Effect of benzimidazole under-dosing on the resistant allele frequency in *Teladorsagia circumcincta* (Nematoda)

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SUMMARY

This experiment was designed to determine the effects of under-dosing on the frequency of benzimidazole resistant allele in the nematode *Teladorsagia circumcincta*. Fenbendazole (FBZ) was tested at 1/32, 1/16, 1/8 and 1/4 of the recommended dose for sheep (5 mg/kg body weight). The fraction of the susceptible homozygote (SS), susceptible heterozygote (RS) and resistant homozygote (RR) genotypes were compared among FBZ dose groups to evaluate differences between SS and RS genotype selective advantage. Almost all SS genotype worms were eliminated by 1/4 of the FBZ recommended dose, whereas a significant fraction of the RS genotype worms survived treatment. The selective advantage was 4·5 times higher for the RS genotype. This selective advantage was determined at 1/4 of the manufacturer's recommended dose of FBZ. This value should be taken as an indictor of the selective advantage of RS over the SS genotype when lambs are under-dosed. A computer simulation was used to study the putative spread of anthelmintic resistance over a range of RS selective advantages (2, 4·5 and 10-fold), with two average sizes of individual host worm population (20 or 2000 worms/host) and two initial R allele frequencies (0·1% or 1%). In all situations, the lowest selective advantage of the RS genotype over the SS genotype was sufficient to promote the spread of resistance in susceptible populations. When the RS genotype had no selective advantage over the SS genotype, genetic drift almost always led to the loss of the R allele, except in the largest populations (average size = 2000 worms).

Key words: anthelmintic resistance, under-dosing, fenbendazole, *Teladorsagia circumcincta*, stochastic model.

INTRODUCTION

Most nematode control programmes rely on broad-spectrum anthelmintic drugs (Dash, 1986), or on the integration of such drugs with grazing management (Strong & Wall, 1990). The benzimidazoles (BZ) are the most commonly used anthelmintics because of their high therapeutic index, the absence of toxic residues in milk or meat, and their low price. BZ resistance has been extensively reported (Prichard, 1990; Borgsteede, 1993) since it was first recorded in 1964, three years after thiabendazole was introduced commercially (Drudge *et al*. 1964). The resistance of *Teladorsagia circumcincta* to BZ is determined by a single major gene, which has both a resistant (R) and a susceptible (S) allele (Roos *et al*. 1990; Geary *et al*. 1992; Kwa *et al*. 1993; Beech, Prichard & Scott, 1994; Lubega *et al*. 1994; Grant & Mascord, 1996). In experimental infections, susceptible homozygous (SS) and heterozygous (RS) genotypes are both eliminated by the recommended dose (Elard, Sauvé & Humbert, 1998). The resistant allele seems to be recessive, as only RR worms were recovered after treatment (Elard *et al*. 1998). Individual BZ resistance is easily defined from the genotype of worms, because there are only 2 phenotypes. However, at the population level, the definition is more quantitative. A resistant population contains homozygous resistant worms, and the level of BZ resistance of the population depends on the frequency of RR worms. Furthermore a wide range of population BZ resistance levels can be obtained, ranging from a few per cent to 100% RR worms. This genetic determinism matches less well in field conditions: RR worms were combined with few heterozygous worms after benzimidazole treatment of naturally infected sheep (Leignel, 2000) or goats (Silvestre, unpublished data).

Although under-dosing has been repeatedly blamed for the build up of resistance (Martin, 1989; Jackson, 1993; Bjorn, 1994; Borgsteede *et al*. 1996; Smith *et al*. 1999), not a single experiment has been done that supports this statement. This hypothesis is supported by the systematic under-dosing of goats, and the fact that for the most widespread nematode species, anthelmintic resistance is much more frequent in goats than in sheep (Cabaret, 2000). Goats are frequently under-dosed if the sheep dose rate is given to goats, because of differences between the bioavailability and efficacy of BZ in goats and in sheep (Hennessy *et al*. 1993). A double dose of BZ is now recommended for treating goats (Chartier &

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Hoste, 1997). Two other factors may cause under-dosing: under-estimating the animal's weight (Edwards *et al*. 1986; Warriss & Edwards, 1995) and using defective drench guns.

