Genetic Variability of the β-Tubulin Genes in Benzimidazole-Susceptible and -Resistant Strains of *Haemonchus contortus*

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ABSTRACT

Benzimidazole anthelmintics are the most common chemotherapeutic agents used to remove intestinal helminths from farm animals. The development of drug resistance within helminth populations is widespread and can render these drugs essentially useless. The mechanism of benzimidazole resistance appears to be common to many species ranging from fungi to nematodes and involves alterations in the genes encoding β-tubulin. During the selection process resulting in resistance, there must be quantitative changes in the population gene pool. Knowledge of these changes would indicate the mechanisms underlying the spread of resistance in the population, which in turn could be used to design more effective drug administration strategies. To this end we have identified allelic variation at two β-tubulin genes in *Haemonchus contortus* using restriction map analysis of individual adults. Extremely high levels of variation were identified at both loci within a susceptible strain. In two independently derived benzimidazole resistant strains, allele frequencies at both loci were significantly different from the susceptible strain but not from each other. The same alleles at both loci, in both resistant strains, were favored by selection with benzimidazoles, suggesting that both loci are involved in determining benzimidazole resistance. These data confirm that changes in allele frequency, rather than novel genetic rearrangements induced by exposure to the drug, explain the changes associated with benzimidazole resistance. These results also show that any DNA based test for the development of benzimidazole resistance must take into account the frequency of alleles present in the population and not simply test for the presence or absence of specific allelic types.

BENZIMIDAZOLES (BZ) are perhaps the most widely used anthelmintics available. Thiabendazole (TBZ) was the first broad spectrum anthelmintic produced and heralded the development of many derivatives which are highly effective against a wide range of parasite species (BROWN *et al.* 1961; MCKELLAR and SCOTT 1990). Unfortunately the widespread use of BZ class drugs has resulted in the appearance of drug resistance (LACEY 1988; PRICHARD 1990; PRICHARD *et al.* 1980). BZ resistance in nematodes has been shown to be heritable and is defined as an increase in frequency of individuals able to tolerate elevated drug doses relative to a normal population (PRICHARD *et al.* 1980). The appearance of drug resistance therefore reflects changes in the composition of a parasite population gene pool. BZ chemotherapy is no longer effective once resistance has developed and alternative methods of control must be used. If we are able to understand the phenomenon of benzimidazole resistance in populations, and the mechanisms responsible for the appearance and spread of alleles which confer resistance, it may be possible to circumvent the spread of drug resistance in the field and continue to control parasitic infections using benzimidazoles.

Investigations into the mechanism of action of BZ have shown that, in the presence of the drug, microtubules are selectively depolymerized (BORGERS and DE NOLLIN 1975; BORGERS *et al.* 1975; QUINLAN *et al.* 1980; SANGSTER *et al.* 1985). This destabilization is a result of BZ binding specifically and with high affinity to β-tubulin (DAVIDSE and FLACH 1977; HAMMERSCHLAG and SISLER 1973; LUBEGA and PRICHARD 1990, 1991b,c). BZ resistance is generally mediated through a decrease in the high affinity binding of BZ to β-tubulin, resulting in an increased tolerance for the drug (LACEY 1988; LACEY and PRICHARD 1986; LUBEGA and PRICHARD 1990; LUBEGA and PRICHARD 1991a,b,c; PRICHARD 1990). The cause of BZ resistance has been examined in organisms ranging from fungi to nematodes. An extensive analysis of spontaneous BZ-resistant mutations in yeast determined that all of 29 mutations tested were due to lesions in a single β-tubulin gene (THOMAS *et al.* 1985). In one case a single amino acid substitution was identified as the cause of resistance. Similarly, in the fungi *Aspergillus nidulans*, *Neurospora crassa* and *Physarum polycephalum*, single amino acid substitutions in the β-tubulin protein confer BZ resistance (FOSTER *et al.* 1987; FUJIMURA *et al.* 1992; JUNG *et al.* 1992; ORBACH *et al.* 1986; SHEIR-NEISS *et al.* 1978). In Physarum, the BZ-resistant form of β-tubulin is incorporated into functional microtubules regardless of the BZ concentration during assembly, indicating that the resistance is mediated through altered properties of a functional protein rather than by inactivation of an intrinsically sensitive isotype (FOSTER *et al.* 1987). A

different conclusion can be drawn from analysis of BZ resistance in the non-parasitic nematode *Caenorhabditis elegans*. Following mutagenesis, all mutations conferring BZ resistance map to one of the three loci known to encode β-tubulin (DRISCOLL *et al.* 1989). The mutations in *C. elegans* responsible for BZ resistance are unusual in that most are deletions or other genetic rearrangements within the coding region of the β-tubulin gene, resulting in loss of function. Inactivation of the susceptible isotype results in a pool of β-tubulin representing the remaining, presumably resistant isotypes. Changes within the β-tubulin genes of *Haemonchus contortus* are therefore of significance for the development of BZ resistance in this parasite.

