Chapter 1

Introduction

Infestations of domestic sheep by intestinal parasitic nematodes are controlled by the administration of anthelmintic drugs. The ability to maintain effective control of parasitism is being seriously threatened by the widespread and apparently inevitable development of resistance. The parasite species of major economic significance in Australia are *Haemonchus contortus, Trichostrongylus colubriformis* and *Ostertagia circumcincta.* Reports of resistance in one or more species to the three classes of broad spectrum anthelmintics, benzimidazoles (BZ), levamisole/morantel and avermectins/milbemycins, are available world wide (Waller, 1985).

The enormity of the resistance problem and the low priority given to the development of new classes of anthelmintics by pharmaceutical companies has generated interest in developing alternative strategies for the control of parasitic infestation in sheep. These include the development of vaccines, selection of breed lines displaying increased immunological responsiveness to helminth infestation and searching for molecular markers associated with disease resistance traits to facilitate identification of useful individuals for breeding programs. Useful outcomes that will be available for rapid adoption by the sheep pastoral industries are not imminent so chemical control remains the only viable option at the present time and susceptibility to the currently available anthelmintics is seen as a resource to be preserved (Grant & Le Jambre, 1993).

The impact of the development of resistance has been reduced by the adoption of integrated pest management schemes, such as "Wormkill" and "Drenchplan", which by taking into account the epidemiology of the nematodes of major importance, *H. contortus*, *T. colubriformis* and *O. circumcincta*, reduce the number of times a drug is administered to those times of the year when the availability of infective larvae is lowest, thus reducing the chance of reinfestation. Using the different classes of anthelmintics in annual rotations or as mixtures have also been tested as a means of slowing or reversing the affects of resistance. However, the lack of sensitive assays to determine the impact of such rotations has made it impossible to accurately assess the long term impact of

such strategies. The assays available for the detection of resistance are all phenotypic assays and are limited either by impracticality or lack of sensitivity.

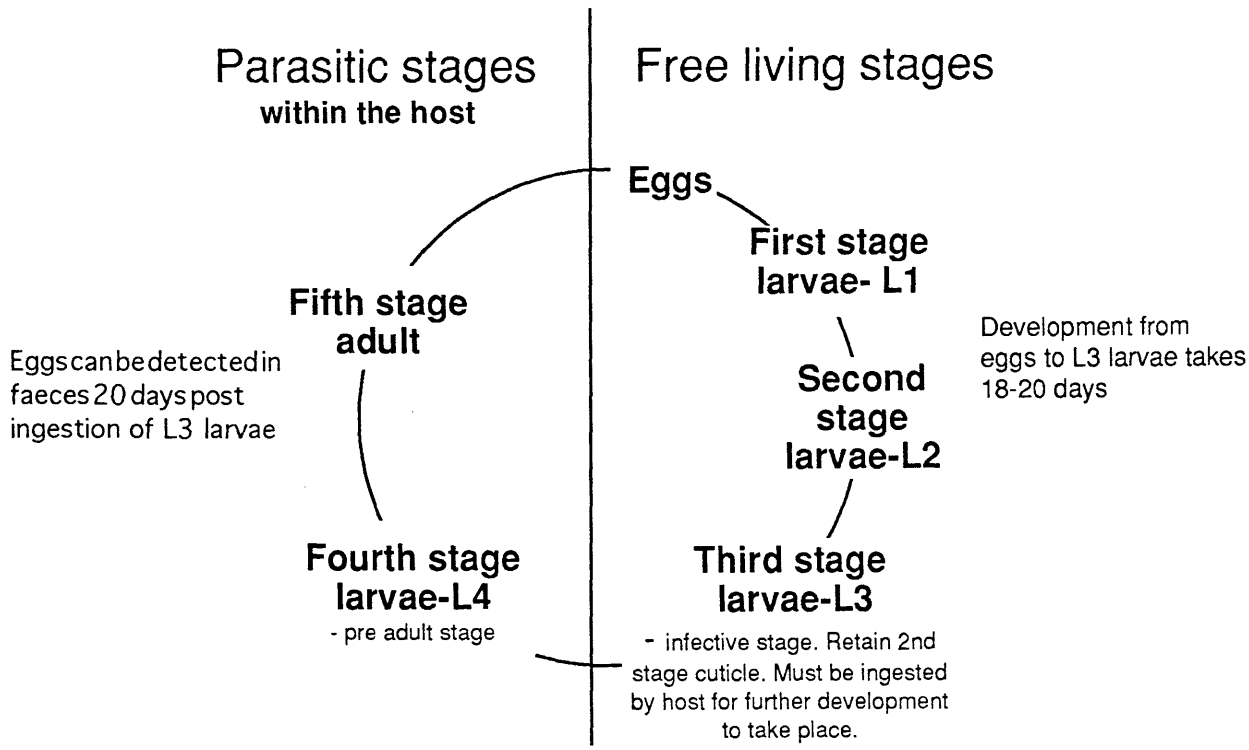
Sophisticated mathematical models, such as that developed by Barnes and Dobson (1990), attempt to predict the long term impact of various control strategies but are also limited by the lack of real biological information. A range of hypothetical values can be entered into the models and hence a range of predictions can be produced but without experimental data there is no way of assessing which prediction is realistic. Use of such models can only reach full potential as management tools when experimentally determined values such as allele numbers and frequencies are included.

One of the principle conclusions of a major symposium convened by CSIRO in 1985 addressing the issue of anthelmintic resistance was that there was a primary need to develop more reliable, sensitive and convenient ways of assaying for anthelmintic resistance. It was also suggested that the best way to develop such assays was to increase the understanding of the genetic and biochemical basis of resistance and that the greatest need in monitoring the affects of various control strategies involving chemotherapy was the ability to detect resistance genes at low frequency (Donald, 1985). This thesis describes the development of an assay that can determine the benzimidazole resistance genotype of individual *T. colubriformis* nematodes. The assay provides a means of detecting resistance alleles when they are present at low frequencies and accurately detects changes in resistance allele frequency associated with changes in anthelmintic efficacy. The *T. colubriformis* species was chosen for this work because of the availability of well characterised resistant and susceptible strains and to compliment work being done elsewhere on BZ resistance genes in *H. contortus*.

Life Cycle

The life cycles of *T. colubriformis*, *H. contortus* and *O. circumcincta* are very similar, differing only in the optimum conditions for larval development and survival on pasture and the site of predilection in the host. Consequently the life cycle of *T. colubriformis* will be described here and can be taken as a general description of the life cycles of the other major species.

T. colubriformis is a parasite of sheep, cattle and goats. It has also been found in camels, gazelles, antelopes and occasionally in the duodenum and stomach of man and some monkeys. It is classed (along with *Haemonchus, Ostertagia* and


Parasitic stages
within the host
Free living stages
Eggs
First stage larvae- L1
Second stage larvae-L2
Third stage larvae-L3
- infective stage. Retain 2nd stage cuticle. Must be ingested by host for further development to take place.
Fourth stage larvae-L4
- pre adult stage
Fifth stage adult
Eggs can be detected in faeces 20 days post ingestion of L3 larvae
Development from eggs to L3 larvae takes 18-20 days

Cooperia species) in the family *Trichostrongylidae* (Clunies-Ross & Gordon, 1936).

The life cycle (Figure 1) begins with the deposition of eggs on pasture in the faeces of sheep infected with adult worms. The free living stage of the nematode is directly affected by environmental conditions, especially temperature and moisture. Hatching of eggs and development of larvae is restricted to those months with a mean air temperature above 10°C. Also, adequate moisture needs to be present both for development of eggs and larvae. Embryonated eggs can withstand desiccation and will hatch when adequate moisture is available. Under favourable conditions eggs will hatch into first stage larvae in one to four days. First stage larvae are small (480 microns), rodlike and feed on bacteria. After a period of growth the larvae moult to second stage larvae. These also feed and grow in the faecal pellet then develop into specialised third stage larvae which are infective to the host. These larvae retain the second stage sheath and must be ingested by the host for further development to take place. Infective larvae are more resistant to adverse conditions than are early larval stages but the majority are destroyed by very high temperatures, desiccation and direct sunlight (Cole, 1986).

Infective larvae are casually ingested by the grazing host and the second stage sheath is lost while in the rumen. On reaching their intestinal habitat (the upper small intestine), third stage larvae moult to fourth stage larvae which subsequently undergo an additional moult to produce the adult nematode. Egg production starts approximately 21 days after infection has occurred (Clunies-Ross & Gordon, 1936).

The principle determinant of infection rate of the host is the number of viable larvae on pasture. The integrated control strategies ("Wormkill" and "Drench plan" mentioned above) have taken this factor into account and treatment with anthelmintics coupled with movement of stock onto decontaminated pasture immediately post treatment is recommended for those times of the year when the availability of infective larvae is lowest.

Distribution of T. colubriformis

The *Trichostrongylus spp.* can be found in sheep grazing areas throughout the world. These nematodes are of serious economic importance in Australia, Europe, the United Kingdom, the United States of America and South Africa. In Australia *Trichostrongylus* spp. are a problem in the southern winter rainfall areas, as well as central and northern NSW. A problem may also arise on the

Western slopes of NSW if seasonal rainfall is high. Sporadic infestations occur also in Southern Queensland (Clunies-Ross & Gordon, 1936). The species *T. colubriformis* is found mainly in the summer rainfall areas of Australia, whereas *T. vitrinus* and *T. rugatus*. occur mainly in the winter rainfall areas. Another species, *T. axei*, is of minor importance as it mainly affects cattle and can be found in both areas (Cole, 1986).

Pathogenic Importance

The most serious effects of *Trichostrongylus* spp. infestations occur in lambs from weaning to 12 months of age. Although adult sheep may carry heavy infestations, symptoms are rarely seen in sheep over 18 months of age as they develop immunity after exposure to infection (Clunies-Ross & Gordon, 1936; Dineen, 1978). Subclinical infections, however, can reduce wool production by 25% in adult sheep (Barger & Southcott, 1975). Disease symptoms range from a significant subclinical inefficiency of production to clinical disease characterised by soft faeces, intermittent or continued diarrhoea, weight loss, listlessness and osteoporosis. Severely affected animals become dehydrated and some deaths may occur (Clunies-Ross & Gordon, 1936).

The adult worms graze the gut mucosa and are found in tunnels beneath the epithelial cells of the small intestine. These lesions are characterised by mucosal and villous atrophy or flattening and sparse stunted microvilli, epithelial hyperplasia. Infiltration of lymphocytes and neutrophils into the damaged area has been observed. This reduces effective gland mass, upsets gut enzymes and hormones all leading to progressive inhibition of gut motility which decreases the movement of digesta. Resulting inappetence reduces availability of energy for maintenance and growth (Symons & Steel, 1978) It is not understood if these effects are due to toxic substances from the worm itself or as a result of non expulsive immune response.

Anthelmintics

There are three classes of broad spectrum anthelmintics. These are the avermectins/milbemycins, levamisole/morantel and the benzimidazoles. Other narrow spectrum anthelmintics are available but will not be discussed.

(i) **Levamisole** Reports indicate that levamisole resistance in *T. colubriformis* and *O. circumcincta* is widespread but resistance is not as common for *H. contortus* (Donald, 1983; Waller, 1985). The basis for this difference is unknown. Levamisole is a ganglion stimulant, acetylcholine-like agonist causing

muscle contraction and paralysis in nematodes (Sangster, Riley & Collins, 1988). Higher concentrations, not likely to be experienced *in vivo*, inhibit the fumarate reductase system (involved in carbohydrate metabolism and energy production) in some nematodes (Behm & Bryant, 1985). Resistance in *T. colubriformis* is reported to be controlled by a single recessive gene on the X chromosome (Martin & McKenzie, 1990)

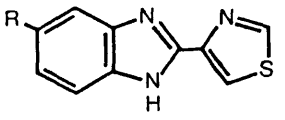
(ii) **Avermectin/milbemycin.** Avermectin is a naturally occurring compound produced by the fungus *Streptomyces avermitilis*. The commercially available product, ivermectin, is a semisynthetic derivative of avermectin. The mode of action of ivermectin is unclear. Arena (Arena, Liu, Paress, Schaeffer & Cully, 1992) reported that the anthelmintic activity of avermectin was mediated via an interaction with a glutamate-gated chloride ion channel. The mechanism of resistance in nematodes is unknown but is unlikely to involve the receptor directly (Shoop pers. comm.). There have already been several reports of ivermectin resistance. A resistant strain of *O. circumcincta* has been isolated in New Zealand (Craig & Miller, 1990) and resistant strains of *Haemonchus contortus* have been isolated on a CSIRO research station (Le Jambre pers. comm.), in South Africa (van Wyk, Malan, Gerber & Alves, 1989) and South America (Echevarria & Trinidade, 1989; Vieira, Berne, Cavalcante & Costa, 1992).

