Parasitol Res (1999) 85: 452–456

ORIGINAL PAPER

L. Elard · J.F. Humbert

Importance of the mutation of amino acid 200 of the isotype 1 β-tubulin gene in the benzimidazole resistance of the small-ruminant parasite *Teladorsagia circumcincta*

Received: 5 October 1998 / Accepted: 16 December 1998

Abstract In this work we demonstrated that the acquisition of benzimidazole (BZ) resistance in the small-ruminant parasite *Teladorsagia circumcincta* is linked to the selection of individuals that are characterized by a tyrosine (Tyr) at amino acid 200 of their isotype 1 β-tubulin gene. This mutation appears to be recessive, since only homozygous mutant (Tyr/Tyr) individuals survived after BZ treatment of two resistant populations in which the three genotypes (rr, rs, ss) were initially present. In comparison with natural BZ-susceptible populations, a decrease in the restriction polymorphism (RFLP) of the isotype 1 β-tubulin gene was observed in natural resistant populations. It seems that this decrease in β-tubulin polymorphism results from the selection of homozygous mutant individuals.

Introduction

The appearance of benzimidazole (BZ) resistance in trichostrongylid nematode communities has stimulated efforts to determine how these parasites become resistant. Knowledge of the molecular changes that confer resistance may lead to the development of rapid, reliable diagnostic protocols and, hence, limit the spread of this phenomenon on farms. The molecular basis of resistance to BZ involves alterations in the β-tubulin gene, β-tubulin being the target of BZ. Two main types of change occur in the trichostrongylid nematodes. One is a mutation at amino acid 200 (Phe-Tyr) of the isotype 1 β-tubulin gene, which leads to BZ resistance in the three dominant species in the temperate zone, *Haemonchus contortus*, *Trichostrongylus colubriformis*, and *Teladorsagia circumcincta* (Kwa et al. 1993b, 1994; Lehrer et al. 1995; Elard et al. 1996). This mutation is functional in the expression of the BZ susceptibility or resistance and is not simply a marker linked to the emergence of resistance (Kwa et al. 1995). The second recorded change in the β-tubulin gene is a loss of allelic diversity of this gene in *H. contortus* and *T. colubriformis* (Roos et al. 1990; Kwa et al. 1993a; Beech et al. 1994; Lubega et al. 1994; Grant and Mascord 1996). This reduction in β-tubulin polymorphism has been interpreted as the result of selection and is considered to be a major means of acquiring BZ resistance.

However, several of these studies were carried out using laboratory populations that have generally been isolated from the field for many generations. There is good evidence that the genetic variability of these strains can decrease in the laboratory due to genetic drift or selection linked to artificial breeding conditions (Gasnier et al. 1992; Nadler 1990). Another problem is that the detected restriction polymorphism can result from mutations in coding or noncoding regions. The high conservation of the coding sequences of the β-tubulin gene, demonstrated by the great similarities observed between the amino acid sequences in different species (Elard et al. 1996), suggests that most of the detected polymorphism in this gene arises from nucleotide substitutions located in the introns or from synonymous substitutions in the coding regions. However, these mutations are selectively neutral and, thus, cannot play a role in the acquisition of resistance. To date, the sole mutation that has been implicated in BZ resistance in trichostrongyle parasites is the mutation of residue 200.

To evaluate the respective role of the two described mechanisms of BZ resistance (mutation and selection of a resistant allele) in *T. circumcincta*, we developed two approaches using natural populations. In the first, the importance of the mutation of residue 200 of the isotype

L. Elard
INRA, Centre de Tours, Station de Pathologie Aviaire et de Parasitologie, F-37380 Nouzilly, France

J.F. Humbert (✉)
INRA, Station d'Hydrobiologie Lacustre, 75 Av. de Corzent, BP 511, F-74203 Thonon, France
E-mail: humbert@thonon.inra.fr
Tel.: +33-4-50-26-78-09,
Fax: +33-4-50-26-07-60