Simulating the development of anthelmintic resistance has shown that the impact of the under-dosing may have a complex effect. Smith *et al*. (1999) showed that the effects of under-dosing on the development of resistance may be either positive or negative, depending on the doses used and the initial frequency of the resistant allele within the population. This was illustrated by a dose that eliminated all the SS and some of the RS genotypes. In a susceptible population, where the R allele was rare, such a dose selected for resistance, whereas in a resistant population, the development of resistance was slowed by the conservation of susceptible alleles in the RS individuals that survived treatment.

Our study was carried out to investigate the effect of under-dosing on the development of resistance to BZ in *T. circimcincta*, as the data available do not quantify the impact of under-dosing on the survival of heterozygous RS under selective pressure due to benzimidazoles, which may play a role in the establishment of resistance in a population. We first infected lambs with a partially resistant *T. circumcincta* strain. We then tested a range of BZ doses to determine the survival rates of the SS and RS genotypes after treatment. These data were used to simulate the spread of resistance and to determine the influence of differences in the survival of SS and RS genotypes after treatment. The selective advantage of each genotype was restricted to its ability to survive the anthelmintic treatment.

MATERIALS AND METHODS

Experimental infection

The *T. circumcincta* strain was collected from a goat farm in central France. This strain comprised 25 % of SS, 50 % of RS and 25 % of RR genotypes (Elard, Cabaret & Humbert, 1999). Two separate experiments were conducted (5–6 lambs each). Parasite-free lambs were treated with triamcinolone acetate, a long-acting corticosteroid (Kenacort®, Squibb), to reduce the influence of lamb variability in the response to nematode infection. Each lamb was infected with 3000 larvae. On day 28 post-infection (p.i.), each lamb was treated with fenbendazole (FBZ) (Panacur®, Hoechst Roussel Vet) according to its weight. FBZ doses corresponded to 1/32, 1/16, 1/8 and 1/4 of the recommended dose in lambs (5 mg/kg body weight). The half dose had been tested previously: it was nearly 100 % effective and was therefore not tested here (Elard, 1999; personal communication). Two lambs were treated with each dose. Two lambs remained untreated (negative control, 1 in each experiment), and 1 lamb was given the therapeutic dose (positive control). Lambs were slaughtered on day 39 p.i. at the INRA abattoir (Nouzilly, France). Abomasa were recovered, the adult worms were examined and counted under a binocular microscope. The total worm burden was estimated from 1/10 aliquots of the whole abomasum washing. The reduction in worm burden was calculated for each FBZ dose from these data as:

$$\% \text{ reduction} = (Nc - Nt)/Nc,$$

where Nc = number of adult worms in control lamb, Nt = number of adult worms in treated lambs.

Extraction of DNA from worms

DNA was rapidly extracted from fresh or frozen worms. Each adult worm was placed overnight at 42 °C in a tube containing 25 μl of extraction buffer (1 mM Tris–HCl, 0·1 mM EDTA, 5 mg/ml proteinase K). Proteinase K was inactivated by incubation at 95 °C for 20 min, and an aliquot (4 μl) of this solution was used for PCR.

Genotyping of worms and data analysis

The BZ resistance genotype of worms was determined as described by Humbert & Elard (1997), based on an allele-specific polymerase chain reaction (AS-PCR). Four primers were used to detect a phenylalanine (TTC, susceptible allele) or a tyrosine (TAC, resistant allele) at residue 200 of the isotype 1 β-tubulin gene. One non-allele specific fragment was amplified as an internal standard. Two allele-specific fragments were generated according to whether the resistant and/or the susceptible alleles were present. Each genotype corresponded to 1 specific electrophoretic profile. Genotype proportions were calculated from at least 50 genotyped worms per lamb, except for the lamb treated at the therapeutic dose, in which all available worms were genotyped as less than 50 worms survived. The observed genotype proportions in populations were compared within each FBZ dose replicate and between all the FBZ doses. Differences were tested for significance by χ^2 test, at $P \leqslant 0{\cdot}05$.