(iii) **Benzimidazoles** The first BZ, thiabendazole, was released in Australia in 1962 and was the first highly efficient broad spectrum anthelmintic. BZ resistance developed rapidly and is widespread in Australia and has been reported from all continents (Donald, 1983; Waller, 1985).

The benzimidazole and benzimidazole-carbamate anthelmintics (figure 2) are based on the same parent molecule, a bicyclic ring structure in which benzene has been fused to the 4- and 5- position of an imidazole heterocycle (Townsend & Wise, 1990). This basic ring structure has no efficacy but various substitutions at the 2- and 5- positions have given rise to a number of clinically and agriculturally useful drugs (Ireland, Gull, Gutteridge, & Pogson, 1979; Townsend & Wise, 1990). Benomyl and thiabendazole are fungicides; albendazole, cambendazole, fenbendazole, mebendazole oxfendazole, oxibendazole, parbendazole and thiabendazole are anthelmintics; nocodazole is an experimental antineoplastic agent (Ireland *et al.*, 1979).

Figure 2. Chemical structure of the benzimidazoles and benzimidazole carbamates (reproduced from Townsend and Wise, 1990).

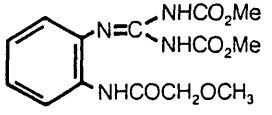
Benzimidazoles



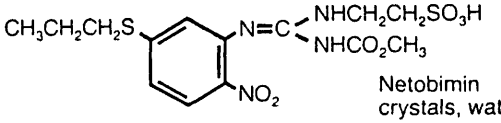
R = H Thiabendazole
crystals, mp 304°C
insoluble in water, slightly soluble in alcohol

R = $(CH_3)_2CHOCONH$- Cambendazole
crystals, mp 238°C
insoluble in water, soluble in alcohol

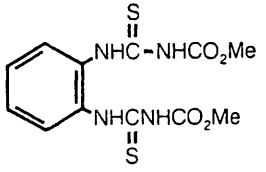
Prodrugs



Febantel
crystals, mp 129°C

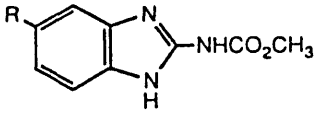


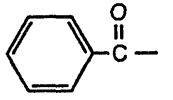
Netobimin
crystals, water soluble

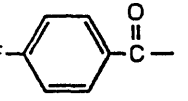


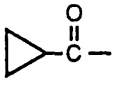
Thiophanate (methyl)
crystals, soluble in alcohol

Benzimidazole Carbamates



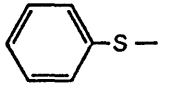
R =  Mebendazole[10]
crystals, acetic acid, mp 288°C
insoluble in water

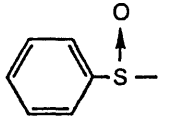
R = F-  Flubendazole[10]
crystals, mp 260°C
insoluble in water

R =  Ciclobendazole[10]
crystals, mp 250°C

R = $CH_3CH_2CH_2S$— Albendazole[10]
crystals, mp 208°C

R = $CH_3CH_2CH_2O$— Oxibendazole[12]
crystals, mp 230°C
practically insoluble in water

R =  Fenbendazole[13]
powder, mp 233°C
insoluble in water

R =  Oxfendazole[14]
crystals, mp 253°C

R = $CH_3CH_2CH_2CH_2$— Parbendazole[9] crystals,
mp 225°C, insoluble in water

Current Methods for Detecting Resistance

There are two classes of assays available for the detection of drug resistance in parasitic nematodes. They are *in vivo* assays and *in vitro* assays (summarised in table 1). Both classes are phenotypic assays and depend on the expression of the resistance phenotype in the stage, whether that be egg, larvae or adult, of the nematode being tested. The *in vivo* anthelmintic efficiency assays are prone to variation between animals due to differences in pharmacokinetics of drug metabolism between animals or variable parasite burdens and parasite fecundity. Taking these effects into account in the data analysis reduces the sensitivity of these assays. *In vitro* assays such as the egg hatch assay, tubulin binding assay and the larval development assay eliminate the effects of the role of host variation as a source of experimental error (Lacey, 1988) but are based upon certain assumptions; (i) the site of action of the drug in eggs or developing larvae are similar to that in the adult nematode; (ii) the drug metabolite used for the assay represents the sole *in vivo* active compound and (iii) comparable dynamics of drug availability *in vitro* and *in vivo* are achieved.

The sensitivity of the faecal egg count reduction test, the *in vitro* egg hatch assay and the tubulin binding assay have been compared by Martin (Martin, Anderson & Jarrett, 1989). These were compared by preparing composite strains of *O. circumcincta* consisting of 0, 1, 10, 25, 50, 75, 90, and 100% of known resistant strains. All tests detected resistance where the resistant strain made up 50% of the mix. However no test unequivocally detected resistance below 25%. This study emphasized the need to develop more sensitive assays for detecting resistance at low levels. A review by Presidente (1985) describes the methods for each assay, except for the more recent larval development assay (LDA) (Lacey, Redwin, Gill, Demargheriti & Waller, 1990), in detail. Only those assays that have been used routinely for the detection of BZ resistance are described below.

Faecal Egg Count Reduction Test (FECRT)

This is an *in vivo* assay and is the usual field procedure for detecting and monitoring resistance. It has also been used widely in laboratory studies. The assay involves comparing faecal egg counts of animals before, and 5 and 10 days after anthelmintic treatment. Failure of the treatment to reduce egg count is an indication of resistance. However, a complication arises if there is a mixed infection as only one species may be resistant and this would not be obvious in such an assay. Presidente pointed out that the FECRT was only an estimate of anthelmintic efficiency because nematode egg output does not always correlate

Table 1. Summary of assay for detecting drug resistance (reproduced from Lacey *et al.*, 1990)

Resistance assay	Reference	Spectrum	Comments
In vivo:			
Comparative efficacy studies		All Anthelmintics	Definitive technique, expensive, time consuming, poor quality data
Faecal egg count reduction		All anthelmintics	Ideal for on-farm investigations, time consuming, poor quality data, can be misleading
In vitro			
Egg hatch	Le Jambre 1976	Most BZs	Easy to perform, poor between-laboratory reproducability (Boersema, 1982)
Egg embryonation	Coles and Simpkin 1977	Most BZs	Measures earlier stage inhibition than egg hatch. Not widely adopted.
Larval paralysis	Martin and Le Jambre 1979 Dobson *et al.*, 1987	Levamisole	Usefulness limited to LVS, timing critical to interpretation (Dobson *et al.*.)
Larval development	Coles *et al.*,. 1988	BZs, Levamisole, not Ivermectin	Assay based on use of drugs in water, larvae fed on *E. coli*
Tubulin binding	Lacey and Snowden 1988	BZs	Good reproducability, easy to perform, requires special reagents
Larval development assay II	Lacey *et al.*, 1990	All anthelmintics	Drugs incorporated into agar plates. Overcomes insolubility of Ivermectin in water
Esterase activity	Sutherland *et al., 1987*	BZs	Mechanism unknown

with actual worm numbers and it only measures the effect on mature females. Egg laying has been shown to be temporarily reduced by drug treatment for both *Ostertagia* sp. and *T. colubriformis* and the relationship between egg count and worm burden is not always linear (Presidente, 1985; Prichard, Hall, Kelly, Martin, & Donald, 1980). In contrast it has been demonstrated that egg count and numbers of worms were well correlated for *H. contortus (ref?)* If the interval after treatment is less than 10 days, the suppressive effect of BZs and levamisole on egg production causes an overestimation of drug efficiency so egg counts then need to be repeated 10 to 14 days after treatment.

In vivo *Anthelmintic Efficiency Assay*

This is considered to be the most reliable method of assessing anthelmintic sensitivity of nematodes as it is based on worm counts in groups of animals which have either been treated with drug or remain untreated. Groups of animals are treated with anthelmintic at 0.5, 1.0 and 2.0 times the recommended dose rate then slaughtered and worm counts are conducted. LD50 and LD90 curves can be determined if at least three different dose rates are used in the test (Prichard *et al.*, 1980). Resistance is generally confirmed when the reduction in worm counts is less than 90%.

This type of assay has low precision and reproducability due to inter-animal variation which alters the drug pharmacodynamics in the host and variability in the efficiency of parasite establishment within the hosts. It is also too costly, labour-intensive and time consuming to be used as a routine assay for the diagnosis of anthelmintic resistance. It has been suggested that any *in vitro* assay needs to be correlated and validated against this assay (Lacey *et al.*, 1990)

In vitro *Egg Hatch Assay*

The egg hatch assay was first described by Le Jambre (1976). BZs prevent egg embryonation and hatching. Eggs isolated from resistant worms hatch in higher concentrations of drug than eggs isolated from susceptible strains of nematodes. The assay is conducted by incubating eggs in serial dilutions of drug. The percentage of eggs that hatch at each concentration is determined and a dose response line of percentage egg hatch corrected for natural mortality from control wells, is plotted against drug concentration. An ED50 can be determined from this plot. Eggs from susceptible nematodes rarely hatch at concentrations > 0.1 micrograms/mL of TBZ. This assay is fast, inexpensive, sensitive and repeatable if a single species of nematode is involved. Eggs, however, need to be at a very early stage of development as once they have passed the ventral-indentation stage then sensitivity is reduced, possibly due to reduced permeability of the drug through the egg shell, and false positives may result. The assay also needed to be standardised as susceptible reference strains varied between laboratories and the conditions under which the assay was performed (Boersema, 1983).

Tubulin Binding Assay

The tubulin binding assay (Lacey & Snowden, 1988) quantifies the amount of radiolabelled BZ that binds to a measured amount of crude protein extracted from L3 larvae (between 25,000 to 500,000 larvae per sample). Results are expressed as a susceptibly factor; that is, the ratio of the amount of BZ binding to the resistant isolate to the amount of drug bound by a standard susceptible strain. The assay can be used routinely and shows good correlation to the resistance status of particular strains determined by the traditional faecal egg count reduction assay. However, Lacey pointed out that the technique for extraction of the protein supernatant needs to be rigorously standardised because if the ratio of tubulin to total crude protein varies from place to place and day to day or the non-tubulin proteins vary from isolate to isolate (due to fungal or other contamination) considerable error can result.

Larval Development Assay

The Larval development assay (LDA) (Lacey *et al.,* 1990) is the most versatile assay presented to date for the detection of drug resistance in parasitic nematodes of sheep and has replaced the egg hatch assay for determination of the genetic basis of drug resistance in nematodes. The assay is a microtitre plate assay where nematode eggs are added to an agar matrix containing the drug of interest. The development of the eggs through hatching and subsequent larval stages to the L3 infective stage are monitored. A dose response curve can be established for inhibition of development in *H. contortus*, *T. colubriformis* and *O. circumcincta* for three of the four major drug classes (BZs, morantel, levamisole but not Ivermectin) available as anthelmintics. It is independent of the need to know the mode of action of the particular drug, which is often not known; for example, ivermectin.

In summary, there are a number of assays available for the detection of BZ resistance all of which are phenotypic assays and rely on the expression of the resistant phenotype. Comparison of the FECRT, *in vitro* egg hatch assay and the tubulin binding assay showed that these tests could not unequivocally detect resistance until the resistance phenotype made up more than 25% of the population being tested. The *in vivo* assays suffer from genetic and environmental variation in the host animal which influences the fecundity of worms and the phenotypic expression of resistance. Therefore both laboratory and field studies would benefit by the development of sensitive molecular assays.

Biochemical Basis of BZ Resistance in Nematodes and the Molecular Basis of BZ Resistance in Lower Eukaryotes

Introduction

The development of resistance to anthelmintic compounds has stimulated research into the pharmacological and genetic basis of drug resistance in nematodes. The mode of action and the pharmacological basis of resistance for levamisole or the avermectins/ mylbemycins is not well understood. However, there is strong experimental evidence that the primary mode of action of the BZs is as inhibitors of microtubule (MT) polymerisation which is a result of drug binding to the heterodimers of tubulin (the principle protein component of MT).