1 β-tubulin gene in the acquisition of BZ resistance was tested by typing of worms from BZ-treated or untreated isolates of the same strains. A polymerase chain reaction (PCR) method recently perfected by us (Humbert and Elard 1997) allows us to determine whether the worms are homozygous (Tyr/Tyr or Phe/Phe) or heterozygous (Tyr/Phe) for the mutation which seems to be decisive in the acquisition of BZ resistance. In the second approach the genetic variability of the isotype 1 β-tubulin gene was evaluated in natural BZ-susceptible and BZ-resistant populations of *T. circumcincta* by a study of the restriction polymorphism of the complete β-tubulin.

Materials and methods

Parasite collection and DNA isolation

Five BZ-susceptible (SuBOU, SuLEL, SuLM, SuPRO, SuTOU) and 4 BZ-resistant (ReCAS, ReGAU, ReGP, ReECH) isolates of *Teladorsagia circumcincta* were collected from goat farms (except for SuBOU, SuPRO, and SuLM, which were isolated from sheep) located in central France. The resistance was estimated by the method of Coles et al. (1992; SuLEL, LD_{50} = 0.03 μg ml^{-1}; ReCAS, LD_{50} = 0.85 μg ml^{-1}; ReECH, LD_{50} = 0.31 μg ml^{-1}; ReGAU, LD_{50} = 0.33 μg ml^{-1}; ReGP, LD_{50} = 0.15 μg ml^{-1}) or in the field by determination of the treatment efficacy (100% reduction in the egg output after BZ treatment of the host in SuBOU, SuPRO, SuTOU, and SuLM populations). The genomic DNA from at least 20 adult male worms in each isolate was prepared as previously described (Humbert and Cabaret 1995).

Detection of mutation of amino acid 200 of the isotype 1 β-tubulin gene in worms recovered from BZ-treated or nontreated isolates

Females of *T. circumcincta* were isolated from two resistant populations (ReCAS and ReGP). Eggs from these females were cultured to obtain infective larvae (L3). For each population, one worm-free lamb was infected with these L3. After 21 days, all feces were collected from these lambs (from day 21 to day 36) and cultured to yield infective larvae. For each isolate, 2 worm-free lambs were then infected with 4000 of these larvae. After 28 days, one of the two lambs was treated with Panacur (fenbendazole) at the commercial dose (5 mg/kg body weight). All lambs were slaughtered at day 35 and the parasites were recovered. The presence of a phenylalanine (TTC) or a tyrosine (TAC) at residue 200 was detected in these worms according to the method described by Humbert and Elard (1997).

Amplification of the isotype 1 β-tubulin gene and restriction enzyme digestion

The isotype 1 β-tubulin gene was amplified in two steps. A 3.6-kb fragment was first amplified with the Expand Long Template PCR System (Boehringer, Mannheim) according to the supplier's instructions with the primers TUB3 (5′-GAG GAG CCC CAT GCC GAG AAG ACG TGG AAG-3′) and TUB5 (5′-ATG CGT GAA ATC GTT CAT GTA CAA GCC GGT-3′). The second step was a nested PCR, which generates a 3.5-kb fragment using the primers N5 (5′-TGT ACA AGC CGG TCA ATG CGG-3′) and N3 (5′-AGC TCT AGC GGG TAT AGC AG-3′). The procedure involved: 50 μl reaction mixture, 5.0 μl 10X *Taq* buffer (Promega), 1.5 U *Taq* polymerase (Promega), and 80 μmol of each deoxynucleoside triphosphate (dNTP; Promega); 30 cycles of 92 °C for 50 s, 58 °C for 60 s, and 72 °C for 90 s; and final extension at 72 °C for 10 min. After control by agarose-gel electrophoresis, only the best amplifications (one band with a good intensity) were kept for the restriction study. These PCR products were digested using restriction enzymes (EcoR1, EcoRV, Hinf1, Dra1, and Rsa1; Eurogentec) and then separated on a 1.5% agarose gel (Gibco-BRL). The electrophoresis gels were analyzed with a BioImage system. Restriction fragments were scored as 1 (present) or 0 (absent) in a data table. Then, a correspondence analysis was performed using the ADE-4 software package (Thioulouse et al. 1997).

