1

2. Specific objectives of this study 4

CHAPTER II. LITERATURE REVIEW....... 6

I. PARASITIC NEMATODE INFECTIONS 6

1. Life cycle of *Haemonchus contortus* 7

2. Distribution and control of nematode infections 8

II. ANTHELMINTICS 9

1. Benzimidazole class 9

2. Levamisole/Morantel class 10

3. Macrocyclic lactone class.......11

A. Chemistry and production 11

B. Mode of action 12
III. ANTHELMINTIC RESISTANCE.. 14
1. Macrocyclic lactone resistance 15
2. Mechanisms of anthelmintic resistance.................................. 16
A. Benzimidazole resistance 17
B. Levamisole resistance 18
C. Macrocyclic lactone resistance 19
3. Detection of anthelmintic resistance 20
IV. GENETIC VARIATION AND THE DEVELOPMENT OF
DRUG RESISTANCE 21
V. P-GLYCOPROTEIN 23
1. Structure and function of P-glycoproteins 24
2. Correlation of P-glycoprotein with drug
resistance in parasitic diseases 27
3. Rationale of Pgp involved in macrocyclic lactone
resistance in nematodes 29

CHAPTER III. MATERIALS AND METHODOLOGY.............. 31
I. EXPERIMENTAL DESIGN 31
II. MATERIALS AND METHODS 32
1. Parasitic nematode strains 32
2. Preparation of parasites 32
3. Extraction of genomic DNA 33
4. Sequences of the primers used in PCR amplification 33
5. Polymerase Chain Reaction (PCR) amplification 34

6. Restriction enzyme digestion and identification of allele patterns 35
7. Allele frequencies and statistical analysis 35
8. Cloning of the Pgp alleles 36
A. Purification of DNA 36
B. Ligation 36
C. Transformation 36
D. Mini-Preparation 37
E. Identification of clones 38
9. Sequencing of the alleles 38

CHAPTER IV. RESULTS OF EVALUATION OF RESTRICTION FRAGMENT LENGTH POLYMORPHISM 40
1. Restriction enzyme digestion analysis 40
2. Comparison of allele frequencies between unselected and IVM- and MOX-selected strains 42
3. Hardy-Weinberg equilibrium analysis 43

CHAPTER V. RESULTS OF CLONING AND SEQUENCING 61
1. Cloning of the alleles 61
2. Sequencing of the alleles 62

CHAPTER VI. DISCUSSION 64

REFERENCES 70

LIST OF FIGURES

CHAPTER IV

Figure 1. Amplified fragment and the position of PCR primers 45

Figure 2. *Dde*I digestion patterns from individual worms of the three *H. contortus* strains: unselected strain (PF17), ivermectin selected (IVF17) and moxidectin selected (MOF17) strains 46

Figure 3. *Alu*I digestion patterns from individual worms of the three different *H. contortus* strains: PF17, IVF17 and MOF17 48

Figure 4. *Hinf*I digestion patterns from individual worms of the three *H. contortus* strains: PF17, IVF17 and MOF17........................ 50

Figure 5. *Rsa*I digestion patterns from individual worms of the three *H. contortus* strains: PF17, IVF17 and MOF17 52

Figure 6. Total *Dde*I digestion patterns from the three strains of worms 54

Figure 7. Total *Alu*I digestion patterns from the three strains of worms 55

Figure 8. Total *Hinf*I digestion patterns from the three strains of worms 56

Figure 9. Total *Rsa*I digestion patterns from the three strains of worms 57

Figure 10. Allele frequencies 58

CHAPTER V

Figure 1. Sequence comparison of alleles A, B, P and X 63

LIST OF TABLES

CHAPTER IV

Table 1. Allele classification 59

Table 2. Genotypes of different strains of worms 60

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responsibilities of all the authors of the co-authored papers. **Under no circumstances can a co-author of any component of such a thesis serve as an examiner for that thesis.**

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LIST OF ABBREVIATIONS

Adenine nucleotide Binding Cassette	ABC
Albendazole	ABZ
Benzimidazole	BZ
BZ-resistance	BZ-R
BZ-susceptibility	BZ-S
Cambendazole	CBZ
Faecal egg count reduction test	FECRT
Fenbendazole	FBZ
Gamma-aminobutyric acid	GABA
Ivermectin	IVM
Levamisole	LEV
Larval development assay	LDA
Mebendazole	MBZ
Morantel	MOR
Moxidectin	MOX
Multidrug resistance	MDR
Nicotinic acetylcholine receptor	nAChR
Oxfendazole	OFZ
P-glycoprotein	Pgp
Phosphate-buffered saline	PBS
Polymerase Chain Reaction	PCR
Pyrantel	PYR
Restriction fragment length polymorphism	RFLP
Thiabendazole	TBZ

This work is dedicated to my parents.

Chapter I

Introduction

1. General introduction

Parasitic nematodes are a major cause of morbidity and mortality in humans and also cause widespread loss of food production by infection of livestock throughout the world (Holden-Dye *et al.*, 1997). The cost of internal parasitism in the high winter rainfall areas alone has been estimated at $89 million annually in animals in Australia (MacLeod *et al.*, 1992). In order to reduce the economic loss, at least for the foreseeable future, to control and to prevent the parasitic infection, and to treat nematode infections in man and animals, will depend on the use of chemotherapeutic agents (Hennessy, 1994), such as anthelmintic drugs, which were developed as a major method for controlling the parasites of domestic ruminants. Even with the introduction of narrow or broad spectrum helminth vaccines, or the breeding of parasite-resistant hosts, tactical (and in many cases, strategic) parasite control will require chemical intervention (Hennessy, 1994).

Current control of important veterinary parasitic nematodes relies mainly on the use of three major classes of broad spectrum anthelmintics: benzimidazole class, levamisole class and the macrocyclic lactone class. Ivermectin (IVM) and moxidectin (MOX) anthelmintics, belong to the newest class - macrocyclic lactones. They are commonly used for the control of nematodes in domestic animals. Ivermectin can bind to and open the alpha subunit of a glutamate-gated chloride channel of nematode cell membrane (Cully *et al.*, 1996) resulting in

the hyperpolarization of neuromuscular cells and to eventual paralysis of the worms. Unfortunately, their frequent use has caused selection for drug resistance. The development of resistance to anthelmintics is widespread in nematode parasites of sheep, goats, horses, cattle and swine, and has become a major impediment to the treatment and control of diseases of parasitic origin in many countries of the world (Prichard, 1994). If resistance to a particular anthelmintic occurs, it is likely that another anthelmintic that has the same mode of action will also be ineffective (cross-resistance) (Martin *et al.*, 1997). So far, resistance has been reported in nematodes to all three broad spectrum anthelmintic classes (Prichard, 1990).

Resistance in sheep and goat nematodes to ivermectin is spreading rapidly in the field (Van Wyk & Malan, 1988; Craig & Miller, 1990; Le Jambre, 1993). The emergence and rapid development of IVM resistance in important nematode species now loom large as a major international threat (Waller, 1994). This is of particular concern to the sheep industry, especially in the major sheep-raising countries of the southern hemisphere. For example, in New Zealand and South Africa, anthelmintic resistance is of major concern. In South America and Africa that lack financial or manpower resources, anthelmintic resistance is almost certain to be widespread (Waller, 1994). Among the gastrointestinal nematodes, *Haemonchus contortus* develops resistance most rapidly (Echevarria & Trindade, 1989). In recent years, anthelmintic resistance has become a significant and growing problem causing loss of production in animal industries in many countries of the world (Bjφrn, 1994).

However, the mechanisms by which macrocyclic lactone resistance is induced are not yet known. The possibility that IVM resistance is due to altered binding to its receptor was investigated. Rohrer *et al* in 1994 showed that ivermectin resistance was not due to an alteration in its binding to the glutamate-gated chloride channel receptor. Currently, Blackhall *et al* (1998b) found that change in allele frequencies of the alpha subunit of a glutamate-gated chloride channel is associated with IVM and MOX resistance in *H. contortus*, but how strong

the relationship between this chloride channel and IVM resistance is still a question. The alteration of IVM effective concentration may also cause the drug resistance; transport of the drug by a mechanism similar to multidrug resistance (MDR, see below) could reduce the local drug concentration resulting in resistance. In recent years, P-glycoprotein (Pgp) has been investigated to determine whether it is involved in IVM resistance in *H. contortus* (Xu *et al.*, 1998; Blackhall *et al.*, 1998a).

P-glycoproteins (Pgps), a group of integral transmembrane proteins, were initially identified to cause multidrug resistance in mammalian tumor cells (Juliano & Ling, 1976; Gottesman & Pastan, 1993). Its overexpression in some forms of drug-resistant cancer cells confers the multidrug resistance phenotype, where additional Pgp molecules are able to pump out a range of structurally and functionally unrelated anti-cancer drugs (van der Bliek & Borst, 1989) from inside of the cells. Evidence that IVM is a good substrate for Pgp comes from the fact that disruption of the mouse *mdr1a* Pgp gene leads to a severe increase in the toxicity of IVM in mice (Schinkel *et al.*, 1994). Furthermore, Didier & Loor (1996) and Pouliot *et al* (1997) have demonstrated biochemically that IVM is an excellent substrate for Pgp in mammalian cells and that it is also a multidrug resistance reversing agent. Recently, a full length Pgp cDNA from *H. contortus* has been cloned and sequenced (Xu *et al.*, 1998). Expression of this P-glycoprotein mRNA was higher in ivermectin-selected strains compared with unselected strains of *H. contortus* (Xu *et al.*, 1998). Different restriction patterns have also been identified between resistant and susceptible strains through Southern blot analysis with this cDNA clone (Xu *et al.*, 1998). These results suggest that Pgp may bind and transport IVM from the lipid bilayer and cytoplasm to outside of the cells before IVM can get into the inside of the cells to exert its toxicity, and the alleles at this locus may respond to selection with ivermectin.

MOX, an agent belonging to the milbemycin family of macrocyclic lactone anthelmintics, has a similar macrocyclic lactone structure as IVM. Shoop *et al* (1993) have suggested that MOX and IVM may share a common mechanism of action and of resistance.

Development of anthelmintic resistance is an evolutionary process that leads to genetic changes in parasite populations in response to drug exposure. It depends on the presence of alleles which can confer resistance. Prior to the introduction of a particular drug, the relevant resistance alleles are usually present at a low frequency in the susceptible population and presumably they have no advantage to compete with the susceptible alleles. Following the introduction of a new drug, small number of worms can survive, and alleles in the small portion of population that survived can then be carried to the next generation and give more and more individuals carrying this resistance allele. Over several generations, the frequency of alleles conferring resistance increases and the resistance allele(s) will then be over represented in the offspring. Therefore, the appearance of drug resistance reflects changes in the composition of a parasite population gene pool (Beech *et al.*, 1994).

Up to now the prevalence of IVM resistance has remained low, but the number of cases has increased during recent years (Geerts, 1995). Therefore, understanding the mechanisms and dynamics of the development of resistance, at both molecular and population genetic levels, is needed in order to detect the development of resistance in *H. contortus* populations at an early stage and to develop adequate control programs.

2. Specific objectives of this study

1). To investigate the genetic variation of a Pgp gene in unselected and IVM- and MOX-selected *H. contortus* populations.

2). To determine the alleles of the Pgp gene in *H. contortus* and to test for a linkage between Pgp and IVM and MOX resistance in *H. contortus.*

3). To identify alleles which are associated with IVM or MOX resistance and susceptibility in *H. contortus.*

4). To develop a marker for detection of early stage IVM and MOX resistance in *H. contortus* populations.

This genetic study allows an understanding of the evolutionary response of the parasite to drug treatment and, therefore to better use anthelmintics to control parasites. This will have important practical implications in preventing the spread of drug resistance and in the control of parasitic infections and in assisting with the design of more effective drug administration strategies for farmers that minimize selection for IVM and MOX resistance.

Chapter II

Literature review

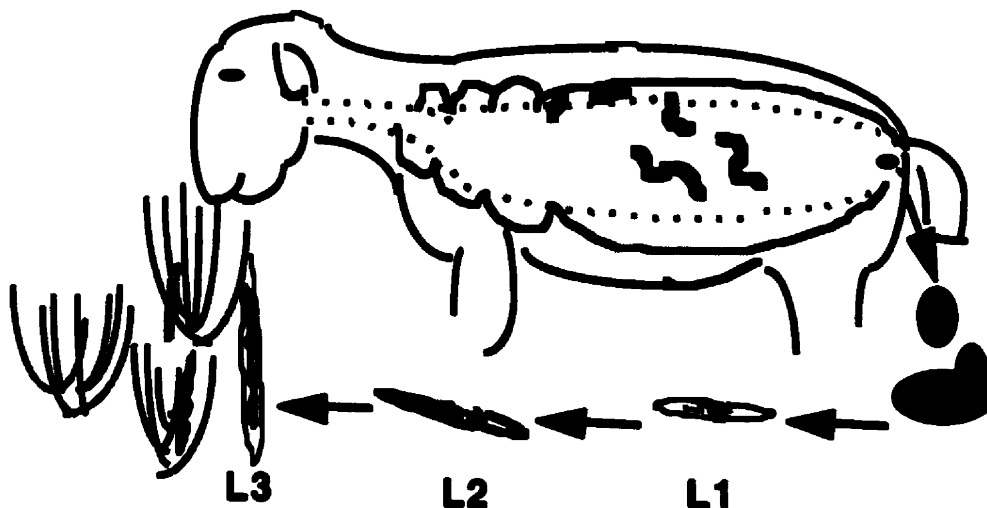
I. Parasitic nematode infections

Diseases caused by parasitic nematodes pose a considerable medical and veterinary health problem. Internal parasites or worms cause economic and production losses to animal producers in many countries of the world. In western countries problems consist mainly of wide spread infections in domestic livestock. Nematodes that feed on blood cause some of the most important parasitic diseases of humans and domestic animals (Aberejola *et al.*, 1979; Georgi, 1980; Preston & Allonby, 1979; Warren, 1988). Anemia is a major problem which can lead to acute disease and death or chronic disease in the parasitized host. Costs of treatment and loss of production in sheep and cattle in Australia are estimated to range between Aus$200 to Aus$400 million annually. Many of the nematode parasites that derive nutrition from host blood are gastrointestinal nematodes, which belong to the order *Strongylida*. One of the most important parasites is the stomach worm of ruminants such as *Haemonchus*. *Haemonchus contortus*, one of the most pathogenic gastrointestinal parasitic nematodes of sheep and goats, is widespread throughout the world. It results in 30-40% mortality in lambs not treated with anthelmintics. Although the disease may occur in all age groups, animals 2-24 months old are more commonly affected. Older sheep may have an acquired resistance to the parasite because of earlier exposure to them.