Most higher eukaryotic organisms have several genes encoding β-tubulin (CLEVELAND and SULLIVAN 1985; SULLIVAN 1988). An extensive examination of an *H. contortus* cDNA library revealed two distinct classes of β-tubulin homologous clones, suggesting that there are two functionally distinct loci which encode β-tubulin, represented by the β8-9 and β12-16 cDNA sequences (GEARY *et al.* 1992). This is consistent with the two distinct isoforms of β-tubulin in *H. contortus* which can be distinguished by their isoelectric point (LUBEGA and PRICHARD 1991b). Evidence from Southern hybridization experiments support the existence of two β-tubulin genes, termed the isotype 1 (equivalent to the β8-9 cDNA) and the isotype 2 (equivalent to the β12-16 cDNA) genes (KWA *et al.* 1993a,b; LUBEGA *et al.* 1993; ROOS *et al.* 1990). The existence of other β-tubulin genes in *H. contortus* has not been ruled out. The mechanism of BZ resistance in *H. contortus* was initially thought to be similar to that in *C. elegans* since DNA prepared from susceptible strains possessed more bands homologous to β-tubulin than did BZ resistant strains. The quantity of DNA it is possible to extract from single *H. contortus* is at the limit of detection by Southern hybridization. Despite this, hybridization of β-tubulin probes to DNA prepared from single individuals has demonstrated that worms from BZ resistant strains have β-tubulin homologous sequences (LUBEGA *et al.* 1993; ROOS *et al.* 1990). DNA sequence analysis of a β-tubulin clone from resistant *H. contortus* has identified substitutions in the amino acid sequence of the isotype 1 β-tubulin thought to mediate BZ resistance (KWA *et al.* 1993b). This may not be the only mechanism for BZ resistance, since a second study of the effects of prolonged severe selection for resistance has demonstrated that the isotype 2 locus may be deleted in highly resistant strains (KWA *et al.* 1993a).

The purpose of the present study was to investigate the genetic diversity of the β-tubulin genes in *H. contortus* and to characterize the quantitative changes which occur as BZ resistance develops. The data available so far on the genetic changes which accompany the development of BZ resistance suggest that the isotype 1 locus plays a dominant role (KWA *et al.* 1993a,b; LUBEGA *et al.* 1993; ROOS *et al.* 1990). By comparing the response of both β-tubulin loci to BZ selection the significance of the isotype 2 locus to BZ resistance can be determined. Furthermore, a detailed analysis of the frequency spectrum of alleles at both loci is necessary before the likelihood of success for a test based on detecting specific β-tubulin alleles for predicting BZ resistance in the field can be assessed.

MATERIALS AND METHODS

Parasite strains: Individuals from three strains of *H. contortus* were used in this study. One BZ-susceptible strain was isolated from the field before extensive BZ use. A second strain derived from this susceptible strain had been selected for BZ resistance by 10 generations of *in vivo* selection with cambendazole (CBZ). The third strain was derived independently from the field on the basis of its resistance to TBZ. The derivation of these strains and analysis with respect to characteristics associated with BZ resistance have been described previously (COLGLAZIER *et al.* 1974; KATES *et al.* 1973; LUBEGA *et al.* 1993; LUBEGA and PRICHARD 1990, 1991a,b,c).

DNA isolation: Adult male *H. contortus* from each strain were taken from the abomasum of single sheep which had been infected with approximately 10,000 L_3 larvae about 21 days previously. Only adult males were used to avoid the possibility of DNA from the eggs present in females contaminating the sample. The worms were washed and stored in water at 4° for no more than 1 week before DNA isolation. DNA was isolated from 59 individuals from the susceptible strain, 60 from the CBZ-resistant strain and 30 from the TBZ-resistant strain. Each worm was transferred to a tube containing 200 μl STE, 0.6 M β-mercaptoethanol and 200 μg/ml protease K (SAMBROOK *et al.* 1989) and incubated at 65° for 1 hr, after which time no remaining tissue was visible. Two phenol chloroform extractions were performed and the DNA precipitated with 2.5 M ammonium acetate and 50% isopropanol following the addition of 10 μg of linear acrylamide as a coprecipitant (GAILLARD and STRAUSS 1990). The DNA pellet was air-dried and redissolved in 50 μl TE.

