Diversity of benzimidazole-resistance alleles in populations of small ruminant parasites

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1. Introduction

The resistance of gastro-intestinal nematodes of small ruminants (sheep and goat) to benzimidazole anthelmintic drugs seems to be linked primarily to a single mutation in the isotype 1 β-tubulin gene. This study was carried out to investigate the origin and diversity of benzimidazole-resistance alleles in trichostrongylid nematodes. We sequenced a 550 bp fragment of the isotype 1 β-tubulin gene from several benzimidazole-resistant Teladorsagia circumcincta populations isolated from dairy goat farms in the central and south-western France. We also sequenced the same β-tubulin fragment from Trichostrongylus colubriformis and Haemonchus contortus populations in south-western France. We found eight benzimidazole-resistance alleles in all T. circumcincta populations studied, six in H. contortus populations, and only one in T. colubriformis populations. In most cases, only one benzimidazole-resistance allele was present in T. circumcincta and H. contortus populations, but two alleles were found in a fewer number of them. Some T. circumcincta populations shared the same benzimidazole-resistance allele whereas some others had a specific benzimidazole-resistance allele. Similar findings were obtained for H. contortus. As no parasites are introduced once the flock of dairy goat farms has been constituted, these data indicate for the three studied species that rare pre-existing benzimidazole-resistance alleles already present before the isolation of populations had been selected. On the other hand, the fact that some benzimidazole-resistance alleles were specific to one population of T. circumcincta or H. contortus, seems to be in agreement with the hypothesis of the selection of spontaneous mutations. Thus, the origin of benzimidazole-resistance alleles in trichostrongylid nematodes seems to involve primarily the selection of rare alleles and possibly of spontaneous mutations. © 2002 Australian Society for Parasitology Inc. Published by Elsevier Science Ltd. All rights reserved.

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by spontaneous mutations. It has been demonstrated that the rate of mutation increases under environmental stress in bacteria (Bridge, 1996; Finkel and Kolter, 1999; Tenaillon et al., 1999) and other organisms (Hofmann and Parsons, 1994). Thus, Baquero and Blazquez (1997) propose that external antibiotic pressure stimulates the generation of antibiotic resistant variants.

The study of the diversity of benzimidazole-resistance alleles in several populations should make it possible to determine the origin of these benzimidazole-resistance alleles in trichostrongyloid communities. On the one hand, a small number of resistance alleles can be expected if these alleles have been dispersed from a population to another by gene flow or if pre-existing alleles were present before the isolation of populations. Historical and geographical data on the emergence of the resistance in populations can be used to distinguish between these two hypotheses (gene flow/selection of a pre-existing allele). On the other hand, a far greater diversity of the resistance alleles can be expected if these alleles result from spontaneous mutations arising after the isolation of the populations.

We tested these hypotheses (gene flow, pre-existing alleles, new mutants) in several French trichostrongyloid populations by sequencing a 550 bp fragment of the isotype 1 β-tubulin gene, including the site responsible for resistance to benzimidazole. A population of a domestic ruminant trichostrongyloid parasite can be defined as all the worms of one parasite species harboured by a whole flock of sheep or goats. In France, Cabaret and Gasnier (1994) have shown that no host movement occurs between dairy goats on different farms, which must dramatically reduce gene flow between parasite populations. This means that benzimidazole-resistance can only become established in a parasite population by the selection of pre-existing alleles or the appearance of new alleles within the worm populations. This situation is very interesting because it contrasts sharply with the findings of studies carried out on the same parasite species in other countries. For example, Blouin et al. (1992, 1995) showed that there is a high degree of gene flow among populations of sheep trichostrongyloid parasites in USA resulting from very large host movements. Gene flow must therefore have played a predominant role in the spread of the benzimidazole-resistant alleles in the USA.

Three trichostrongyloid species were investigated in this study: *T. circumcincta*, *T. colubriformis* and *H. contortus*. *Teladorsagia circumcincta* worms from eight populations located in central France were sequenced to compare the genetic variability of a 460 bp fragment of the isotype 1 β-tubulin gene in benzimidazole-susceptible and benzimidazole-resistant worms and to find out if the resistant alleles were markedly distinct from the susceptible ones. In addition, *T. circumcincta* worms isolated from 13 populations from south-western France were also sequenced to compare their resistant alleles with those found in populations from central France. We chose to investigate *H. contortus* and *T. colubriformis* worms because these two species have very different population dynamics and these can have a considerable impact on the diversity of resistant alleles in worm populations.