1. Life cycle of *Haemonchus contortus*

The life cycle of *H. contortus* consists of several stages as is shown in figure 1. *Haemonchus contortus* does not require an intermediate host. Adult male and female worms live in the abomasum (or true stomach) of ruminant animals. The female can lay 5,000 to 10,000 eggs per day which pass out of the host with the feces. The eggs in the feces can develop into first, second and third stage larvae. Eggs and newly hatched larvae (L1 and L2) remain in the feces where they feed on bacteria. The larvae in these stages are not resistant to cold and desiccation. Third stage larvae do not feed and are highly resistant to drying and cold temperatures. They climb to the tips of blades of grass in moist conditions and the definitive host becomes infected by ingesting third stage larvae on herbage. After infection, the young worms pass into the abomasum where they burrow into the mucosa and develop into forth stage larvae. It takes about 48 hours. They then begin to feed on blood of the infected host and develop to adulthood. Mating of adults occurs and egg production commences 15 days later. Enormous numbers of larvae may accumulate on heavily grazed pastures.

Figure 1. Life Cycle of *Haemonchus contortus*



The lambs are most severely affected by the parasite. Even relatively low infection levels already affect growth rate, weight gain and wool production. When large numbers of larvae infect sheep, death can occur suddenly, while the sheep still appear to be in good health, and cause significant losses in production (Gibbs, 1985; Janssens *et al.*, 1989). Therefore, *H. contortus* is one of an economically important group of gastrointestinal nematodes of sheep which are closely related taxonomically, epidemiologically and pathologically (Soulsby, 1982). It is regarded as one of the most pathogenic helminths in domesticated animals (Soulsby, 1982).

2. Distribution and control of nematodes infections

H. contortus is found worldwide. Larval development of *H. contortus* occurs optimally at relatively high temperatures. *Haemonchosis* is primarily a disease in warm, humid climates wherever sheep are raised for food or wool (Soulsby, 1982). This is the most important worm infection in the areas of the tropics and subtropics such as Australia, Brazil, the Middle East and Nigeria.

Different strategies have been developed to control these parasitic nematode infections. Anthelmintics, the antiparasitic agents, have been discovered as a major method to control nematodes of domestic animals, but the emergence of drug resistance is becoming a serious problem. Progress also continues to be made with non-chemotherapeutic approaches such as vaccination (Emery, 1996), breeding for hosts that display increased tolerance or resistance to the parasite (reviewed by Gray & Gill, 1993; Gill *et al.*, 1994) and biological control (Grønvold *et al.*, 1996), but practical application has been slow to develop.

II. Anthelmintics

Anthelmintics prevent, control and treat many types of parasitic nematodes in both humans and domestic animals. Factors that favored the use of these anthelmintic drugs include the fact that these drugs usually act against a broad spectrum of different nematode species, are readily available and are relatively cheap (Bird, 1991). Anthelmintics currently on the market can be grouped into three distinct modern broad-spectrum classes: the macrocyclic lactone class; benzimidazole class and levamisole/morantel class. They will continue to be used until effective vaccines are produced and/or hygiene standards have been improved (Martin *et al.*, 1997).

1. Benzimidazole class

Benzimidazole (BZ) anthelmintics, introduced in the 1960s, are the most common chemotherapeutic agents used to remove intestinal helminths (nematodes, cestodes and trematodes) from animals. Various analogs also display distinct antifungal activity. The members of the class include thiabendazole (TBZ), albendazole (ABZ), fenbendazole (FBZ), mebendazole (MBZ), cambendazole (CBZ), and oxfendazole (OFZ). TBZ was the first broad spectrum anthelmintic which is highly effective against a wide range of parasite species (Brown *et al.*, 1961; McKellar & Scott, 1990). Since the introduction of the first BZ, new derivatives have appeared regularly. Investigations of the mode of action of BZ drugs in parasitic nematodes have indicated that BZs induce disintegration of the microtubular framework in nematode cells (Borgers & De Nollin, 1975; Bughio *et al.*, 1994). They are believed to interact with the cytoskeletal protein tubulin and act by specifically binding with high affinity to nematode β-tubulin which alters the tubulin-microtubule equilibrium that

prevents polymerization during microtubule assembly and causes depolymerization of microtubules in intestinal cells of nematodes (Sangster *et al.*, 1985; Lacey, 1988). Tubulin is a soluble protein found in most eukaryotic cells that binds to the drug colchicine. α- and β-tubulins are tightly associated forming a heterodimer, and are the main components of microtubules. Microtubules, built of tubulin, are critical to chromosomal movement during cell division. Usually microtubules depolymerize into stable αβ dimers (tubulin). Tubulin does not dissociate into α and β monomers unless denaturing agents are added (Darnell *et al.*, 1990).

2. Levamisole/Morantel class

This group of anthelmintics have been in use since the mid 1960s. In addition to levamisole (LEV) and morantel (MOR), pyrantel (PYR) also belongs to this class. They are cholinergic agonists with a selective pharmacology for nematode receptors. These chemicals are structurally related and act on the same site on nematode muscle (Coles *et al.*, 1975; Harrow & Gration, 1985). LEV is a potent anthelmintic which acts on nicotinic acetylcholine receptors (nAChR) of nematodes, thereby disrupting neuromuscular transmission resulting in paralysis of worms. Paralysis is due to sustained muscle contraction (Coles *et al.*, 1975; Harrow & Gration, 1985). nAChRs are well characterized multi-subunit cation channels activated by binding of the neurotransmitter acetylcholine (Hoekstra *et al.*, 1997). In vertebrates, the muscle nAChRs are composed of four different types of subunits forming a pentameric structure, whereas the neuronal nAChRs consist of only two types of subunits: α- and non-α-subunits. The α-subunits contribute the major part of a binding site for acetylcholine (Conti-Tronconi *et al.*, 1994). The nAChR subunits are encoded by multigene families with a homology between subunits and across species leading to a

functional diversity of nAChRs (Le Novere & Changeux, 1995; Hoekstra *et al.*, 1997). LEV also inhibits the migration of third stage larvae of susceptible and resistant isolates of *H. contortus* (Sangster *et al.*, 1988).

3. Macrocyclic lactone class

Macrocyclic lactones, a group of structurally related anthelmintic compound, include the avermectins such as ivermectin (IVM), abamectin, doramectin and the milbemycins such as moxidectin (MOX). They are widely used for the treatment of both internal and external parasites, specifically nematodes and arthropods in humans, domestic animals and plants. They are highly effective against different stages and species of susceptible strains of most gastrointestinal nematodes of sheep (Benz *et al.*, 1989; Bisset *et al.*, 1991).

A. Chemistry and production

Macrocyclic lactones are the most widely used compounds and derived from fermentation products of soil-dwelling bacteria of the genus *Streptomyces*. They are a group of chemically related 16-membered macrocyclic lactones with a similar ring system and are acaricidal, insecticidal, and nematicidal (Steel, 1993; Clark *et al.*, 1994). The avermectins were first isolated from a soil actinomycete microorganism, *Streptomyces avermitilis* (Burg *et al.*, 1979), and were discovered in the mid 1970s because of their potent anthelmintic activity (Egerton *et al.*, 1979; Chabala *et al.*, 1980) and marketed for veterinary use in 1981. Avermectins have been tremendously successful anthelmintic agents because of their ability to kill parasites without affecting the host organism (Ostlind *et al*, 1979; Putter *et al.*, 1981). IVM is the mostly used drug in the avermectin group.

IVM, a semi-synthetic member of the avermectin group of drugs, is the 22, 23-dihydro derivative of avermectin B1. It was the first broad spectrum endectocide with a wide margin of safety against a wide range of parasites (Campbell *et al.*, 1983; Egerton *et al.*, 1980). It was released for registration in 1981, and has been approved for use in over 60 countries for control of parasites in humans, cattle, sheep, horses, goats, swine, dogs, and other mammals (Di Netta, 1989; Campbell *et al.*, 1983). It is particularly effective against arrested and fourth larval stages of economically important gastrointestinal nematodes in sheep (McKeller & Marriner, 1987; Echevarria *et al.*, 1992) and cattle (Barth & Preston, 1987). It has also been shown to be effective against both benzimidazole and levamisole/morantel resistant nematodes in sheep (Waller & Donald, 1983). In general, IVM administered at a dose of 0.2 mg/kg orally to sheep results in efficacies of 97-100% against adult and fourth stage larval forms of *H. contortus* (Campbell & Benz, 1984).

MOX is structurally related to ivermectin. It is a chemically modified derivative of a macrocyclic lactone and is isolated from *Streptomyces cyaneogreseus noncyanogenus*. It has been shown to have a prolonged period of activity in sheep (Rendell & Callinan, 1996). At 0.2 mg/kg, nematocidal concentrations of IVM were maintained for 28 days and of MOX for 42 days in sheep (Taylor *et al.*, 1997).

B. Mode of action

The mechanism of action of ivermectin and related drugs on nematodes is not entirely elucidated. It is thought to specifically bind to and open glutamate-gated chloride channels located on muscles of the pharynx and, possibly, the somatic musculature (Cully *et al.*, 1996; Sangster, 1996) in the nematode and arthropods cell membrane, increasing the Cl^- permeability and leading to the hyperpolarization of the target neuromuscular cell (Cully *et al.*,

1994; Arena *et al.*, 1995); eventually paralyzing or starving the nematodes (Sangster, 1996). However, the identity of the target ion-channel has been controversial.

Initial reports suggested that ivermectin acts by opening Cl^- channels associated with gamma-aminobutyric acid (GABA) receptors on the somatic musculature of parasitic nematodes, resulting in a flaccid paralysis (Kass *et al.*, 1980; 1984; Turner & Schaeffer, 1989). Later, Arena *et al* in 1991 showed that ivermectin activates a Cl^- channel in *Xenopus laevis oocytes* injected with mRNA obtained from the free-living nematode *Caenorhabditis elegans*, this channel was not sensitive to GABA. It acts by opening a glutamate-gated chloride channel of neuromuscular membranes of both nematodes and arthropods (Rohrer *et al.*, 1992; Cully *et al.*, 1996) and results in the paralysis of worms. Studies on *C. elegans* have shown that IVM inhibits pharyngeal pumping with a half-maximal effect at approximately 5 nM (Avery & Horvitz, 1990). Geary *et al* in 1993 reported that ivermectin concentrations between 10^{-10} - 10^{-8} M paralyzed the pharynx of adult *H. contortus.*

Ligand-gated ion channels are multimeric membrane-spanning proteins which play a critical role in the function of neuronal and muscular systems. Binding of agonists results in rapid opening of an ion channel pore that is selective for either anions or cations. The glutamate-gated chloride channels are found in nematodes and insects (Cully *et al.*, 1996). It was first reported from the locust (*Schistocerca gregaria*) leg muscle preparations (Cull-Candy & Usherwood, 1973; Lea & Usherwood, 1973a, b). The hyperpolarizing response is mediated via an increase in chloride conductance through activation of receptors. In nematodes the synapse between interneurons and excitatory motor neurons is the primary site of action. The chloride ion influx lowers cell membrane resistance and causes hyperpolarization of the resting potential of postsynaptic cells. This makes neurotransmission more difficult so that transmission of stimuli to muscles is prevented. Binding to the

glutamate-gated chloride channel results in a flaccid paralysis of affected parasites followed by their death or expulsion (Courtney & Roberson, 1995).

Studies on the mechanism of action of moxidectin were conducted by measuring the changes in membrane conductance in leg muscle fibrae of the common shore crab using standard micro-electrode techniques. It was concluded from these experiments that moxidectin and ivermectin share a common mechanism of action (Shoop, 1993).

III. Anthelmintic resistance

The extensive use of these anthelmintic drugs has led to wide spread drug resistance causing a serious threat to the effective control of nematode infections throughout the world (Coles *et al.*, 1994). Resistance to all of the broad spectrum macrocyclic lactone compounds has been encountered in most regions of the world where the compounds are used routinely in animal production. It is common for parasitic nematodes to develop resistance to anthelmintics, especially when they are used in continuous, intensive treatment programs (Pankavich *et al.*, 1992). It is now over 30 years since resistance to thiabendazole, the first true broad spectrum anthelmintic, was reported by Drudge *et al* (1964) in *H. contortus* infections of sheep in the USA. Anthelmintic resistance in parasitic nematodes of sheep and goats is becoming an increasingly important subject in veterinary, parasitological and farming circles in many countries. There is no question of the seriousness of the problem in Australia, South Africa and the humid semi-tropical regions of South America where 300 million sheep are raised, and the scientific literature is well served with detailed reviews of the prevalence, the rate of spread and the increase in magnitude of the resistance (Martin, 1986; Waller, 1986, 1987; Prichard, 1990; Boray *et al.*, 1990). It is a continuing problem for control of nematodes in sheep (Christine *et al.*, 1992). Anthelmintic resistance has compromised the

control of parasitic nematodes in several animal based industries. In the United Kingdom, Australia, and New Zealand field surveys have documented development of drug-resistant strains of *H. contortus* and *Ostertagia circumcinta* (Christine *et al.*, 1992). There are also extensive reports on anthelmintic resistance in the USA, Europe, South Africa, Kenya, Indian, Malaysia, Latin American. In fact, virtually wherever it has been investigated anthelmintic resistance has been found.

In addition to resistance to thiabendazole reported in the early 1960s (Drudge *et al.*, 1964; Conway, 1964; Shelton, 1968), side resistance to other benzimidazoles rapidly occurred (Hambry *et al.*, 1986; Miller & Craig, 1988; Craig & Miller, 1990). Benzimidazole resistance in *H. contortus* has been extensively reported in Australia (Smeal *et al.*, 1968; Webb & McCully, 1979; Riffkin *et al.*, 1984; Edwards *et al.*, 1986), New Zealand (Vlassoff & Kettle, 1980; Kettle *et al.*, 1981) and the USA (Craig & Miller, 1990). Resistance to the anthelmintic levamisole has also been reported (Santiago & Costa, 1979; Green *et al.*, 1981; Kettle *et al.*, 1981; Edwards *et al.*, 1986). Resistance to the benzimidazole, levamisole/morantel group, ivermectin, closantel and multiple resistance have been found in gastrointestinal nematodes of sheep, goats, cattle and horses in many areas of the world where repeated treatment with anthelmintics is common.

1. Macrocyclic lactone resistance

Resistance to IVM arose very rapidly. *H. contortus* is the parasite most commonly identified as resistant although other isolates of parasitic resistance to IVM are emerging too (Sangster, 1996). Clinical resistance remains rare in most countries but has been reported in South Africa and Latin America. Drug resistance to IVM, has become a widespread problem, particularly in nematodes of sheep, goats and cattle. In some parts of the world, the survival

of commercial animal production is threatened by the development of anthelmintic resistance.

The first indication of IVM resistance by *H. contortus* in sheep under field conditions occurred in 1986 in South Africa (Van Wyk & Malan, 1988), 33 months after the drug was introduced there (Carmichael *et al.*, 1987). In 1988, four additional isolates of *H. contortus* from sheep in South Africa were reported to be resistant to ivermectin (Van Ayk & Malan, 1988). By 1989, a further five were reported (Van Wyk *et al.*, 1989). IVM resistance has also been reported from other regions of the world. An ivermectin-resistant strain from sheep raised on a pastoral experimental station was isolated in Southern Brazil (Echevarria & Trindade, 1989; Vieira *et al.*, 1992), and Craig and Miller (1990) isolated an ivermectin-resistant strain from an angora goat flock in Southern Texas. IVM resistance was also been reported in New Zealand (Badger & McKenna, 1990) and Scotland (Jackson *et al.*, 1992). Under laboratory conditions, nematodes that are resistance to ivermectin may also show reduced sensitivities to moxidectin (Conder *et al.*, 1993).

