

# P-glycoprotein selection in strains of *Haemonchus contortus* resistant to benzimidazoles

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## Abstract

Anthelmintic resistance in parasitic nematodes of livestock is a chronic problem in many parts of the world. Benzimidazoles are effective, broad-spectrum anthelmintics that bind to and selectively depolymerise microtubules. Resistance to the benzimidazoles, however, developed quickly and is caused by genetic changes in genes encoding  $\beta$ -tubulins, subunits of microtubules. In *Haemonchus contortus*, resistance to avermectins has been correlated with genetic changes at a gene encoding a P-glycoprotein, a cell membrane transport protein that has a very high affinity for ivermectin. The substrate specificity of P-glycoprotein is very broad, and resistance to benzimidazoles can be modulated by lectins specific for P-glycoprotein. We investigated the possibility that genetic changes in P-glycoprotein might be correlated with benzimidazole resistance in nematodes. An analysis of restriction fragment length polymorphisms of a P-glycoprotein gene from a sensitive and a cambendazole-selected strain of *H. contortus*, derived from the sensitive strain, showed a significant difference in allele frequencies between strains. The frequency of one allele in particular increased substantially. The same allele was also found at a high frequency in an independently derived thiabendazole-selected field isolate. We present genetic evidence of selection at a P-glycoprotein locus during selection for benzimidazole resistance in *H. contortus*.

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## 1. Introduction

Before development of the macrocyclic lactones (ML), benzimidazoles (BZ) were the most widely used anthelmintics for controlling parasitic nematodes in livestock. Extensive use eventually led to the development of resistance to BZ. The development of anthelmintic resistance is an evolutionary process where individuals that survive drug treatment contribute

their genes to the next generation. Over many generations, the frequency of survivors will increase in the population. How the survivors tolerate the effects of an anthelmintic may be due to different mechanisms. Some allelic variants of a protein, to which a drug binds, may have a lower affinity for the drug (Anderson et al., 1998). Alternatively, allelic or expression differences in a gene may reduce a drug's effect on the target protein without affecting binding affinity. Reducing the concentration of an anthelmintic at its target site by a transport or efflux mechanism could enhance survival (Sangster, 1996). Another possibility for enhancing survival is the differential ability to metabolically modify the anthelmintic, thereby reducing its effectiveness (Coles, 1989). Whatever the means of survival,

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genetic changes will occur at the population level over time, and the effectiveness of anthelmintic treatment will diminish.

BZ acts by binding specifically and with high affinity to  $\beta$ -tubulin (Hammerschlag and Sisler, 1973; Davidse and Flach, 1977; Lubega and Prichard, 1990, 1991a,b), thereby selectively depolymerising microtubules (Borgers and de Nollin, 1975; Borgers et al., 1975; Quinlan et al., 1980). The molecular characterisation of genes encoding  $\beta$ -tubulin from the parasitic nematode *Haemonchus contortus* has shown that BZ resistance is correlated with genetic changes at these loci (Roos et al., 1990; Kwa et al., 1993a,b, 1994; Beech et al., 1994). Similar correlations between the  $\beta$ -tubulin gene sequence and BZ sensitivity are also found in such phylogenetically diverse organisms as fungi (Sheir-Neiss et al., 1978; Thomas et al., 1985; Orbach et al., 1986; Foster et al., 1987) and the free-living nematode *Caenorhabditis elegans* (Driscoll et al., 1989). The effect of BZ on tubulin polymerisation is thus likely to be the principal mechanism of BZ resistance in parasitic nematodes.

Some evidence suggests that resistance to an anthelmintic may develop by multiple mechanisms, even within a single population. ML drugs are known to bind to and gate glutamate-gated chloride (GluCl) channels in *C. elegans* (Cully et al., 1994), leading to hyperpolarisation of neuromuscular cells and paralysis. Allele-frequency changes in a gene encoding an alpha subunit of the GluCl channel from ivermectin (IVM)- and moxidectin-selected strains of *H. contortus* (Blackhall et al., 1998a), and an IVM-resistant strain of *Cooperia oncophora* (Njue and Prichard, 2004), are consistent with the involvement of GluCl channels in a mechanism of resistance to these drugs, likely due to changes in the drug target. Similar changes in allele frequencies in the *H. contortus* strains have been found in a gene encoding a  $\gamma$ -aminobutyric acid (GABA) receptor (Blackhall et al., 2003), implicating a second mechanism of resistance to ML which, as with the GluCl channel, could involve alterations to the drug target. ML anthelmintics were subsequently shown to bind and gate GluCl channels in *H. contortus* (Forrester et al., 2002), and substitutions of single amino acids could modulate drug sensitivity of GluCl channels from *C. oncophora* (Njue et al., 2004) and GABA receptors from *H. contortus* (Feng et al., 2002). A third mechanism, one that does not involve changes in the drug target, may be contributing to IVM resistance. P-glycoprotein (Pgp), a membrane transport protein responsible for some cases of multidrug resistance in human cancer cells (Gottesman and Pastan, 1993), may also contribute to ML resistance in *H. contortus*. ML