Several population genetic studies have been conducted to determine the genetic basis of BZ resistance. For *T. colubriformis* resistance was reported to be influenced by more than one gene and was inherited as an incompletely recessive trait with a significant maternal effect (Martin, McKenzie, & Stone, 1988). The apparent polygenicity (multiple genes which each contribute a small component to the overall expression of resistance) has been hard to reconcile with the biochemical data (summarised in the following paragraphs) which clearly indicates alterations in tubulin as a major determinate of BZ resistance in nematodes. In contrast BZ resistance in *H. contortus* has been reported to be an autosomic, monogenic trait (Le Jambre, Royal, & Martin, 1979) despite the apparently common biochemical basis for resistance in both species (Lacey, 1988).

The application of molecular genetic techniques to the study of parasitic nematode genetics will greatly facilitate research into the molecular basis of drug resistance but little work has been done in this area. However, the molecular basis of BZ resistance has been better characterised in eukaryotic model systems such as *Aspergillus nidulans*, *Neurospora crassa* and *Caenorhabditis elegans* where in all cases mutations in genes coding for the β-tubulin component of MT have been implicated in drug resistance. This literature review describes the pharmacological and molecular information upon which the decision to investigate the role of β-tubulin genes in BZ resistance in *T. colubriformis* was based.

Biochemistry of BZ Resistance in Nematodes

Benzimidazoles are inhibitors of the *in vitro* polymerisation of microtubules in mammals (Laclette, Guerra & Zetina, 1980), nematodes (Friedman & Platzer,

1978) and fungi (Davidse & Flach, 1977). Cytological examination of the nematode *Ascaris suum* showed that exposure to BZ resulted in the disappearance of cytoplasmic microtubules in the intestinal cells of the nematode (Borgers & DeNollin, 1975). Sangster (Sangster, Prichard, & Lacey, 1985) reported the same phenomenon in *T. colubriformis*. These observations have led to the biochemical analysis of the BZ/ tubulin interaction.

Benzimidazoles appear to interact with tubulin at the colchicine binding site on tubulin dimers preventing polymerisation into microtubules. Colchicine, which is highly toxic to mammals (1-5mg/kg, in vivo; <10µM, *in vitro* cell culture) shows little toxicity in nematodes (up to 1mM) in contrast to BZs which are toxic in lower eukaryotes at 1-10mg/kg *in vivo* or 10µM *in vitro* but virtually non toxic (>50mg/kg) in mammals (Lacey, 1986).

The differential sensitivity of MT mediated functions in fungi and nematodes in comparison to higher eukaryotes and the reduction in the disruption of these functions in resistant mutants was an indication that alterations in the BZ/tubulin interaction were responsible for BZ resistance in both organisms.

The most abundant source of tubulin for biochemical studies has been the vertebrate brain: up to 20% of soluble protein extracted from the brain is tubulin due to the high density of microtubules in the axons and dendrites of nerve cells. A number of biochemical techniques have been used to determine the nature of BZ/tubulin interaction and the choice of technique largely depends on the amount of tubulin that can be obtained from the organism of interest. These have been summarised by Lacey (1986) and include: (i) inhibition or induction of polymerisation of isolated tubulin; (ii) inhibition or induction of depolymerisation of microtubules; (iii) direct binding studies with the radioactively labelled compounds using DEAE paper absorption, charcoal extraction, gel filtration or equilibrium dialysis; (iv) displacement of known labelled inhibitors by test compounds. Techniques (i) and (ii) require large amounts of tubulin and hence have mainly been applied in characterising the qualities of mammalian brain tubulin. Large amounts of tubulin cannot be obtained from nematodes and therefore techniques (iii) and (iv) have been used to characterise the BZ/ tubulin interaction in *T. colubriformis* and *H. contortus*.

The direct binding studies with radioactively labelled compounds compare the amount of drug that remains bound to protein fractions after free drug has been removed by one of the extraction techniques (for example, charcoal extraction or gel filtration). The extraction techniques can be applied at different stringencies and therefore give a measure of the "strength" of the association of

the drug and the protein fraction. To increase the stringency of such assays detergents or solvents are included in the extraction step. The ability of a drug to remain bound under these conditions usually indicates formation of covalent bonds between the drug and the protein. The BZ/tubulin interaction in nematodes is not stable under these stringent conditions and therefore covalent bonds are not formed but under the conditions imposed by charcoal extraction the interaction is not readily dissociated and therefore has been described by Lacey (1988) as a pseudo-irreversible interaction.

The interaction of colchicine and BZs with tubulin has been shown to be temperature dependent (Lubega & Prichard, 1991; Russell & Lacey, 1991). Russell reported that the extent of BZ binding in susceptible and resistant strains of nematodes, including the parasitic nematodes and the free-living nematode *C. elegans*, was temperature dependent. BZ susceptible isolates of *T. colubriformis* showed maximum charcoal stable drug binding at 37°C in contrast to resistant strains that exhibited in total a reduced level of charcoal stable binding compared to susceptible strains and a reduction in the temperature to 4-10°C for optimum drug binding. Optimum drug binding for both susceptible and resistant strains of *C. elegans* occurred at 4°C. The *in vivo* efficacy of these drugs as anthelmintics has therefore been attributed to the higher affinity of these drugs for nematode tubulin compared to the readily reversible binding to mammalian tubulin at the physiological temperature of 37°C (Russell, Gill, & Lacey, 1992)

It has been demonstrated that increased resistance to BZ in nematodes *H. contortus* and *T. colubriformis* correlates to decreased charcoal stable binding of BZ to crude supernatants of L3 larvae (Lacey & Prichard, 1986). This observation led to the development of the tubulin drug binding assay (Lacey & Snowden, 1988). Binding of BZ to crude protein extracted from nematodes shows two components; low affinity binding and high affinity binding. Partial purification of crude nematode protein into fractions using poly-lysine chromatography has allowed the high affinity binding to be ascribed to the tubulin containing fraction. Binding studies are used to determine the association constant.(Ka), a measure of strength of the interaction, and the Bmax which is a measure of the concentration of the receptor(s) per milligram of protein. Tubulin extracted from resistant strains of *T. colubriformis* and *H. contortus* shows a reduction in the amount of high affinity binding (Bmax or pmol/mg of protein) when compared to drug binding of tubulin extracted from susceptible strains. The association constant (Ka) did not differ between susceptible and resistant strains (Russell & Lacey, 1992). In addition, the altered mobility of a particular

tubulin isotype in resistant strains using 2D gel electrophoresis has been reported (Lubega & Prichard, 1991).

Lubega and Prichard (Lubega & Prichard, 1990) have also demonstrated that the amount of drug bound varies with the stage of the nematode examined; eggs bind more BZ than larvae which bind more BZ than adult worms. This is correlated with the amount of tubulin present at the particular stage as there is a gradual reduction in the amount of tubulin present from eggs to adults. Development of eggs is inhibited *in vitro* (the basis of the egg hatch assay), *in vivo* and *in utero* by exposure to BZs (embryonated eggs are less sensitive to BZ exposure due to reduced permeability of the egg shell). Developing eggs contain more tubulin than adult stages (this may be related to the developmental requirement for the mitotic spindle in the dividing cells of the egg) and bind more drug/mg of protein than larvae or adults. In resistant strains of *H. contortus* there is a reduced amount of BZ binding at all developmental stages when compared to the respective stages in the susceptible strain.

Examination of BZ binding to extracts from individual larvae from a BZ susceptible population of *H. contortus* showed that there was a range of phenotypes with respect to drug binding ranging from susceptible to that of fully resistant worms reflecting the mixture of genotypes for drug resistance present in the population prior to drug selection (Lacey, 1988).

The various BZ compounds show different anthelmintic potencies indicated by the variation in the recommended dose rates for each compound. The degree of anthelmintic potency is also reflected in the binding constants (IC50, Ka, Bmax) determined for the interaction of each compound with the tubulin fraction extracted from nematodes (Lacey, Snowden, Eagleson & Smith, 1987; Lubega & Prichard, 1991). The IC50 value is the concentration of unlabelled drug required to inhibit 50% of the labelled drug binding, the Ka and Bmax were defined above. Two compounds, oxfendazole (OFZ) and albendazole sulphoxide (ABZSO) had lower Ka values than would be expected from the observed anthelmintic potency. These two compounds are broken down by the rumen microflora into the active components, fenbendazole and albendazole respectively. Oxfendazole cannot be used in the egg hatch assay for the same reason.

In summary, BZ resistance in *T. colubriformis* and *H. contortus* is associated with a reduction in high affinity binding to tubulin extracted from eggs, larvae or adults of resistant strains when compared to tubulin extracted from susceptible strains. Associated with this reduction in drug binding is a dramatic reduction in

the temperature from 37°C in susceptible strains to 4-10°C in resistant strains at which optimum drug binding occurs. The lower optimum temperature of drug binding to tubulin extracted from resistant strains more closely resembles the optimum temperature at which tubulin extracted from more resistant organisms such as *C. elegans* and mammals takes place. The association constants of the various BZs, except those converted in the rumen to the active form, for tubulin binding reflect their anthelmintic potency *in vivo*. Therefore there is strong biochemical evidence that alterations in tubulin are involved in the mechanism of resistance to benzimidazoles in parasitic nematodes.

Molecular Genetics of BZ Resistance

Microtubules

Microtubules are proteinaceous structures found in all eukaryotic cells. No such structure has been identified in prokaryotic organisms. Microtubules are formed by the polymerisation of dimers of two highly conserved proteins α- and β-tubulin. In 1991 a report was published describing a third class of tubulins, γ-tubulin, which is found only in the spindle pole bodies of cells and appears to interact directly with β-tubulin (Oakley & Oakley, 1989).

All three classes of tubulin α, β and γ are highly conserved molecules both between each of the three classes and also between species. All three classes show the greatest degree of sequence divergence both in length and nucleotide content at the carboxy-terminus from aa 430 to the stop codon (the average size of all three classes is approximately 455 amino acids) (Little & Seehaus, 1988).

Microtubules have many functions in eukaryotic cells. They (i) form the mitotic spindle during cell division (ii) along with actin filaments make up the major components of the cell cytoskeleton maintaining cell shape (iii) are involved in cellular motility (cilia are constructed from microtubules) (iv) transport organelles and other molecules around the cell by interaction with microtubule associated proteins (MAPS) such as kinesin and dynein.

Microtubules are very labile structures and are either undergoing slow growth or rapid disassembly, a steady state is not observed. When tubulin is assembled into microtubules, linear protofilaments (pf) are formed. These protofilaments show polarity and have a plus end (the end where tubulin dimers are being added during MT growth) and a minus end. The minus end is usually embedded in a microtubule organising centre (MTOC) and the plus end of the microtubule extends out from the MTOC. MTOCs include the basal bodies or centrioles of

cilia, the centrosome in interphase cells or the spindle pole bodies in dividing cells.

Most eukaryotes have microtubules containing 13 pf (Tilney, Bryan, Bush, Fujiwara, Mooseker, Murphy *et al.*, 1973). The 13 pf are arranged in a cylindrical structure with a hollow centre. Nematodes form an exception to this generalisation with the free-living nematode *C. elegans* having 11pf microtubules and 15pf microtubules. The 15pf microtubules are unique to six cells in *C. elegans*, the touch receptor neurons and may be uniquely sensitive to colchicine (Savage, Hamelin, Culotti, Coulson, Albertson & Chalfie, 1989). *T. colubriformis* has been shown to have a mixture of both 11 and 12pf microtubules in all cells (Davis & Gull, 1983).

DNA sequence analysis shows that β-tubulin is a highly conserved molecule

β-tubulin genes have been cloned from many and diverse organisms including both higher and lower eukaryotes. Isoelectric focussing of proteins, screening with antibodies and DNA sequence analysis has shown that both α- and β-tubulin genes constitute multigene families (Cleveland & Sullivan, 1985). That is, individual organisms usually contain multiple β-tubulin genes. DNA sequence analysis of cloned genes shows that both α- and β-tubulin genes are highly conserved molecules (Little & Seehaus, 1988). The greatest degree of divergence for both classes of tubulin is seen at the extreme carboxy terminus.

The tertiary conformation of the tubulin proteins has not been determined and this has led some researchers to put forward hypotheses about the structure and function of tubulins based on sequence analysis (Burns, 1991; Burns and Surridge, 1990).

Burns (1991) has compared the sequences of conserved regions of all three tubulin classes in order to hypothesise on the structural constraints of these molecules concluding that all three are so similar that they would have similar tertiary structures. Comparison of 160 tubulin sequences, not including the variable carboxy-terminus showed homologies between α/β tubulin of 63%, α/γ 51% and between β/γ 59% (Figure 3).