2. Mechanisms of anthelmintic resistance

To interfere with parasite reproduction, a drug must find its target within the parasite. After the drug associates with the target, the parasite must be sufficiently incapacitated to be killed by the host defense or to die spontaneously. Each of these steps provides the parasite with opportunities to interfere with drug action, resulting in drug resistance.

Historically, IVM-resistant worms, which have survived IVM treatment, remain susceptible to MOX. Against nematodes MOX is a more potent anthelmintic(Sangster, 1995). Pankavich *et al* (1992) and Craig *et al* (1992) showed that moxidectin was effective against

ivermectin-resistant strains of *H. contortus*. The mode of action of MOX is thought to be similar to that of IVM. It acts against nematodes at a relatively low dose-0.2 mg/kg or less. It is likely that milbemycins and avermectins act on helminths in the same way although they do differ in potency and spectrum (Sangster, 1996).

A. Benzimidazole resistance

Molecular studies at both the protein (Lubega & Prichard, 1991b) and the DNA level (Roos, 1990; Roos *et al.*, 1990; Beech *et al.*, 1994) of the mechanism of BZ resistance in nematodes provide evidence that BZ resistance is associated with an alteration in β-tubulin genes which reduces or abolishes the high affinity binding of BZs for tubulin in the organisms, and not in α-tubulin. This decreases the β-tubulin-BZ interaction. In this way, the resistant parasite is able to tolerate higher drug concentration. So far, two distinct β-tubulin isotype classes have been reported in *H. contortus* (Roos *et al.*, 1990; Geary *et al.*, 1992). They can be discriminated by their amino acid sequences at the carboxy-terminus and also by their DNA sequence, because probes constructed from these genes do not cross-react in a Southern blot due to a difference in codon usage (Geary *et al.*, 1992). Using (fragments of) these genes as probes to screen *H. contortus* populations by restriction fragment length polymorphism (RFLP), a clear difference in RFLP pattern between susceptible and resistant populations was obtained (Roos *et al.*, 1990; Lubega & Prichard, 1990; 1991a). It was suggested that β-tubulin isotype 1 locus has multiple alleles (Roos *et al.*, 1990; Roos, 1990) and this was proven by Beech *et al* (1994). Isotype 2 β-tubulin genes are likely coded by a different locus from isotype 1. These studies on the sheep parasite *H. contortus* have shown that resistance to benzimidazole drugs is correlated with selection for individuals in the population possessing a specific β-tubulin isotype 1 gene (Lacey, 1988; Roos, 1990; Roos *et*

al., 1990; Kwa *et al.*, 1993a; Kwa *et al.*, 1993b). Sequence analysis of this β-tubulin locus in both BZ-susceptible (BZ-S) and BZ-resistant (BZ-R) *H. contortus* populations showed three amino acid differences at positions 76, 200 and 368 (Geary *et al.*, 1992; Kwa *et al.*, 1993b). The Phe 200 (BZ-S) to Tyr 200 (BZ-R) change is of importance, since it is commonly conserved between different BZ-S and BZ-R *H. contortus* populations and functional analysis of β-tubulin genes from *H. contortus* showed that the single Phe to Tyr mutation at position 200 in β-tubulin isotype 1 coupled with either on alteration in isotype 2 β–tubulin or the deletion of the isotype 2 β–tubulin gene may confer BZ-resistance in *H. contortus* (Kwa *et al.*, 1994; Lubega & Prichard, 1991a; Beech *et al.*, 1994) .

B. Levamisole resistance

Levamisole is a cholinergic agonist. The pharmacological evidence showed that an alteration in acetylcholine receptor is associated with LEV resistance in *H. contortus*. LEV resistance is thought to be associated with reduction in the number of nicotinic acetylcholine receptors or a change in the binding affinity of these receptors (Sangster *et al.*, 1988; Hoekstra *et al.*, 1997). The resistant nematodes either have fewer acetylcholine binding sites or their affinity for LEV is reduced (Sangster, 1990). Although several genes encoding nAChR subunits have already been isolated from the *C. elegans*, it remains to be elucidated which genes are associated with levamisole resistance in parasitic nematodes in the field (Hoekstra *et al.*, 1997).

C. Macrocyclic lactone resistance

IVM appears to be a glutamate agonist. So far, the mechanism of macrocyclic lactone resistance in *H. contortus* is not clear. There are several possibilities: (1) alteration of the macrocyclic lactone binding site (Coles, 1989) such as the glutamate-gated chloride channel receptor could cause conformation changes which prevents binding or decreases affinity of ivermectin to the receptor. *In vitro* studies of IVM resistant nematodes suggest that IVM receptors are located on pharyngeal and somatic muscle; (2) the alteration of ivermectin effective concentration also can cause the resistance, transport of the drug by a mechanism similar to MDR could reduce the local drug concentration resulting in resistance; (3) Coles (1989) also hypothesized that hydrolysis of the lactone ring could probably cause avermectin resistance. For the reason outlined below the second of these alternatives appears to have occurred in ivermectin resistant *H. contortus.*

Some studies showed that MOX is effective against IVM-resistant *H. contortus* (Craig *et al.*, 1992; Pankavich *et al.*, 1992). They suggested that this is due to either different modes of action of these two drugs or differences in response to a resistance mechanism. Some studies showed that IVM and MOX share co-resistance and concluded that apparent efficacy of MOX against IVM-resistant parasites may be dose-dependent (Conder *et al.*, 1993; Shoop *et al.*, 1993). Nevertheless, structural similarity, the same target site and a concentration-dependent cross-resistance suggest that ivermectin and moxidectin may share common mechanisms of resistance in nematodes. However, factors which modulate drug concentration at the site of action may determine relative expression of resistance between ivermectin and moxidectin.

3. Detection of anthelmintic resistance

In general, the reduction in anthelmintic potency is used to detect resistance. There are no quick effective methods to test ivermectin and moxidectin resistance in an *H. contortus* population. The *in vivo* fecal egg count reduction test (FECRT) is widely used and considered the test of choice for sheep parasites in the field. It is a simple and relatively noninvasive method. In this case, resistance is defined as the failure of a commercially recommended dose of anthelmintic to reduce egg counts by >95%; but there were false positive LEV-resistance and false negative IVM-resistance diagnostic problems, because IVM can suppress egg laying so that worms resistant to this class of drugs appear to be susceptible in egg count reduction tests (Sangster, 1996). The eggs of the different species involved in resistance are morphologically similar, so tests based on egg counts lack species specificity (Sangster, 1996). This test has poor sensitivity and may fail to detect resistance even when it affects 25% of the worm population (Martin *et al.*, 1989). *In vitro* tests are preferred and ideally performed on eggs or larvae and produce results quickly. The larval development assay (LDA), probably the best *in vitro* test currently available, works well with BZ, LEV and MOR, but some of the assays have problems with IVM (Bjφrn, 1994). Eggs and larvae can be readily recovered or grown from experimentally-infected animals or from those in the field and incubated in the presence of a range of concentrations of solubilized drug. The effects on development or motility are then measured (Sangster, 1996). *In vivo* tests such as worm count reduction tests are available. Infected hosts are treated with drug and worm numbers compared with untreated controls, but it is an expensive approach and several animals have to be slaughtered for each assay; alternatives are more common (Sangster, 1996). These methods are insufficiently sensitive to monitor the development of resistance before it is overt. If a way could be found to detect resistance when gene frequencies are low, then alternative

measures (such as the use of a narrow-spectrum drug) could be used to kill the specific resistance population and extend the life of the broad-spectrum anthelmintic classes.

IV. Genetic variation and the development of drug resistance

Genetics is the study of how physical and physiological features of an organisms are passed down to their offspring. Genetic variation is expressed as forms of a gene with slightly different sequences, which are known as alleles. Individuals with two different alleles at a particular locus are said to be heterozygous, and with two identical alleles are homozygous. Although any one individual can have a maximum of two different alleles at a locus, the number of different alleles in a population can be much greater than two. Drug resistance has been defined as 'a change in the allele frequency of a population that is produced by drug selection whereby more drug is required to exact some effect than was required prior to selection' (Shoop, 1993). Selective removal of susceptible members in a population by drug toxicity leaves resistant members to contribute the greater share of genes to the next generation. Apparently, resistance has arisen as a result of selection of parasites during therapeutic treatment (Martin, 1990; Jackson, 1993). Survivors of a treatment, being the most resistant component of the population, carry resistance genes which they pass on to their offspring (Sangster, 1996).

During the selection process resulting in resistance, there must be some genetic variation in the population gene pool. An absolute pre-requisite for a genetic response to a drug selective pressure is genetic variation within the population under selection. Before a new drug is first used, the allele(s) which are associated with resistance are usually present at a very low frequency in the susceptible population, and presumably they have no advantage compare with the susceptible alleles and allele variation should be higher in a susceptible

population compare to drug resistant strains (Roos *et al.*, 1990; Beech *et al.*, 1994). The evolution of resistance will proceed rapidly when the initial population is very variable. When the alleles which are present in the resistant strain also exist in the susceptible strain, the resistance may arise rapidly. However, the speed with which drug resistance develops depends on the change in frequency of worms carrying resistance alleles. The rate at which resistance develops are dependent on the amount of genetic variation in the population and the intensity of selection (May, 1993). It is impossible for any drug to be 100% efficacious, against 100% of parasite species, 100% of the time. Therefore, on each occasion when an anthelmintic is used, the small number of surviving worms, which are the resistance portion of the population, have an increased opportunity to contribute to the next generation. Thus, one of the existing alleles may give a few individuals carrying this allele a survival advantage. Over several generations, alleles conferring resistance accumulate, so that more worms in the population survive treatment (Craig, 1993). This allele will be over represented in the offspring. Selection for resistance removes the susceptible alleles from the population and eventually only resistant alleles remain. This is consistent with the model which proposes that a susceptible population is genetically and phenotypically heterogeneous (McKenzie, 1985) and, importantly, does not require that resistance arises as a result of a new mutational event (Beech *et al.*, 1994). The evolution of resistance will proceed rapidly when the initial population is very variable.

When drug pressure is applied repeatedly and intensively, successive populations move directionally towards less and less susceptibility, because the frequency of the resistance allele increases through drug selection and the frequency of other alleles decreases. Overall, development of resistance is an evolutionary response.

For an anthelmintic to initially be highly efficacious, the frequency of resistance alleles must be low in the population. Resistance alleles will be initially low in a population because

those alleles are less advantageous, or at least confer no selective advantage, in the absence of anthelmintic pressure. Under anthelmintic pressure, they do confer a selective advantage and will increase in frequency. If the anthelmintic pressure is withdrawn for a long period, one could expect that the frequency of the resistance alleles would decrease if they were less advantageous than susceptibility alleles in the absence of anthelmintic pressure. Therefore, if a population of worms is no longer exposed to that anthelmintic, one would expect a reversion to susceptibility. However, the rate of reversion will be fast or slow in relation to the selective disadvantage of the resistance alleles in the absence of the anthelmintic pressure.

V. P-glycoprotein

P-glycoprotein was initially identified 20 years ago through its ability to confer multidrug resistance in mammalian tumor cells by Juliano and Ling (1976). It is a high molecular weight (approximately 140-170kDa) polytopic membrane glycoprotein which is located in the plasma membrane at the cell surface consistent with its function as a multidrug transport protein (Willingham *et al.*, 1987). It is recognized that the Pgp-encoding genes belong to a superfamily of genes that encode ATP-binding membrane transport proteins [Adenine Nucleotide Binding Cassette (ABC)] in diverse species (Childs & Ling, 1994). The importance of Pgps for drug resistance in organisms ranging from bacteria to human cells has also been found in many other organisms, such as the invertebrates *Drosophila melanogaster* and *C. elegans* (Wu *et al.*, 1991; Lincke *et al.*, 1992; Gerrard *et al.*, 1993; Broeks *et al.*, 1995) and parasitic protozoa *Leishmania tarentolae*, *Plasmodium falciparum* (Foote *et al.*, 1989; Wilson *et al.*, 1989; Ouellette *et al.*, 1990; Samuelson *et al.*, 1990; Borst & Ouellette, 1995), *Entamoeba* (Descoteaux *et al.*, 1992).

The cloning of Pgp or MDR cDNA has revealed that Pgps are encoded by small gene families: there are two members in human-MDR1 and MDR3 (also known as MDR2), three members in rodents-mouse *mdr1a* (*mdr3*), *mdr1b* (*mdr1*) and *mdr2*, rat *pgp1*, *pgp2* (*mdr1b*) and *pgp3* and hamster *pgp1*, *pgp2* and *pgp3*. Transfection experiments have shown that these genes are functionally different (Gros *et al.*, 1986a, Ueda *et al.*, 1987; Schinkel & Borst, 1991). The human MDR1, mouse *mdr1a* and *mdr1b*, hamster *pgp1* and *pgp2*, rat *pgp1* and *pgp2* can cause multidrug resistance (Chen *et al.*, 1986; Gros *et al.*, 1986b; Devault & Gros, 1990) while human MDR3, mouse *mdr2*, hamster *pgp3* and rat *pgp2* and *pgp3* cannot (Schinkel & Borst, 1991; Gros *et al.*, 1988), but the latter Pgps have an essential role in the secretion of phosphatidylcholine into bile and it has been hypothesized that they may be phospholipid transport proteins or phospholipid flippases (Smith *et al.*, 1993). Amplification or overexpression of the Pgp gene(s) has been described in some clinically resistant isolates, and transfection of some parasite Pgp genes conferred resistance in wild-type parasites (Ouellette *et al.*, 1994).

The multidrug resistance phenotype mediated by Pgp can be reversed by a large member of compounds (Georges *et al.*, 1990) called chemosensitizers (or reversing agents). They are noncytotoxic agents. These compounds can bind to Pgp by competed with drugs, thus they block the binding of cytotoxic drugs to Pgp and enhance the cytotoxicity of drugs in multidrug resistant cells. The binding of these agents will result in reversal of multidrug resistance (Pearce *et al.*, 1989).

1. Structure and function of P-glycoproteins

Pgp has been found to be causative of the MDR phenotype in many human cancers and parasitic diseases by actively extruding out of cells a variety of structurally and

functionally unrelated hydrophobic amphipathic molecules which are not negatively charged (Ambudkar *et al.*, 1995). Pgp contains more than 1200 amino acids (Chen *et al.*, 1986). Based on a computer-assisted amino acid sequence comparison with prokaryotic transport proteins, a structural model was predicted. No X-ray crystal structure is available at present. It was known that Pgp consists of two tandem duplicated homologous halves, each half contains six predicted hydrophobic transmembrane domains and one intracellular hydrophilic ATP binding site (Chen *et al.*, 1986; Gros *et al.*, 1986b). The ATP binding domains reside in the cytoplasm and provide the energy by hydrolysis for Pgp to remove drugs. It is believed to belong to the ATP-binding cassette superfamily. Therefore, these ATP-binding sites are very important in extruding drugs from cells, because binding and hydrolysis are essential for substrate transport.