treatment is associated with changes in allele frequencies (Blackhall et al., 1998b; Eng and Prichard, 2005) and levels of expression (Xu et al., 1998; Huang and Prichard, 1999; Roulet and Prichard, personal communication) of Pgp in *H. contortus* and *Onchocerca volvulus*. Since BZ can act as substrates for human Pgp (Nare et al., 1994), and multidrug-reversing agents can enhance the toxicity of BZ in eggs of *H. contortus* (Beugnet et al., 1997), Pgp may contribute to BZ resistance in adult nematodes.

The present study is an analysis of genetic variation in a Pgp gene, *PGP-A*, from cambendazole (CBZ)-sensitive and CBZ-selected strains, and a thiabendazole (TBZ)-selected field isolate of *H. contortus*. An analysis of a gene unlikely to be involved in BZ resistance, a GluCl channel alpha-subunit gene, showed no evidence for any genetic change associated with BZ resistance.

## 2. Materials and methods

### 2.1. Parasite populations

Three populations of *H. contortus* were used in this study. One of these populations (CBZ-sensitive) had no prior exposure to CBZ. The second population (CBZ-selected), derived from the CBZ-sensitive strain, was treated with CBZ for 10 generations (Kates et al., 1973; Colglazier et al., 1974). After 10 generations, CBZ treatment at twice the recommended dose had an efficacy of 45% in the treated strain compared to 99.8% in the sensitive strain (Colglazier et al., 1974). A third population (TBZ-selected) was independently derived from the field on the basis of its resistance to TBZ (Lubega and Prichard, 1990). After selection with TBZ, this field isolate required 4.4 times more TBZ than a sensitive strain to cause a 50% inhibition in egg hatching.

### 2.2. DNA isolation

The isolation of DNA used in this study has been described previously (Beech et al., 1994).

### 2.3. Polymerase chain reaction (PCR) amplification

A sense primer, PGP2S, 5' GAAATGACTCAAG-CACAAG 3', designed based on the cDNA sequence of *PGP-A* reported from *H. contortus* (Xu et al., 1998), and an antisense primer, MX-D, 5' AGACAAAGACATT-CAGAG 3', designed from unpublished sequence data (M. Xu, personal communication), were used to obtain a genomic PCR product approximately 870 base pairs in

length from 30 individuals of each strain. The reaction mixture contained 5 µl 10X *Taq* buffer, 5 µl 2 mM dNTPs, 4 µl 25 mM MgCl<sub>2</sub>, 1 µl 20 mM primer solutions, 1 unit *Taq* polymerase, approximately 2 ng DNA template, and water to a final volume of 50 µl. Amplification reactions were performed on an MJ Research, Inc. PTC-100 Programmable Thermal Controller (Watertown, MA). Amplification conditions were: 95° for 4 min followed by 40 cycles of 95° for 15 s, 53° for 30 s, and 70° for 1 min with a final extension step at 70° for 5 min. A fragment of a *GluCl* channel subunit gene was amplified for single-strand conformation polymorphism (SSCP) analysis as described in Blackhall et al. (1998a). PCR products were visualised on a 1% agarose gel containing 0.5 µg ml<sup>-1</sup> ethidium bromide under UV illumination.

2.4. Restriction fragment length polymorphism analysis

PCR products were digested with the restriction enzymes DdeI, HinfI, RsaI, and AluI as described by the

manufacturer (Promega Corporation, Madison, WI). Ten microlitre of each PCR product were digested in a total volume of 20 µl. The digestion products were electrophoresed through a 6% nondenaturing polyacrylamide gel, stained with ethidium bromide, visualised under UV illumination, and photographed. Alleles were identified with each enzyme as homozygotes or as consistently occurring fragment patterns in heterozygotes. An example of PCR products digested with HinfI and RsaI is shown in Fig. 1.

2.5. SSCP analysis

PCR products of the *GluCl* channel subunit gene were processed and analysed as described in Blackhall et al. (1998a).