Polymerase chain reaction (PCR) amplification: PCR reactions were performed in a Geneamp 9600 (Perkin-Elmer Cetus) with reagents and *Taq* DNA polymerase supplied by Promega. Primers used in PCR amplifications were sense primers RBE6 (CGTGAAATCGTTCATGTG), RBE9 (CACGTTCAGGCCGGACAG), RBE10 (ATGCGGNAAYCARATYGG), RBE13 (TCATACAAAGGAGAGAGC) and RBE15 (TGTTTACTACAATGARGC); the antisense primers were RBE7 (CTCCTCGGGATATGCCTC), RBE8 (AACGAAAGGAGTCCATCG), RBE14 (TCAGAGATAACCTCCCAG) and RBE16 (TGTAGTAMACATTGATYC). These primers were based on sequences of the isotype 1 (β8-9) and isotype 2 (β12-16) cDNAs (GEARY *et al.* 1992). Amplification conditions for all pairs of primers were as follows: 95° for 5 min to ensure denaturation of the template DNA followed by 40 cycles of 15 sec at 95°, 30 sec at 55° and 2 min at 72°. Fifty-microliter PCR were performed using 1 μl (approximately 2 ng) of the genomic DNA solution prepared from each individual with either RBE6-RBE8, to amplify the isotype 1 locus, or RBE9-RBE8, to amplify the isotype 2 locus. A second amplification using 1 μl of the primary amplification as a template was performed using RBE10-RBE8 for products from both loci. The primer pairs RBE13-RBE8, RBE15-RBE8, RBE9-RBE14 and RBE9-RBE16 were also used to determine whether an isotype 2-specific amplification product could be obtained from the

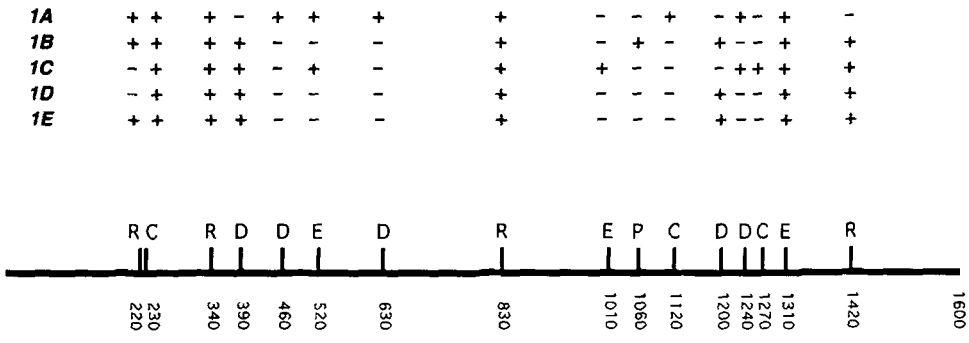

FIGURE 1.—Summary restriction map of alleles identified at the β-tubulin isotype 1 locus. Restriction sites are indicated by a letter: C, *Cfo*I; D, *Dde*I; E, *Hae*III; P, *Hpa*II; and R, *Rsa*I. The position in nucleotides from the 5′ end of the PCR product of each restriction site is indicated under each map. The presence or absence of the restriction sites in each of the five allelic types are indicated above the map.

individual presumed to be homozygous for the allele *2X* (see RESULTS).

Restriction enzyme digestion and mapping of alleles: Restriction enzymes and reagents were supplied by Promega and digestion was carried out as described by the manufacturer. Ten microliters of the secondary PCR product from either locus was digested with *Cfo*I, *Dde*I, *Hae*III, *Hpa*II or *Rsa*I. The resulting fragments were separated on a non-denaturing, 5% polyacrylamide gel and visualized by staining with 0.5 μg/ml ethidium bromide. The identity of the different alleles was deduced, where possible, by identifying a homozygote, where the sum of the fragment sizes equalled the size of the original PCR fragment, or by identifying those bands in heterozygotes which consistently occurred together. Restriction maps of the different alleles were constructed by single and double digestion of the PCR product from individuals which appeared to be homozygous or by assuming a minimum number of changes from alleles which could be mapped. Restriction fragment sizes are available upon request.