P-glycoprotein has been shown to actively transport a wide variety of hydrophobic agents, including vinca alkaloids, colchicine, antibiotics and anthracyclines, out of cells by an ATP-dependent mechanism, thus reducing their cytosolic concentration (Gottesman & Pastan, 1993; Doige & Sharom, 1992; Nielsen & Skovsgaard, 1992). Pgp is expressed in some chemotherapy-resistant tumors (Pastan & Gottesman, 1991) and in many normal tissues (Thiebaut *et al.*, 1987; Cordon-Cardo *et al.*, 1989; Buschman *et al.*, 1992), in which its physiological function still remains unclear in spite of suggested roles in detoxification by excluding toxins from normal cells and secretion of metabolites into bile, urine and the gastrointestinal tract (Thiebaut *et al.*, 1987; Cordon-Cardo *et al.*, 1989; Smith *et al.*, 1993). MDR1 Pgp is found in epithelial cells of the liver, kidney, pancreas, small and large intestines, in cells of the adrenal cortex, and in endothelial cells of the brain and testis, as well as in the placenta (Chin & Liu, 1994). The role of Pgp as an energy-dependent efflux pump has been proposed from its structural homology to the bacterial transport protein.

Very high levels of Pgp were found in brain capillaries (Cordon-Cardo *et al.*, 1989; Jette *et al.*, 1993), where it interacts with various drugs (Jette *et al.*, 1995) and appears to play a critical role in host defense against certain lipophilic toxins (Schinkel *et al.*, 1994). Immunohistological studies have suggested that Pgp is localized in the luminal membrane of endothelial cells forming brain blood vessels (Sugawara *et al.*, 1990; Tanaka *et al.*, 1994). High levels of P-gp in the luminal membranes of brain capillary endothelial cells suggests that it may play an important role in limiting the access of anti-cancer drugs to the brain (Beaulieu *et al.*, 1997). It may thus contribute to multidrug resistance in brain tumors by expelling drugs from the endothelial cells into the circulation in addition to extruding them from tumoral cells (Henson *et al.*, 1992) and may also contribute greatly to the properties of the blood-brain barrier, where blood vessels are less permeable than in other tissues such as the heart and lungs. High expression levels of Pgps in the adrenal gland (Thiebaut *et al.*, 1987; Croop *et al.*, 1989) and in the endometrium of the gravid mouse uterus (Arceci *et al.*, 1988) have suggested involvement in transport of steroid hormones. The expression study of *pgp-1* and *pgp-3* in the nematode *C. elegans* shows that Pgp is tightly controlled and restricted to intestinal cells. This indicates that the presence of Pgps in the digestive tract is an evolutionarily conserved feature of these proteins (Lincke *et al.*, 1993). These proteins (Pgps) are therefore thought to provide a protective mechanism to the body by eliminating exogenous toxins or their toxin metabolites before they have a chance to exert their cytotoxic effects.

It is known that drug resistance is caused by the ability of Pgps to extrude drugs against a concentration gradient, resulting in a decrease of the intracellular drug concentration in contact with the drug target. There are two ways for Pgp to remove substrates from cells resulting in decreased uptake and increased efflux. One way is by binding of substrates intracellularly to specific domains of the Pgp and the substrates are released on the extracellular side of the membrane (resulting in an increase in efflux of drugs). Another way

is by removing substrates directly from the lipid bilayer before they get into the cells (resulting in decreased accumulation of drugs).

In independent drug-resistant clones, multiple copies of the transfected plasmid are associated with high levels of expression of mdr cDNA. These results establish a direct relationship between the expression of the cloned mdr cDNA and the multidrug resistant phenotype (Gros *et al.*, 1986a). These suggest that overexpression and/or amplification of Pgp gene in mammalian cells confer multidrug resistance.

2. Correlation of P-glycoprotein with drug resistance in parasitic diseases

P-glycoprotein is widely represented in the animal kingdom and parasites (Ouellette *et al.*, 1994; Upcroft, 1994), but only a small minority of these Pgps have been found in a wide range of some parasitic protozoans selected for drug resistance. However, the functions of most of these Pgps are not known. Amplification of Pgp-encoding genes has been associated with resistance to a spectrum of hydrophilic agents in *Leishmania* (Ouellette *et al.*, 1990; Ellenberger & Beverley, 1989). Two genes related to P-glycoprotein have been described: *Leishmania pgpA* and *mdr*1 gene. *Leishmania pgpA* is frequently amplified in arsenite-resistant mutants (Ouellette *et al.*, 1991; Grondin *et al.*, 1993), and transfection of the gene indicated that it was involved in resistance to oxyanions such as arsenite and antimonite (Papadopoulou *et al.*, 1994). Sequence analysis of *mdr*1 gene in *Leishmania* has shown that it is closely related to the mammalian P-glycoproteins and transfection experiments indicated that it was conferring resistance to drugs included in the multidrug resistance spectrum (Henderson *et al.*, 1992; Chow *et al.*, 1993).

Genes homologous to the mammalian mdr genes have now been identified in *Plasmodium* . Amplification of Pgp-encoding genes has been associated with chloroquine and mefloquine resistance in *Plasmodium falciparum* (Foote *et al.*, 1989; Wilson *et al.*, 1989). In *P. falciparum* which causes human malaria, two mdr gene homologues *pfmdr1* and *pfmdr2* have been cloned and sequenced (Foote *et al.*, 1989; Wilson *et al.*, 1989; Zalis *et al.*, 1993). *Pfmdr1* gene was found to be associated with drug resistance. Resistance was associated with a point mutation and gene amplification (Foote *et al.*, 1990; Cowman *et al.*, 1994; Peel *et al.*, 1994; Basco *et al.*, 1996).

Only recently have Pgp homologs been investigated in nematodes. A family of Pgp homologs have been described in the nematode, *C. elegans*. *C. elegans* is a free living soil nematode. It can directly contact toxins that are present in the natural environment as well as those released through industry and agriculture (Leonard & Belgium, 1991). In *C. elegans,* three full length Pgp gene and one partial Pgp gene homologs, *pgp-1, 2, 3* and *4*, have been cloned, sequenced and mapped to chromosomes I, IV and X (Lincke *et al.*, 1992). They were identified in both the apical membrane of excretory cell and in the apical membrane of intestinal cells (Lincke *et al.*, 1992). *Pgp-1*, when deleted along with a multidrug resistance-associated protein homologue, *mrp-1*, confers hypersensitivity to heavy metals (Broeks *et al.*, 1996) and *Pgp-3,* when deleted, confers sensitivity to colchicine and chloroquine (Broeks *et al.*, 1995). The Pgp gene family of *C. elegans* shares extensive structural homology with their mammalian counterparts (Lincke *et al.*, 1992).

In 1993, Sangster *et al* suggested several partial genes for Pgp in parasitic nematode *H. contortus,* although sequence information was missing. In 1998, Xu *et al* have identified a Pgp gene in *H. contortus* . They showed that the product encoded by this gene may play a role in ivermectin resistance in *H. contortus.* They have also cloned and sequenced a full length of cDNA encoding a Pgp homolog from *H. contortus.* The analysis of this sequence

showed 61-65% homology to the other multidrug resistant protein sequences, found in mice, humans and *C. elegans*. Xu *et al* (1998) also showed that expression of this Pgp mRNA was higher in eggs of an IVM-selected strain than an unselected strain of *H. contortus*. Different restriction patterns have been identified between unselected strains and IVM- or MOX-selected strains through Southern blot analysis.

3. Rationale of Pgp involved in macrocyclic lactone resistance in nematodes

Based on the above information, the reasons for choosing Pgp gene as a candidate gene in macrocyclic lactone resistance in the nematode *H. contortus* are summarized as follows:

a. Pgp has been shown to cause multidrug resistance in mammalian tumor cells (Gottesman & Pastan, 1993) and some parasitic diseases.

b. The overexpression of a Pgp homolog gene in IVM selected strains of *H. contortus* was observed (Xu *et al.*, 1998).

c. A full length of Pgp cDNA from *H. contortus* has been cloned and sequenced (Xu *et al.*, 1998).

d. Different restriction patterns between unselected and IVM selected strains in *H. contortus* were also found (Xu *et al.*, 1998).

e. *In vivo* experiments have shown that disruption of the mouse *mdr*1, a P-glycoprotein gene, leads to an impairment in the blood-brain barrier and to increased

sensitivity to IVM in these mice (Schinkel *et al.*, 1994). Mice with deletion of *mdr*1a were 50-100 times more sensitive to ivermectin than normal mice.

f. IVM can strongly interact with the Pgp drug binding site possibly due to its high hydrophobicity (Pouliot *et al.*, 1997). This evidence shows that IVM is a good substrate for Pgp.

g. Furthermore, the multidrug resistance reversing agent verapamil can increase the efficacy of IVM and MOX against a MOX selected *H. contortus* strain (Xu *et al.*, 1998).

This evidence shows that Pgp may contribute to ivermectin and possibly moxidectin resistance in the parasitic nematode *H. contortus*. The present study, therefore, is designed to investigate the genetic variation of the Pgp gene in the nematode *H. contortus* and its association with resistance to IVM and MOX, using molecular biology techniques. The study provides important information to better understand the genetic basis of drug resistance in the parasite, which can be used as a marker for diagnosis of IVM and MOX resistance at an early stage and provide scientific information for development strategies for controlling the spread of the drug resistance. This information may help to reduce the economic losses associated with anthelmintic resistance and prolong the life of anthelmintic drugs.

Chapter III

Materials and Methodology

I. Experimental Design

This study consisted of the following experimental techniques: DNA extraction, Polymerase Chain Reaction (PCR), restriction enzyme digestions, cloning and sequencing. In the study, we designed the specific Pgp primers to look for the genetic variation of the Pgp gene in *H. contortus*. Three strains of adult *H. contortus* parasites were obtained from sheep that were infected with each strain of the infective stage larvae: PF17, IVF17 and MOF17 respectively. Genomic DNA was extracted from forty individual male worms of each strain and PCR reactions were performed to amplify an approximately 876 bp long fragment by using the specific Pgp primers. The PCR products were then digested with four different restriction enzymes: *Dde*I, *Alu*I, *Hinf*I and *Rsa*I. The restriction patterns of four different enzymes were observed and alleles of the three strains at the Pgp locus were analyzed. The alleles which are associated with susceptibility and IVM- and MOX-resistance were detected using analysis of frequencies of the alleles. The alleles that are related to IVM- and MOX-resistance, or susceptibility were then cloned and sequenced from all three strains of the parasites.

II. Materials and Methods

1. Parasitic nematode strains

Three laboratory strains of *H. contortus* were used in this study. All of them were derived from the same parent isolate (it means that infective larvae (L3) of *H. contortus* were originally sensitive to both ivermectin and moxidectin). The concentrations of individual compounds were selected on the basis of achieving an efficacy of 80-95% in order to allow sufficient egg production for cultivation of L3 for subsequent passages. One strain is the Cyanamid unselected strain (PF17) which was obtained after 17 passages in sheep without any anthelmintic exposure at Cyanamid; one is Cyanamid ivermectin selected strain (IVF17) which was exposed to ivermectin for 17 passages, and another one is Cyanamid moxidectin selected strain (MOF17) which was exposed to moxidectin for 17 passages of *H. contortus*. PF17, IVF17 and MOF17 were passaged in parallel. The drug dosages in the final generation were 0.15 mg/kg for IVM and 0.015 mg/kg for MOX. At the twelfth generation, ED95 was calculated for each strain. It was 0.01125 mg/kg body weight for IVM and 0.00179 mg/kg body weight for MOX in PF12. The ED95 for IVF12 was 0.10874 mg IVM/kg body weight, and for MOF12 was 0.00949 mg MOX/kg body weight (Wang *et al.*, 1995).

2. Preparation of parasites

The larvae of the above three strains of *H. contortus* were provided by Fort Dodge Animal Health, Princeton, NJ, USA. Sheep were infected with 10,000 third infective stage larvae. After about a month, the sheep were killed and adult male worms were collected from the abomasum. Worms were then washed with sterile phosphate-buffered saline (PBS) at 37°C several times and were ready for extraction of DNA. If DNA was not isolated

immediately after worms were collected, the worms were frozen in liquid nitrogen and stored at -80°C until used.

3. Extraction of genomic DNA

H. contortus genomic DNA was extracted from fresh or frozen individual adult male worms by using standard procedures. Only adult male worms were used to isolate DNA to avoid the possibility of DNA contamination from fertilized eggs or sperm present in female samples. Worms were washed with clean water, male worms were isolated under the microscope, and then transferred into a 1.5 ml eppendorf tube that containing 200 μl STE (0.1 M NaCl, 10 mM Tris-Cl, pH 8.0, 1.0 mM EDTA, pH 8.0), 10 μl β-mercaptoethanol, 10 μl of 20% SDS and 2.0 μl of protease K (20 mg/ml), incubated at 37°C for 2 hours to completely digest the worm (Beech *et al.*, 1994). DNA was extracted with phenol/chloroform/isoamyl alcohol (25:24:1) once and then precipitated with isopropanol: ammonium acetate (2:1) following an addition of coprecipitant with 4 μl of 2.5 mg/ml linear acrylamide (Gaillard & Strauss, 1990). The DNA pellet was washed with 70% alcohol once, then air dried and resuspended in 100 μl TE buffer (10 mM Tris-HCl, pH 8.0, 1.0 mM EDTA, pH 8.0) and stored at -20°C.

4. Sequences of the primers used in PCR amplification

PCR reactions were performed to amplify a fragment approximately 876 bp long using specific primers designed from the cDNA sequence of the *H. contortus* Pgp gene. The sense primer PGP2S (5'GAAATGACTCAAGCACAAG3') (designed by Dr. M. Xu and modified by Bill Blackhall in our lab) and the antisense primer MX-D

(5'AGACAAAGACATTCAGAG3') (designed by Dr. M. Xu) and PGP1A (antisense primer 5'AGTGAACTAAGATGGGGT3') were used in the PCR reactions. Initial DNA amplification using PGP2S and MX-D primers on DNA from individual worms was not efficient enough for restriction enzyme analysis. Therefore, two rounds of PCR reaction was set up to obtain sufficient product for restriction enzyme digestion. The first PCR reaction was set up by using antisense primer PGP1A located 50 bp downstream from MX-D on the Pgp cDNA with PGP2S to produce a 200 bp longer fragment than that from the primers PGP2S and MX-D, the first PCR product was then used as template to be reamplified with primers PGP2S and MX-D for a second round of PCR reaction. The PCR product obtained with PGP2S and MX-D from genomic DNA was approximately 876 bp in length.

5. Polymerase Chain Reaction (PCR) amplification

The genomic DNA (approximately 2.0 μg) was used to amplify an approximately 876 bp fragment by PCR in the final volume of 25 μl in the presence of 10X Taq buffer 2.5 μl, 25 mM $MgCl_2$ 2.5 μl, 20 μM primers PGP2S and MX-D 1.0 μl respectively, Taq polymerase 1 Unit and H_2O up to 25 μl. The PCR amplification reaction was performed on an MJ Research, Inc. PCR-100 Programmable Thermal Controller. Amplification conditions for the first PCR reaction were 95°C for 3 min 55 sec to denature the template DNA completely, followed by 40 cycles of 95°C for 10 sec, 53°C for 20 sec, 72°C for 1 min 30 sec. The final extension temperature was 72°C for 5 min. 1.0 μl (approximately 1.0 ng) of genomic DNA from each individual worm was amplified in a total volume of 25 μl. The second PCR amplification condition was similar to the first, except 30 cycles were used instead of 40 cycles. After amplification and before restriction enzyme digestion, the PCR products were

visualized on a 1.0% agarose gel containing 0.5 µg/ml of ethidium bromide with a base pair size marker (made by our own lab) under UV illumination.