Cleveland and Sutherland (1985) were able to show by detailed analysis of chicken β-tubulin genes that individual isotypes that constitute the β-tubulin family have diversified within species but that individual isotypic forms have been highly conserved between species. Consequently they were able to divide vertebrate β-tubulins into six classes according to the variable C-terminal

Figure 3. Comparison of α-, β-, and γ-tubulins, excluding the highly variable C-terminal peptide (reproduced from Burns, 1991).
(A) the amino acid sequence, in single letter code, of mouse α1, chick β1, and *Aspergillus nidulans* γ-tubulin. The insertions required for optimal alignment of the sequences are shown as dashes.

(B) Comparison of α-tubulin sequences with β- and γ-tubulins, and β-tubulin with γ-tubulin. The box indicates a residue common to the appropriate mouse α1, chick β1, or *A nidulans* γ-sequences. The circle indicates the residue can be the same in the paired comparisons by selection of an α-, β- or γ-tubulin sequence from the appropriate organism(s). The absence of a symbol indicates that the respective pairs always differ at the residue position, while the dashes indicate the insertions required to align the sequences.

(C) Comparisons of α-, β-, and γ- tubulin sequences. The box indicates an invariable residue in the analysed α-, β-, or γ- tubulin sequences; dashes indicate the insertions required to align the sequences as in A.

(D) Location of proline residues in at least one of the known tubulins

(E) Distribution of known anti α- or anti β-tubulin antibodies.

(F) The alternative models for the GTP-binding site.

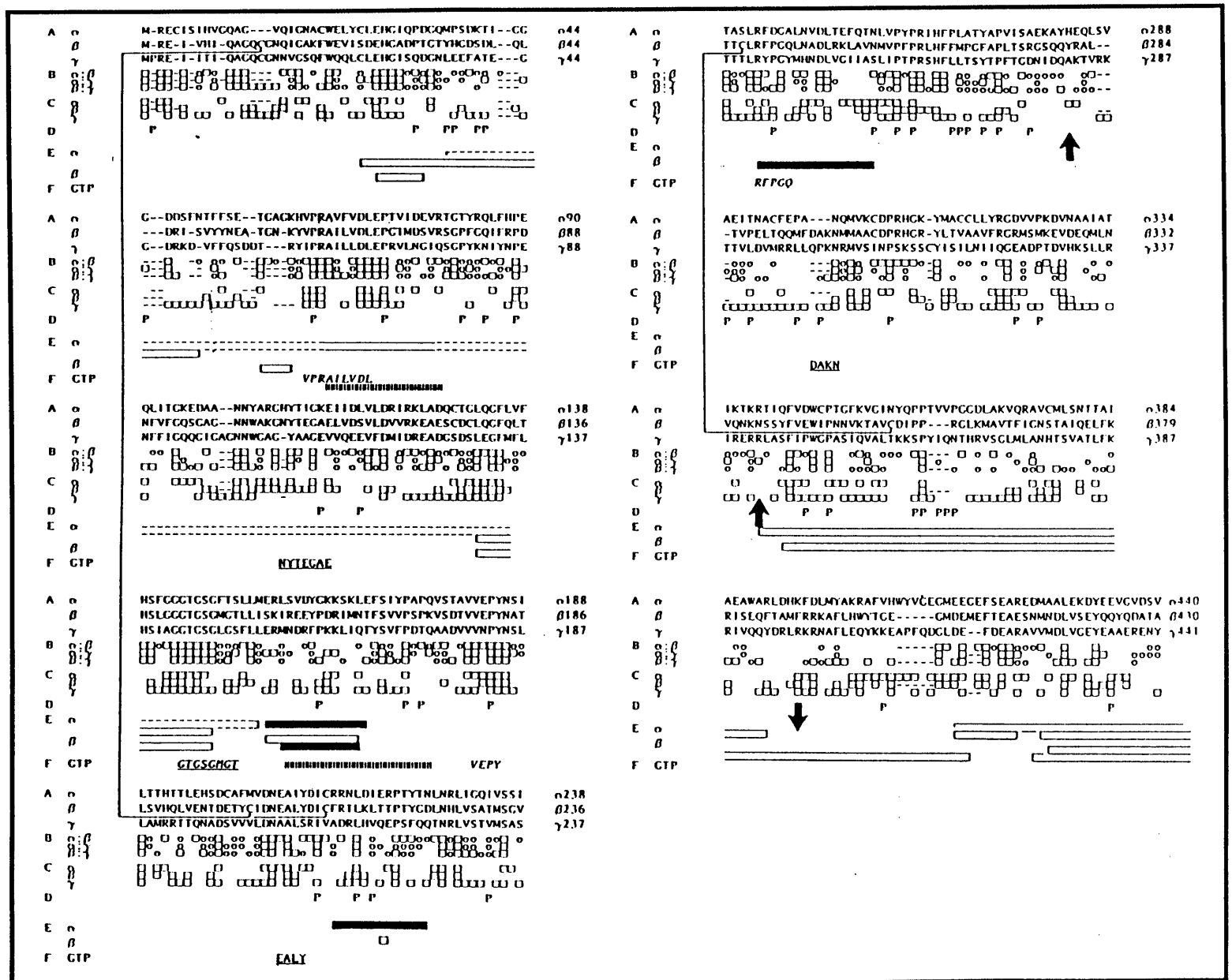
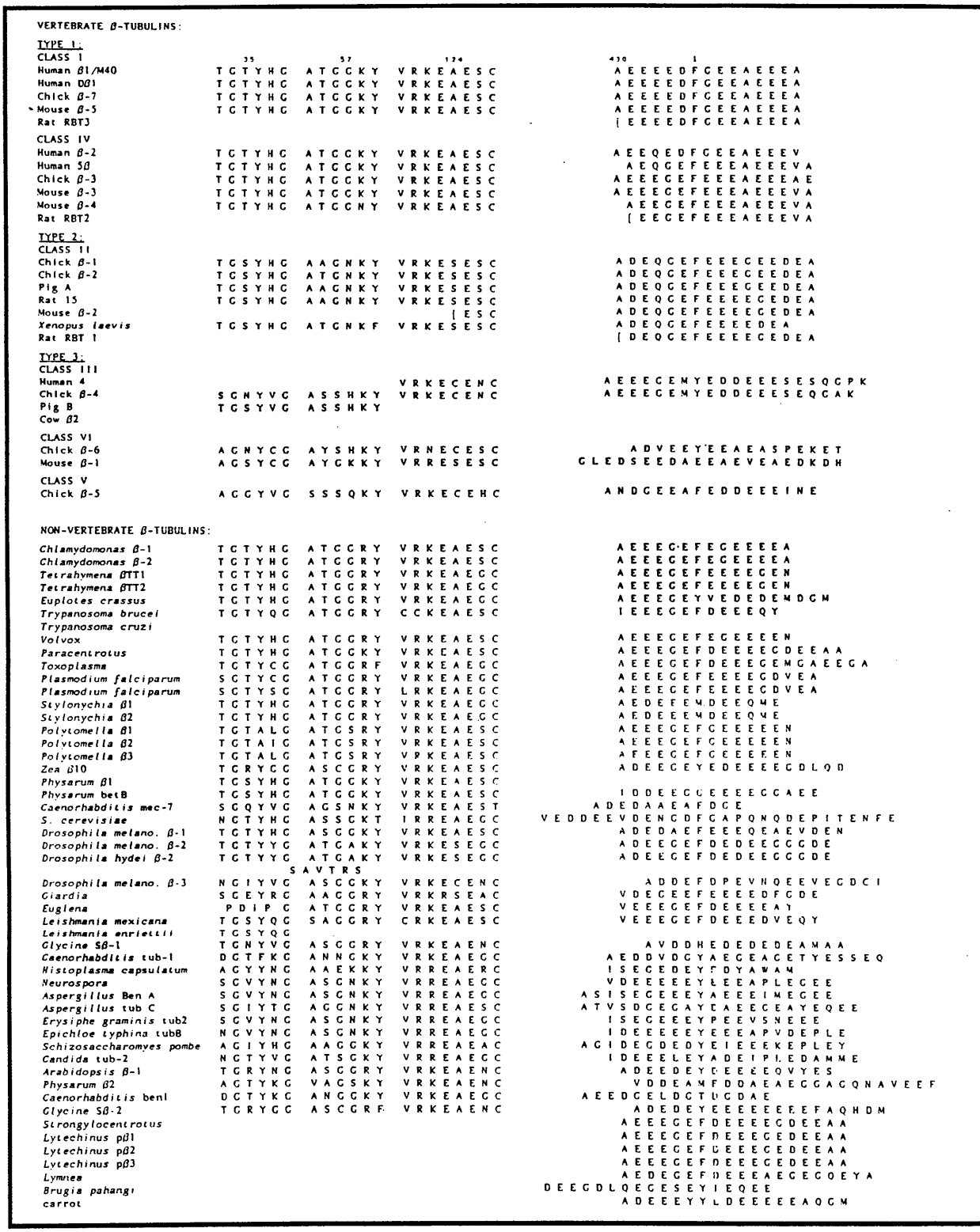

VPRAILVDL
RFPGQ
DAKN
NYTEGAE
GTGSGMGT
VEPY
EALY

Figure 4. Compilation of vertebrate and non-vertebrate β-tubulin sequences around the regions showing heterogeneity at residues 35, 55-57, and 124, along with the C-terminal peptides from amino acid 430 (the last of the conserved residues). The vertebrate sequences are aligned under the classes assigned by Cleveland. The C-terminal peptide is aligned relative, when present, to the aromatic residue (phenylalanine F or tyrosine Y) (reproduced from Burns and Surridge, 1990).


VERTEBRATE β-TUBULINS:
TYPE 1:
CLASS I
Human β1/M40
Human Dβ1
Chick β-7
Mouse β-5
Rat RBT3
CLASS IV
Human β-2
Human 5β
Chick β-3
Mouse β-3
Mouse β-4
Rat RBT2
TYPE 2:
CLASS II
Chick β-1
Chick β-2
Pig A
Rat 15
Mouse β-2
Xenopus laevis
Rat RBT 1
TYPE 3:
CLASS III
Human 4
Chick β-4
Pig B
Cow β2
CLASS VI
Chick β-6
Mouse β-1
CLASS V
Chick β-5
NON-VERTEBRATE β-TUBULINS:
Chlamydomonas β-1
Chlamydomonas β-2
Tetrahymena βTT1
Tetrahymena βTT2
Euplotes crassus
Trypanosoma brucei
Trypanosoma cruzi
Volvox
Paracentrotus
Toxoplasma
Plasmodium falciparum
Plasmodium falciparum
Stylonychia β1
Stylonychia β2
Polytomella β1
Polytomella β2
Polytomella β3
Zea β10
Physarum β1
Physarum betB
Caenorhabditis mec-7
S. cerevisiae
Drosophila melano. β-1
Drosophila melano. β-2
Drosophila hydei β-2
Drosophila melano. β-3
Giardia
Euglena
Leishmania mexicana
Leishmania enrietii
Glycine Sβ-1
Caenorhabditis tub-1
Histoplasma capsulatum
Neurospora
Aspergillus Ben A
Aspergillus tub C
Erysiphe graminis tub2
Epichloe typhina tub8
Schizosaccharomyes pombe
Candida tub-2
Arabidopsis β-1
Physarum β2
Caenorhabditis ben1
Glycine Sβ-2
Strongylocentrotus
Lytechinus pβ1
Lytechinus pβ2
Lytechinus pβ3
Lymnea
Brugia pahangi
carrot

sequences. Burns and Surridge (1990) extended this sequence analysis comparing a larger number of β-tubulin genes (25 vertebrate and 51 invertebrate sequences) showing that all β-tubulin sequences, both vertebrate and invertebrate, were highly conserved with regions of heterogeneity (also observed by Cleveland and Sutherland) confined to four regions of the gene, the carboxy-terminus, and amino acid positions 35, 55-57 and 124.(Figure 4).

In vertebrates, particular sequences at the aa35, 55-57 and 124 positions correlated with particular C-terminal variable sequence of the particular isotype class. They further noted that the variable C-terminal sequences of vertebrates always contained either a phenylalanine or tyrosine residue and that there was a strong correlation between this amino acid and the amino acids present at positions 217 and 218. Burns and Surridge modified the six classes of vertebrate β-tubulins put forward by Cleveland and Sutherland into three types according to the conservation of the sequences appearing at the four heterogeneous regions.

The invertebrate β-tubulins are also highly conserved molecules with the regions of microheterogeneity being observed at the same residues as vertebrates β-tubulins. They have not been divided into classes as have the vertebrate tubulins because the same conservation of C-terminal sequences has not been observed between species nor do the variable regions show coordinated substitutions with the same C-terminal sequences. Some invertebrate sequences; for example *Chlamydomonas, Tetrahymena* and *Trypanosoma* do, however, show similarity to the vertebrate class 1 β-tubulins in all four heterogenous regions. The conservation of an aromatic amino acid, phenylalanine or tyrosine, in the C-terminal sequence is conserved for invertebrates (figure).