Computer simulation of resistance development

The stochastic simulation model used here was first described by Saul (1995) to study the spread of the anthelmintic-resistant allele in the nematode *Ascaris lumbricoides*, according to external selection pressure. The programme consisted of a succession of generations that were submitted to anthelmintic treatment or to putative genetic drift only. This model was used to determine the spread of BZ resistance in a population of *T. circumcincta*. The main part of the

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programme was stochastic, but several parameters were calculated deterministically. The programme stepped through the time to be modelled calculating the numbers of each type of egg produced (SS, RS and RR) during each time-period, and the number of worms that died. The host population was divided into a series of subpopulations, by a non-linear regression using a Gauss-Newton stepwise fit. It was assumed that the worms in each subpopulation have a Poisson distribution. Thus, the whole population distribution of worms follows a negative binomial distribution. The size of the flock was set at 100 lambs and 2 average levels of infestation were tested: 20 worms per host or 2000 worms per host. At the beginning of the simulation, we specified life-cycle parameters. They were based on published data and/or our laboratory results for *T. circumcincta*. The life-span of the mature worms was defined by a first-order mortality rate, which gave them an average life-expectancy. The programme allowed each genotype to have different death rates so that different selective pressures could be modelled. In the absence of anthelmintic treatment, the genotypes had the same life-expectancy, set at 6 months. The rate at which a *T. circumcincta* female lays eggs was assumed to depend on the total number of worms present in the host (Smith & Galligan, 1988). The exponential frequency-dependent fecundity of worms specified in Saul's model (Saul, 1995) was adjusted to adapt this parameter to *T. circumcincta* ($Z = 0{\cdot}99899$). After eggs had been deposited, successive moults produced the infective larvae, following a specified delay. The free-living stages have a limited life-span, and were assumed to die with a first-order mortality rate constant. The first-moult larvae matured to infective larvae after 17 days, and the free-living stages survived on pastures for 33 days (Callinan, 1978; Rossanigo, 1992). Following infection, a global reproduction efficiency (time to reach sexual maturity and death rate of immature worms) was defined as the number of female worms in the host population as a result of the mating of a pair of mature worms over their life-time. In field conditions, 1 female *T. circumcincta* of the same strain as that used in the experimental infection generated 20 females per year (Leignel, 2000; personal communication). The worm reproduction rate was set at 20 (i.e. 1 female produced 20 female offspring during a grazing season).

The programme uses the initial R allele frequency, Hardy-Weinberg equilibrium and average worm burden to determine the initial effective number of each worm genotype in each subpopulation. At each generation, the number of each genotype is determined. As this figure corresponds to the mean effective number of this genotype in each subpopulation, this value may be less than one worm. The programme calculates genotype effective number using the life-cycle parameters specified by the user. The step size used to calculate the number of each genotype at each parasitic state was set at 15 days. This time-step needs to be shorter than the duration of the life-cycle (17 days in this case) and short in proportion to the life-time of the worms, to permit the accurate calculation of effective numbers. Simulation began in March, when the lambs are turned out to pastures. The lambs were not treated during the first year so as to obtain a stable worm burden. The simulation was run for 12 years. One year represents 24 steps (one step corresponding to 15 days). Two anthelmintic treatments per year (in July and November) with the same benzimidazole were simulated from the second to the twelfth year (corresponding to 12 grazing seasons). Thus, each year the worms were subjected to selection pressure during 2 steps, and to genetic drift during 22 steps. The lambs stayed on the same pasture after anthelmintic treatment. The impact of the anthelmintic treatments was reflected by an instantaneous decrease in the number of adult worms.

The initial frequency of the resistant allele was 1% in a first group of simulations, as in previous studies (Elard *et al.* 1999) the frequency of the RS genotype ranges from 0 to 2% in natural 'susceptible' populations. The frequency of the R allele was 0·1% in a second group of simulations, as specified in other simulations (Barnes, Dobson & Barger, 1995). The selective advantage of the heterozygote was simulated as an increase in the average life-span of the RS genotype compared to that of the SS genotype at the time of the treatment. The average life-expectancies of the RR and SS genotypes were fixed at 0·5 years and fell to 0·01 year at the time of the treatment. In the simulations, the whole flock was under-dosed. However, in practice, under-dosing actually affects only a small proportion of animals in the flock. So to allow for this, a second simulation was performed with a lower RS selective advantage, which produced only a slightly longer life-expectancy than the SS genotype. To test the effect of genetic drift on the evolution of R allele frequency, we simulated both a large and a low worm burden (2000 and 20 worms per host respectively). Each simulation was run 10 times for each set of input parameters.

RESULTS

Effect of under-dosing on worm burden

The effects of under-dosing on the efficacy of FBZ are shown in Table 1. The percentage reduction in the number of worms was calculated for each lamb. The therapeutic dose eliminated 95% of the worms. Doses lower than 1/4 of the recommended dose caused the worm burden to be reduced by less than 50%. Very low doses gave widely varying results

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Table 1. Percentage reduction of *Teladorsagia circumcincta* worm numbers according to the dose of fenbendazole

No. of experiment	FBZ dose	Total no. of worms	% reduction
1	0*	1568	—
	1/32	1616	0
	1/32	1543	2
	1/16	960	39
	1/16	1322	16
	1†	80	95
2	0*	504	—
	1/8	498	1
	1/8	466	7
	1/4	265	47
	1/4	286	43

* Not treated.
† Recommended dose (5 mg/kg body weight).