6. Restriction enzyme digestion and identification of allele patterns

The PCR product from genomic DNA was digested with four different restriction enzymes-*Dde*I, *Alu*I, *Hinf*I and *Rsa*I which were purchased from Promega. The fragments of restriction enzyme digestion were separated by 6% non-denaturing polyacrylamide gel (mixture of 45 ml of 6% acrylamide (0.5X TBE), 15 µl TEMED and 400 µl of 10% ammonium persulphate) electrophoresis, where they migrate according to size. The DNA fragments were stained directly with 0.5 µg/ml ethidium bromide, and visualized under ultraviolet light. The identity of the different alleles were deduced, where possible, by identifying a homozygote, where the sum of the fragment sizes equal the size of the original PCR fragment, or by identifying those bands in heterozygotes which consistently occur together (Beech *et al.*, 1994).

7. Allele frequencies and statistical analysis

For the purpose of this study, the frequency of an allele is calculated simply as its percentage of the total of all alleles detected among the samples examined. A Chi-square test was used to analyze whether frequencies of alleles between IVM- and MOX-selected strains, between IVM selected and unselected strains and between MOX selected and unselected strains were significantly different. Allele classes which number less than 5 were pooled to make sure that there was a minimum expected number of at least 5 per class per strain.

8. Cloning of the Pgp alleles

A. Purification of DNA

Approximately 876 bp of fragment from genomic DNAs which contain the alleles that are associated with IVM or MOX resistance and susceptibility were amplified by using the same primers as we used before (PGP2S and MX-D) in a large volume of PCR reaction (50 or 100 μl). The 50 or 100 μl of PCR product was loaded on a thick 1% agarose gel, and the band was excised under the UV light and purified by using the Geneclean Kit (BIO/CAN Scientific), or DNA was purified with the 1-10 μl white plugged pipet tips by cutting the bands into small pieces, putting the pieces into white plugged pipet tips, and placing the tips into 1.5 ml tubes, then spinning at high speed for 30 sec. The DNA in the liquid at the bottom of the tube was collected and stored at -20°C.

B. Ligation

The purified DNA from above was ligated into the pGEM-T vector from the ligation kit of Promega. In the ligation reaction, the 5.0 μl of purified DNA was mixed with 0.5 μl of pGEM-T vector, 1.0 μl of 10X ligase buffer and 1.0 μl of ligase (3 U/μl), then H_2O up to 10 μl was added and the reaction was ligated at 4°C overnight.

C. Transformation

The resulting recombinant plasmid from the ligation was then used for transformation by following the protocol below. One tube of competent cells (*E. coli*, prepared by ourselves) was thawed slowly on ice. 5.0 μl of ligated plasmid was mixed with 100 μl of competent

cells by gentle flicking. They are left on ice for 20 min, then heated in a 42°C water bath for exactly 90 sec.; immediately replacing them on ice for 2 min. Transformed cells were subsequently transferred into a tube which contained 3-4 times SOB (or SOC) medium and shaken at 37°C for one hour. Approximately 200 µl of cell culture was mixed with 10 µl of X-gal, 4.0 µl of IPTG and 2.0 µl of ampicillin (100 mg/ml), mixed gently and spread on the LB Agar plate, and then incubated at 37°C overnight.

D. Mini-Preparation

Twelve single white colonies of each sample from the above plates were picked up and grown each in 5 ml of SB medium at 37°C overnight in a shaker. The clones which contained recombinant plasmids were then identified by the mini-preparation. The SB culture was centrifuged at 2000 rpm for 10 min, and the supernatant was removed. The pellet was resuspended with 200 µl of GTE. The resuspended solution was then transferred into an new Eppendorf tube; 400 µl of fresh NaOH-SDS (50 µl of 20% SDS, 910 µl of H_2O and 40 µl of 5 N NaOH) were added, vortexed quickly and placed on ice about 5 min. Then 300 µl of 7.5 M ammonium-acetate was added, and the mixture vortexed and spun for 2 min. The liquid was transferred into a tube containing 500 µl of phenol/chloroform/isoamyl alcohol (25:24:1), vortexed and spun for 2 min. The top layer was subsequently transferred into a tube containing the 600 µl of isoproponal, vortexed and spun for 10 min. The pellet was dried by air and resuspended in the 50 µl of TE. 1.0 µl of the above plasmid DNA was run on the 1% Agarose gel with one plasmid DNA which had no insert obtained from the blue colony as a negative control and one size marker. The positive plasmid DNA containing an insert has a larger size than the negative one dose. If the plasmid DNA was contaminated by RNA, 1.0 µl of RNase was added into each tube and digested at 37°C for 1-2 hours.

E. Identification of clones

To identify the clones which contained the alleles associated with IVM resistance and susceptibility, a PCR reaction was performed by using plasmid DNA which had a correct size on the Agarose gel. 10X Taq reaction buffer 5.0 µl, 2 mM dNTPs 5.0 µl, 25 mM $MgCl_2$, 20 µM primers PGP2S and MX-D 1.0 µl respectively, Taq polymerase (10 U) 0.1 µl, Plasmid DNA 0.2 µl, H_2O up to 50 µl. PCR amplification conditions were 95°C for 5 min to completely denature the template DNA, followed by 35 cycles of 95°C for 15 sec, 53°C for 20 sec, 72°C for 1 min 30 sec. The final extension temperature was 72°C for 5 min.

Restriction enzyme digestion reactions were then applied by using the same method explained above to identify the correct alleles. According to the restriction pattern, clones that contained the alleles which associated with susceptibility and IVM and MOX resistance were found.

9. Sequencing of the alleles

The clones which contained the alleles of interest were sequenced by the Sheldon Biotech Center of McGill University. A small part of the clones were sequenced in our lab. Sequencing reactions were performed by using the cycle sequencing pGEM Kit from Promega. The following protocol was used: 2.0 µl of d/ddNTP (A, C, G, T), 1.0-2.0 µl of DNA, 1.0 µl of [α–^{35}S]dATP (10 µCi/µl), 1.5 µl of 2 µM universal forward primer PGP2S and reverse primer MX-D respectively, 5.0 µl 5X sequencing buffer, 1.0 µl sequencing grade Taq DNA polymerase, and H_2O up to 16 µl of total volume. One drop of mineral oil was added to avoid evaporation. The PCR machine was preheated to 95°C, the reaction tubes were then placed in it. Thirty cycles of sequencing were performed as following: 95°C, 2

min; 95°C, 30 sec; 42°C, 30 sec; 70°C, 1 min. After completion of the reaction, 3.0 µl of stop solution was added and tubes were heated at 70°C for another 2 min or heated before loading on the sequencing gel. Each sample was run on a short gel and a long gel with 1X TBE buffer under 200 volts. The film was exposed for 3-5 days before reading the sequences.

Chapter IV

Results of Evaluation of Restriction Fragment Length Polymorphism

1. Restriction enzyme digestion analysis

Genetic variation at the Pgp locus was investigated in IVM- and MOX-selected and unselected strains. Forty individual adult male worms from each strain were surveyed. The specific primers were designed according to the PGP-A cDNA sequence (Xu *et al.*, 1998) for PCR amplification of an approximately 876 bp fragment from individual genomic DNA. The position of the primers and the amplified fragment are shown in Figure 1. Insufficient PCR product was obtained to do restriction enzyme digestion when genomic DNA was initially amplified with the primers PGP2S and MX-D. Therefore, the primer PGP1A was used with PGP2S to amplify an approximately 200 bp longer fragment as a first step. This PCR product was then employed as template for reamplification by using the initial primers PGP2S and MX-D. The use of nested PCR resulted in sufficient product for restriction enzyme digestion analysis. The different restriction patterns were seen when this fragment was digested with four different restriction enzymes (*Alu*I, *Rsa*I. *Dde*I, and *Hinf*I) respectively (Figure 2-5).

Different restriction patterns from three strains were observed when digested with the above four different restriction enzymes. A common restriction pattern in the three strains was observed, especially in the two drug selected strains. The pattern from each strain is indicated in figures 2-5. When digested with *Dde*I, thirty-two worms in the ivermectin selected strain

(IVF17) and twenty-seven worms in the moxidectin selected strain (MOF17) contained at least one common pattern (data was not shown), while only seven worms in the unselected strain (PF17) contained this pattern. This pattern is shown in column A in figure 6. When digested with *Alu*I, thirty-nine worms in both IVF17 and MOF17 strains were found to have one common restriction pattern (figure 3) - column A in figure 7, and twenty-eight worms in the PF17 strain contained this common pattern. When digested with *Hinf*I, twenty-nine worms in the IVF17 strain, twenty-five in the MOF17 strain contained a common restriction pattern, see figure 4, which is shown as column A in figure 8, while only seven worms in the PF17 strain contained this pattern. Using *Rsa*I, thirty-six worms in the IVF17 and thirty-eight worms in the MOF17 strain had a common restriction pattern - shown as column A (see figures 5 and 9), and twenty-eight worms in the PF17 strain had this pattern.

The different number of digestion patterns from all three strains were also detected when digested with the four different restriction enzymes (Table 1). There were five restriction patterns observed when digested with *Dde*I, with five patterns found in PF17, three found in IVF17 and four detected in MOF17 (data not shown). The PF17 strain which contained all five restriction patterns found in the three strains is shown in figure 6. A total of three patterns were observed when digested with *Alu*I: two in IVF17, three in MOF17 and three in PF17 as a representative shown in figure 7. When digested with *Hinf*I, a total of five digestion patterns were found: four in IVF17, five in MOF17 and four in PF17. PF17 strain, as a representative is shown in figure 8. In order to show all the patterns digested with *Hinf*I, an additional E digestion pattern, which was absent from PF17, but present in MOF17 strain was added in figure 8. There were six restriction patterns found when digested with *Rsa*I: four in IVF17, four in MOF17 and four in PF17 strain. The PF17 strain, as a representative, is shown in figure 9. The additional C and F patterns which were absent in PF17, but present in IVF17 and MOF17 were added in figure 9 to show all six *Rsa*I digestion patterns. The

PCR and restriction enzyme digestions were repeated to confirm the results obtained in this study.

2. Comparison of allele frequencies between unselected and IVM- and MOX-selected strains

Restriction polymorphism was observed in the three strains following digestion of the approximately 876 bp PCR product with four restriction enzymes. When the IVM- and MOX-selected strains were compared with the unselected strain, it was observed that the unselected strain contained more restriction patterns than both selected strains, especially when digested with *Alu*I and *Rsa*I. A reduction in genetic variability in both selected strains, as measured by the number of alleles present in the population, was apparent. A total of 23 alleles were identified based on a combination of the four restriction enzyme digestion patterns among the three strains by restriction fragment length polymorphism (RFLP) analysis (Table 1). These alleles have been named from A to e arbitrarily. Comparing the unselected strain with the selected strains, the number of alleles observed in the unselected strain was higher than in the selected strains. Seventeen alleles were found in the unselected strain, while 12 were seen in the ivermectin selected strain and only 7 were found in the moxidectin selected strain.

The frequencies of the alleles from the three strains were analyzed and the results are shown in Figure 10. Only 5 alleles, A, B, O, X, and Y, in the total of 23 alleles found, were common to all three strains. Our result also showed that some alleles appeared in higher frequency or were only present in either the IVF17 or the MOF17 strain but were not present at a high frequency, or detected at all, in the unselected strain. For example, alleles A, L, M, N, P, Z, and e were more abundant in the selected strains than in PF17. Allele A was found with extremely high frequency in both IVM- and MOX-selected strains compared with the

unselected strain. Its frequency increased from 0.063 in PF17 to 0.5 in IVF17 and to 0.438 in MOF17. This suggested that allele A is correlated with IVM and MOX resistance. Some alleles had higher frequencies or were present only in the unselected strain, such as R, S, T, U, V, W, X, a, b, c, and d. Allele X had a higher frequency in the PF17 compared with both selected strains. Its frequency decreased from 0.2 in PF17 to 0.063 in IVF17 and to 0.075 in MOF17. This suggests that allele X is associated with susceptibility. Allele B also had a higher frequency, but it was common in all three strains. The frequencies of most alleles among the three strains were very low.

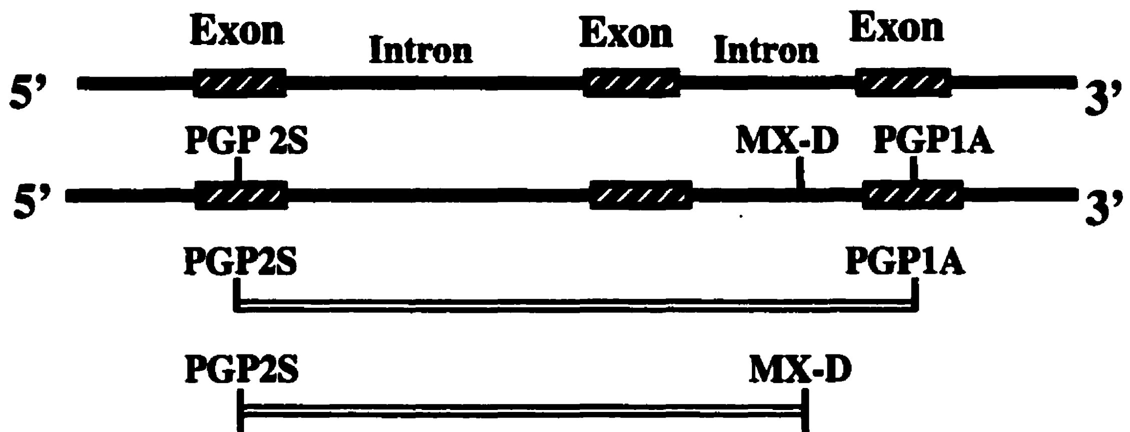
The results of the statistical analysis showed that allele frequencies were highly significant different between IVF17 and PF17 ($P<0.0005$). There was also a highly significant different between MOF17 and PF17 ($P<0.0005$). Allele frequencies between the IVF17 and MOF17 strains was not significant difference ($P>0.25$).