Results

Typing of worms from BZ-treated or un-treated isolates from resistant populations

The results of the BZ treatment experiment on two resistant populations (Table 1) show that the most resistant population (ReCAS, LD_{50} = 0.85 μg ml^{-1}) was characterized without treatment by the high prevalence of homozygous resistant individuals (Tyr/Tyr for residue 200). In the treated lambs this prevalence increased to 100% (LD_{50} = 1.05 μg ml^{-1}). The other resistant population (ReGP, LD_{50} = 0.15 μg ml^{-1}) was characterized without treatment by a great proportion of heterozygous (Phe/Tyr for residue 200) and homozygous resistant (Tyr/Tyr) individuals. After BZ treatment, only homozygous resistant individuals were observed in the worm population, and the LD_{50} was estimated to 0.97 μg ml^{-1}.

For the ReGP population the restriction polymorphism of the isotype 1 β-tubulin gene of the worm populations collected from BZ-treated or untreated lambs was compared using Rsa1, Dra1, EcoRI, and EcoRV restriction enzymes. The multienzyme patterns were determined for each individual by addition of the results obtained with these four enzymes. In all, 12 patterns were observed in 18 individuals of the untreated population, whereas only 4 patterns were observed in 18 individuals of the treated population.

Table 1 Genotyping for amino acid 200 of the isotype 1 β-tubulin of worms in two BZ-resistant strains of *Teladorsagia circumcincta* treated or untreated with BZ. For each assay, more than 100 individuals were typed

		Tyr/Tyr (BZ-resistant[a])	Tyr/Phe (Susceptible)	Phe/Phe (Susceptible)
Without BZ treatment	*Re*CAS	*96.4*	*3.6*	*0*
	ReGP	35.8	52.6	11.6
After BZ treatment	*Re*CAS	*100*	*0*	*0*
	ReGP	100	0	0