2. Materials and methods

2.1. Parasite collection and evaluation of benzimidazole-resistance or -susceptibility

Eight dairy goat farms in central France and 13 in south-western France were studied. All the farms in the same region were located within a radius of 30 km, apart from the farms 4 and 5, which were 150 km away from the other farms in central France (Fig. 1). The parasite community of a flock corresponds to the combination of all the parasite species harboured by the hosts in the whole flock. Resistance to benzimidazole was evaluated for the whole parasite community of a flock by an egg hatch assay, which determined the dose of benzimidazole needed to kill 50% of the eggs (LD50), or by a faecal egg count reduction test (Coles et al., 1992).

We studied eight farms in central France to compare the diversity and the sequences of benzimidazole-resistant or susceptible alleles. The parasite communities of four farms were benzimidazole-susceptible (faecal egg count reduction test: 100% reduction of the egg excretion after benzimidazole treatment of the host in the parasite communities of the farms 5, 7 and 8; farm 6, LD50 = 0.03 μg ml⁻¹) whereas the parasite communities of the other four farms were benzimidazole-resistant (farm 1, LD50 = 0.85 μg ml⁻¹; farm 2, LD50 = 0.31 μg ml⁻¹; farm 3, LD50 = 0.15 μg ml⁻¹; farm 4, LD50 = 0.33 μg ml⁻¹) (Elard et al., 1999). Thirteen parasite communities from the south-west of France were sampled. Only two of them in farms 9 and 18 were benzimidazole-susceptible, with a faecal egg count reduction test of over 95%. The other 11 communities (farms 10, 11, 12, 13, 14, 15, 16, 17, 19, 20 and 21) were benzimidazole-resistant, with a faecal egg count reduction test of 25.7–64.3% (Silvestre, 2000).

Both methods (egg hatch assay and faecal egg count reduction test) give an estimation of the benzimidazole-resistance or -susceptibility of a parasite community as a whole, but cannot be used to assess the proportion of benzimidazole-resistant worms in a particular species. We therefore performed an allele-specific polymerase chain reaction (allele specific-PCR) to determine the benzimidazole-resistance or -susceptibility of individual worms in each species in the worm community (Humbert and Elard, 1997). A simple PCR method for rapidly detecting defined point mutations. Technical Tips Online, http://tto.trends.com, T40076; Elard et al., 1999). Briefly, four primers were used to detect the phenylalanine (TTC, susceptible allele) or the tyrosine (TAC, resistant allele) at residue 200 of the isotype 1 β-tubulin gene. This system can generate three fragments: one non-allele specific fragment was amplified as an internal standard plus two allele-specific fragments generated
according to whether the resistant and/or susceptible alleles were present. We used the specific electrophoretic profile to identify three genotypes: susceptible homozygote (TTC/TTC, SS), susceptible heterozygote (TTC/TAC, Sr) and resistant homozygote (TAC/TAC, rr). Only adult male worms were used in order to avoid the risk of unreliable DNA amplification from the eggs of female worms. Genomic DNA was prepared from individual worms from central France (Humbert and Cabaret, 1995), whereas, for the other populations, PCRs were performed directly on DNA crudely extracted by incubating with proteinase K from individual worms with no further purification (Silvestre and Humbert, 2000). The allele-specific PCR identified a few resistant worms in benzimidazole-susceptible communities (farms 9 and 18). The homozygous susceptible (SS) and resistant (rr) T. circumcincta worms from central France were then sequenced, whereas only homozygous resistant (rr) T. circumcincta, H. contortus and T. colubriformis worms from south-western France were sequenced.