3. Hardy-Weinberg equilibrium analysis

Based on the allele information, the genotypes of the three strains were analyzed and the results are presented in Table 2. Genetic variation can also be measured by Hardy-Weinberg equilibrium of the number of homozygotes in the parasite population. An increase in homozygosity is generally associated with a reduction in variability, assuming the alleles are in Hardy-Weinberg equilibrium. A Chi-square analysis of the observed and expected homozygote and heterozygote frequencies showed that the two selected strains were in Hardy-Weinberg equilibrium but that the unselected strain was not. The excess of homozygotes in this strain may indicate the presence of one or more alleles that failed to amplify during the PCR reaction, so that the result from the restriction enzyme digestions would appear as homozygotes when, in fact, the worms were heterozygotes. Because of this,

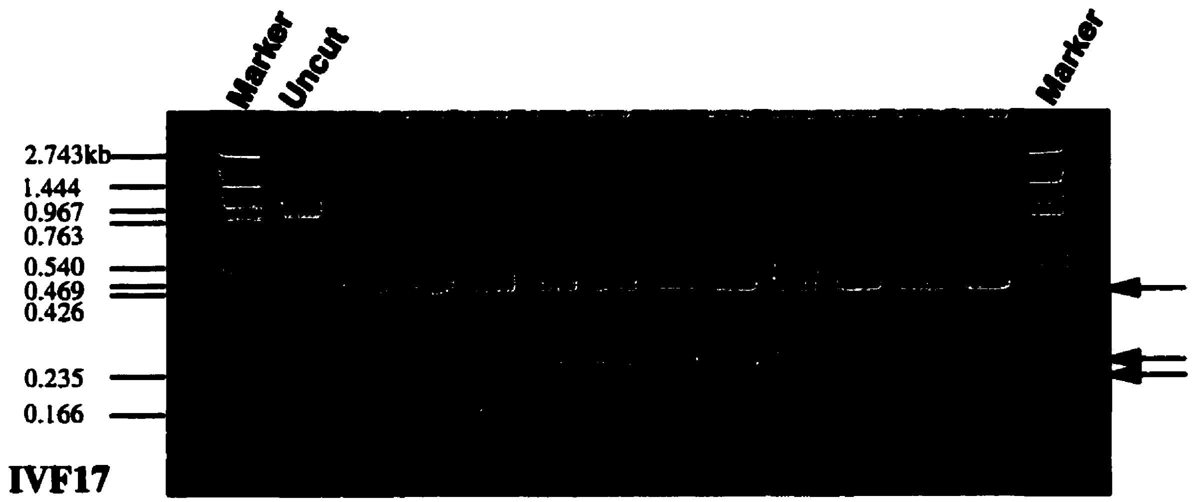
the number of homozygotes observed cannot be used as an indicator of any change in genetic variability between strains.

Figure 1. Amplified Fragment



This diagram shows the amplified fragment from genomic DNA of individual *Haemonchus contortus* and the positions of the primers used in this study.

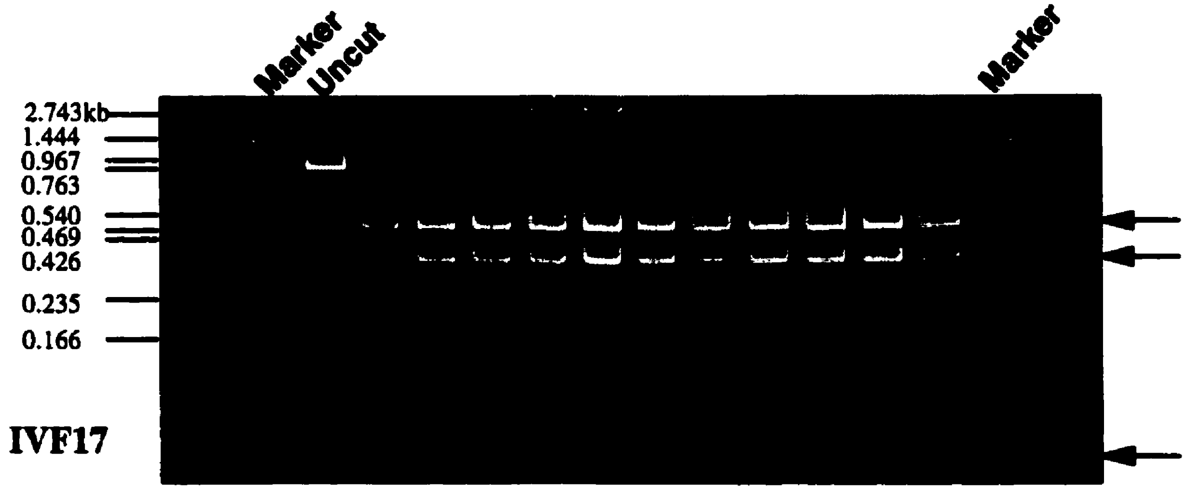
Figure 2. *Dde*I digestion patterns from individual worms of the three *Haemonchus contortus* strains: ivermectin selected (IVF17), moxidectin selected (MOF17) and unselected (PF17) strains. Arrows indicate a common restriction pattern of the three strains when digested with *Dde*I restriction enzyme. DNA size marker is shown where indicated.


Marker
Uncut
Marker
2.743kb
1.444
0.967
0.763
0.540
0.469
0.426
0.235
0.166
IVF17

Marker
Uncut
Marker
MOF17

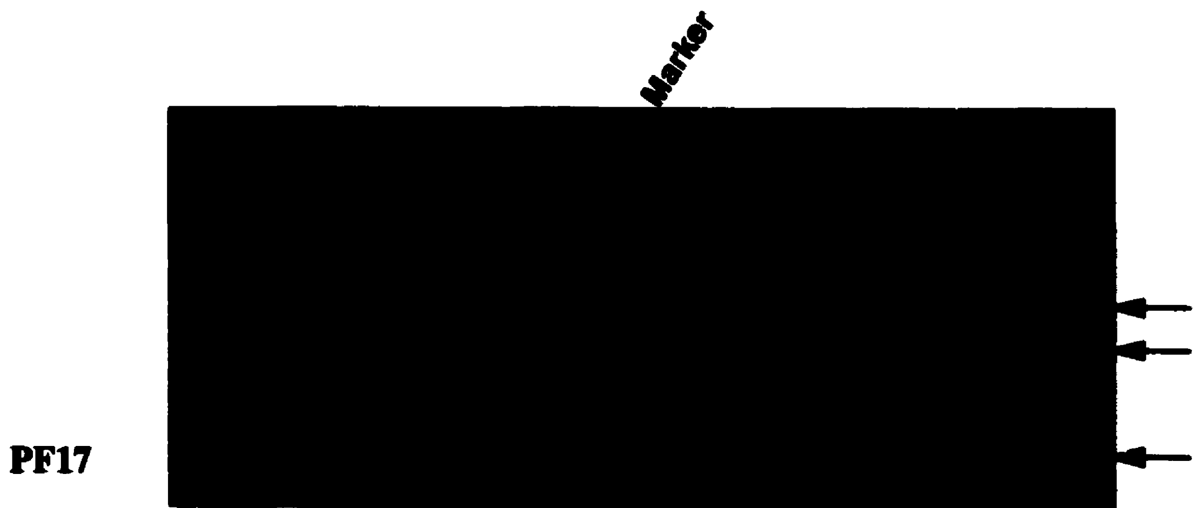
Marker
PF17

Figure 3. *Alu*I digestion patterns from individual worms of the three different *Haemonchus contortus* strains: PF17, IVF17 and MOF17. Arrows indicate a common restriction pattern of the three strains when digested with *Alu*I restriction enzyme. The numbers on left side are the standard DNA size marker.



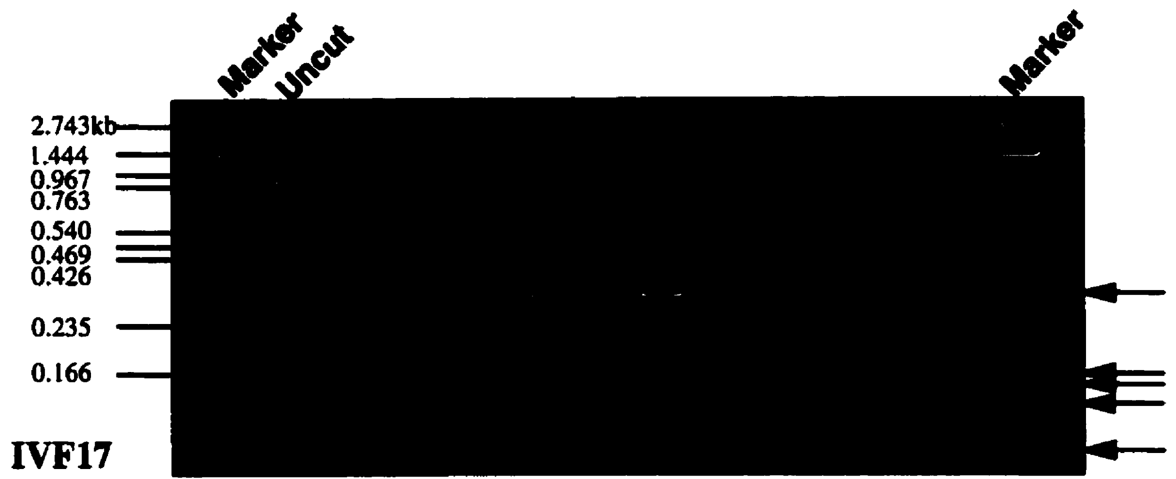
Marker
Uncut
Marker
2.743kb
1.444
0.967
0.763
0.540
0.469
0.426
0.235
0.166
IVF17

Marker
Uncut
Marker
MOF17



Marker
PF17

Figure 4. *HinfI* digestion patterns from individual worms of the three different *Haemonchus contortus* strains: PF17, IVF17 and MOF17. Arrows indicate a common restriction pattern of the three strains when digested with *HinfI* restriction enzyme. The numbers on left side are the standard DNA size marker.


Marker
Uncut
Marker
2.743kb
1.444
0.967
0.763
0.540
0.469
0.426
0.235
0.166
IVF17

Marker
Uncut
Marker
MOF17

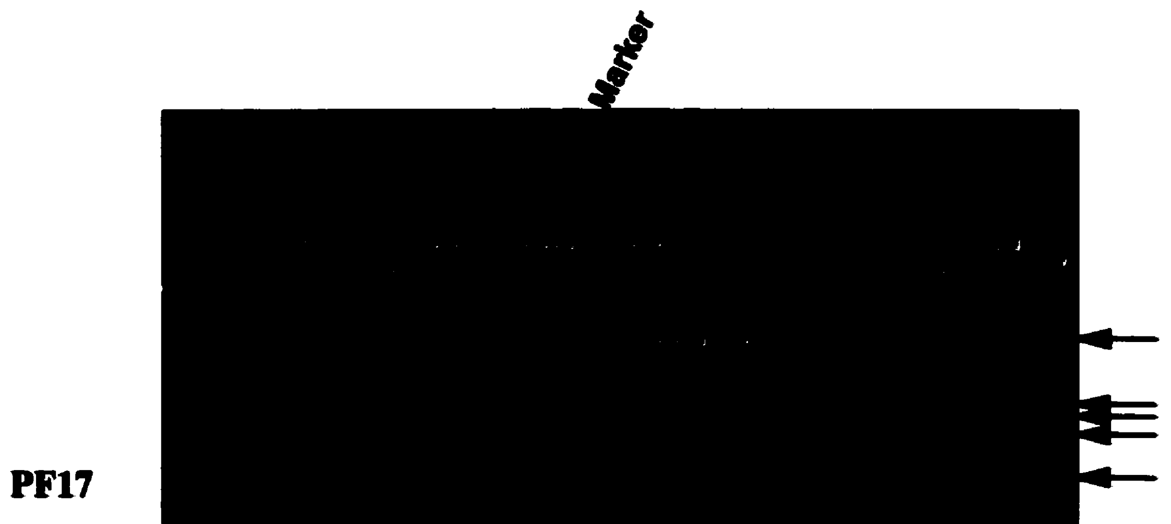

Marker
PF17

Figure 5. *RsaI* digestion patterns from individual worms of the three *H. contortus* strains: ivermectin selected (IVF17), moxidectin selected (MOF17) and unselected (PF17) strains. Arrows indicate a common restriction pattern of the three strains when digested with *RsaI* restriction enzyme. DNA size marker is shown where indicated.

Marker
Uncut
Marker
2.743kb
1.444
0.967
0.763
0.540
0.469
0.426
0.235
0.166
IVF17

Marker
Uncut
Marker
MOF17

Marker
Uncut
Marker
PF17

Figure 6. *Dde*I restriction patterns of individual worms from the PF17 strain. The bands in the gel (left) and letters in the schematic (right) show the five different digestion patterns obtained with *Dde*I restriction enzyme digestion from the three strains. Lanes1 and 6 contain B and D patterns; 2, 11, 17 and 18 contain two C patterns; 3 and 15 contain two D patterns; 4 contains A and C; 5, 7 and 8 contain two B patterns; 10, 12, 13 and 14 have B and C; 16 contains A and D while lane 19 contains A and B restriction patterns.

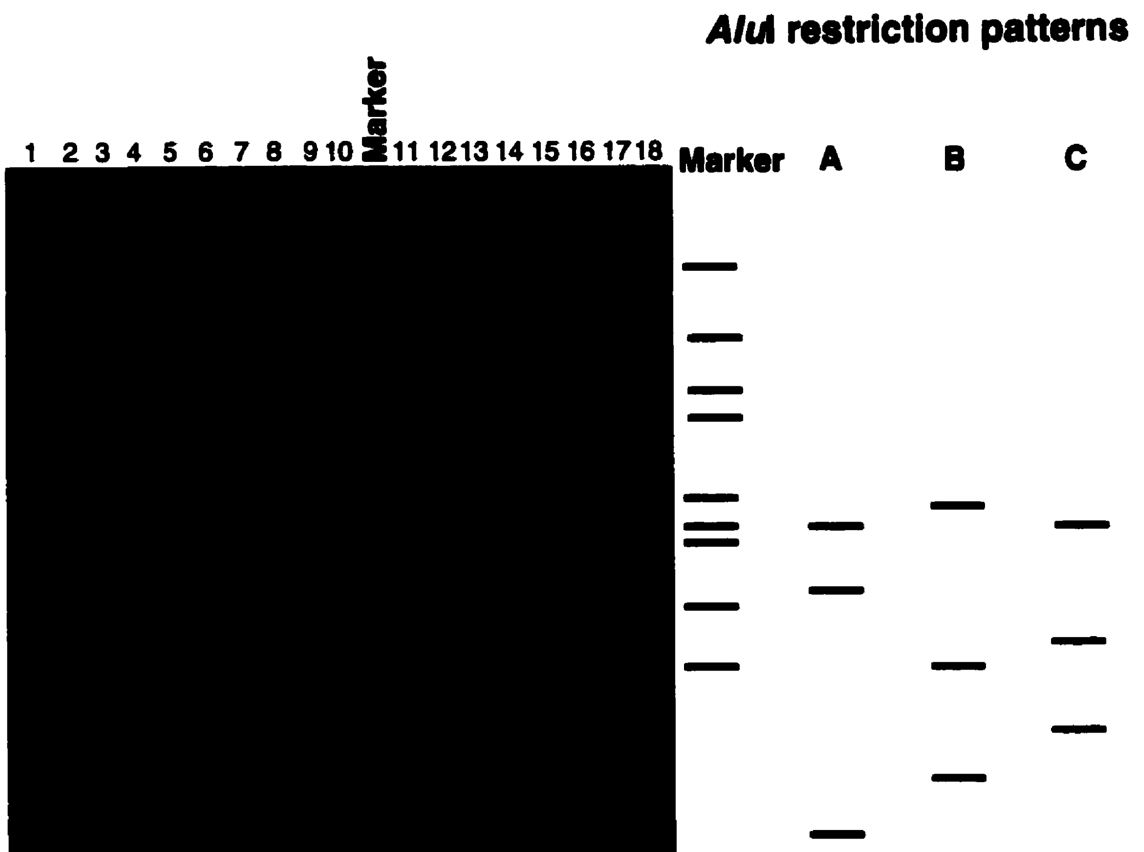


Figure 7. *Alu*I restriction patterns of individual worms from the PF17 strain. The bands in the gel (left) and letters in the schematic (right) show the three different digestion patterns obtained with *Alu*I restriction enzyme digestion from the three strains. Lanes 1, 6, and 16 contain patterns A and C; 2, 11, 17 and 18 contain two B patterns; 3 and 15 contain two C patterns; 4, 10, 12, 13 and lane 14 contains A and B patterns while lanes 5, 7, 8 and 9 contain two A patterns.