[a] Phenotype

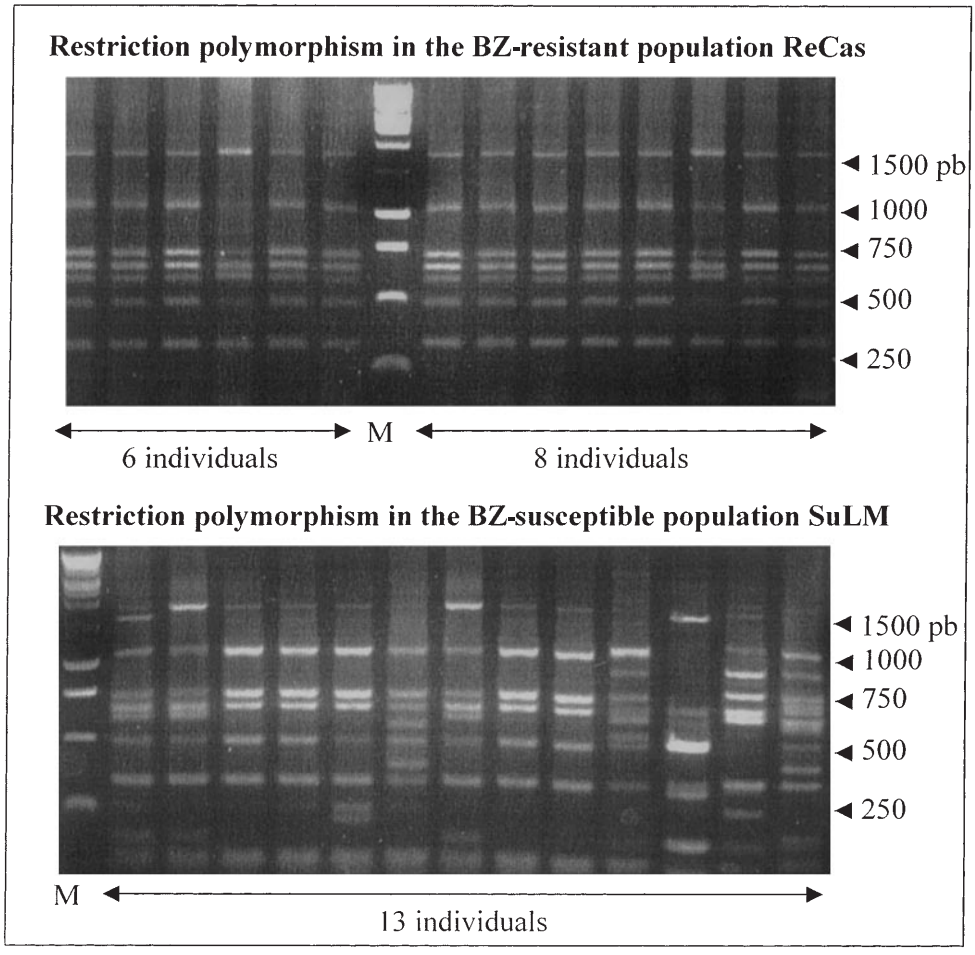

Fig. 1 Restriction patterns obtained with the Rsa1 enzyme on two natural populations of *Teladorsagia circumcincta*: ReCAS and SuLM (*M* Molecular-weight marker – (1-kb DNA ladder, Promega)

Restriction polymorphism of the isotype 1 β-tubulin gene in natural BZ-susceptible and BZ-resistant populations

Digestion of the amplified fragment (3.5 kb) gave 2–12 bands in restriction profiles that varied with the populations and the restriction enzymes used (Fig. 1). The multienzyme patterns were determined for each individual by addition of the results obtained with five enzymes (Dra1, EcoR1, EcoRV, Hinf1, Rsa1). The results (Table 2) show that the number of distinct restriction patterns is greater ($P < 0.05$, Mann-Whitney U-Test) in BZ-susceptible populations than in the resistant. In addition, the most resistant population (ReCAS, $LD_{50} = 0.85$ μg ml^{-1}) was characterized by the presence of only 2 different restriction patterns in the 18 individuals studied. Conversely, the ReGP population, which was characterized by the lower LD_{50} value (0.15 μg ml^{-1}) in the resistant populations, presented 10 different restriction patterns (for 17 individuals studied). The multienzyme patterns were not the same in all of the BZ-resistant populations, but several of these patterns were observed in different BZ-resistant populations.

The correspondence analysis on these restriction data (Fig. 2) confirm these observations. The subsets of individuals (populations) are summarized by ellipses that

Table 2 Number of multienzyme patterns observed in the BZ-susceptible and BZ-resistant populations of *T. circumcincta*

	ReCAS $n = 18$[a]	ReECH $n = 13$	ReGAU $n = 10$	ReGP $n = 17$	SuBOU $n = 15$	SuLEL $n = 8$	SuLM $n = 15$	SuPRO $n = 11$	SuTOU $n = 9$
Number of patterns	2	2	4	10	13	5	10	10	6
	(0.11[b])	(0.15)	(0.40)	(0.59)	(0.87)	(0.63)	(0.67)	(0.91)	(0.67)