Alterations in β-tubulin genes result in BZ resistance in lower eukaryotes

Compounds, such as colchicine, podophyllotoxin and the Vinca alkaloids, used as tools for the study of microtubule structure and function in higher eukaryotes (plants and mammals) show little activity in fungi therefore the demonstration that BZs interacted with fungal tubulin stimulated the use of these compounds as tools for research into the structure and function of fungal tubulins. Isolation of mutants in the fungal genetic model systems *Aspergillus nidulans* and *Neurospora crassa* resistant to BZs has led to the cloning of both α- and β-tubulin genes in lower eukaryotes. Identification at the molecular level of the genetic changes associated with BZ resistance in these organisms have demonstrated that particular changes in β-tubulin genes result in benzimidazole resistance in lower eukaryotes.

- *The fungi* Aspergillus nidulans *and* Neurospora crassa

Mutants of *A. nidulans* resistant to the antifungal antimitotic drugs benomyl and/or thiabendazole were first isolated and studied genetically by Van Tuyl (1977). Mutations conferring drug resistance mapped to three unlinked loci, *benA*, *benB*, and *benC*. Out of the 28 benomyl resistant mutations Van Tuyl analysed 26 mapped to the *benA* locus and 24 of these conferred resistance to both benomyl and TBZ. However, two alleles from the *benA* locus, *benA*16 and *benA*19, conferred resistance to TBZ and supersensitivity to benomyl. The *ben*A locus was identified as a β-tubulin gene (Sheir-Neiss, Lai & Morris, 1978). Of the 26 *benA* mutants 18 strains possessed electrophoretically abnormal β-tubulins and at least three strains were temperature sensitive for growth. The isoelectric points of mutant β-tubulins were altered by plus or minus one or two charge units. These changes were consistent with single amino acid substitutions in the β-tubulin genes (Morris, 1980; Sheir-Neiss *et al.*, 1978).

Davidse and Flach (1977) showed that radioactively labelled methyl benzimidazole carbamate (MBC) the biologically active breakdown product of benomyl had altered binding affinity for tubulin extracts of certain *benA* mutants. A mutant *benA15*, which is resistant to benomyl, had a decreased binding affinity for MBC, and *benA16*, which is resistant to TBZ but supersensitive to benomyl had an increased binding affinity for MBC.

Biochemical analysis of *A. nidulans* suggested there were two α-tubulin and two β-tubulin genes. Sheir-neiss *et al.* (1978) reported that the *benA* gene coded for two β-tubulin polypeptides as 16 out of 26 of the *benA* mutants identified by Van Tuyl that showed electrophoretically altered tubulins showed a simultaneous alteration of two tubulin peptides identified using two dimensional gel electrophoresis. A third β-tubulin peptide was identified in the *benA* mutants. The shift in the β-tubulin peptides observed in most of the *benA* mutants allowed the detection of an unaltered β-tubulin peptide (Weatherbee & Morris, 1984). This β-tubulin was demonstrated to be produced by the *tubC* gene (Davidse, 1986).

Vegetative growth of *benA* mutants is normal in the presence of benomyl except conidiation (the formation of asexual spores) is blocked. If the product of the *tubC* gene is disrupted in the benomyl resistant *benA* mutants then normal conidiation takes place in the presence of benomyl. This suggested that the *benA* products can substitute for the *tubC* product (May, Gambino, Weatherbee, & Morris, 1985) and that there is a degree of functional redundancy amongst

members of the tubulin multigene family. This is also apparent in *C. elegans* (see below).

Cloning and sequencing of the two mutants, *benA16* and *benA19*, that confer both resistance to TBZ and supersensitivity to benomyl revealed that these two mutants contain different nucleotide changes that cause the same single amino acid substitution, valine for alanine at aa 165 (Jung, 1990). Since these BZs differ only in the R2 group this suggested that the region around aa165 was involved in BZ binding.

The third class of tubulins, γ-tubulin, was first described in *Aspergillus nidulans* (Oakley & Oakley, 1989). Three mutant *mipA* alleles were isolated as extragenic suppressors of benA33, a heat sensitive β-tubulin mutation. The *mipA* (microtubule interacting protein) gene was cloned. Sequence was obtained from the wild type genomic version of *mipA* DNA and all or portions of six cDNA clones. An ORF of 454aa was identified and comparison with α- and β-tubulins revealed striking homology. There was a 32.3-35.2% homology to 29 β-tubulins from mammals, birds, insects, algae, protozoans and fungi and 28.9-31.7% homology to α-tubulins. Greatest divergence from the α-or β-tubulin genes was apparent at the C-terminal end of the gene. A later report describes the localisation of expression of γ-tubulin to the spindle pole bodies of *A. nidulans* and disruption of gene expression is lethal (Oakley, Oakley, Yoon, & Jung, 1990). Two additional reports have described the subcloning and sequencing of γ-tubulins from *Xeanopus laevis*, *Schizosaccaromyces pombe* and partial genes from maize, diatom, budding yeast (Stearns, Evans, & Kirschner, 1991), *Drosophila melanogaster* and *Homo sapiens* (Zheng, Jung, & Oakley, 1991). Both confirm that expression of γ-tubulin is localised exclusively in the spindle pole bodies of fungi or the pericentriolar material of the centrosome in animal cells. It is known that microtubule nucleation takes place in the pericentriolar material and it is proposed that γ-tubulin attaches microtubules to the spindle pole and determines microtubule polarity with the negative end proximal to the centrosome by direct interaction with β-tubulin.

Borck and Braymer (Borck & Braymer, 1974) used UV irradiation to generate benomyl resistant mutants of *N. crassa*. Fifteen out of a total of 41 resistant mutants were used for mapping. All 15 mutations mapped to a single locus. The mutants were dominant and varied in degree of tolerance to the drug. This gene was cloned and sequenced (Orbach, Porro, & Yanofsky, 1986) and resistance shown to be associated with a T- to-A transversion at base 1020 resulting in a phenylalanine to tyrosine change at amino acid position 167.

- *The slime mould* Physarum polycephalum

The life cycle of *P. polycephalum* has distinct phases including uninucleate myxamoebae, which under appropriate conditions can transform into flagellates (the flagellates can return to the myxamoebal cell type again). Myxamoebae can also develop, usually sexually, into multinucleate syncytial plasmodia. Thick walled uninucleate spores can form in the plasmodia, which under appropriate conditions germinate to produce the uninucleate myxamoebae. The myxamoebal and flagellate stages of the lifecycle are more sensitive to the action of BZs than the plasmodial phase (Davidse, 1986).

Mutations conferring resistance to BZ in *P. polycephalum* mapped to four unlinked loci, *benA, benB, benC,* and *benD* (Burland, Schedl, Gull & Dove, 1984). The mutations were expressed differentially during development. The *benA* and *benC* mutations conferred resistance only to myxamoebae. The *benB* and *benD* mutations were expressed in both the myxamoebae and plasmodia. One of the *benD* mutants, *benD210,* showed altered electrophoretic mobility for a β-tubulin on 2D gels. The *benD* locus was consequently identified as a β-tubulin structural gene and shown to be linked to the β-tubulin *betB* locus. The altered mobility of the β-tubulin from the *benD210* mutant revealed a second β-tubulin electromorph present in wild type myxamoebae. Myxamoebae were shown, therefore, to express at least two β-tubulins whereas plasmodia expressed only the one β-tubulin from the *benD* locus as only the altered electromorph in plasmodia was seen in the *benD210* mutants (Burland *et al.*, 1984).

RFLP analysis and mendelian genetics have been used to determine the number of α- and β-tubulin genes in *P. polycephalum.* Analysis of the assortment of DNA fragments homologous to β-tubulin genes among progeny indicated that there were four unlinked α-tubulin genes (*altA, altB, altC and altD*) and at least three unlinked β-tubulin loci (*betA, betB and betC*). The β-tubulin locus *betB* was shown to be allelic with the resistance locus *benD*, the *betA* locus was shown to be linked to *benA* and the α-tubulin locus *altA* was shown to be linked to the *benC* BZ resistance locus (Schedl, Owens, Dove & Burland, 1984).

- *The yeasts* Saccharomyces cerevisiae *and* Schizosaccharomyces pombe

Saccharomyces cerevisiae has only one β-tubulin (*tub2*) gene which has been cloned and sequenced. It is essential for viability. There are no introns in the 457 aa genomic sequence (Neff, Thomas, Grisafi & Botstein, 1983).

Of 173 *benR* mutants isolated by Thomas *et al.* (Thomas, Neff & Botstein, 1985) the majority mapped to the single *tub2* β-tubulin locus. Six of the mutants

were cold sensitive for growth and three temperature sensitive for growth in the absence of benomyl. Four of the cold sensitive *benR* mutants were subject to more detailed mapping. In all cases the cold sensitive and *benR* phenotypes mapped to the same restriction fragment. Sequencing of this fragment from one of these mutants showed a single base pair change from the wild type, resulting in an amino acid substitution from arginine to histidine at amino acid position 241 (Thomas *et al.*, 1985). It was concluded that this single change was responsible for both the cold sensitivity and drug resistance phenotypes.

Truncation of the 3' carboxy variable region of the *tub2* gene results in temperature sensitive growth and supersensitivity to BZs in cells expressing the truncated tubulin. The C-terminus was not necessary for viability. Over expression of the *tub2* gene, however, results in non-viable cells. The 3' variable end appeared to be necessary for viability under suboptimal conditions and therefore diversification at the carboxyl termini of β-tubulins in higher vertebrates might differently affect functions and stability of microtubules under specific conditions

BZs have also been demonstrated to inhibit nuclear division in the fission yeast *Schizosaccharomyces pombe* and subsequently mutant strains resistant to TBZ were isolated and characterised. Resistance mapped to three separate linkage groups *ben1*, *ben2* and *ben3* (Yamamoto, 1980). All the strongly TBZ-R mutants and about half of the supersensitive ones were mapped at the *nda3* locus, a cold sensitive mutant specifically arrested during nuclear division at 22°C. The *nda3* locus has been mapped to the *ben1* locus. The *nda3* gene was cloned and sequenced and found to be the single β-tubulin gene present in *Schizosaccharomyces pombe* (Hiraoka, Toda & Yanagida, 1984).

- *The nematodes* Caenorhabditis elegans, Haemonchus contortus *and* Brugia pahangi

Three β-tubulin genes (*ben-1, tub-1,* and *mec-7*) have been cloned from the free-living nematode *C. elegans* (Driscoll, Dean, Riley, Bergholz & Chalfie, 1989; Gremke, 1986; Savage *et al.*, 1989). The position of each of the three genes has been identified on the *C. elegans* physical map. The *ben-1* and *tub-1* genes are located on chromosome III and the *mec-7* gene is on the X chromosome.

The *mec-7* gene codes for the β-tubulin isotype that is present in the 15pf microtubules seen in only six cells in the worm, the touch receptor neurons (Savage *et al.*, 1989). Mutations in the *mec-7* gene render the worm touch

insensitive and cytological observation shows that the 15pf microtubules are replaced by loose or abnormal arrays of 11pf microtubules. These microtubules appear not to be affected by BZ treatment but may be uniquely sensitive to colchicine. When *C. elegans* is exposed to colchicine animals are completely touch insensitive and the touch cells lack microtubules but the animals develop at the same rate and have as many progeny as untreated controls (Chalfie & Thomson, 1982).

The function of the *tub-1* gene has not been determined. It is possible that this gene is essential for viability and mutations in this gene are either lethal or mutationally silent (no obvious phenotype is associated with its loss).

The *ben-1* gene has been identified as coding for a β-tubulin that is uniquely sensitive to benzimidazoles (Driscoll et al., 1989). In some mutants the ben-1 gene had been deleted from the genome. Animals lacking the gene appeared to be wildtype except for BZ resistance and it was concluded that the ben-1 product is functionally redundant. All mutations (28 were analysed) were dominant at 25°C. However, heterozygote resistance was not observed below 15°C nor at high drug concentrations (12μM/mL).

The *ben-1* (Driscoll *et al.*, 1989) , *mec-7* (Savage *et al.*, 1989) and *tub-1* (Gremke, 1986) genes have been cloned and sequenced. The *ben-1* gene shows 96% homology to *tub-1* gene and 92% homology to the *mec-7* gene. All three genes are highly divergent at the C-terminal end beyond amino acid 430. The *mec-7* product is one of the shortest reported β-tubulin genes.