2.2. Sequencing and cloning of the central part of the isotype 1 β-tubulin gene

A total of 156 worms were sequenced. To study benzimidazole-resistance allele diversity, we chose to sequence a few number of homozygous resistant worms (generally five worms) from a larger number of farms. This strategy made it possible to identify the commonest alleles. However, we sequenced more worms (15 worms) in two randomly chosen populations. A 550 bp fragment from the central part of the isotype 1 β-tubulin gene was amplified by PCR (25 μl reaction mixture, 2.5 μl 10× Taq buffer (Promega), 1.0 U Taq polymerase (Promega), 80 μmol of each dNTP, 25 ng of DNA) with two primers (25 pmol of each). For T. circumcincta, H. contortus and T. colubriformis, the forward primer was M1: 5'-CCAAGGACGATTCTTTGG-3', M3: 5'-CCATGACCGATTCTCAGT-3' and M4: 5'-CCAACTACGGATTTGGG-3', respectively. The reverse primer (M2: 5'-GATCAGCATTCAGCTGGTCC-3') was the same for the three species. PCR was carried out in an MJ Research thermal cycler using the following conditions: one cycle of 94 °C for 90 s, 55 °C for 60 s, 72 °C for 90 s; 33 cycles of 92 °C for 30 s, 55 °C for 60 s, 72 °C for 90 s; final extension 72 °C for 10 min. The PCR products were purified on QIAquicks columns (Qiagen). The PCR products were directly sequenced using the forward primers (M1, M3 or M4), but some of them were cloned in the pGEM-T vector (Promega) according to the Supplier’s instructions and sequenced using M13 Forward and Reverse sequencing primers on an Applied Biosystems 377 automated DNA sequencer (Applied Biosystems, Perkin Elmer). Only the resulting 460 bp double-strand fragments were subsequently aligned. The GCG package (Genetics Computer Group Inc.) and GeneDoc (Nicholas and Nicholas, 1997. GeneDoc: a tool for editing and annotating multiple sequence alignments. Distributed by the author:
www.cris.com/~ketchup/genedoc.shtml) were used for sequence alignment.

3. Results

3.1. Diversity of benzimidazole-resistance and -susceptibility alleles in *T. circumcincta* populations from central France

As previously reported by Leignel et al. (2002), two isotype 1 β-tubulin types (Type I and Type II) were present in the *T. circumcincta* populations (Fig. 2). Each type was characterised by having synonymous mutations in coding regions, three indels and many mutations in introns. Both types comprised benzimidazole-resistant and benzimidazole-susceptible alleles with a TAC or a TTC codon in position 200, respectively.

All benzimidazole-resistant alleles were characterised by a TAC codon at amino-acid 200, which was in accordance with our PCR typing. The same benzimidazole-resistance allele (tcr1) was found for the first isotype 1 β-tubulin type (Type I), in three benzimidazole-resistant populations (farms 1, 3 and 4) (Table 1). This allele had a different nucleotide at position 363 (A/G) of the fragment than any of the other benzimidazole-susceptible sequences (Fig. 2). Another benzimidazole-resistance allele (tcr2) was found in the farm 2 population (Table 1). Unlike the first allele, this allele had no specific nucleotide site (excepted for the codon in position 200 involved in benzimidazole-resistance), when compared with benzimidazole-susceptible sequences (Fig. 2).

One benzimidazole-resistance allele (tcr6) for the second isotype 1 β-tubulin type (Type II) was found in the five resistant worms in the *T. circumcincta* population of farm 3 (Table 1). Compared with benzimidazole-susceptible sequences, this tcr6 allele had a specific nucleotide at two positions (71:T/A or G and 379:T/C) (Fig. 2).

3.2. Diversity of benzimidazole-resistance alleles in *T. circumcincta* populations from south-western France

Six of the 13 *T. circumcincta* populations studied in south-western France were benzimidazole-resistant. They contained both isotype 1 β-tubulin types (Type I and Type II) as found in populations from central France, but the benzimidazole-resistance alleles were different from those identified in central France. The same benzimidazole-resistance allele (tcr3 in Fig. 2) was found for the isotype 1 β-tubulin Type I, in the farm 11 *T. circumcincta* worm population (15 worms sequenced), in two individuals of the farm 12 worm population and in one individual of the farm 14 worm population (Table 1). A second benzimidazole-resistance allele (tcr4) was observed in two individuals of the farm 12 worm population and a third (tcr5), in two individuals of the farm 13 worm population and in five of the farm 14 worm population (Table 1).

Two other benzimidazole-resistance alleles belonging to
the isotype 1 β-tubulin Type II were found: the first (tcr7 in Fig. 2) in the farm 10 T. circumcincta worm population (15 worms sequenced) and the second (tcr8) in the farm 15 worm population (four worms partially sequenced) (Table 1).

3.3. Diversity of benzimidazole-resistance alleles in H. contortus populations in south-western France

We found six benzimidazole-resistance alleles (Table 1 and Fig. 3) in the seven H. contortus populations studied. Two of them occurred in several populations: hcr1 in farm 10 H. contortus worm population (one worm), farm 11 worm population (four worms), farm 12 worm population (one worm) and hcr3 in farm 9 worm population (four worms) and farm 17 worm population (two worms). Each of the other four was only found in one population: hcr2 in farm 12 worm population (two worms), hcr4 and hcr6 in farm 18 worm population (two worms), and finally hcr5 in farm 16 worm population (one worm). Benzimidazole-resistance alleles differed from each other by several point mutations or indels, except for alleles hcr4 and 5, which were very similar (only one difference) (Fig. 3). Several heterozygous (benzimidazole-hcr1/benzimidazole-hcr2) worms were found in the farm 12 H. contortus worm population.