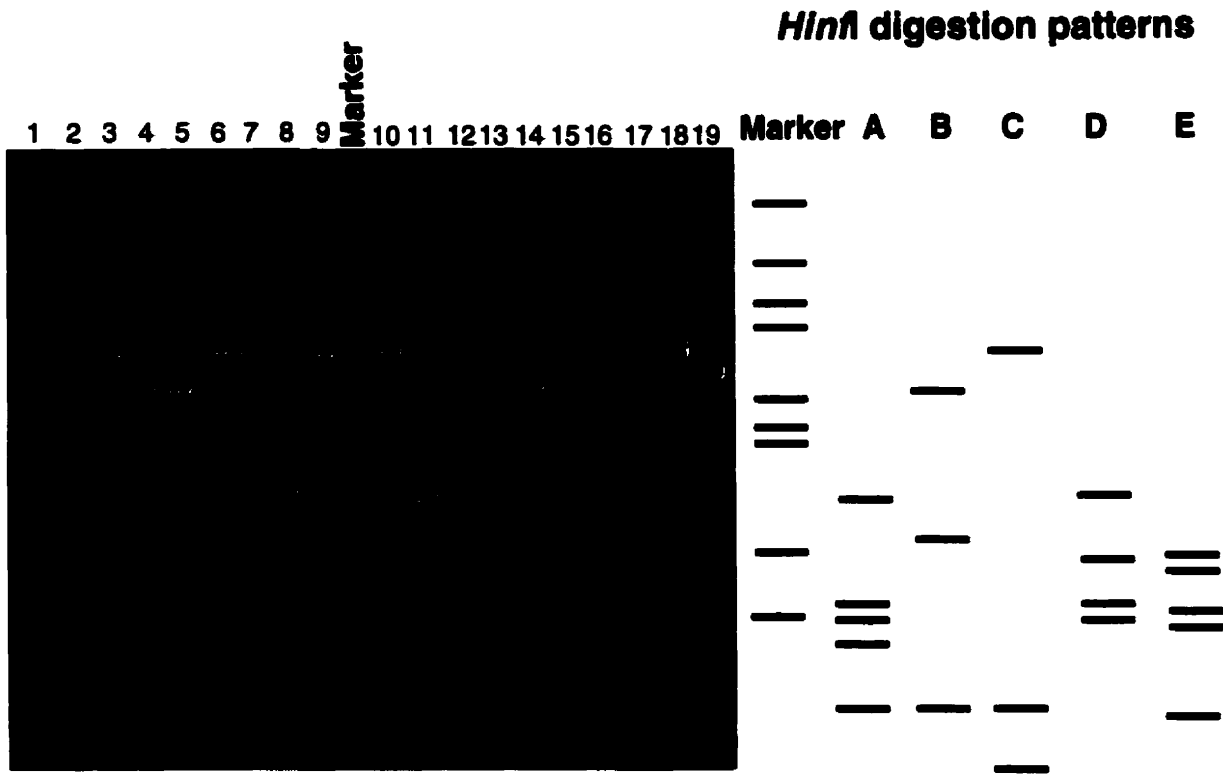


Figure 8. *Hinf*I restriction patterns of individual worms from the PF17 strain. The bands in the gel (left) and letters in the schematic (right) show the five different digestion patterns obtained with *Hinf*I restriction enzyme digestion from the three strains. Lane1 contains A and D patterns; 2, 3, 9 and 18 contain two C patterns; 4 contains A and B patterns; 5 and 7 contain two B patterns; 6, 7, 8 and 10 contain C and D patterns; 11 contains two A patterns; 12,13 and 15 contain two D patterns; 14 and 19 contain B and D patterns while lane 16 contains A and C patterns.

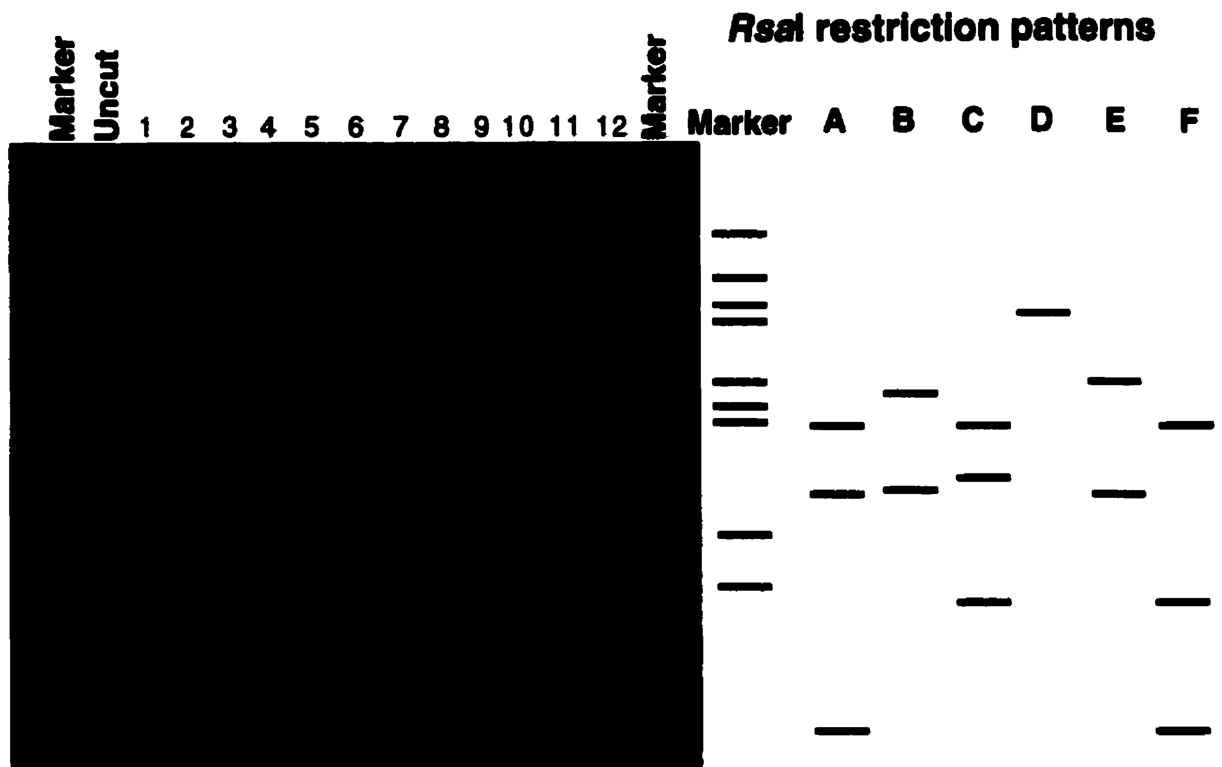


Figure 9. *Rsa*I restriction patterns of individual worms from the PF17 strain. The bands in the gel (left) and letters in the schematic (right) show the six different digestion patterns obtained with *Rsa*I restriction enzyme digestion from the three strains. Lane 1, 4, 5 and 6 contain A and B restriction patterns; 2 and 10 contain two A patterns; 3, 8 and 12 contain patterns A and E; lane 7 contains two D patterns and lane 9 contains two E patterns.

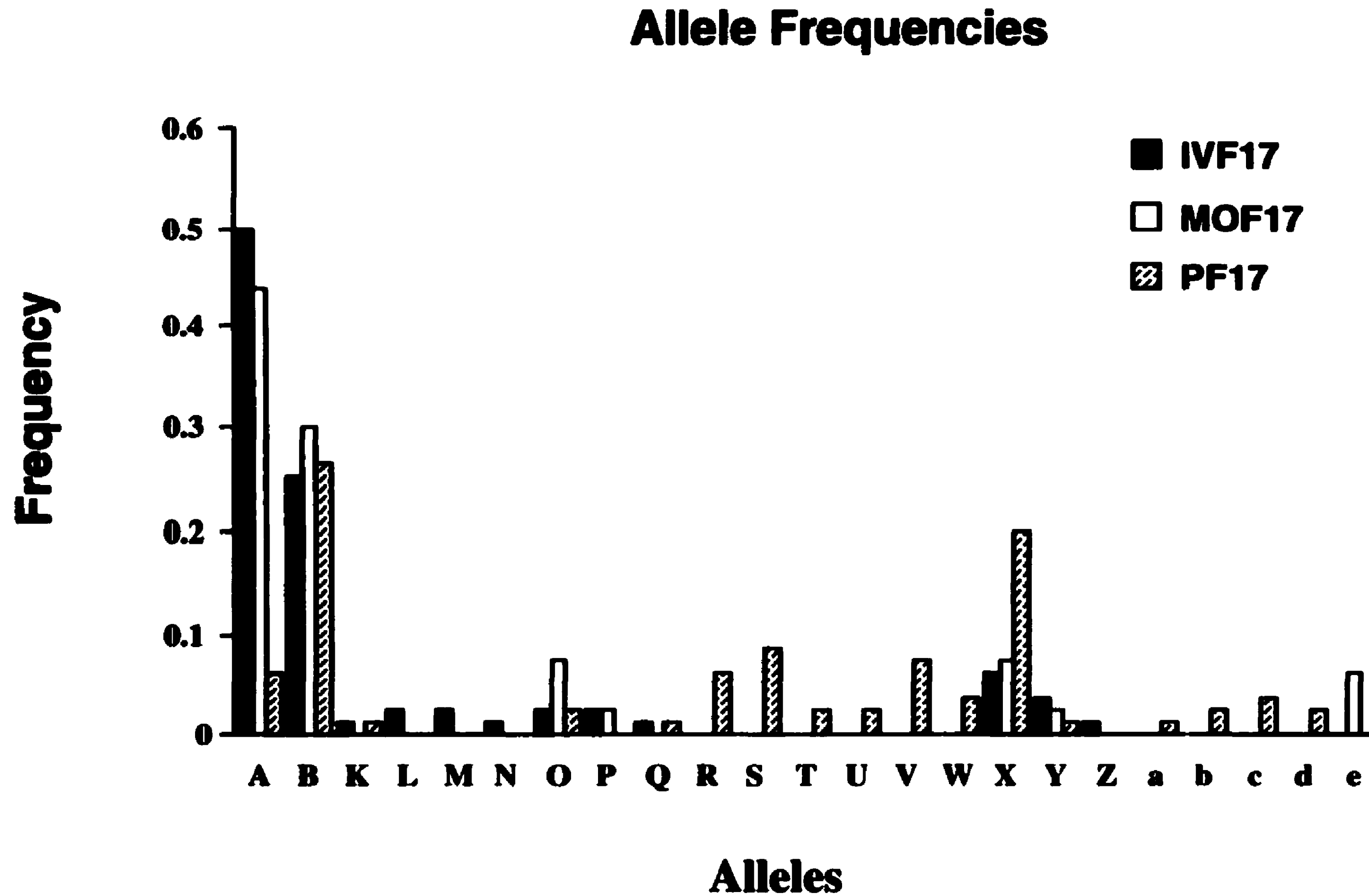


Figure 10. Frequency of P-glycoprotein alleles in unselected (PF17) and ivermectin-(IVF17) and moxidectin-selected(MOF17) strains of *Haemonchus contortus.*

Table 1. Allele Classification

	Name of alleles	*Dde*I	*Alu*I	*Hinf*I	*Rsa*I
1	A	A	A	A	A
2	B	B	A	D	A
3	K	B	A	A	A
4	L	A	A	A	B
5	M	A	A	A	D
6	N	B	A	D	B
7	O	C	B	C	A
8	P	B	A	E	F
9	Q	D	C	B	D
10	R	D	C	C	D
11	S	B	A	B	A
12	T	A	A	C	A
13	U	C	B	A	B
14	V	D	C	D	D
15	W	C	B	B	B
16	X	C	B	C	B
17	Y	A	A	D	A
18	Z	A	A	E	A
19	a	A	B	A	A
20	b	B	A	C	A
21	c	C	B	D	A
22	d	E	B	C	B
23	e	B	A	E	C

Table 1. Alleles and allele patterns of P-glycoprotein in both IVM- and MOX- selected strains and the unselected strain of *H. contortus*. Each restriction enzyme produces a specific restriction pattern. Alleles are identified by taking into account the patterns with all four restriction enzymes (*Dde*I, *Alu*I, *Hinf*I and *Rsa*I). The first column indicates the total of 23 alleles found in the three strains. The remaining columns refer to the patterns produced by four different enzymes.

Table 2. Genotypes of the Different Strains of Worms

IVF17 Worm #	Genotype	MOF17 Worm #	Genotype	PF17 Worm #	Genotype
1	AQ	1	AA	1	KV
2	LL	2	AA	2	XX
3	MM	3	BX	3	RR
4	AA	4	BX	4	AW
5	AB	5	AX	5	SS
6	XX	6	AB	6	BR
7	AA	7	AO	7	Bb
8	BN	8	Ae	8	XX
9	AB	9	AA	9	Bb
10	AA	10	Be	11	TT
11	AA	11	AB	12	BX
12	BB	12	AB	13	UU
13	AA	13	AA	15	Bc
14	AA	14	AA	18	Bc
15	BB	15	Be	19	Sc
16	AA	16	AA	21	VV
17	BB	17	XX	22	AR
18	KZ	18	AA	23	WW
19	AA	19	AB	25	XX
20	AB	20	Be	28	SY
21	AB	22	AB	30	BO
22	BX	23	AO	31	RX
23	AA	24	BP	32	BB
24	BB	27	AB	33	AQ
25	AA	28	BO	34	BB
26	AA	29	AO	37	XX
27	AO	30	AO	38	Ba
28	AA	32	BB	39	BB
29	AB	34	BY	40	SV
30	AA	36	BB	41	BB
31	PP	37	AA	43	BB
32	BY	39	AO	44	SV
33	AB	40	AB	45	XX
34	AX	42	Ae	46	SV
35	AA	43	BB	47	dd
36	BY	44	PX	48	AA
37	AX	47	BY	49	BO
38	BY	48	BB	50	BX
39	AO	49	AA	51	BX
40	AB	50	AA	52	XX

Chapter V

Results of Cloning and Sequencing

1. Cloning of the alleles

Based on the RFLP analysis, the alleles associated with susceptibility and IVM and MOX resistance were identified. The alleles A, B, P and X from all three strains were cloned. Allele A and X were associated with IVM and MOX resistance and susceptibility, respectively, while allele B had a similar frequency in all three strains, and allele P was present only in the two selected strains. Two heterozygous worms for each of these alleles from each strain were chosen to separate these alleles from others for subsequent sequencing. Four restriction enzymes were used to digest the approximately 876 bp fragment produced by PCR in this study. If more restriction enzymes had been used for RFLP analysis, possibly more restriction recognition sites would have been found. In addition, more alleles may have been identified and more heterozygous worms could be observed from some of the apparent homozygotes. The result of the Hardy-Weinberg equilibrium analysis also showed that one or more alleles failed to amplify during the PCR reaction. Based on this information, we know that if some of the apparently homozygous worms were chosen, it would be hard to know whether they were true homozygotes or heterozygotes.