[a] Number of individuals studied
[b] Number of patterns/number of individuals

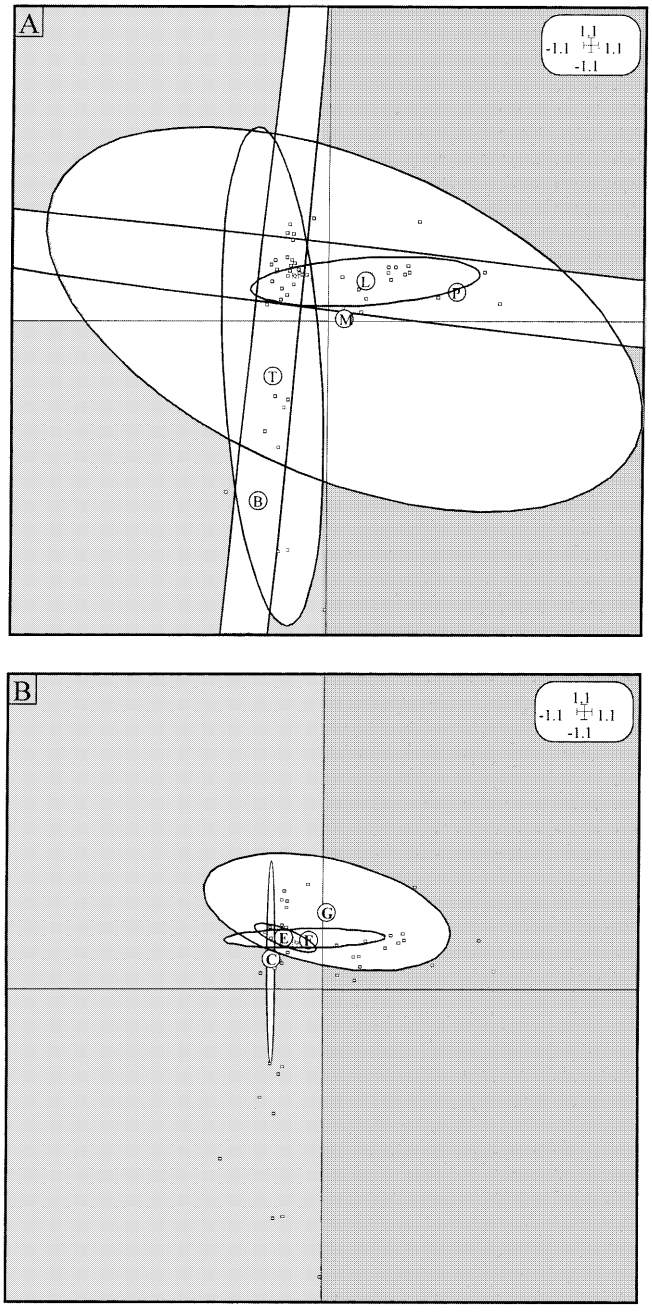

Fig. 2A, B Correspondence analysis (axis 1 = 11% of variance, axis 2 = 9% of variance) on the restriction fragments of the isotype 1 β-tubulin gene. **A** Projection in the plane defined by the first two axes of the susceptible populations (*B* SuBOU, *L* SuLEL, *M* SuLM, *P* SuPRO, *T* SuTOU). **B** Projection in the same plane of the resistant populations (*C* ReCAS, *E* ReECH, *G* ReGAL, *F* ReGAU)

contain 90% of the individuals according to a Gaussian distribution. The comparison of these ellipses clearly shows that the genetic variability of the isotype 1 β-tubulin gene, as estimated by restriction polymorphism, is lower in the BZ-resistant populations than in the susceptible.