Data analysis: Genotype frequencies were tested for Hardy-Weinberg equilibrium by a 1-tailed test for an excess of homozygotes, calculating exact probabilities from a binomial distribution based on the observed allele frequencies (SOKAL and ROHLF 1981). Differences in allele frequencies between strains were tested for significance using a *G* test for heterogeneity, pooling allele classes where necessary to ensure a minimum expected number of at least 3 (SOKAL and ROHLF 1981). Significance was taken at the 5% level. Maximum likelihood frequency estimates for isotype 2 alleles were calculated according to the "EM" algorithm (DEMPSTER *et al.* 1977; YASUDA and KIMURA 1968). Sequence divergence and the standard error between pairs of alleles were calculated using Equations 8 from NEI and LI (1979) and Equations 19 and 20 in NEI and TAJIMA (1981). Nucleotide diversity and the standard error within strains were calculated using Equations 11 and 18 in NEI and TAJIMA (1981). Estimates of the proportion of polymorphic sites and theta were calculated from HUDSON (1982) assuming no recombination.

RESULTS

An initial amplification of the entire genomic isotype 1 locus was performed with RBE6 and RBE7 using DNA prepared from approximately 0.5 g adult *H. contortus* from the CBZ-resistant strain. A product of 3.6 kb was identified, and restriction mapping confirmed the identity of the product as closely related to the published isotype 1 genomic DNA sequence (KWA *et al.* 1993b). DNA amplification was too inefficient, however, to amplify the entire gene from single adults. Amplification was obtained using DNA from single individuals using RBE8 as the antisense primer. This region was chosen for amplification since it contained approximately 1400 nucleotides (nt) of intron derived sequence within the 200 nt of coding sequence.

Isotype 1 locus: PCR amplification of genomic DNA from single adult worms resulted in an isotype 1-specific product 1600 nt in length. A total of five isotype 1 alleles were identified, based on their restriction patterns with the five enzymes used in this survey. These alleles have been named, arbitrarily, *1A* to *1E*. A summary restriction map indicating the location of the restriction sites is shown in Figure 1. A total of 16 restriction sites were surveyed, representing 4.0% of the PCR product length, 12 of these sites were polymorphic within the sample. The presence or absence of these restriction sites in each of the five alleles is shown in Figure 1. The frequencies of the five alleles in each strain are shown in Table 1. No strain differed significantly from Hardy-Weinberg equilibrium. Sequence divergence (and the standard error) between alleles ranged from 0.015 (0.015) between alleles *1B* and *1E* to 0.188 (0.075) between alleles *1A* and *1D*.

Isotype 2 locus: An amplification product 1450 nt in length was produced from all individuals except one from the susceptible strain. Despite giving an apparently normal isotype 1-specific amplification product this individual gave no isotype 2-specific product with the primer pairs RBE9-RBE8, RBE9-RBE14, RBE9-RBE16, RBE10-RBE8, RBE13-RBE8 and RBE15-RBE8. This individual was designated as being homozygous for a hypothetical allele, *2X*, which failed to serve as a template for DNA amplification under these PCR conditions. A total of 12 more isotype 2 alleles, named *2A* to *2L* arbitrarily, were identified based on their restriction patterns. A summary restriction map and the presence or absence of the variation detected in each of the alleles is shown in Figure 2. A total of 27 restriction sites were surveyed, which represented approximately 7.4% of the PCR product length. Of these, 22 were polymorphic within the sample. In addition to restriction site polymorphism, alleles *2C* and *2E* differed by small insertion-deletion events from the other alleles (*a* and *b* in Figure 2). The observed frequencies of all 13 isotype 2 alleles are shown in Table 2. Neither resistant strain showed

TABLE 1

Frequencies of the β-tubulin isotype 1 alleles in the three strains of *H. contortus*

Allele	Susceptible	CBZ-resistant	TBZ-resistant
1A	0.458	0.975	0.933
1B	0.305	0.025	0.000
1C	0.093	0.000	0.000
1D	0.085	0.000	0.000
1E	0.059	0.000	0.067

significant departure from Hardy-Weinberg equilibrium. The departure of the susceptible strain from Hardy-Weinberg equilibrium at the isotype 2 locus was highly significant. This departure was probably due to the presence of allele *2X* in the sample. Individuals heterozygous for the *2X* allele would appear to be homozygous and would consequently be misscored. Although neither resistant strain showed evidence of this allele, the maximum likelihood frequency estimates for the different alleles in all three strains, assuming the presence of allele *2X*, are given in Table 2. Sequence divergence (and the standard error) between alleles, ignoring the variation due to insertion-deletion events, ranged from 0.009 (0.009) between alleles *2A* and *2J* to 0.193 (0.113) between alleles *2C* and *2D*.

The frequencies of all genotypes observed, with respect to both β-tubulin loci are given in Table 3.