3.4. Diversity of benzimidazole-resistance alleles in T. colubriformis populations in south-western France

Only one benzimidazole-resistance allele (cor1) was found in the 11 T. colubriformis populations studied (one in farm 11 worm population, six in farm 12, one in farm 13, five in farm 14, five in farm 15, five worms in farm 16, two in farm 17, five in farm 18, five in farm 19, five in farm 20 and four in farm 21) (Table 1). And there was only one polymorphic site in the second intron (G/A) of four worms of the farm 20 T. colubriformis worm population.

4. Discussion

We investigated the diversity and origin of the benzimidazole-resistance alleles in trichostrongylid parasite populations to try to find out if the spread of benzimidazole-resistance within trichostrongylid parasite populations results from gene flow, the selection of pre-existing benzimidazole-resistant alleles or the selection of new benzimidazole-resistant alleles arising as spontaneous mutations.

Because of the hypotheses under investigation, we chose to sequence the benzimidazole-resistance alleles in a few worms from a large number of farms, rather than from a large number of worms from fewer farms. However, in farm 10 we sequenced all the homozygous resistant T. circumcincta available and found only one benzimidazole-resistance allele in these worms (Table 1). This allowed us to conclude that the main benzimidazole-resistance alleles had been sequenced in the farms studied. A small number of resistant worms from several populations of H. contortus and T. colubriformis were sequenced, but molecular genotyping detected only a few homozygous resistant worms in these populations. Consequently, the benzimidazole-resistance alleles identified can be taken to be the main ones.

Table 1

<table>
<thead>
<tr>
<th>Region</th>
<th>Farm no.</th>
<th>Teladorsagia circumcincta</th>
<th>Haemonchus contortus</th>
<th>Trichostrongylus colubriformis</th>
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<tr>
<td>Centre</td>
<td>1</td>
<td>tcr1 (6 rr)</td>
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</tr>
<tr>
<td></td>
<td>2</td>
<td>tcr2 (5 rr)</td>
<td>ND</td>
<td>ND</td>
</tr>
<tr>
<td></td>
<td>3</td>
<td>tcr1 (1 rr), tcr6 (5 rr)</td>
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<td>ND</td>
</tr>
<tr>
<td></td>
<td>4</td>
<td>tcr1 (4 rr)</td>
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<td>ND</td>
</tr>
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<td>5</td>
<td>5 SS</td>
<td>ND</td>
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<td></td>
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<td>hcr1 (1 rr)</td>
<td>–</td>
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<td>hcr1 (4 rr)</td>
<td>–</td>
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<td></td>
<td>12</td>
<td>tcr3 (2 rr), tcr4 (2 rr)</td>
<td>hcr1 (1 rr), hcr2 (2 rr)</td>
<td>cor1 (6 rr)</td>
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<td>–</td>
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<td>cor1 (5 rr)</td>
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<td>17</td>
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<td>cor1 (2 rr)</td>
</tr>
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<td>–</td>
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<td>–</td>
<td>–</td>
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<tr>
<td></td>
<td>21</td>
<td>–</td>
<td>–</td>
<td>cor1 (4 rr)</td>
</tr>
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</table>

*a Benzimidazole-resistance alleles found in more than one farm are indicated in bold. ND, not determined.
*b No resistant worms were collected on the farm.
present in farms. We investigated fewer *H. contortus* (19 worms) than *T. circumcincta* (67 resistant worms) or *T. colubriformis* (44 worms); however, benzimidazole-resistance alleles in *H. contortus* were very diverse, whereas only one benzimidazole-resistance allele was identified in a large number of *T. colubriformis* worms. Consequently, sequencing a large number of *H. contortus* worms could identify new benzimidazole-resistance alleles, but this should not affect our main conclusions.