Table 2. *Teladorsagia circumcincta* genotypes recorded (number of worms genotyped and mean genotype proportion) at each fenbendazole dose

FBZ dose	SS genotype	RS genotype	RR genotype	χ^2 value (significance, $P < 0.05$).
0*	14	34	14	0·16 (N.S.)
0	13	34	16	
Mean	21·6 %	54·4 %	24 %	
1/32	27	46	29	0·58 (N.S.)
1/32	31	50	40	
Mean	26 %	43 %	31 %	
1/16	11	74	51	3·66 (N.S.)
1/16	18	55	47	
Mean	11·3 %	50·4 %	38·3 %	
1/8	19	40	53	6·92 ‡
1/8	10	51	34	
Mean	14 %	44 %	42 %	
1/4	1	13	80	0.18 (N.S.)
1/4	1	10	53	
Mean	1·6 %	14·6 %	84·2 %	
1†	0	0	39	—
Mean	0 %	0 %	100 %	

* Not treated.
† Recommended dose (5 mg/kg body weight).
‡ Significant difference ($P \leqslant 0·05$).
N.S., Not significant.

and the worm burden was reduced by 0–39 %. The number of established worms in the second experiment was smaller than in the first one.

Effect of under-dosing on genotype composition

Larvae were genotyped before infection to determine the proportions of each genotype in the initial wild population. Untreated populations were not significantly different from the initial population ($\chi^2 = 0·57$, at $P < 0·05$): 31 infective larvae were genotyped, and 4 SS, 20 RS and 7 RR genotypes were recovered. For each dose level, the proportions of genotypes in the population of both lambs were compared (Table 2). There was no significant difference between 2 lambs treated with the same dose, except for the dose 1/8 of the recommended dose. Data for the same dose were pooled. Almost all the SS genotype worms were eliminated by 1/4 of the recommended dose of FBZ, but some of the RS genotype worms survived. The genotype proportions at different doses were compared (Table 3): the genotype compositions were significantly different, except in two cases. There was no significant

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Table 3. χ^2 values for comparisons of genotype proportions between fenbendazole doses

	1/32	1/16	1/8	1/4	1‡
0*	4·18	11·4§ (2·3 %)†	11·6§ (15·3 %)	106·5§ (32·3 %)	70·4§ (30 %)
1/32		17·3§ (7·9 %)	11·3§ (0·2 %)	109·4§ (22 %)	65·3§ (25·7 %)
1/16			2	84·3§ (40·5 %)	51·8§ (38 %)
1/8				68·3§ (36·6 %)	44·1§ (38·9 %)
1/4					7·1§ (80 %)

* Not treated.
† Contribution of RS proportion in χ^2 value in parentheses.
‡ Recommended dose (5 mg/kg body weight).
§ Significant difference ($P \leqslant 0{\cdot}05$).

Table 4. Influence of different selective advantages (1, 2, 4·5 and 10) of the RS genotype over the SS genotype in the selection of resistant worms

(R and S represent the fixed allele (frequency exceeding 95 %) at the end of the 12-year-simulation.)

Worm burden	R allele frequency	RS = SS*	RS = 2 × SS	RS = 4·5 × SS	RS = 10 × SS
20	0·1 %	S (50/50)[a]	S (8/10) R (2/10)	S (4/10) R (6/10)	S (2/10) R (8/10)
	1 %	S (7/10)[b] R (3/10)	R (10/10)	R (10/10)	R (10/10)
2000	0·1 % 1 %	S (30/30) R (10/10)[c]	R (10/10) R (10/10)	R (10/10) R (10/10)	R (10/10) R (10/10)

* [a]In one case, f(S) = 0·70; [b]in one case, f(S) = 0·83; [c]in one case, f(R) = 0·72.

difference between populations that were untreated or treated with 1/32 of the recommended dose ($\chi^2 = 4{\cdot}18$, $P < 0{\cdot}05$). Neither was there any significant difference in the genotype proportions in lambs treated with 1/16 and 1/8 of the recommended dose ($\chi^2 = 2$, at $P < 0{\cdot}05$).