Comparison of susceptible to resistant strains: The number of alleles observed in the susceptible strain was higher at both loci than in the resistant strains and allele frequencies were significantly different between the susceptible and resistant strains, but not between resistant strains. Only two of the five isotype 1 alleles in the susceptible strain were observed in the CBZ-resistant strain. The *1A* allele, which was most frequent in the susceptible strain had increased almost to fixation. The *1B* allele, which was the second most frequent allele in the susceptible strain, was still present but at a greatly reduced frequency. This pattern was very similar to that seen in the TBZ resistant strain. Again, the *1A* allele had increased in frequency to a similar degree, but in this case the other allele observed was *1E*.

The pattern of variability of the isotype 2 locus was more complex than at the isotype 1 locus. In the susceptible strain 11 alleles were observed (including *2X*). In the CBZ resistant strain, three of these persisted, *2A*, *2B* and *2C*, with the appearance of two alleles specific for the resistant strain, *2K* and *2L*. The pattern of alleles in the TBZ resistant strain appeared to be very similar to the CBZ resistant strain, with alleles *2A*, *2B*, *2C* and *2E* persisting and the appearance of allele *2L*. In both resistant strains allele *2A* had increased in frequency to approximately 75%, whereas in the susceptible strain this was only the third (or fourth, taking the maximum likelihood frequencies) most frequent allele. Estimates of the variability within the three strains, in terms of the probability that a site was polymorphic (p) and two different estimates (θ and π) of the probability that two sequences drawn at random would differ for any given nucleotide, are shown in Table 4.

DISCUSSION

Variability in the susceptible strain: DNA sequence variation at both β-tubulin loci was much greater than has been reported previously for nuclear DNA. Surveys of nuclear DNA variation within populations have produced estimates of nucleotide diversity ranging from 0.0003 up to 0.0057 (LYNCH and CREASE 1990). Nucleotide diversity at both β-tubulin loci was an order of magnitude higher than these at 0.094 and 0.091 for the isotype 1 and isotype 2 loci, respectively (Table 4). Assuming selective neutrality and a population at equilibrium, nucleotide diversity (π) is an estimate of $4N\mu$, where N is the effective population size and μ the mutation rate. Increased variability could be explained if either the effective population size or the neutral mutation rate were elevated.

In this case it seems unlikely that an elevated mutation rate alone can explain the increased variability. Mitochondrial DNA is known to evolve approximately 10 times faster than the majority of nuclear genes (THOMAS and WILSON 1991; WILSON *et al.* 1985). Mitochondrial DNA variation has been examined in two parasitic nematodes with geographic distributions broadly similar to that of *H. contortus*. In Ascaris from both humans and pigs the nucleotide diversity of mitochondrial DNA is estimated to be 0.016 (ANDERSON *et al.* 1993). In the cattle parasite, *Ostertagia ostertagi*, the diversity was slightly higher at 0.022 (BLOUIN *et al.* 1992). The variability of both β-tubulin genes was higher than both of these mitochondrial DNA estimates. If an increased mutation rate were responsible for the differences in variability, the mutation rate of the β-tubulin genes would have to be higher than that of mitochondrial DNA.

It would seem more likely that the increased variability is a result of a high effective population size for the parasite. This need not be simply the result of a large number of individuals in the population. Elevated diversity estimates can also be the result of introgression between populations which have been subdivided for a significant period in the past. When separate subpopulations evolve independently from each other the average sequence divergence between alleles from different subpopulations increases with time. If migration then brings these highly divergent alleles into the same population the estimate of sequence diversity increases because of the unusually high sequence divergence within the sample (SLATKIN 1982, 1989; STROBECK 1987). Observations of high sequence diversity within populations are rare. In the threespine stickleback, *Gasterosteus aculeatus*, from the Queen Charlotte Islands mitochondrial DNA types with approximately 2.5% sequence di-