2.6. Data analysis

Fisher's exact tests were performed on allele frequencies in the CBZ-sensitive and CBZ-selected strains for both genes examined. No analyses were

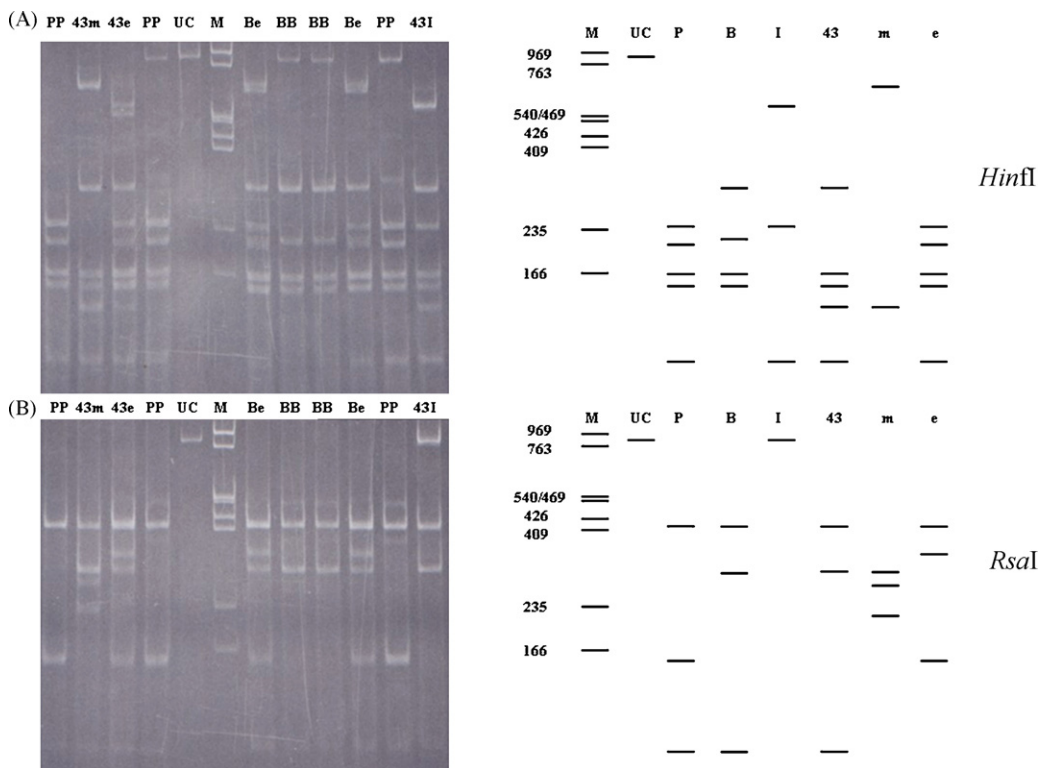


Fig. 1. *Left*: Examples of Pgp PCR products digested with HinfI (A) and RsaI (B), and electrophoresed on a 6% nondenaturing polyacrylamide gel. The genotypes of the samples are indicated at the top of the lanes. Bands not identified in the diagram on the right represent partial digestion products. *Right*: (A) and (B) diagrams showing the fragment sizes of the various Pgp alleles cut with HinfI or RsaI, respectively. The numbers represent the sizes of the marker (M) bands in base pairs. UC = uncut PCR product. No single enzyme was able to identify all alleles and the four restriction enzymes, DdeI, HinfI, RsaI, and AluI were used to identify the different alleles.

performed between the CBZ-sensitive strain and the TBZ-selected field isolate since they were independently derived. Hardy–Weinberg exact tests were performed on all strains to test for Hardy–Weinberg equilibrium. All tests were performed with Genepop Version 3.4 (Raymond and Rousset, 1995).

### 3. Results

A total of 6 Pgp alleles was found in the populations (30 worms each) examined (Fig. 2). The CBZ-sensitive strain possessed 4 alleles, with allele 43 being the most common, having a frequency of 0.783. Allele I was the second most common allele at a frequency of 0.15. The CBZ-selected strain had a very different allelic profile. The frequency of allele 43 decreased to 0.167. Allele B increased in frequency. Allele P, which was not seen in the CBZ-sensitive strain, was the most common allele, with a frequency of 0.5. Allele P may exist in the CBZ-sensitive strain, but at a sufficiently low frequency as to be absent in a random sample of only 30 worms. A Fisher's exact test indicated a highly significant difference in allele frequencies between the CBZ-sensitive and CBZ-selected strains. In the TBZ-selected isolate, allele P also had a frequency of 0.5, with frequencies of the other alleles being comparable to those of the CBZ-selected strain. An apparent selection for allele P has occurred during the course of BZ treatment.

None of the three populations were in Hardy–Weinberg equilibrium at the Pgp locus. All exhibited an excess of homozygotes.