H. contortus is another economically important parasite of sheep, closely related to *T. colubriformis*. It resides in the abomasum and is blood sucking, causing anaemia and death if unchecked. Two β-tubulin genes, designated the isotype 1 and isotype 2 genes, have been cloned and sequenced in *H. contortus* (Geary, Nulf, Favreau, Tang, Prichard, Hatzenbuhler, et al., 1992; Kwa, Venstra & Roos, 1993). As with other β-tubulin genes these genes show high sequence homology except at the 3' C-terminus where the amino acid sequences diverge.

One of these genes, the isotype 1 gene, has been clearly linked to BZ resistance in this species. Using the isotype 1 gene as probe in detailed RFLP analyses, Roos demonstrated that RFLPs associated with this gene could be detected between three independent susceptible and nine independent resistant populations of worms isolated from Europe, Britain, Africa and the USA (Roos & Boersema, 1990). Not only were RFLPs between susceptible and resistant populations of worms present but each population had its own specific banding

pattern. A number of restriction enzymes were used in the analysis. In each case the susceptible populations showed 5-10 bands depending on the enzyme used whereas the resistant populations showed only 1-2 bands. The bands present in the resistant population were also present in the susceptible populations when the RFLP patterns of susceptible and resistant strains derived from the same geographic regions were compared. This presented the possibility that no new mutations were necessary for resistance and that the gene/s that confer resistance were present in worm populations as a common polymorphism before exposure to BZs and that selection for resistance would therefore be rapid as had been observed in the field.

To further confirm these results Roos carried out a selection experiment over six generations starting from a BZ susceptible population. Using the enzyme HpaI and the isotype 1 β-tubulin gene probe the number of polymorphic bands was reduced from five to one as a result of selection. The analysis was then extended to RFLP analysis between individual worms to establish if all worms contained the same bands or if individuals contained a subset of bands. Only male worms were compared to avoid the complication of the presence of eggs. There was extensive polymorphism between individuals from the susceptible population with seven different hybridisation patterns present between 14 different susceptible males. Susceptible males showed 1-4 bands with different combinations in each. Individuals from the resistant population showed predominantly only a 9kb fragment with some individuals having an additional band (Roos, Boersema, Borgsteede, Cornelissen, Taylor & Ruitenberg, 1990).

Geary *et al.* (1992) published a report describing two *H. contortus* cDNA β-tubulin clones (b8-9 and b12-16) and RFLP analysis using these as probes. The b8-9 clone had the same sequence as the isotype 1 gene cloned by Roos. The b12-16 (isotype 2 gene) clone showed 79% homology at the nucleotide level but 93.5% at the amino acid level to the b8-9 clone. The cDNA clones differ completely at the carboxy terminus between amino acids 436-445 and under stringent conditions the cDNA clones gave distinct banding patterns. Geary *et al.* reported similar results to Roos with b8-9 showing an RFLP between the resistant and susceptible strains of *H. contortus* with the restriction enzyme SpeI. Also with extended exposure of blots bands that appeared in the resistant population were present in the susceptible population.

Two β-tubulin genes have been identified in the filarid nematode *Brugia pahangi*. One of the genes (B1) has been sequenced (Guenette, Prichard, Klein & Matlashewski, 1990) and shares 94% and 90% homology with the *ben-1* and

tub-1 β-tubulins from *C. elegans*. The second gene (Guenette *et al.*, 1990) has not been fully sequenced but shows different banding patterns to genomic DNA when compared to the B1 gene. The two genes are differentially expressed with the B2 gene being expressed predominantly in adult male worms and the B1 gene expressed in microfilariae and adult worms (Guenette, Prichard & Matlashewski, 1992). Infections by adult worms of *B. pahangi* are apparently not controlled by BZ treatment but the microfilariae are susceptible to BZs.

Tubulin extracted from adult nematodes has been shown to have a greater binding affinity for BZ than mammalian tubulin but no further information is available concerning the involvement of either of these β-tubulin genes in BZ susceptibility in this nematode.

Since there is strong pharmacological and molecular evidence for the involvement of β-tubulin in BZ resistance then it becomes the obvious choice for determining the involvement of β-tubulin genes in BZ resistance in parasitic nematodes such as *T. colubriformis* and investigating the feasibility of developing a sensitive molecular assay for detecting β-tubulin alleles associated with BZ resistance in parasitic nematodes.

Aims of This Study

The aims of this study are:

(i) to determine whether β-tubulin genes are involved in the development of BZ resistance in *T. colubriformis;*

(ii) determine the particular β-tubulin locus/loci at which selection for BZ resistance is taking place; and

(iii) use the information gained from (i) and (ii) to develop a molecular assay for BZ resistance in *T. colubriformis*.

Chapter 2

Materials and Methods

T. colubriformis Nematode Strains

T. colubriformis is an obligate parasite with a heterosexual mating system. This means that genetically homogeneous populations or strains of nematodes cannot be generated by cloning individuals *in vitro.* So, even though particular isolates of nematodes are called strains, the individuals that make up a given strain are not necessarily genetically identical. The drug susceptible strain, therefore, used in this project could contain many alleles for any given locus reflecting the genotypes of the genetically diverse individuals originally cultured to generate the strain. It has been demonstrated using RFLP analysis with random, single copy probes that particular strains of nematodes contain as much intra-strain polymorphism as inter-strain polymorphism (Grant & Whitington, 1994). Strains of the parasite used in this study were supplied as third stage larvae:

1. McMaster susceptible -This strain is BZ naive. The strain has been used by CSIRO Division of Animal Health as a standard strain for BZ susceptibility.
2. McMaster resistant- This strain was selected from the McMaster susceptible strain during the course of a laboratory selection experiment (Dobson, Griffiths, Donald, & Waller, 1987; Waller, Dobson, Donald, Griffiths & Smith, 1985). Samples from each of 21 generations of this experiment have been preserved. The twelfth generation of this experiment was examined for this piece of work.
3. NERT- New England Resistant *T. colubriformis*.- A BZ resistant strain isolated from a private property on the New England Tablelands, Australia (provided by Dr. P. Martin - CSIRO Division of Animal Health Parkville).
4. KST- Kybebolite susceptible *T. colubriformis* isolated in Victoria. This was a field strain which appeared susceptible but may have been exposed to BZs. (Martin *et al.,* 1988)
5. KRT- Kybebolite resistant *T. colubriformis* . This strain was selected from the KST strain in a laboratory selection experiment by Dr P. Martin

6. BCRT- A BZ resistant *T. colubriformis* strain isolated at the CSIRO research station at Badgery's Creek (Lacey *et al.*, 1990).
7. 8TBZ-A BZ resistant strain isolated at the CSIRO Badgery's Creek research station from an experiment where eight treatments of BZ were administered annually between 1982 and 1986 (Waller, Donald, Dobson, Lacey, Hennessy, Allerton *et al.*, 1989).
8. Arthursleigh- a BZ resistant field strain of *T. colubriformis* (Lacey *et al.*, 1990; Waller, Dobson & Haughey, 1990)

T. colubriformis Genomic Library

The library used to clone the β-tubulin clones for this study was constructed using the McMaster susceptible strain described above and donated by Dr Keith Savin from CSIRO Biomolecular Engineering.

The EMBL3 Lambda vector was used for the construction of the genomic library. The EMBL3 vector is a replacement vector where the portion of the lambda genome not required for replication and packaging of the lambda chromosome into infective phage particles is replaced by DNA from a different source; in this case *T. colubriformis* genomic DNA fragments. The cloned fragments are flanked by polylinkers in the EMBL3 vector that are in inverse orientation (Sal1-BamH1-EcoR1-central fragment-EcoR1-BamH1-Sal1). A 'complete' library is obtained by cutting high molecular weight DNA in a pseudo-random manner by partial cleavage with a restriction enzyme, usually Sau3A, that cuts frequently compared with the desired insert size. Using Sau3A to digest the genomic DNA also generates cohesive ends that are compatible with the BamHI site in the vector arms. Consequently an overlapping set of DNA fragments, representative to a first approximation of the entire genome is obtained.

Only phage genomes in the 40-52kb range are packaged into phage particles with high efficiency. To avoid packaging of non-contiguous (chimeric) clones the exogenous DNA is fractionated for size and only large fragments purified for cloning into the vector; therefore chimeric clones will be oversized and not packaged. Desirable insert size is between 18-22 kb however fragments can be from 9-22kb. Cloned fragments can be released from the Lambda vector arms by restriction enzyme digestion with the enzymes SalI, EcoRI and sometimes with Bam HI (the BamHI site being reconstituted after cloning one in six on average) (Sambrook, Fritsch & Maniatis, 1989)

Plasmid Clones

TABLE 2: Plasmid clones generated during the course of this project

Clone name	Insert size	Cloning site in mcs	Description
pWG 21	12kb	SalI	original β-tubulin genomic clone. 12kb fragment cloned from the lambda clone 1.5a
pWG 22	7.5kb	SalI	β-tubulin fragment cloned from lambda clone 2.9-additional *tcb-1* clone
pWG 23	8kb	SalI	β-tubulin fragment cloned from lambda clone 2.3. Represents an additional allele of the *tcb-1* locus
pWG 24		SalI	β-tubulin fragment cloned from lambda clone 3.5. Represents the *tcb-2* locus
pWG 30		SalI	β-tubulin fragment subcloned from the lambda clone 2.1. Represents the *tcb-3* locus
pWG 31		SalI	β-tubulin fragment subcloned from the lambda clone 2.12. Represents the *tcb-4* locus
pWG 34	2.9kb	PstI	Subcloned from the pWG21 clone. This clone was used for partial sequencing of the *tcb-1* gene
pWG 35	1.4kb	PstI-EcoRV	Subcloned from the pWG34 clone. Contains coding and intervening sequence from aa 44 to aa175 of the *tcb-1* locus
pWG 36	1.1kb	EcoRV	ScaI fragment subcloned from pWG34. contains coding sequence and intervening sequences from aa 55 to approximately aa 324. Used in the original RFLP analysis
pWG 37	0.8kb	PstI-EcoRV	ScaI-PstI fragment subcloned from pWG34. Contains coding and intervening sequence from aa 348 into the 3' noncoding region of the *tcb-1* locus

T. colubriformis tcb-1 β-tubulin Clone

A putative β-tubulin clone from *T. colubriformis* designated pWG21 was supplied by Dr Warwick Grant. The clone was obtained by screening the *T. colubriformis* genomic library at low stringency with a 1.25kb SalI fragment from a *C. elegans* β-tubulin clone of the *tub-1* locus. The *T. colubriformis* pWG21 clone consists of a 12kb SalI insert in the Bluescript SK+ (Stratagene) plasmid vector.

T. colubriformis tcb-2 β-tubulin cDNA Clone

A cDNA clone of a β-tubulin gene designated *tcb-2* was donated by Le Jambre and Lenane (CSIRO Pastoral Research Laboratory-Armidale NSW).

Bacterial Strains

The following bacterial strains were used as noted:

1. P2392-(LE 392 (P2 lysogen))

This was used for growth of the lambda library. Fresh overnight culture was used for each manipulation of phage. Cells were grown at 37°C in NZCYM media supplemented with 0.2% maltose (the phage tails bind to the *lam*B (maltose-binding receptor protein) on the bacterial cells, which is induced by including maltose in the growth media). Cells were grown overnight in 20mL volumes then spun down and resuspended in half the original culture volume of 10mM $MgSO_4$.

2. XL-1 Blue (recA1,endA1,gyrA96,thi-1,hsdR17,supE44,relA1,lac,[F'proAB,lacIq ZΔ M15,Tn10(tet^r)])

This was used as the host strain for transformation of plasmid.

3. DH-5α (F-,phi80dlacZΔM15,recA1,endA1,gyrA96,thi-1,hsdR17(r_K,m_{K+}), supE44,relA1,deoR,Δ(lacZYA-argF)U169)

Used also for transformation.

DNA Preparation

Genomic DNA

Genomic DNA was prepared from L3 stage larvae. Larvae were cleaned by allowing them to wriggle through muslin suspended in a beaker of water. After the larvae settled at the bottom of the beaker, the water was decanted and larvae

transferred to a 15mL Falcon tube. Larvae were compacted by centrifugation at 3000rpm. Usually 0.5 to 1.0 mL of compacted larvae were digested each time. Larvae were digested at 65°C for at least two hours in 5mL of worm lysis buffer (proteinase K (100mg/mL final concentration.), 1.0% SDS, 1% B-mercaptoethanol solution). It was found that omission of 2-mercaptoethanol from the lysis solution prevented lysis/breakdown of the larvae. The solution containing the lysed larvae was extracted 2-4 times with phenol-chloroform-isoamyl alcohol (50:49:1). Two extractions with chloroform followed with DNA being precipitated with two volumes of ethanol. The DNA pellet was rinsed in 70% ethanol then air-dried briefly. The DNA pellet was then resuspended in 100µL of TE (10mM Tris HCl (pH7.6), 1mM EDTA).