The colonies which contained alleles A, B, P and X were obtained by using one or two of the restriction enzymes we had used before which can recognize the different alleles (based on the first restriction patterns) to digest the plasmid DNA that contained the fragment

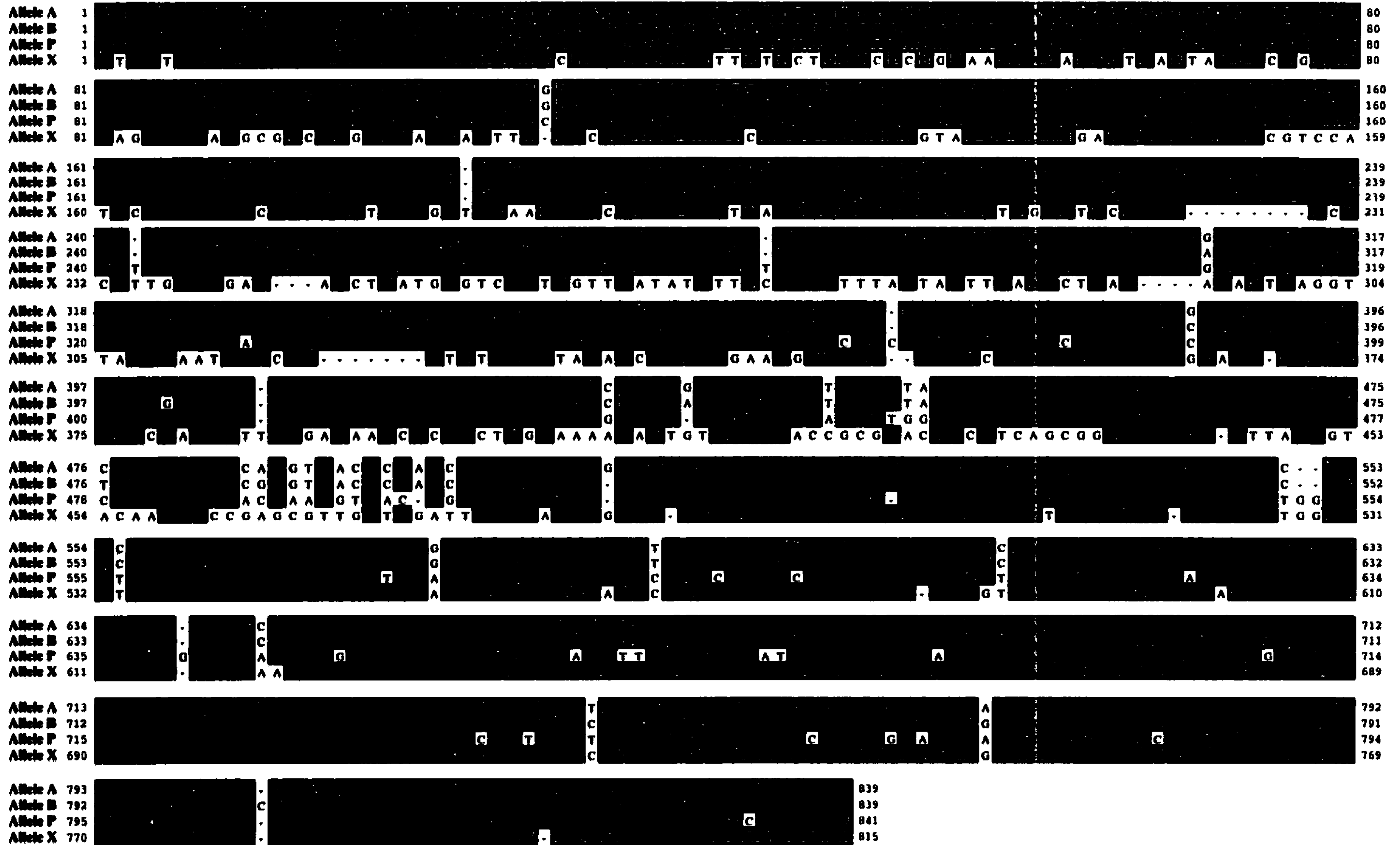
we had amplified. The exact same restriction patterns as had been observed before were found. These clones were prepared for subsequent sequencing.

2. Sequencing of the alleles

After cloning, the two alleles from heterozygous worms, which contain two different alleles such as A and B or A and X, were separated from each other. Three clones of each allele from each of two worms were chosen to sequence in order to obtain accurate sequencing information. The three sequences of each allele from each strain were compared with each other first. In general, most parts of the three sequences were identical except for the very occasional base change. Base pairs which were identical between two sequences were chosen as representative. We then compared sequences of each allele among the three strains and also compared different alleles. Most of the sequences of each allele was identical between the three strains. Only a few base pairs were different from each other. These few differences could be due to real differences between the strains or could have arisen during PCR amplification such as the fact that primer MX-D is in an intron. The sequences of allele A, B, and P had the same length while allele X was 24 bp shorter than alleles A, B, and P. The first 500 bp of sequence in allele X was quite different from the sequences of alleles A, B, and P, especially in the mid part of the sequence. The differences in the sequences of these alleles could help in making primers to differentiate between each sequence. The sequence comparison of the alleles A, B, P and X is shown in figure 11.

These sequence information will be useful for the future designing a marker that can detect the development of macrocyclic lactone resistance at an early stage by using the sensitive technique of the PCR reaction with genomic DNA from individual worms.

Figure 1. Sequence comparison of the alleles A, B, P and X



Chapter VI

Discussion

Chemotherapeutic treatment of medical, veterinary and plant parasitic nematode infections is increasingly impaired by the development of drug resistant parasite populations (Bird, 1991; Jackson, 1993). The failure of many parasitic infections to be treated effectively or cured by chemotherapeutics has led to a search for the genetic alterations in parasites which are responsible for resistance to cytotoxic drugs. Anthelmintic resistance is a significant and growing production problem in the animal industries in many countries of the world.

The mechanism of anthelmintic resistance in nematodes is remarkably complex. Benzimidazole resistance in *H. contortus* is caused by alterations of β-tubulin genes (Lubega & Prichard, 1990; 1991a; 1991b). In a study of benzimidazole resistance in *H. contortus* it was also found that BZ-R correlated in some cases with the selection for one specific allele of β-tubulin isotype 1 locus (Roos, 1990; Roos *et al.*, 1990; Kwa *et al.*, 1993b; Beech *et al.*, 1994). The frequency distribution of alleles was significantly different between the unselected and selected strains, but not between two BZ-selected strains (Beech *et al.*, 1994). Binding experiments have shown that an alteration in the acetylcholine receptor is associated with levamisole resistance in *H. contortus* (Sangster, 1996). The mechanisms of resistance to the macrocyclic lactone drugs in *H. contortus* are still not clear. Recent findings in our group suggest that resistance to an anthelmintic drug may be developed by multiple mechanisms. Blackhall *et al* (1998b) showed that allele frequency changes in a gene encoding a putative alpha subunit of the glutamate-gated chloride channel from ivermectin- and moxidectin-

selected strains of *H. contortus* were associated with ivermectin and moxidectin resistance. In addition, the multidrug resistance protein, P-glycoprotein, was investigated in recent years, and found to contribute to ivermectin resistance in *H. contortus* (Xu *et al.*, 1998). Pgp has already been well characterized in mammalian cells to recognize and transport various structurally and functionally unrelated drugs from inside cells to the outside of cells (see [Gottesman & Pastan, 1993] for review). Pgp has also been shown to be involved in drug resistance in some parasitic protozoa, such as *Leishmania donovani* (Hendrickson *et al.*, 1993; Legare *et al.*, 1994); *Entamoeba histolytica* (Descoteaux *et al.*, 1995) and *Plasmodium falciparum* (Foote *et al.*, 1989). In *C. elegans*, a Pgp, which may lead to drug resistance, has also been reported (Broeks *et al.*, 1995; 1996). Evidence indicated that Pgp may be involved in some cases of drug resistance in arthropods. An insecticide has been shown to be a substrate for rat Pgp (Lanning *et al.*, 1996). Since BZs can act as substrates for human Pgp (Nare *et al.*, 1994), Pgp may also contribute to BZ resistance in nematodes and has been investigated in our group. Blackhall *et al* (1998c) analyzed the genetic variation in a Pgp gene from cambendazole sensitive and cambendazole selected, and a thiabendazole selected strain of *H. contortus* and suggested that Pgp may contribute to resistance to classes of anthelmintics other than the macrocyclic lactones.

However, relatively little information is available concerning the biochemical and molecular mechanisms that contribute to IVM and MOX resistance in nematodes. Our study of the genetic variation of the P-glycoprotein gene, using unselected and IVM- and MOX-selected strains, provides further evidence that Pgp is associated with IVM and MOX resistance in the nematode *H. contortus*, but not proof that Pgp is involved in the mechanism of resistance to these drugs. We have compared the total number of alleles among the three strains and the high level of genetic variability present in the unselected and ivermectin- and moxidectin-selected *H. contortus* strains. The susceptible population was highly

heterogeneous with respect to the 876 bp fragment. It suggested that the allele variation is greater in *H. contortus* populations before a macrocyclic lactone anthelmintic is used. Restriction Fragment Length Polymorphism data demonstrated that selection for resistance correlated with a selection for one specific allele of the Pgp locus. Allele frequencies at the Pgp locus change significantly under selection for macrocyclic lactone resistance. Variability was greatly reduced in the number of alleles observed in the two selected strains. In both selected strains, similar changes in allele frequencies had occurred, with allele A increasing greatly in frequency. It indicated that allele A is strongly associated with ivermectin and moxidectin resistance in the nematode *H. contortus*. It should be noted, however, that allele A is present in the susceptible population but at a low frequency before IVM or MOX are applied. It was clearly shown that the selection for IVM- and MOX-resistance was associated with a significant increase in the frequency of one of the alleles already presented in the susceptible population. This was also found in the study of Merck unselected and IVM selected *H. contortus* strains (Blackhall *et al.*, 1998a). These data are very important for investigation into the genetic changes associated with the development of IVM and MOX resistance. Other alleles such as X, R, S, and V appeared to be related to macrocyclic lactone susceptibility. Statistic analysis showed that there was a highly significant difference in the frequencies of alleles between both drug selected strains and the unselected strain. Based on these results, allele A and allele X could be used as diagnostic markers for detecting the development of IVM and MOX resistance in *H. contortus* in the future.

How Pgp is involved in ivermectin and moxidectin resistance in *H. contortus* is not clear. There is clear evidence in mammalian cells that Pgp can transport various hydrophobic drugs from the inside of the cells to the out side of the cells. Studies have demonstrated that ivermectin is a good substrate for Pgp transport in mammalian cells (Didier & Loor, 1996; Pouliot *et al.*, 1997). These data, taken together, suggested that Pgp may transport IVM and

MOX mainly from the lipid bilayer and cytoplasm of the nematode cells before they exert their toxicity, thus decreasing the concentration of IVM in the cell membrane.

A DNA based assay for the alleles which were associated with resistance is potentially a useful tool for the detection of IVM and MOX resistance in *H. contortus*. PCR uses small amounts of DNA (e.g., 10-50 ng DNA from individual worms) and less than the amounts necessary for restriction fragment length polymorphism analysis by Southern hybridization (5 μg) (Radford *et al.*, 1993). Restriction enzyme digestion is an indirect approach to estimate the variation at the nucleotide level. Without question, nucleic acid sequencing represents the highest level of resolution for studies of genetic variation. Allele sequencing will reveal the nucleotide differences between alleles (Kreitmom, 1983). The sequences of the alleles which are associated with susceptibility and ivermectin and moxidectin resistance will provide information to obtain better markers by using a sensitive diagnostic technique, such as PCR, to test the spread of the ivermectin resistance in the field. The PCR technique can be used to multiply a fragment of the genome which allows the use of very little starting material, such as the diagnosis of a single worm. Therefore, molecular techniques are expected to offer many possibilities in diagnosis of anthelmintic resistance and in studying the mechanisms of anthelmintic resistance against different drugs. If resistance in an *H. contortus* population can be detected when an allele (which is associated with resistance) is at a low frequency, then alternative control measures (such as the use of a narrow spectrum drug) could be used to kill the specific resistant population and extend the life of the broad spectrum class. This has important practical consequences for the future.

This genetic study on both selected populations and an unselected population showed that the allele A at the Pgp locus is associated with selection for IVM and MOX resistance. The findings from this study will allow an understanding of the evolutionary response of the parasite to drug treatment and give insight into the molecular mechanisms of macrocyclic

lactone resistance in *H. contortus*. Allele A which has a high frequency in drug selected strains can be a marker in the future to detect the development of resistance at an early stage, to help minimize the frequency of the resistance alleles in parasite populations and provide a warning for the proper use of anthelmintics or more effective and appropriate drug administration strategies.

The usual approach for the laboratory selection of resistance strains is to infect sheep and recover the worms' eggs surviving treatment. These are cultured infective larvae under controlled conditions and used to infect sheep for the next round of selection. Usually more than 10 selection cycles are required. It is possible that this kind of protocol exerts different selection pressures on the worms and that isolates derived in this way differ from field isolates (Martin, 1990; Le Jambre *et al.*, 1995). This genetic study was only done with laboratory strains.

While BZ resistance in *H. contortus* is associated with the selection for specific alleles of β-tubulin genes, Pgp may also be involved. Blackhall found a specific Pgp allele associated with BZ resistance suggesting Pgp may be involved in a MDR phenotype (Blackhall, personal communication). Based on these findings, if the Pgp gene is involved in ivermectin resistance in *H. contortus* , allele frequency changes during the development of ivermectin resistance should be detectable. One or more alleles, which are associated with resistance may be already present in this nematode as a result of previous selection by BZs.

The fact that anthelmintics with novel modes of action may not be available in a short period of time underlines the need to regard existing anthelmintics as extremely valuable resources in worm control in the foreseeable future. Early diagnosis of the development of drug resistance by means of sensitive and reliable tests may provide tools for developing

strategic drug treatment programs, which may delay or prevent the selection for irreversible resistance, such as the rotation of drugs with different modes of action.

Summary

This genetic population study using molecular techniques has provided important information for investigation of the mechanisms of resistance to ivermectin and moxidectin in the sheep nematode, *H. contortus* and the spread of ivermectin and moxidectin resistance in parasitic nematode populations. Our results provided further evidence that Pgp may play a role in ivermectin resistance in *H. contortus* in sheep. The RFLP data indicated that allele A is associated with ivermectin and moxidectin resistance and allele X is associated with susceptibility in *H. contortus*. This has practical implications. It can be used to develop markers to diagnose the spread of macrocyclic lactone resistance at an early stage and provide the information for farmers and veterinarians to develop strategies for controlling drug resistance and reducing economic losses and prolonging the life of anthelmintic drugs.

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