The SSCP analysis of the GluCl channel subunit gene identified a total of 5 alleles (Fig. 3) in the 90

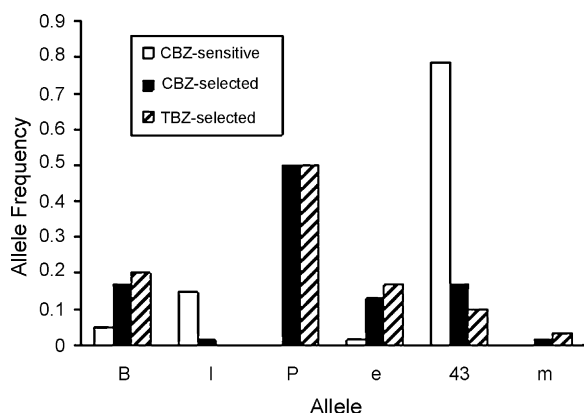


Fig. 2. Allele frequencies of a Pgp gene in a CBZ-sensitive strain, a CBZ-selected strain which was derived from the CBZ-sensitive strain, and an independent TBZ-selected isolate of *H. contortus* (30 worms each).

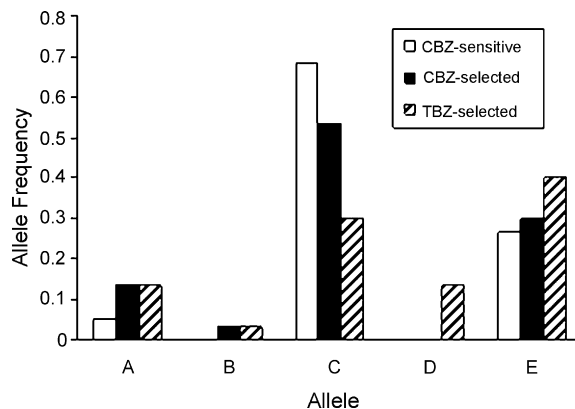


Fig. 3. Allele frequencies of a GluCl-channel subunit gene in a CBZ-sensitive strain, a CBZ-selected strain which was derived from the CBZ-sensitive strain, and an independent TBZ-selected isolate of *H. contortus* (30 worms each).

worms examined. Allele C was the most common allele in the CBZ-sensitive and CBZ-selected strains, with frequencies of 0.683 and 0.533, respectively. Allele E was the next most common allele in these two strains. A Fisher's exact test indicated that allele frequencies of this gene were not significantly different between the CBZ-sensitive and CBZ-selected strains. In the TBZ-selected isolate, allele E was most common, with a frequency of 0.4. The second most common allele, C, had a frequency of 0.3. All three populations were in Hardy–Weinberg equilibrium at the GluCl-channel locus.

### 4. Discussion

This study presents evidence suggesting that, while the principal cause of BZ resistance is alteration of beta-tubulin, other mechanisms independent of the drug-binding target are contributing to BZ resistance in *H. contortus*. Previous studies on the effects of BZ drugs on microtubule polymerisation and alterations in tubulin genes of resistant parasites implicate microtubule stability as a major mechanism in the development of BZ resistance, one involving alteration of the drug target. The changes in allele frequencies of a Pgp gene reported here suggest the contribution of a different mechanism, one that involves limiting the amount of drug reaching its target. This mechanism may involve an increase in expression in intestinal cells, thereby reducing the access of the drug to the tissues of the worms. Increased expression could occur through an increase in copy number of the gene or by the selection of an allele that exhibits enhanced expression, perhaps through alteration of promoter or other regulatory

sequences. Alternatively, BZ treatment may select for an allele of Pgp that possesses a higher binding affinity for BZ.

The selection for a specific allele of Pgp, allele P, in the CBZ-selected strain and the TBZ-selected isolate of *H. contortus* is apparent. Allele P was not seen in the sensitive strain. Allele P has also been found to occur at very low frequencies in other, independently derived strains of *H. contortus* (Blackhall et al., 1998b). The substantially higher frequency of this allele in both the CBZ-selected strain and the TBZ-selected field isolate is unlikely to be due to a stochastic process.

The deviation from Hardy–Weinberg equilibrium seen at the Pgp locus in all three populations was characterised by an excess of homozygotes. Selection, the presence of null alleles (alleles that fail to amplify during PCR), inbreeding (also via bottlenecks), and population subdivision can all lead to an excess of homozygotes. Gene amplification might also affect an analysis of Hardy–Weinberg equilibrium (Hartl and Clark, 1989), but the state of PGP-A copy number in *H. contortus* is unknown. Selection is apparent in the CBZ- and TBZ-selected populations, but Hardy–Weinberg equilibrium is quickly restored through mating in the absence of selection pressure. The populations used in this study have been maintained in sheep without drug treatment, so selection is unlikely to be responsible for the observed excess of homozygotes. The Hardy–Weinberg disequilibrium at the Pgp locus is also not likely to be due to the effects of inbreeding or population subdivision, as none of the three populations deviated from equilibrium at the GluCl-channel subunit locus. The excess of homozygotes may thus indicate the presence of a null Pgp allele in these populations. The frequency of such a null allele, however, would be variable among different populations of *H. contortus*, as no Pgp null alleles, from the same pair of PCR primers, were apparent in five other strains of *H. contortus* examined elsewhere (Blackhall et al., 1998b). Whatever the cause of the excess of homozygotes at this locus, the presence of allele P at substantial frequencies in the two independently derived drug-selected populations relative to the unselected population represents strong evidence for the selection of this allele during BZ treatment.