The significant differences in genotype proportions after treatment with different doses of FBZ could be attributed mainly to a decrease in the SS genotype and/or an increase in the RR genotype. We calculated the contribution of the RS genotype in the χ^2 values (Table 3) in order to assess the importance of the RS genotype for resistance. In untreated lambs, and those treated with 1/32 of the recommended dose, the contribution of the RS genotype in the χ^2 value was not important (between 0·2 and 30 %). However, the contribution of the RS genotype in the χ^2 value increased at 1/16, 1/8 or 1/4 of the recommended dose treatment (between 36·6 and 40·5 %). In the lambs treated with 1/4 of the recommended dose, the contribution of RS genotype in the χ^2 value was very considerable (80 %). The significant difference between the untreated lambs and those given 1/4 of the recommended dose was therefore mainly due to an increase in the fraction of worms with the RS genotype.

The relative selective advantage of the RS genotype over the SS genotype was calculated. The initial population contained 54·4 % RS and 21·6 % SS genotype worms. Anthelmintic treatment at 1/4 of the recommended dose resulted in 14·6 % RS and 1·3 % SS genotype worms. This corresponded to a reduction of 16·6-fold for the SS genotype worms and 3·7-fold for the RS genotype worms. The selective advantage of the RS genotype was therefore estimated to be 4·5 times greater than that of the SS genotype when lambs were under-dosed with 1/4 of the recommended FBZ dose. This highlighted the need to evaluate a range of the selective advantages of the RS and SS genotypes, as well as the long-term influence of this selective advantage, using a simulation programme.

Computer simulation of resistance development

The survival of the RS genotype was set at 2, 4·5 and 10 times greater than that of the SS genotype. The case of no selective advantage of RS genotype over SS genotype was also tested. All simulation results are summarized in Table 4.

In large worm burdens (2000 worms per host), the R allele was always selected unless the initial frequency of this allele was low (i.e. 0·1 %) and there was no selective advantage of the RS over the SS genotype. In this case, the R allele was lost due to genetic drift. For the other situations, the 4·5 selective advantage of the RS over the SS genotype was the situation that conducted the most rapidly to the fixation of the R allele. With a smaller selective advantage of the RS genotype over the SS genotype

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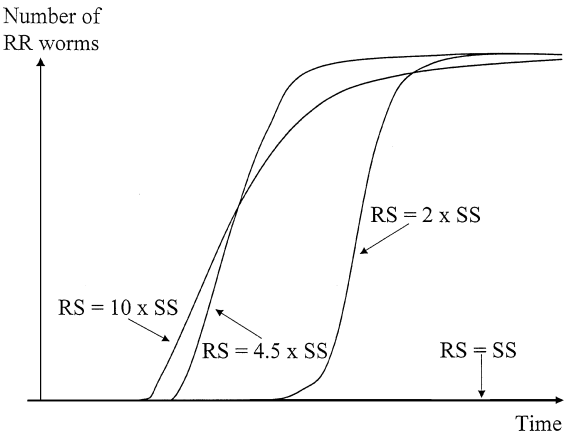

Fig. 1. Evolution of the RR genotype number in a worm population according to the selective advantage of the RS genotype over the SS genotype: when the RS genotype had no selective advantage over the SS genotype: RS = SS; when it was twice as great: RS = 2 × SS; when it was 4·5 times as great: RS = 4·5 × SS, and when it was 10 times as great: RS = 10 × SS. The number of hosts was 100, the average number of worms was 2000 and the initial R allele frequency was 0·1 %.

(RS = 2 × SS), the increase in the R frequency took longer while, with a larger selective advantage of the RS genotype (RS = 10 × SS), the increase in the R frequency began more rapidly, but heterozygous worms made it possible for the S allele to be maintained for longer (Fig. 1). For each situation tested, the results of the simulations were always highly repeatable. In small size populations (20 worms per host), genetic drift led to the loss of the R allele in 71 out of 120 cases. When the initial R allele frequency was low (i.e. 0·1 %), the selective advantage of RS genotype had a marked impact on the probability of selecting a resistant allele in the worm population (the R allele was selected in 2 cases out of 10 when RS = 2 × SS, in 6 cases out of 10 when RS = 4·5 × SS and in 8 cases out of 10 when RS = 10 × SS). In small populations in which the initial R allele frequency was low, genetic drift had a greater effect than in large population size, and the results of the simulations were more stochastic.