Lambda DNA

Liquid Lysates

Liquid lysates were prepared in 50ml volumes of NZCYM medium in conical flasks with cotton wool plugs for good aeration. 10^5 pfu were mixed with 0.1mL of the P2392 culture and incubated at 37°C for 10 minutes then added to the media. Lysis occurred at approximately six hours.

The protocol for the purification of lambda DNA is a combination of three protocols (Chisholm, 1989; Kaslow, 1988; Verma, 1989) and was carried out as follows

1. The Lambda DNA was extracted from 50 mL liquid lysates in 50 mL falcon tubes.

2. RNase (100µg/mL) and DNase (100µg/mL) were added and the lysate incubated at 37°C for 30 minutes.

3. 2.992 g NaCl (1.0M) was added to each tube and dissolved. The tube was then spun at 6000 rpm for 10 mins.

4. The lysates were then transferred to clean tubes containing 5g PEG (BDH 6000) The PEG was dissolved thoroughly on a wheel, then the tubes placed on ice for one hour.

5. The tubes were then spun at 6000 rpm for 20 minutes..

6. The supernatant was then discarded and the pellet thoroughly drained. The pellet was then resuspended in 1.0mL SM and transferred to a 2.2mL eppendorf tube. This was then extracted twice or more with an equal

volume of chloroform to remove residual PEG. Each extraction was spun for 5 min and the upper phase retained.

7. The RNase and DNase incubation was repeated for 15 min. Samples were then spun for 10 min and then transferred to a clean tube.

8. 1.0ml of saturated NH_4SO_4 was then added to each sample and then chilled on ice for 10-15 min then spun for 20 minutes.

9. The pellet was then resuspended in 1.0mL lysis buffer (6.0M Guanidine-HCl, 2.5% Sarkosyl, 25mM EDTA, 500mM Ammonium acetate) and 10µL 20 mg/ml proteinase K was added to each sample.

10. Tubes were then incubated at 55 °C for one hour.

11. Tubes were then cooled to room temperature and 1.0mL of isopropanol was added. This was mixed thoroughly and then spun down for 20 minutes. The pellet was then washed in 70% ethanol, pulsed in centrifuge and traces of ethanol removed but the pellet was not dried.

12. The pellet was then left overnight in 100 µL TE to gently dissolve.

13. 3-5 µL of DNA was digested with restriction enzyme and the digest run out on 0.8% agarose gels to determine the concentration.

Plasmid DNA Preparation

Plasmid DNA was isolated using a modified protocol of the alkaline lysis procedure (Morelle, 1989) first published by Birnboim and Doly (1979). The lysozyme was omitted as the alkali solution lysed the bacterial cells.

A scaled up version of this procedure was used to prepare plasmid for double stranded sequencing.

Restriction Endonuclease Digests

Standard reactions were carried out in 20µL volume using the buffers recommended by the manufacturer (Promega, Boehringer Mannheim, NEB, Toyobo). BSA was added at a concentration of 0.5mg/mL and RNase at 10mg/mL. All reactions were carried out at 37°C or the suppliers recommended temperature.

Gel Electrophoresis

DNA was routinely separated by electrophoresis in 0.8% agarose (Sigma) gels submerged in 1x TAE buffer (0.04M tris-acetate, 0.001M EDTA). Gel formats were either 6x10cm or 15x10cm and run at 5V/cm. After electrophoresis gels were stained in ethidium bromide (0.5 mg/mL) and visualised under UV light and results recorded on Polaroid 667 film.

Southern Blots

Capillary blotting of DNA from agarose gels onto Amersham hybond N^+ membrane was carried out according to the protocol of Reed and Mann (1985). This membrane was chosen in preference to nitrocellulose due to convenience as neither baking or UV crosslinking is necessary to bind the DNA to the membrane and membranes can be readily stripped of signal by boiling the membrane in 0.1% SDS ,0.1x SSC(1x SSC solution is 0.15M NaCl, 0.015M sodium citrate),1% SDS, 65°C. Final [Na^+] of 0.2M) and reprobed. Membranes were rinsed and stored in the fridge in 2x SSC until needed.

Gels with genomic DNA in them were depurinated by 2x5 minute washes in 0.25M HCl then blotted for at least 3 to 16 hours in 0.4M NaCl. Depurination fragments the DNA which promotes the transfer of larger fragments of DNA out of the gel matrix onto the membrane. Over depurination can cause excessive binding of the DNA to the membrane which can lower the sensitivity of the subsequent hybridisation reaction. Gels with lambda or plasmid DNA were blotted directly onto membrane without depurination.

Hybridisations

All southern blots were hybridised for 16 hours at 65°C in an aqueous buffer (2x SSPE(0.18mM NaCl, 0.01M sodium phosphate, pH7.7, 0.001 EDTA), 0.5% Blotto, 0.5 mg/mL sheared salmon sperm DNA and 7% SDS.) except for the first rounds of library screening for which a formamide buffer (50% formamide, 0.1% SDS, 5x Denhardts solution, 6xSSC, 0.001M EDTA and 0.5% sheared salmon sperm DNA) was used for hybridisation. Incubation of the library screen blots were carried out at 42°C for 16 hours.

Washes

Two room temperature washes in 2xSSC, 0.1% SDS. Then two washes at 65°C in 2xSSC, 0.1% SDS. The concentration of SSC was reduced by half for subsequent washes if counts were still high (greater than 100 counts /second).

Exposure

Blots were exposed onto Kodak X-Omat film. The length of exposure and use of intensifying screens depended on the number of counts per second that could be detected using a Geiger counter. For example, blots with cloned DNA as the target needed only two to three hours exposure at room temperature whereas southern blots with genomic DNA required exposure of at least three days at -70°C with one screen.

Library Screening

Library Screening

The library was plated out on NZCYM agar plates using top agarose to spread the phage. The titre of the library which is measured in plaque forming units (pfu) per mL was determined by serial titrations onto NZCYM plates (Sambrook *et al.*, 1989).

After the titre of the library was determined phage were plated at 5,000 plaques per 9cm petri dish. Presuming that the genome size of *T. colubriformis* is similar to *C. elegans* which is approximately 10^8 bp and that lambda clones have on average 20kb inserts then 10,000 plaques should be two genome equivalents. For each library screening four plates were screened which is approximately four genome equivalents.

Plaque lifts were carried out according to a CP lift membrane protocol from Amersham. The membrane used was Amersham Hybond N+. All lifts were done in duplicate.

Membranes were prehybridised for 1 hour in the formamide hybridisation buffer described previously. The membranes were then placed in fresh hybridisation solution containing ^{32}P-labelled probe and incubated for 16 hours at 42°C. Hybridisations were carried out in plastic bags with pairs of membranes back to back (DNA side (the front) was in contact with the filter paper) interleaved with Whatman 542 filter papers .

The probe was mixed in with the hybridisation solution and a small amount used to wet each filter paper between each pair. Volumes of hybridisation solution varied with numbers of filters (approximately 1mL per pair of membranes plus an extra 3mL).

Washes

Hybridised membranes were washed at low stringency with two fifteen minute washes at room temperature and one 20-minute wash at 65°C in 2x SSPE, 0.1 % SDS.

Exposure

Membranes were exposed onto Kodak X-Omat AR film overnight at -70°C. If necessary a longer exposure of three days at -70°C was used in order to detect a signal.

Positive Plaques

Signals were considered to be positive if the duplicate membranes had matching signals. Positive plaques were picked and eluted in SM buffer (50mM Tris-HCl, pH7.5, 100mM NaCl, 8mM $MgSO_4$, 0.01% gelatin).

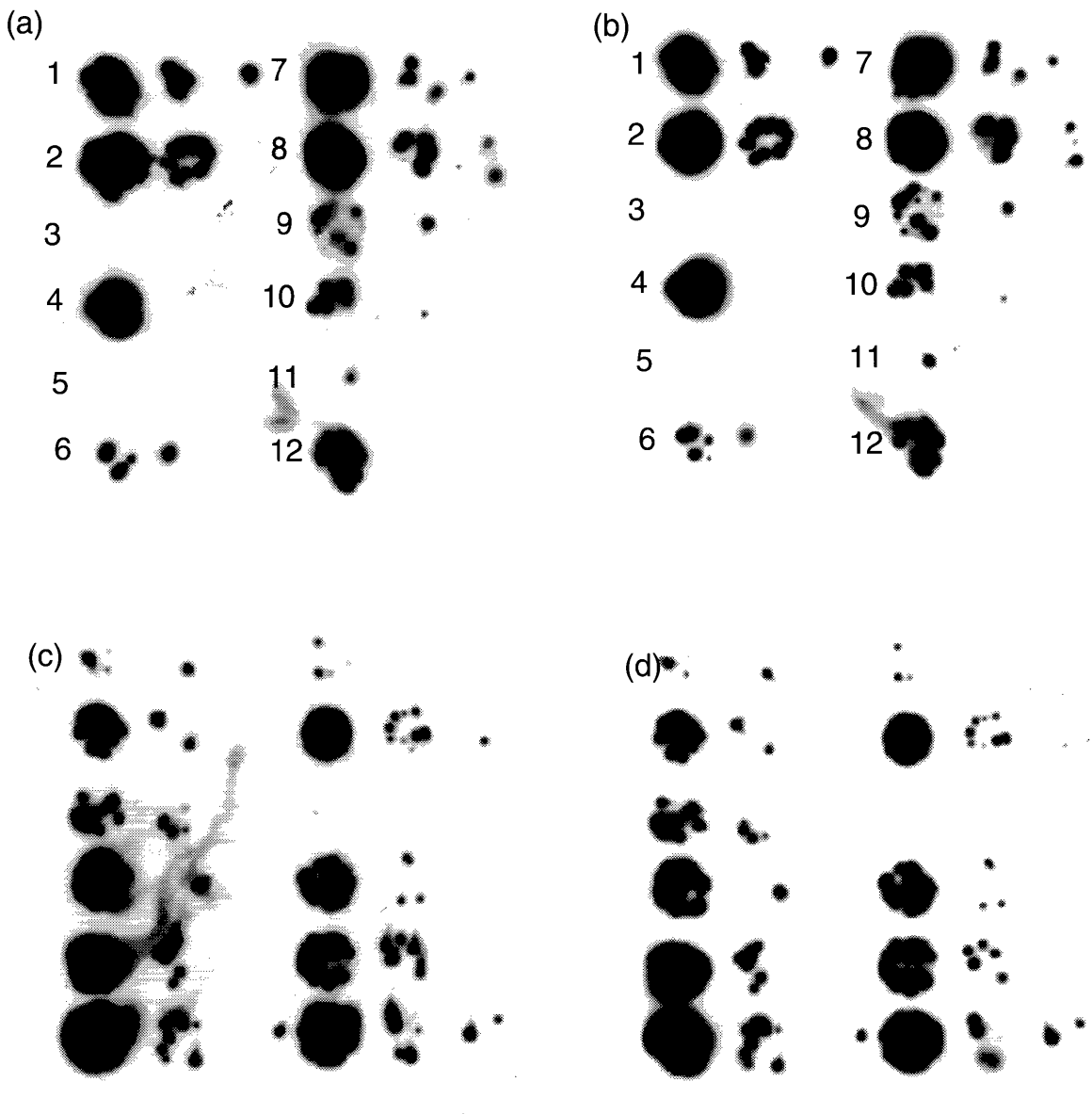
Purification of Positive Plaques

Plaques were purified according to the procedure by Carlock (1986) (see Figure 5). Each positive was put through three rounds of screening. On the final screening purity was indicated by every plaque on the plate hybridising to the probe used in the initial library screening.

Macroplaque Experiments

A differential hybridisation experiment was conducted to compare the hybridisation of the lambda clones isolated from the library screening to various probes from the *tcb-1* locus and the *tcb-2* locus. Aliquots (5µL-which usually resulted in confluent growth) of each purified clone was spotted onto an NZCYM agar plate in a grid formation. The bacteria were already spread onto the plate by mixing 200µL of an overnight culture in top agarose and spreading this evenly over the warmed agar plates. Phage were then incubated overnight at 37°C. Large plaques of approximately 5mm diameter grew as a result. Twenty six clones were grown on one 9cm plate. Duplicate lifts were taken of each plate as for library screenings and probed with various probes as described in the results.