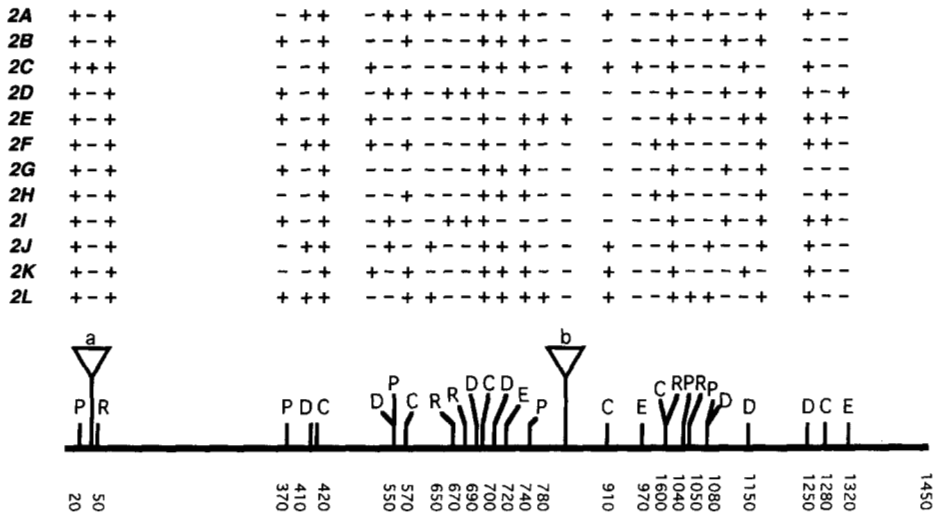

FIGURE 2.—Summary restriction map of alleles identified at the β-tubulin isotype 2 locus. Letters indicating the position of restriction sites are the same as in Figure 1. Letters a and b denote small insertion-deletion events of 15 and 35 nt, respectively. The position in nucleotides from the 5′ end of the PCR product of each restriction site is indicated under the map. The presence or absence of the features in each of the 12 allelic types is indicated above the map.

TABLE 2

Frequencies of the β-tubulin isotype 2 alleles in the three strains of *H. contortus*

Allele	Susceptible	Susceptible[a]	CBZ-resistant	CBZ-resistant[a]	TBZ-resistant	TBZ-resistant[a]
2A	0.119	0.090	0.758	0.694	0.766	0.680
2B	0.305	0.252	0.083	0.071	0.100	0.088
2C	0.271	0.221	0.125	0.109	0.067	0.053
2D	0.119	0.083	0.000	0.000	0.000	0.000
2E	0.051	0.043	0.000	0.000	0.050	0.035
2F	0.059	0.036	0.000	0.000	0.000	0.000
2G	0.017	0.017	0.000	0.000	0.000	0.000
2H	0.008	0.008	0.000	0.000	0.000	0.000
2I	0.017	0.017	0.000	0.000	0.000	0.000
2J	0.017	0.017	0.000	0.000	0.000	0.000
2K	0.000	0.000	0.008	0.008	0.000	0.000
2L	0.000	0.000	0.025	0.025	0.017	0.017
2X	0.017	0.216	0.000	0.093	0.000	0.127

[a] Maximum likelihood frequency estimates.

vergence were found within the same lakes (O'REILLY *et al.* 1993). The explanation for this appears to be that many of the streams on the island disappeared during the last major glaciation. Recolonization of streams by marine stickle back occurred later and in some cases the colonizing fish came in to contact with small populations of freshwater stickleback which had remained isolated in glacial refugia. In a survey of mitochondrial DNA in Ascaris, introgression of haplotypes specific for pig derived Ascaris into the human derived Ascaris population was observed in a few cases. Discounting these alleles reduced the within-human Ascaris nucleotide diversity estimate from 0.016 to 0.004 (ANDERSON *et al.* 1993). A characteristic of introgression is the presence of large discontinuities in the divergence estimates between alleles (ANDERSON *et al.* 1993; O'REILLY *et al.* 1993; STROBECK 1987). In *H. contortus* there was little evidence for such discontinuities. This was also found to be the case for mitochondrial DNA from Ostertagia (BLOUIN *et al.* 1992). The populations of *H. contortus* and Ostertagia may simply consist of a very large number of individuals which contribute to the mating population.

Comparison of the susceptible and resistant strains: Allele frequencies at both β-tubulin loci change significantly under selection for BZ resistance. Variability was greatly reduced in the two resistant strains, both in the number of alleles observed and nucleotide diversity. In both resistant strains similar changes in allele frequencies had occurred, with alleles *1A* and *2A* increasing greatly in frequency. Of particular interest was allele *2X*, which failed to serve as a template for PCR amplification. The reason for the failure of amplification is unclear. Given the high divergence between β-tubulin alleles, there may have been point mutations within the primer annealing sites which prevented efficient amplification. Although this possibility can not be excluded, a total of six different pairs of primers were used with this individual and in no case was an isotype 2 specific product obtained. An alternative explanation is that allele *2X* represents a deletion for the isotype 2 gene. Such a deletion has been identified in strains of *H. contortus* and appears to have reached fixation in strains which have been exposed to long term intense selection for BZ resistance (KWA *et al.* 1993a).