No significant change in allele frequencies between the CBZ-selected and CBZ-unselected strains occurred at the GluCl-channel locus. This observation suggests that genome-wide changes have not occurred during the process of strain development. Similar findings at three neutral loci in two other sets of laboratory strains of *H. contortus* (Blackhall et al., 1998a, 2003) support the

hypothesis that the development of such strains does not cause genome-wide changes. Changes at the Pgp locus in the present study are therefore likely to represent change due to drug selection rather than to effects such as inbreeding, population bottlenecks, or stochastic processes.

Nare et al. (1994) found that BZ is a substrate for the human Pgp, ABCB1. The BZ they used was a photoaffinity-labelled analogue of fenbendazole. Unlabelled fenbendazole, however, was found not to be a substrate for human Pgp in vitro (Merino et al., 2005), along with albendazole and oxfendazole. Merino et al. (2002) also determined albendazole not to be a substrate of human Pgp. In *H. contortus*, multidrug-reversing agents can enhance the toxicity of BZ, including thiabendazole, in eggs of *H. contortus* (Beugnet et al., 1997; Kerboeuf et al., 2002), implicating an interaction between BZ and efflux pumps in this species. Recently, evidence has been presented that the uptake of the BZ, triclabendazole, may be regulated in *Fasciola hepatica* via a Pgp efflux pump (Mottier et al., 2006). Humans have 2 genes encoding Pgp, but *H. contortus* may have at least 12 Pgp genes (Beech, unpublished data), so the potential for a broad range of Pgp substrates exists in *H. contortus*.

Overexpression of ABCB1 is responsible for some cases of multidrug resistance in human cancer cells (Gottesman and Pastan, 1993). A defining characteristic of multidrug resistance is that selection for resistance to one drug confers resistance to other, unrelated drugs. Resistance to BZ in *H. contortus*, however, does not confer use-level resistance to IVM (Wescott and LeaMaster, 1982; Swan et al., 1984; Taylor et al., 1990; Waruiru et al., 1996), moxidectin (Besier et al., 1993; Oosthuizen and Erasmus, 1993; Bauer and Conraths, 1994; Kerboeuf et al., 1995), levamisole (Colglazier et al., 1971; Berger, 1975; Kelly et al., 1977; Borgsteede and Duyn, 1989), closantel (Oosthuizen and Erasmus, 1993), morantel (Colglazier et al., 1972; Kelly et al., 1977), or pyrantel (Kelly et al., 1977). The contribution of Pgp in BZ resistance, then, does not implicate Pgp for conferring use-level multidrug resistance in *H. contortus*. Although overexpression of Pgp has been found in an IVM-selected strain of *H. contortus* (Xu et al., 1998), it is not known if this strain shows any evidence of cross-resistance to other classes of anthelmintics. An alteration and/or overexpression of Pgp may contribute to anthelmintic resistance, but not in itself be sufficient for resistance to be apparent at recommended anthelmintic dose rates. These recommended dose rates are sometimes 2–50 times the ED<sub>95</sub> concentrations. Thus to find evidence of a contribution

of Pgp selection to cross-resistance, dose–response titrations to look for shifts in ED<sub>95</sub> or ED<sub>50</sub> levels would be required. Such studies have yet to be performed.

## 5. Conclusion

We present data that indicate an association between a specific allele of Pgp and survival of BZ treatment. A functional link between Pgp and BZ drugs has previously been demonstrated in *Haemonchus* (Beugnet et al., 1997; Kerboeuf et al., 2002). We cannot rule out the possibility of genetic hitch-hiking being responsible for the changes in allele frequencies. Genetic characterisation of allele P, together with functional studies, will be necessary to establish a causal link with resistance. Assuming this P-glycoprotein locus is not genetically linked to a  $\beta$ -tubulin locus, our data provides further evidence for the involvement of Pgp, in addition to target modification, in resistance to BZ drugs. By reducing the amount of drug able to reach its target, Pgp could act as a general defensive mechanism against xenotoxins. This proposal is supported by our earlier work indicating an association between a specific Pgp allele and survival of ML treatment (Blackhall et al., 1998b). Target modification, then, may play a major role in resistance only when sufficient drug is able to reach these targets.