DISCUSSION

We have attempted to find out whether the nematode RS genotype has a greater selective advantage than the SS genotype when lambs are treated with anthelmintics at less than the recommended dose. We then used a computer simulation to study the development of resistance when the RS genotype had a range of selective advantages over the SS genotype. The reduction in the worm burden was unsatisfactory when lambs were treated with 1/32, 1/16, 1/8 and 1/4 of the recommended dose, as drug efficacy was less than 50 %. The therapeutic dose was more effective than any of the low doses. The considerable difference in the establishment rate of worms between the negative control in Exp. 1 and that in Exp. 2 may be explained by the age of the lambs. In the first experiment the lambs were less than 5 months old, whereas they were 7 months old in the second experiment. Bishop *et al*. (1996) showed the presence of genetic variation for acquired resistance to *T. circumcincta* infection in lambs, between 1 and 6 months old. Bouix *et al*. (1998) demonstrated that resistance to nematode infection was clearly observed in 6 to 7-month-old lambs. Due to the small size of the host groups, efficacies based on intensities are only indicative of trends: under-dosing reduced the worm population in several cases, but the level at which under-dosing influenced efficacy of the drug differed considerably between our first (1/16) and second experiments (1/4).

Effect of under-dosing on genotype composition

Experimental infection showed that the genotype composition of the population before and after infection (larvae and untreated worm populations respectively) was not significantly different. This means that all the genotypes became established at similar rates, confirming published data (Elard *et al*. 1998). Elard *et al*. (1998) found that worms of all genotypes had the same selective advantage (establishment rate of infective larvae, worm fertility and worm life-expectancy) in the absence of anthelmintic treatment. Under our conditions FBZ treatment was therefore the only factor that affected genotype survival, and hence genotype composition.

Genotyping the worms was the only reliable way of assessing their resistance, as an egg hatch assay or a faecal egg count reduction test can only be interpreted if at least 25 to 50 % of the worms in a population are resistant (Elard *et al*. 1999). FBZ at 1/4 of the recommended dose killed some of the RS genotype worms and almost all the SS genotype worms. This is the first time that an experimental infection has been used to demonstrate that under-dosing results in a selective advantage for the RS genotype over the SS genotype. The frequency of the R allele is very low in a 'susceptible' population, and the majority of R alleles occurs in the RS genotype (Elard *et al*. 1999). As the first RR genotype probably originates from RS genotype mating, under-dosing may favour resistance to anthelmintics. Although the model of 1 major gene, with 2 alleles, of which the resistant allele is recessive matches with therapeutic doses, the data clearly show that it matches less well with sub-therapeutic doses. The mechanism of action of BZ and the structure of the microtubules may explain the recessive nature of the mutation at therapeutic doses and the apparent 'co-dominance' at sub-therapeutic

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doses. The spontaneous association of subunits of α and β-tubulin gives the dimers that polymerize into microtubules. In the presence of BZ, the drug binds to susceptible dimers and inhibits their polymerization. In a heterozygous worm, dimers constitute a heterogeneous pool of susceptible and resistant dimers. At therapeutic doses, the drug binds all the susceptible dimers, and insufficient resistant dimers are available for tubulin polymerization to maintain the integrity of the microtubule, wheras at sub-therapeutic doses, drug availability is reduced. Consequently, a fraction of the susceptible dimers may not be exposed to BZ but may participate in tubulin polymerization. In this situation, a heterozygous worm could survive anthelmintic treatment given at a sub-therapeutic dose. This experiment was conducted on 1 generation only. To study the long-term effect of under-dosing, we used the computer simulation described by Saul (1995).

Development of resistance : computer simulation

The change in the proportion of the RR genotype in the population was simulated using 2 initial R allele frequencies (1 % and 0·1 %), a range of RS genotype selective advantages and 2 sizes of worm population. The programme allowed us to change the selective advantage by modifying the average life-expectancy. We used a worm average life-expectancy of 0·045 year for the RS genotype, based on our experimental data (RS genotype survival was 4·5 times greater than of the SS genotype, taken to be 0·01 year). As different levels of under-dosing can occur, this selective advantage was taken as a rough indicator of the potential selective advantage of the RS over the SS genotype. We tested different selective advantages of the RS genotype over SS genotype: RS average life-expectancy set at 0·02 and 0·1 year corresponded to survival rates 2 and 10 times greater than that of the SS genotype, respectively.