TABLE 3

Genotype frequencies, with respect to both β-tubulin loci, observed in each of the *H. contortus* strains

Genotype	Susceptible	CBZ-resistant	TBZ-resistant
1A1A 2A2A	0.000	0.583	0.500
1A1A 2A2B	0.000	0.083	0.133
1A1A 2A2C	0.034	0.133	0.067
1A1A 2A2E	0.000	0.000	0.033
1A1A 2A2K	0.000	0.017	0.000
1A1A 2A2L	0.000	0.050	0.033
1A1A 2B2B	0.068	0.033	0.033
1A1A 2B2C	0.051	0.017	0.000
1A1A 2B2E	0.017	0.000	0.000
1A1A 2B2F	0.017	0.000	0.000
1A1A 2C2C	0.000	0.033	0.033
1A1A 2D2D	0.051	0.000	0.000
1A1A 2E2E	0.000	0.000	0.033
1A1B 2A2A	0.034	0.033	0.000
1A1B 2A2G	0.034	0.000	0.000
1A1B 2B2B	0.034	0.000	0.000
1A1B 2B2C	0.034	0.000	0.000
1A1B 2B2D	0.017	0.000	0.000
1A1B 2B2H	0.017	0.000	0.000
1A1B 2B2I	0.017	0.000	0.000
1A1B 2C2C	0.051	0.017	0.000
1A1B 2C2D	0.017	0.000	0.000
1A1B 2C2E	0.017	0.000	0.000
1A1B 2C2J	0.017	0.000	0.000
1A1B 2E2E	0.017	0.000	0.000
1A1B 2F2F	0.017	0.000	0.000
1A1B 2X2X	0.017	0.000	0.000
1A1C 2C2C	0.017	0.000	0.000
1A1D 2B2C	0.034	0.000	0.000
1A1E 2A2A	0.000	0.000	0.133
1A1E 2B2B	0.017	0.000	0.000
1A1E 2C2C	0.034	0.000	0.000
1B1B 2A2A	0.017	0.000	0.000
1B1B 2B2E	0.017	0.000	0.000
1B1B 2C2C	0.017	0.000	0.000
1B1C 2A2A	0.017	0.000	0.000
1B1C 2B2E	0.017	0.000	0.000
1B1C 2D2D	0.017	0.000	0.000
1B1C 2F2F	0.017	0.000	0.000
1B1D 2A2C	0.017	0.000	0.000
1B1D 2C2C	0.017	0.000	0.000
1B1E 2A2I	0.017	0.000	0.000
1B1E 2B2B	0.034	0.000	0.000
1B1E 2B2C	0.017	0.000	0.000
1C1C 2B2B	0.017	0.000	0.000
1C1C 2D2D	0.017	0.000	0.000
1C1D 2B2D	0.017	0.000	0.000
1C1D 2F2F	0.017	0.000	0.000
1D1D 2C2C	0.017	0.000	0.000
1D1D 2D2J	0.017	0.000	0.000

The frequency distribution of alleles was significantly different between the susceptible and resistant strains, but not between the two resistant strains. Selection with CBZ or TBZ had induced similar shifts in allele frequency at both β-tubulin loci. If the changes in frequency had occurred solely as a result of genetic drift, caused by a reduction in effective population size during the selection process, the most abundant allele would be expected to approach fixation. At the isotype 2 locus the *2A* allele, which had increased in frequency in parallel in both resistant strains, was only the fourth most frequent allele in the susceptible strain. This strongly suggests that the frequency changes were a consequence of drug selection. The change in allele frequencies was even greater at the isotype 1 locus which is already known to be involved in determining BZ resistance (KWA *et al.* 1993a,b; LUBEGA *et al.* 1993; ROOS *et al.* 1990). It is not known whether the alleles identified at both β-tubulin loci encode functional β-tubulin protein.

It is important to remember that the DNA surveyed from each of the two β-tubulin loci represented mostly non-coding, intron derived sequence. The high sequence variability of the intron derived sequence increases the number of allelic types it is possible to classify each copy of the gene into, thereby decreasing the possibility of heterogeneity within any allelic class. The *1A* allele in the susceptible strain, for example, could be a mixture of different types which were indistinguishable with the enzymes chosen. Unfortunately this problem could only be solved by sequencing the β-tubulin loci completely in each individual which presents technical difficulties because the individuals are diploid and cloning of the PCR amplified products would be necessary. The approach used here allowed many individuals to be screened rapidly at the expense of a loss in resolution. Given the high sequence divergence between alleles at both loci the possibility of heterogeneity within the designated allelic classes is minimal.