With regard to the origin of the benzimidazole resistance in worm populations, our findings suggest that benzimidazole-resistant alleles pre-existed in worm populations before the constitutions of the herds, but also that new alleles were generated as a result of spontaneous mutations. In support of the first hypothesis (pre-existing alleles), we found the same benzimidazole-resistance allele in several *T. circumcincta* populations: tcr1 in three populations from central France (Fig. 2) and tcr3 and 5 in several populations from south-western France. A similar situation was observed in the *H. contortus* populations: hcr1 and 3 were found in several populations from south-western France. As no infected animals were introduced after the flock of dairy goats had been constituted (Cabaret and Gasnier, 1994), the fact that the same allele is shared by different populations suggests that these alleles were present in the worm populations prior to the creation of the herds and so, before the worm populations were constituted. This is strongly supported by epidemiological data (Silvestre et al., 2000), which showed that benzimidazole-resistance in nematode populations was positively correlated with the diversity of origin of the animals purchased when the dairy goat herd was constituted. The high degree of similarity among different benzimidazole-resistance alleles in *H. contortus* populations (e.g. hcr4 and 5) suggests that these benzimidazole-resistance alleles have a common origin, and that they diverged later as a result of the selection of a new synonymous mutation which occurred in one of them. Only one benzimidazole resistance allele was found in all populations of *T. colubriformis*, which was consistent with the hypothesis of the presence of a pre-existing resistance allele in the worm populations.

In support of the second hypothesis (new alleles arising by spontaneous mutation), we found that several populations of *H. contortus* and *T. circumcincta* had specific benzimidazole-resistance allele. The tcr2, tcr7 and tcr8 benzimidazole-resistance alleles were found in only one *T. circumcincta* population, from central (tcr2) and south-western France (tcr7 and 8). Similarly, hcr4, 5 and 6 were found in only one *H. contortus* population from south-western France. It is unlikely that these alleles had originally been present in other farms and then lost by genetic drift. Roos et al. (1990) found that the polymorphism of the α-tubulin gene was no lower in benzimidazole-resistant populations than in benzimidazole-susceptible ones. In addition, a sequencing study of the ND4 gene of the mtDNA (Leignel and Humbert, in press) also showed an
apparent lack of genetic drift in benzimidazole-susceptible and -resistant populations of *T. circumcincta*, which was explained by the breeding management practices and the large size of the worm populations.

The second goal of our work was to study benzimidazole-resistance allele diversity in relationship to the life traits of trichostrongyloid species. If some benzimidazole-resistance alleles result from spontaneous mutations, species with a rapid ‘turn-over’ (highly prolific but with a short life expectancy of the adult worms) should be more likely to have benzimidazole-resistance alleles resulting from spontaneous mutations. Our results clearly show that *H. contortus* and *T. circumcincta* populations harboured more diverse benzimidazole-resistance alleles (six and eight benzimidazole-resistance alleles, respectively) than *T. colubriformis*, in which only one benzimidazole-resistance allele was found, despite the large number of farms studied. This is consistent with what we know about the demographic traits of the two former species. *Haemonchus contortus* is very prolific (5000 eggs laid per female and per day) and although *T. circumcincta* is less prolific (500 eggs/female/day), it is still five times more prolific than *T. colubriformis* (Cabaret and Ouhelli, 1984). Assuming an average of 1000 females per host and an average of 100 hosts per flock, $5 \times 10^8$, $5 \times 10^7$ and $10^7$ larvae are produced per day and per population of *H. contortus*, *T. circumcincta* and *T. colubriformis* species, respectively. This means that, with a mutation rate per base pair per replication of $2 \times 10^{-10}$ (value proposed for the nematode *Caenorhabditis elegans* by Drake et al., 1998), one resistant worm is produced in the worm population of a flock every 20 days for *H. contortus*, every 200 days for *T. circumcincta* and every 1000 days for *T. colubriformis*. As *T. circumcincta* also has the most regular seasonal pattern of infective larvae abundance on pastures, which leads to a longer potential period of reproduction (Kerboeuf, 1985), the difference between the benzimidazole-resistance allele diversity of *H. contortus* and *T. circumcincta* and that of *T. colubriformis* is compatible with all these observations.

In conclusion, it appears that resistance to benzimidazole in trichostrongyloid parasites of small ruminants can result from the selection of pre-existing alleles and probably for *Teladorsagia* and *Haemonchus* genera, of new alleles arising by spontaneous mutation. This makes it difficult to prevent the establishment of benzimidazole resistance in worm populations and emphasises the importance of breeding management practices, and especially of anthelmintic use in determining the rate of resistance allele selection in worm populations. As recently suggested by Sangster (2001), schemes for integrated parasite management are particularly needed to prevent and to manage anthelmintic resistance.

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