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## References

- Anderson, T.J.C., Blouin, M.S., Beech, R.N., 1998. Population biology of parasitic nematodes: applications of genetic markers. *Adv. Parasitol.* 41, 219–283.
- Bauer, C., Conraths, F.J., 1994. Comparative efficacy of moxidectin and mebendazole against gastrointestinal nematodes in experimentally infected lambs. *Vet. Rec.* 135, 136–138.
- Beech, R.N., Prichard, R.K., Scott, M.E., 1994. Genetic variability of the  $\beta$ -tubulin genes in benzimidazole-susceptible and -resistant strains of *Haemonchus contortus*. *Genetics* 138, 103–110.
- Berger, J., 1975. The resistance of a field strain of *Haemonchus contortus* to five benzimidazole anthelmintics in current use. *J. S. Afr. Vet. Assoc.* 46, 369–372.
- Besier, R.B., Lyon, J., Kieran, P.J., 1993. The effect of moxidectin against benzimidazole- and levamisole-resistant nematodes of sheep in Western Australia. *Aust. Vet. J.* 70, 422–423.
- Beugnet, F., Gauthey, M., Kerboeuf, D., 1997. Partial in vitro reversal of benzimidazole resistance by the free-living stages of *Haemonchus contortus* with verapamil. *Vet. Rec.* 141, 575–576.
- Blackhall, W.J., Pouliot, J.-F., Prichard, R.K., Beech, R.N., 1998a. *Haemonchus contortus*: selection at a glutamate-gated chloride channel gene in ivermectin- and moxidectin-selected strains. *Exp. Parasitol.* 90, 42–48.
- Blackhall, W.J., Liu, H.Y., Xu, M., Prichard, R.K., Beech, R.N., 1998b. Selection at a P-glycoprotein gene in ivermectin- and moxidectin-selected strains of *Haemonchus contortus*. *Mol. Biochem. Parasitol.* 95, 193–201.
- Blackhall, W.J., Prichard, R.K., Beech, R.N., 2003. Selection at a  $\gamma$ -aminobutyric acid receptor gene in *Haemonchus contortus* resistant to avermectins/milbemycins. *Mol. Biochem. Parasitol.* 131, 137–145.
- Borgers, M., de Nollin, S., 1975. Ultrastructural changes in *Ascaris suum* intestine after mebendazole treatment *in vivo*. *J. Parasitol.* 61, 110–122.
- Borgers, M., de Nollin, S., de Brabander, M., Thienpont, D., 1975. Influence of the anthelmintic mebendazole on microtubules and intracellular organelle movement in nematode intestinal cells. *Am. J. Vet. Res.* 36, 1153–1166.
- Borgsteede, F.H., Duyn, S.P., 1989. Lack of reversion of a benzimidazole resistant strain of *Haemonchus contortus* after six years of levamisole usage. *Res. Vet. Sci.* 47, 270–272.
- Coles, G.C., 1989. The molecular biology of drug resistance. In: Bennet, E., Behm, C., Bryant, C. (Eds.), *Comparative Biochemistry of Parasitic Helminths*. Chapman and Hall, London, pp. 125–144.
- Colglazier, M.L., Kates, K.C., Enzie, F.D., 1971. Activity of levamisole, pyrantel tartrate, and rafoxanide against two thiabendazole-tolerant isolates of *Haemonchus contortus*, and two species of *Trichostrongylus*, in sheep. *Proc. Helm. Soc. Wash.* 38, 203–205.
- Colglazier, M.L., Kates, K.C., Enzie, F.D., 1972. Activity of cambendazole and morantel tartrate against two species of *Trichostrongylus* and two thiabendazole-resistant isolates of *Haemonchus contortus* in sheep. *Proc. Helm. Soc. Wash.* 39, 28–32.
- Colglazier, M.L., Kates, K.C., Enzie, F.D., 1974. Cambendazole-resistant *Haemonchus contortus* strain in sheep: further experimental development. *J. Parasitol.* 60, 289–292.
- Cully, D.F., Vassiliatis, D.K., Liu, K.K., Pares, P.S., Van Der Ploeg, L.H.T., Schaeffer, J.M., Arena, J.P., 1994. Cloning of an avermectin-sensitive glutamate-gated chloride channel from *Caenorhabditis elegans*. *Nature* 371, 707–711.
- Davidge, L.C., Flach, W., 1977. Differential binding of methyl benzimidazol-2-yl-carbamate to fungal tubulin as a mechanism of resistance to this antimitotic agent in mutant strains of *Aspergillus nidulans*. *J. Cell. Biol.* 72, 174–193.
- Driscoll, M., Dean, E., Reilly, E., Bergholz, E., Chalfie, M., 1989. Genetic and molecular analysis of a *Caenorhabditis elegans*  $\beta$ -tubulin that conveys benzimidazole sensitivity. *J. Cell. Biol.* 109, 2993–3003.
- Eng, J.K., Prichard, R.K., 2005. A comparison of genetic polymorphism in populations of *Onchocerca volvulus* from untreated- and ivermectin-treated patients. *Mol. Biochem. Parasitol.* 142, 193–202.
- Feng, X.-P., Hayashi, J., Beech, R.N., Prichard, R.K., 2002. Study of the nematode putative GABA type-A receptor subunits: evidence for modulation by ivermectin. *J. Neurochem.* 83, 870–878.
- Forrester, S.G., Prichard, R.K., Beech, R.N., 2002. A glutamate-gated chloride channel subunit from *Haemonchus contortus*: expression in a mammalian cell line, ligand binding, and modulation of