Simulations showed that genetic drift effects were important in small populations, when the RS genotype had no selective advantage over the SS genotype, whatever the initial R allele frequency, and when the RS genotype survived twice as long as the SS genotype and the initial R allele frequency was low (i.e. 0·1 %). In large populations, genetic drift effects were only observed when initial R allele frequency was low (0·1 %) and the RS genotype had no selective advantage over the SS genotype. Under these conditions, the R allele was lost in several cases as a consequence of the genetic drift. In the other situations, even the slightest selective advantage of the RS genotype favours the development of resistance within a susceptible population subjected to slight selective pressure (2 treatments per year).

We also tested a worm average life-expectancy of 0·33 year (instead of 0·5 year) to represent the shorter worm life-span in summer (Gibson & Whitehead, 1981). Both the initial R allele frequencies were also tested (1 % and 0·1 %). In each case, resistance spread more slowly when average worm life-expectancy was 0·33 year. This could be because the selection pressure depends on the frequency of anthelmintic treatment and on the worm life-expectancy. The longer worms stay in the host, the greater the risk of its being subjected to anthelmintic treatment.

We validated the programme by determining the worm burden according to worm average life-expectancy (0·5 and 0·1 year), without any anthelmintic treatment. We found that the worm burden stabilized at 50000 and 10000 worms, respectively, after the first grazing season on pastures. These values agreed with classical field data. It is worth noticing that Saul's programme takes genetic drift into account and that it may have impeded resistance spread in worm populations. Nevertheless, Saul's model simulates a farm without any introduction of hosts (i.e. resistant worms introduction) and does not take into account breeding management practices that could interfere with the build up of resistance (Silvestre *et al*. 2000). The local breeding management practices will modify greatly the selection pressure exerted on R alleles. For example, when hosts are treated and then kept indoors for the winter, at turn out, they will excrete resistant eggs that will contribute strongly to pasture contamination in the spring (Martin, 1989). This situation may dramatically increase the R allele frequency. Saul's model is also not entirely realistic due to several simplifications, such as a constant life-cycle time for example, as one knows that hypobiotic larvae are frequent in the winter time and this increases the duration of the life-cycle. Thus the conclusions drawn from simulations must be viewed with caution, but they do have the merit of showing that a slight selective advantage of heterozygous (RS) over homozygous (SS) worms can strongly influence the development of the resistance to BZ in a worm population.

In conclusion, the experimental infection showed that RS genotype survival at 1/4 of the recommended dose was 4·5 greater than that of SS genotype worms. Simulations using Saul's stochastic model indicated that anthelmintic resistance will develop rapidly in a natural 'susceptible' worm population if the RS genotype has the slightest selective advantage over the SS genotype. We therefore suggest that under-dosing at 1/4 of the recommended dose of FBZ for sheep may be widespread in goat herds, due to the different way anthelmintics are metabolized in sheep and goats. This might, in part, explain why goats have greater resistance to anthelmintics than sheep. This work was undertaken in *T. circumcincta*, which has medium life-cycle traits, such as fertility, survival of adult worms and pathogenicity. A small selective advantage of the RS over SS genotype

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might produce different effects in very fertile worms with a short life-expectancy, such as *Haemonchus contortus*, or in low fertility worms with a long life-expectancy, such as *Trichostrongylus colubriformis*.

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REFERENCES

BARNES, E. H., DOBSON, R. J. & BARGER, I. A. (1995). Worm control and anthelmintic resistance: adventures with a model. *Parasitology Today* **11**, 56–63.

BISHOP, S. D., BAIRDEN, K., MCKELLAR, Q. A., PARK, M. & STEAR, M. J. (1996). Genetic parameters for faecal egg count following mixed, natural, predominantly *Ostertagia circumcincta* infection and relationships with live weight in young lambs. *Animal Science* **63**, 423–428.

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.

BJORN, H. (1994). Workshop summary: anthelmintic resistance. *Veterinary Parasitology* **54**, 321–325.

BORGSTEEDE, F. H. M. (1993). Anthelmintic resistance in nematodes of sheep and goats. *Anthelmintic Resistance in Nematodes of Farm Animals. A Seminar Organised for the European Commission, Brussels, Belgium* **8**, 1–16.

BORGSTEEDE, F. H. M., ROOS, M. H., SMITH, G. & PRICHARD, R. K. (1996). Workshop summary: anthelmintic resistance. *Veterinary Parasitology* **64**, 129–132.