These data are an important extension of previous investigations into the genetic changes associated with the development of BZ resistance. When DNA prepared from many individuals is hybridized with probes specific for the isotype 1 and isotype 2 loci many bands appear in susceptible strains, most of which disappear in resistant strains (GEARY *et al.* 1992; LUBEGA *et al.* 1993; ROOS *et al.* 1990). By examining the pooled DNA in this way it is impossible to determine whether the changes are the result of loss of alleles or deletion of loci from a multigene family. Southern hybridization to DNA from single adult worms was used in order to try to resolve this issue (LUBEGA *et al.* 1993; ROOS *et al.* 1990). It is apparent that individuals from the resistant strains contain DNA homologous to the isotype 1 and isotype 2 loci. This suggests a loss of allelic variation, rather than deletions are responsible for resistance. Due to limitations in the sensitivity of Southern hybridization only a single restriction enzyme could be used with each sample, so only a single base substitution could be observed in each. The advantage of the technique used here is that many different restriction enzymes could be used to survey each locus. A total of 64 and 108 sites were screened for the isotype 1 and isotype 2 loci respectively giving a much more representative estimate of the variability present at the two loci than has been achieved previously, since the number of nucleotide sites screened is the major scource of variance in the estimates of variability (LYNCH and CREASE 1990). The data we present here demonstrate for the first time that the isotype 1 and isotype 2

TABLE 4

Estimates of variability within the three strains of *H. contortus*

	p	θ	π
Susceptible isotype 1	0.150 (0.043)	0.072 (0.090)	0.094 (0.002)
CBZ-resistant isotype 1	0.118 (0.039)	0.057 (0.087)	0.008 (0.004)
TBZ-resistant isotype 1	0.111 (0.039)	0.062 (0.099)	0.019 (0.008)
Susceptible isotype 2	0.172 (0.037)	0.083 (0.080)	0.091 (0.003)
CBZ-resistant isotype 2	0.129 (0.033)	0.062 (0.073)	0.037 (0.005)
TBZ-resistant isotype 2	0.133 (0.033)	0.075 (0.087)	0.041 (0.008)

The proportion of polymorphic sites (p), the heterozygosity per nucleotide site (θ) and nucleotide diversity (π) are given. Standard errors are given in parentheses.

β-tubulins represent distinct loci, with many alleles segregating at each locus.

We have clear evidence here that specific alleles at both the isotype 1 and isotype 2 loci are involved in BZ resistance and that the alleles which are most closely associated with BZ resistance were already present in the *H. contortus* population before the BZ class drugs were developed. In contrast to the report of a deletion of the isotype 2 locus in highly BZ resistant strains there was no evidence for an increase in frequency of such an allele in either resistant strain. To reconcile these results it is necessary to infer either two different mechanisms underlying BZ resistance (KWA *et al.* 1993a), or chance fixation of the isotype 2 locus deletion in strains which undergo very strong selection for resistance. It has been shown that a shift in frequency of the alleles present in a susceptible strain can result in resistance. Once most of the variation is lost, however, the potential for further increase in the level of BZ resistance becomes limited. Deletion of the isotype 2 locus would therefore provide a second mechanism for increasing the level of resistance. If allele *2X* does represent a deletion of at least part of the isotype 2 locus then the deletion is already present at high frequency in the susceptible strain. Since the frequency of *2X* decreases in both resistant strains it appears to be associated with BZ susceptibility, rather than resistance.

Despite the different conditions under which the two BZ-resistant strains were developed, the allele frequency distributions at the two β-tubulin loci are remarkably similar. Selection with different drugs within the BZ family at different times and locations has resulted in selection for the same allelic types. In a survey of several different BZ-resistant strains from around the world using Southern hybridization with DNA prepared from many adult worms, banding patterns are found to be very similar (KWA *et al.* 1993b). Although Southern hybridization is not sensitive enough to detect all but the most common allelic types, and so the presence of other alleles at low frequencies is unknown, the general pattern is that BZ resistant strains have the same alleles at very high frequency, irrespective of the drug used or the conditions under which resistance arises. In these circumstances a DNA based assay for the alleles at both β-tubulin loci which were associated with resistance is potentially a useful tool for detecting BZ resistance in *H. contortus*. If detection of the *1A* and *2A* alleles alone were used as the basis for a test, the chance of falsely diagnosing resistance would be high since these alleles are both present, in quite high frequency, in a known susceptible strain. Any DNA based test must therefore take into account the frequency spectrum of the alleles present.

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