- anthelmintic binding by glutamate. *Biochem. Pharmacol.* 63, 1061–1068.
- Foster, K.E., Burland, T.G., Gull, K., 1987. A mutant  $\beta$ -tubulin confers resistance to the action of benzimidazole-carbamate microtubule inhibitors both *in vivo* and *in vitro*. *Eur. J. Biochem.* 163, 449–455.
- Gottesman, M.M., Pastan, I., 1993. Biochemistry of multidrug resistance mediated by the multidrug transporter. *Ann. Rev. Biochem.* 62, 385–427.
- Hammerschlag, R.S., Sisler, H.D., 1973. Benomyl and methyl-2-benzimidazolecarbamate (MBC): biochemical, cytological and chemical aspects of toxicity in *Ustilago maydis* and *Saccharomyces cerevisiae*. *Pestic. Biochem. Physiol.* 3, 42–54.
- Hartl, D., Clark, A., 1989. *Principles of Population Genetics*, second ed. Sinauer Associated, Inc., Sunderland, MA, USA.
- Huang, Y.J., Prichard, R.K., 1999. Identification and stage-specific expression of two putative P-glycoprotein coding genes in *Onchocerca volvulus*. *Mol. Biochem. Parasitol.* 102, 273–281.
- Kates, K.C., Colglazier, M.L., Enzie, F.D., 1973. Experimental development of a cambendazole-resistant strain of *Haemonchus contortus* in sheep. *J. Parasitol.* 59, 169–174.
- Kelly, J.D., Hall, C.A., Whitlock, H.V., Thompson, H.G., Campbell, N.J., Martin, I.C., 1977. The effect of route of administration on the anthelmintic efficacy of benzimidazole anthelmintics in sheep infected with strains of *Haemonchus contortus* and *Trichostrongylus colubriformis* resistant or susceptible to thiabendazole. *Res. Vet. Sci.* 22, 161–168.
- Kerboeuf, D., Hubert, J., Cardinaud, B., Blond, F., 1995. Efficacy of oral moxidectin against benzimidazole-resistant isolates of gastrointestinal nematodes in sheep. *Vet. Rec.* 136, 16–17.
- Kerboeuf, D., Guégnard, F., Le Vern, Y., 2002. Analysis and partial reversal of multidrug resistance to anthelmintics due to P-glycoprotein in *Haemonchus contortus* eggs using *Lens culinaris* lectin. *Parasitol. Res.* 88, 816–821.
- Kwa, M.S.G., Veenstra, J.G., Roos, M.H., 1993a. Molecular characterization of  $\beta$ -tubulin genes present in benzimidazole-resistant populations of *Haemonchus contortus*. *Mol. Biochem. Parasitol.* 60, 133–144.
- Kwa, M.S.G., Kooyman, F.N.J., Boersema, J.H., Roos, M.H., 1993b. Effect of selection for benzimidazole resistance in *Haemonchus contortus* on  $\beta$ -tubulin isotype 1 and isotype 2 genes. *Biochem. Biophys. Res. Commun.* 191, 413–419.
- Kwa, M.S.G., Veenstra, J.G., Roos, M.H., 1994. Benzimidazole resistance in *Haemonchus contortus* is correlated with a conserved mutation at amino acid 200 in  $\beta$ -tubulin isotype 1. *Mol. Biochem. Parasitol.* 63, 299–303.
- Lubega, G.W., Prichard, R.K., 1990. Specific interaction of benzimidazole anthelmintics with tubulin: high-affinity binding and benzimidazole resistance in *Haemonchus contortus*. *Mol. Biochem. Parasitol.* 38, 221–232.
- Lubega, G.W., Prichard, R.K., 1991a. Interaction of benzimidazole anthelmintics with *Haemonchus contortus* tubulin: binding affinity and anthelmintic efficacy. *Exp. Parasitol.* 73, 203–213.
- Lubega, G.W., Prichard, R.K., 1991b. Specific interaction of benzimidazole anthelmintics with tubulin from developing stages of thiabendazole-susceptible and -resistant *Haemonchus contortus*. *Biochem. Pharmacol.* 41, 93–101.
- Merino, G., Alvarez, A.I., Prieto, J.G., Kim, R.B., 2002. The anthelmintic agent albendazole does not interact with p-glycoprotein. *Drug Metab. Dispos.* 30, 365–369.