Discussion

The first goal of this work was to evaluate the importance of the mutation of residue 200 of the isotype 1 β-tubulin gene in the development of BZ resistance. The typing of worms (Table 1) recovered from BZ-treated or untreated resistant strains (ReGP, ReCAS) shows that only homozygous individuals (Tyr/Tyr) for residue 200 were resistant and that homozygous (Phe/Phe) and heterozygous (Phe/Tyr) individuals were eliminated from the worm population after BZ treatment. The LD_{50} value, as estimated on the egg hatching (Coles et al. 1992), correlated with the proportion of homozygous resistant individuals occurring in the worm populations. Thus, the mutation (Phe to Tyr) at residue 200 of the isotype 1 β-tubulin gene confers BZ resistance and appears to be recessive. This result is in full agreement with the observation of Kwa et al. (1995), who had proposed this hypothesis after experiments of transformation of a susceptible *Caenorhabditis elegans* strain with a BZ-resistant *Haemonchus contortus* allele. The comparative study of the β-tubulin sequences in different organisms supports the hypothesis that the nature of the amino acid at position 200 is fundamental for the development of BZ susceptibility or resistance. Numerous studies showed that BZ altered the microtubules of the parasites but not those of the vertebrate host. An examination of the β-tubulin sequences of vertebrates has indicated that a tyrosine is present at position 200 in five (classes I–V) of the six isotype classes present in mammals (Sullivan 1988). Conversely, Katiyar et al. (1994) have observed that Phe-200 is a strong predictor of BZ susceptibility for protozoan parasites.

The second goal of this study was to determine whether a decrease in the polymorphism of the isotype 1 β-tubulin gene was present in natural BZ-resistant populations of *Teladorsagia circumcincta*. Even if the number of individuals in some populations was low due to difficulties in obtaining a good amplification of the complete isotype 1 β-tubulin sequence, all the results obtained in this restriction-fragment-length polymorphism (RFLP) study were very consistent. The BZ-resistant populations were characterized by a low diversity of the restriction profiles in comparison with the susceptible populations. This reduction is more important in the resistant population (ReCAS), which presented the higher LD_{50} value, than in the other. Thus the genetic variability of the isotype 1 β-tubulin gene is lower in the natural resistant populations of *T. circumcincta* than in the susceptible populations. This result confirms those reported on laboratory strains of two other trichostrongyle nematodes, *Haemonchus contortus* (Roos et al. 1990; Kwa et al. 1993a, b; Beech et al. 1994; Lubega et al. 1994) and *Trichostrongylus colubriformis* (Grant and Mascord 1996).

If this mutation of residue 200 is the main factor involved in the acquisition of BZ resistance, the decrease in the polymorphism of the isotype 1 β-tubulin gene

observed by RFLP in the resistant populations must then be interpreted as the selection of homozygous mutant individuals (rr). This hypothesis is supported by the RFLP results we obtained in the BZ-treated or untreated strains of the ReGP population; we observed that the selection of homozygous resistant worms (from 36% in the untreated population to 100% in the treated population) correlated with a strong decrease in the restriction polymorphism of the β-tubulin gene.

The small genetic variability observed in the isotype 1 β-tubulin gene of the resistant populations can be explained by two hypotheses. First, bottlenecks could occur in resistant populations when a strong selection pressure is exerted by BZ treatments. In this case, a decrease in heterozygosity would inevitably be observed in neutral loci of resistant populations. Second, hitchhiking effects on the isotype 1 β-tubulin gene could be associated with BZ resistance. In this case, no decrease in polymorphism would necessarily be observed in neutral loci, which are not linked with the β-tubulin gene. To test these hypotheses a comparative study is needed of the genetic variability that actually develops on neutral markers of BZ-susceptible or BZ-resistant populations. One other question linked to this work concerns the possible consequences of this mutation on the fitness of the parasites. The β-tubulin gene is one of the most highly conserved genes, which shows that mutations in coding regions of this gene are counterselected. A comparison of the fitness of Tyr-200 versus Phe-200 parasites in the absence of BZ selection pressures would be very interesting because it has many implications in the management of BZ resistance in the field.

Acknowledgements L. Elard was supported by a grant from the Région Centre and the INRA. We thank C. Sauvé for technical assistance, J. Cabaret and N. Gasnier for providing parasite populations, and J. Cabaret for stimulating discussions. The English text was checked by Dr. Owen Parkes.