Figure 5. An example of the method used to carry out the second round of screening and purification of lambda phage after the initial library screening experiment. Each sample is diluted by three factors of 10. Aliquots (5μL) of each dilution were spotted out onto a square grid plate. In this way 12 eluates can be screened per plate and single hybridising plaques picked from the dilutions for third round screening.


(a)
1
2
3
4
5
6
7
8
9
10
11
12
(b)
1
2
3
4
5
6
7
8
9
10
11
12
(c)
(d)

Isolation of DNA Fragments

DNA fragments were isolated by excising the relevant band from agarose gels and the DNA recovered using the "Geneclean " kit from BIO 101 (Struhl, 1985).

Subcloning

Vectors

The vectors used for subcloning were the Bluescript set of plasmids from Stratagene. The SK + plasmid was used for subcloning.

The plasmid vector was digested with the appropriate restriction enzyme then treated with alkaline phosphatase. The phosphatase minimises the background of religated plasmid as it removes the terminal phosphate group from the 5' end of the plasmid. The T4 ligase enzyme used in the ligation reactions requires a terminal phosphate to ligate fragments therefore the phosphatased plasmid cannot be religated to itself. The phosphate group required for ligation of fragments is provided by the fragment being cloned. To remove the alkaline phosphatase the treated plasmid was electrophoresed in an agarose gel and then the Geneclean kit was used to recover the plasmid from the gel.

Ligations

The vector and fragments for cloning were combined in an approximately 3:1 molar ratio (fragment :vector). The volume of ligations was between 10 and 14μL. Reactions were incubated overnight at 14°C.

After transformation (see below) the ligation products were plated out onto isopropylthio-b-D-galactoside (IPTG-200mg/mL)/ 5-bromo-4-chloro-3-indoyl-b-D-galactoside (Xgal-20mg/mL), Ampicillin(50μg/mL) plates which allows the use of the blue/white colour selection to detect recombinant transformants. Colonies that contain inserts remain white whereas colonies that are blue do not contain cloned fragments.

The blue/white colour selection is made possible by α-complementation of the β-galactosidase (*lacZ*) gene. The vector carries a fragment that codes for the regulatory sequences and the first 146 amino acids of the β-galactosidase gene. Within this fragment is a polycloning site or multiple cloning site (mcs) that does not disrupt the reading frame of the β-galactosidase gene. The vector is then transformed into a bacterial strain that carries the sequence for the carboxy-

terminal portion of β-galactosidase. These fragments can then associate in the bacteria to form a functional protein. The IPTG inactivates the *lac* repressor and allows expression of the carboxy-terminus of the *lacZ* gene. The Xgal is turned blue by the enzymatic activity of the functional β-galactosidase. If a fragment has been cloned into the mcs the β-galactosidase gene is disrupted and colonies remain white (Sambrook *et al.*, 1989).

Transformations

Transformations were done using calcium chloride prepared competent cells (Sambrook *et al.*, 1989). DNA is mixed with the competent cells and the mixture heated at 42°C for 90 seconds for transformation. The bacterial strains used for transformations were either XL1-Blue or DH5α.

DNA Labelling

All probes were labelled using random-primed oligo-labelling kit from Bresatec (Feinberg & Vogelstein, 1983) and with α-^{32}P-dCTP as label. Incorporation was monitored using PEI-cellulose thin layer chromatography sheets (Merck). Unincorporated nucleotides were not removed.

Sequencing

DNA sequencing was carried out using the Sanger dideoxy chain termination method (Sanger, Nicklen, & Coulson, 1977) as described in the USB Sequenase kit. The radiolabel was α-^{35}S dATP.

Sequencing reactions were performed using double stranded DNA as template. Double stranded plasmid was denatured according to the Promega protocols guide second edition, and resuspended denatured plasmid in 7μL of water. This was used directly with the Sequenase kit according to the manufacturers (USB) instructions.

All sequencing reactions were run on 50 cm, 5% polyacrylamide gels containing, 134mM Tris, 44mM Boric acid, 25mM EDTA and 8mM urea with 1xTBE as buffer(10x TBE is 0.89M Tris base pH8.0, 0.89M boric acid, and 20mM EDTA). Gels were run at constant power of 45 Watts at 50-55°C with wedge spacers and a sharkstooth comb.

The first gels performed were fixed in 10% acetic acid for ten minutes then soaked in 20% ethanol for five minutes, drained, transferred onto blotting paper and dried under vacuum at 80°C. Later it was found that fixing of the gels was

unnecessary and so gels were directly transferred onto blotting paper after running and dried under vacuum at 80°C as before. Gels were exposed overnight onto Kodak X-Omat film at room temperature.

Sequencing of 3' ends of β-tubulin clones was done by using a primer (P1) specifically designed to hybridise in the conserved region of β-tubulin loci. The sequence of this primer is shown in Table 3 along with the sequence present in the three β-tubulin loci of *C. elegans*. The P1 primer includes aa 400 to 406. This allows the sequence of some conserved DNA and then the 3' unique sequence to be read. Other primers used for sequencing are described in Table 4.

TABLE 3: Sequence of primer P1. The sequence is shown here with sequence from the same region of the three β-tubulin loci of *C. elegans*

Species	β-tubulin locus	Nucleotide sequence aa400-406
C. elegans	*ben-1*	5' GC GAG GGC ATG GAC GAG ATG
	tub-1	5' GC GAA GGA ATG GAC GAG ATG
	mec-7	5' GA GAA GGA ATG GAC GAG ATG
T. colubriformis	*tcb-2*	5' GT GAA GGT ATG GAC GAA ATG
Primer P1		5' GT GAG GGA ATG GAC GAG ATG

TABLE 4: Custom made primers used for sequencing subclones

Primer #	Sequence	Priming Direction	Clone sequenced
P1	GTGAGGGCATGGACGAGATG	5'-3'	ALL
LM3	ATGTTCTATACTTGATGC	5'-3'	3.2, 3.5 (*tcb-2*)
I 11	TATGCGCAGTACGATAGCT	3'-5'	3.2,3.5 (*tcb-2*)
LM5	CAATGCTACGGGTCATTG	3'-5'	2.12 (*tcb-4*)
LM6	GATTTGATCCTCCAGCCA	5'-3'	2.1 (*tcb-3*)
LM7	CGGACCATTCATTCACCTTCA	3'-5'	*tcb-1*, 2.9,2.3
LM8	GAAGCTGGGCAACGAATGAAG	3'-5'	2.1 (*tcb-3*)
LM11	GCTGCGATTAATCCTTCAAAC	3'-5'	2.1 (*tcb-3*)

Heteroduplex Analysis

Heteroduplexes were first observed as an artefact when amplified products from PCR reactions were run on polyacrylamide gels. The phenomenon was not observed for the same samples when electrophoresed in agarose gels (Nagamine, Chan & Lau, 1989). The phenomenon is observed when products amplified from homologous sequences that have slight sequence variations are run out on polyacrylamide gels. For example products amplified from two homologous sequences from different regions of the genome (such as the mYfin sequences in the mouse which has two positions one in Zinc-finger y-1 and zfy-2) or in individuals that are heterozygous at a particular locus for a particular sequence variant (Keen, Lester, Inglehearn, Curtis & Bhattacharya, 1991). The expected products are observed on the gels as well as additional bands depending on the number of sequences amplified in the reaction (usually only two products when testing for heterozygous individuals and therefore two additional bands are observed) that migrate at a different rate compared to the expected product. The expected bands are the homoduplexes (double stranded DNA with identical nucleotide sequence) and the additional bands are heteroduplexes (double stranded DNA with nucleotide mismatches where the sense strand of one product has annealed with the antisense strand of the second product and the second additional band where the antisense strand of the first product has annealed with the sense strand of the second product). The heteroduplexes show altered mobility in the gels due to mismatches in the double stranded DNA causing an alteration in the physical conformation of the molecule which changes the way the molecule migrates through the gel matrix. The extent of the mobility shift depends on whether the mismatch is due to nucleotide substitution or deletion. Keen *et al.* (1991) reported that three different known single base pair substitutions (G-A, G-T, and C-T) in exon 11 of the cystic fibrosis gene could be readily detected as heteroduplexes on a commercially available product (Hydrolink from AT biochem). Heteroduplex analysis is not recommended for fragments larger than 900 bp.

It is a simple technique where the product can be heated and cooled then run on the gel or equal amounts of two PCR reactions to be compared are mixed and heated to 95°C and allowed to cool slowly to room temperature. This allows the products to reanneal at random. The reannealed product is then run on the a non-denaturing MDE (AT Biochem) gel in a sequencing gel rig overnight at 800V (according to the manufacturers instructions). Gels are stained in ethidium

bromide for 10 minutes then viewed over UV light. Products that are identical run as single bands as they reanneal without physical alterations to the molecule whereas products that have nucleotide differences, either deletions or substitutions reanneal containing mismatches which alter the molecular shape, run as two or three bands close together in the one lane.

Polymerase Chain Reaction

Preparation of Individual Larvae as Templates for PCR Reactions

L3 stage larvae were used in all PCR reactions. Using 1.5 mL eppendorf tubes individual larvae were picked into 10µL of 1x PCR buffer. Larvae were then frozen at -70°C for at least one hour then snap thawed in a heat block at 95°C for 10 minutes. Larvae were cooled then Proteinase K added at a concentration of 450µg/mL. Tubes were then placed in a 50°C water bath overnight. Proteinase K was inactivated by placing tubes into a 95°C hot block for 15 minutes and the tubes containing digested larvae were then stored at -70°C. until required for PCR.

PCR Reaction Conditions

A Perkin Elmer PCR buffer(20mM Tris pH 8.5, 50mM KCl, 0.1% Tween 20)) was used to carry out PCR reactions. Nucleotides (Amrad-Pharmacia) were added at a concentration of 200µM per 100 µL reaction. The primer concentration per 100µL reaction was 50pM for each primer. 2.5U of Taq polymerase were added to each reaction. Samples were overlayed with an equal volume of mineral oil to prevent evaporation. Reactions were carried out in 0.5mL tubes in a Hybaid thermocycler.

The optimal $MgCl_2$ concentration for each primer pair was determined by carrying out reactions in increasing concentrations of $MgCl_2$ (concentration was increased in 0.5 mM increments in the range 1.0mM to 3.0mM). The recommended 1.5m $MgCl_2$ concentration was found to be the most appropriate concentration.

A nested PCR protocol was applied to amplifying the 3' region of the *tcb-1* locus. Products were first amplified directly from individual nematode larvae using the primers P1 and LM7. Products were run out on 8% acrylamide gels to check for successful amplification. The products from the first amplification reaction were diluted in water by 10 then 1µL of these dilutions was added to 30µL reactions containing the primers WG5 and WG6. 5µL aliquots from this

second amplification reaction were digested with the restriction enzymes HpaII and EcoRV.

Primers Used in the PCR Analysis

TABLE 5: The primers used in PCR reactions

Primer #	Sequence 5'-3'	Reaction
P1	GTGAGGGCATGGACGAGATG	Reaction 1 nested PCR, heteroduplex & RE
LM7	GCTGCGATTAATCCTTCAAAC	R1, heteroduplex & RE
WG5	TTCTGCATCGAGATCGCCGAT	Reaction 2 nested PCR
WG6	GTTCTCCTATGCTTGATGTCTCCG	Reaction 2 nested PCR

The annealing temperature of reactions using P1 and LM7 was determined by estimating the melting temperature of the primer and dropping this by 2-4°C. An estimation of the melting temperature of the primers was calculated according to the following formula Tm=2(A+T)+4(G+C) The annealing temperature using WG5 and WG6 was determined by experiment as these have a mismatch close to the 3' end of the primer and the effect of this on melting temperature was not known. Reaction conditions are shown in Table 6.

TABLE 6: The reactions using the primer pairs P1/LM7 and WG5/WG6 had the following regime

	Denature 94°C	Anneal 58°C	Extend 72°C	Number of cycles	
				P1-LM7	WG5-WG6
Stage 1	2.00 min	40 sec	1.00 min	1	1
Stage 2	30 sec	40 sec	1.00 min	33	19
Stage 3	30 sec	40 sec	2.00 min	1	1

Gel Electrophoresis

All PCR products were analysed by electrophoresis in 8% non-denaturing polyacrylamide minigels run in 1xTBE buffer. Gels were stained 10 minutes in ethidium bromide and viewed under UV light. Gels were prepared and run using the Biorad mini Protean system.