- Merino, G., Jonker, J.W., Wagenaar, E., Pulido, M.M., Molina, A.J., Alvarez, A.I., Schinkel, A.H., 2005. Transport of anthelmintic benzimidazole drugs by breast cancer resistance protein (BCRP/ABC2). *Drug Metab. Dispos.* 33, 614–618.
- Mottier, L., Alvarez, L., Fairweather, I., Lanusse, C., 2006. Resistance-induced changes in triclabendazole transport in *Fasciola hepatica*: ivermectin reversal effect. *J. Parasitol.* 92, 1355–1360.
- Nare, B., Liu, Z., Prichard, R.K., Georges, E., 1994. Benzimidazoles, potent anti-mitotic drugs: substrates for the P-glycoprotein transporter in multidrug-resistant cells. *Biochem. Pharmacol.* 48, 2215–2222.
- Njue, A.I., Prichard, R.K., 2004. Genetic variability of glutamate-gated chloride channel genes in ivermectin-susceptible and -resistant strains of *Cooperia oncophora*. *Parasitology* 129, 741–751.
- Njue, A.I., Hayashi, J., Kinne, L., Feng, X.-P., Prichard, R.K., 2004. Mutations in the extracellular domains of glutamate-gated chloride channel  $\alpha$ 3 and  $\beta$  subunits from ivermectin-resistant *Cooperia oncophora* affect agonist sensitivity. *J. Neurochem.* 89, 1137–1147.
- Oosthuizen, W.T., Erasmus, J.B., 1993. Efficacy of moxidectin against a strain of *Haemonchus contortus* resistant to ivermectin, a benzimidazole and a salicylanilide. *J. S. Afr. Vet. Assoc.* 64, 9–12.
- Orbach, M.J., Porro, E.B., Yanofsky, C., 1986. Cloning and characterization of the gene for  $\beta$ -tubulin from a benomyl-resistant mutant of *Neurospora crassa* and its use as a dominant selectable marker. *Mol. Cell. Biol.* 6, 2452–2461.
- Quinlan, R.A., Pogson, C.I., Gull, K., 1980. The influence of the microtubule inhibitor, methyl benzimidazole-2-yl-carbamate (MBC) on nuclear division and the cell cycle in *Saccharomyces cerevisiae*. *J. Cell Sci.* 46, 341–352.
- Raymond, M., Rousset, F., 1995. GENEPop (version 1.2): population genetics software for exact test and ecumenicism. *J. Hered.* 86, 248–249. <http://wbiomed.curtin.edu.au/genepop>.
- Roos, M.H., Boersema, J.H., Borgsteede, F.H.M., Cornelissen, J., Taylor, M., Ruitenber, E.J., 1990. Molecular analysis of selection for benzimidazole resistance in the sheep parasite *Haemonchus contortus*. *Mol. Biochem. Parasitol.* 43, 77–88.
- Sangster, N., 1996. Pharmacology of anthelmintic resistance. *Parasitology* 113, S201–S216.
- Sheir-Neiss, G., Lai, M.H., Morris, N.R., 1978. Identification of a gene for  $\beta$ -tubulin in *Aspergillus nidulans*. *Cell* 15, 639–647.
- Swan, G.E., Schroder, J., Carmichael, I.H., Louw, J.P., Harvey, R.G., Penderis, I., 1984. Efficacy of ivermectin against internal parasites of sheep. *J. S. Afr. Vet. Assoc.* 55, 165–169.
- Taylor, M.A., Hunt, K.R., Wilson, C.A., Baggott, D.G., 1990. Efficacy of ivermectin against benzimidazole-resistant nematodes of sheep. *Vet. Rec.* 127, 302–303.
- Thomas, J.H., Neff, N.F., Botstein, D., 1985. Isolation and characterization of mutations in the  $\beta$ -tubulin gene of *Saccharomyces cerevisiae*. *Genetics* 111, 715–734.
- Waruiru, R.M., Weda, E.H., Otieno, R.O., Ngotho, J.W., Bogh, H.O., 1996. Comparative efficacies of closantel, ivermectin, oxfendazole, thiophanate and levamisole against thiabendazole resistant *Haemonchus contortus* in sheep. *Trop. An. Health Prod.* 28, 216–220.
- Wescott, R.B., LeaMaster, B.R., 1982. Efficacy of ivermectin against naturally acquired and experimentally induced nematode infections in sheep. *Am. J. Vet. Res.* 43, 531–533.
- Xu, M., Molento, M., Blackhall, W., Ribeiro, P., Beech, R., Prichard, R., 1998. Ivermectin resistance in nematodes may be caused by alteration of P-glycoprotein homolog. *Mol. Biochem. Parasitol.* 91, 327–335.