References

Beech RN, Pritchard RK, Scott ME (1994) Genetic variability of the β-tubulin genes in benzimidazole-susceptible and -resistant strains of *Haemonchus contortus*. Genetics 138: 103–110

Coles GC, Bauer C, Borgsteede FHM, Geerts S, Klei TR, Taylor MA, Waller PW (1992) World Association for the Advancement of Veterinary Parasitology (W.A.A.V.P.) methods for the detection of anthelmintic resistance in nematodes of veterinary importance. Vet Parasitol 44: 35–44

Elard L, Comes AM, Humbert JF (1996) Sequences of β-tubulin cDNA from benzimidazole-susceptible and -resistant strains of *Teladorsagia circumcincta*, a nematode parasite of small ruminants. Mol Biochem Parasitol 79: 249–253

Gasnier N, Cabaret J, Moulia C (1992) Allozyme variations between laboratory reared and wild populations of *Teladorsagia circumcincta*. Int J Parasitol 22: 581–587

Grant WN, Mascord LJ (1996) Beta-tubulin polymorphism and benzimidazole resistance in *Trichostrongylus colubriformis*. Int J Parasitol 26: 71–77

Humbert JF, Cabaret J (1995) Use of random amplified polymorphic DNA (RAPD-PCR) for identification of trichostrongylid nematodes. Parasitol Res 81: 1–5

Humbert JF, Elard L (1997) A simple PCR method for rapidly detecting point mutations. Technical Tips Online [http://tto.trends.com, T40076]

Katiyar SK, Gordon VR, McLaughlin GL, Edlin TD (1994) Antiprotozoal activities of benzimidazoles and correlations with β-tubulin sequence. Antimicrob Agents Chemother 38: 2086–2090

Kwa MS, Kooyman FNJ, Boersema JH, Roos MH (1993a) Effect of selection for benzimidazole resistance in *Haemonchus contortus* in β-tubulin isotype 1 and isotype 2 genes. Biochem Biophys Res Commun 191: 413–419

Kwa MS, Veenstra JG, Roos MH (1993b) Molecular characterisation of β-tubulin genes present in benzimidazole-resistant populations of *Haemonchus contortus*. Mol Biochem Parasitol 60: 133–144

Kwa MS, Veenstra JG, Roos MH (1994) Benzimidazole resistance in *Haemonchus contortus* is correlated with a conserved mutation at amino acid 200 in β-tubulin isotype 1. Mol Biochem Parasitol 63: 299–303

Kwa MS, Veenstra JG, Dijk MD, Roos MH (1995) β-Tubulin genes from the parasitic nematode *Haemonchus contortus* modulate drug resistance in *Caenorhabditis elegans*. J Mol Biol 246: 500–510

Lehrer S, Davey H, Watson T, Wilkins RJ (1995) Sensitive PCR for detecting benzimidazole resistant sub populations of ovine nematodes in the Waikito. Proc N Z Soc Anim Prod 55: 209–210

Lubega GW, Klein RD, Geary TG, Pritchard RK (1994) *Haemonchus contortus*: the role of two β-tubulin gene subfamilies in the resistance to benzimidazole anthelminthics. Biochem Pharmacol 47: 1705–1715

Nadler S (1990) Molecular approaches to studying helminth population genetics and phylogeny. Int J Parasitol 20: 11–29

Roos MH, Boersema JH, Borgsteede FHM, Cornelissen J, Taylor M, Ruitenberg EJ (1990) Molecular analysis of selection benzimidazole resistance in the sheep parasite *Haemonchus contortus*. Mol Biochem Parasitol 43: 77–88

Sullivan KF (1988) Structure and utilization of tubulin isotypes. Annu Rev Cell Biol 4: 687–716

Thioulouse J, Chessel D, Doledec S, Olivier JM (1997) ADE-4: a multivariate analysis and graphical display software. Stat Comput 7: 75–83