BOUIX, J., KRUPINSKI, J., RZEPECKI, R., NOWOSAD, B., SKRZYZALA, I., ROBORZYNSKI, M., FUDALEWICZ-NIEMCZYK, W., SKALSKA, M., MALCZEWSKI, A. & GRUNER, L. (1998). Genetic resistance to gastrointestinal nematodes parasites in Polish long-wool sheep. *International Journal for Parasitology* **28**, 1797–1804.

CABARET, J. (2000). Anthelmintic resistance in goats: from fiction to facts. *Proceeding of the 7th International Conference on Goats, Tours-Poitiers, France* **2**, 793–794.

CALLINAN, A. P. L. (1978). The ecology of the free-living stages of *Ostertagia circumcincta*. *International Journal for Parasitology* **8**, 233–237.

CHARTIER, C. & HOSTE, H. (1997). La thérapeutique anthelminthique chez les caprins. *Le Point Vétérinaire* **28**, 1907–1914.

DASH, K. M. (1986). Control of helminthosis in lambs by strategic treatment with closantel and broad-spectrum anthelmintics. *Australian Veterinary Journal* **63**, 4–8.

DRUDGE, J. H., SZANTO, J., WYANT, Z. N. & ELAM, G. (1964). Field studies on parasitic control in sheep: comparison of thiabendazole, ruelene, and phenothiazine. *American Journal of Veterinary Research* **25**, 1512–1518.

EDWARDS, J. R., WORTH, R., CHANEET, G. C. D., BESIER, R. B., KARLSSON, J., MORCOMBE, P. W., DALTON-MORGAN, G. & ROBERTS, D. (1986). Survey of anthelmintic resistance in Western Australian sheep flocks. 1. Prevalence. 2. Relationship with sheep management and parasite control practices. *Australian Veterinary Journal* **63**, 135–144.

ELARD, L., CABARET, J. & HUMBERT, J. F. (1999). PCR diagnosis of benzimidazole-susceptibility or -resistance in natural populations of the small ruminant parasite, *Teladorsagia circumcincta*. *Veterinary Parasitology* **80**, 231–237.

ELARD, L., SAUVÉ, C. & HAMBERT, J. F. (1998). Fitness of benzimidazole-resistant and -susceptible worms of *Teladorsagia circumcincta*, a nematode parasite of small ruminants. *Parasitology* **117**, 571–578.

GEARY, T. G., NULF, S. C., FAVREAU, M. A., TANG, L., PRICHARD, R. K., HATZENBUHLER, N. T., SHEA, M. H., ALEXANDER, S. J. & KLEIN, R. D. (1992). Three beta-tubulin cDNAs from the parasitic nematode *Haemonchus contortus*. *Molecular and Biochemical Parasitology* **50**, 295–306.

GIBSON, T. E. & WHITEHEAD, J. D. (1981). Changes in the worm burden of lambs under continuous infection with *Ostertagia circumcincta*. *British Veterinary Journal* **137**, 192–195.

GRANT, W. N. & MASCORD, L. J. (1996). Beta-tubulin gene polymorphism and benzimidazole resistance in *Trichostrongylus colubriformis*. *International Journal for Parasitology* **26**, 71–77.

HENNESSY, D. R., SANGSTER, N. C., STEEL, J. W. & COLLINS, G. H. (1993). Comparative pharmacokinetic behaviour of albendazole in sheep and goats. *International Journal for Parasitology* **23**, 321–325.

HUMBERT, J. F. & ELARD, L. (1997). A simple method for rapidly detecting defined point mutations. http://tto.trends.com. Technical Tips Online.

JACKSON, F. (1993). Anthelmintic resistance – the state of play. *British Veterinary Journal* **149**, 123–138.

KWA, M. S. G., KOOYMAN, F. N. J., BOERSEMA, J. H. & ROOS, M. H. (1993). Effect of selection for benzimidazole resistance in *Haemonchus contortus* on beta-tubulin isotype 1 and isotype 2 genes. *Biochemical and Biophysical Research Communications* **191**, 413–419.

LEIGNEL, V. (2000). Diversité génétique et résistance aux benzimidazoles chez *Teladorsagia circumcincta* (Nematoda, Trichostrongylidae), parasite de petits ruminants. Montpellier Université, Montpellier II, 170.

LUBEGA, G. W., KLEIN, R. D., GEARY, T. G. & PRICHARD, R. K. (1994). *Haemonchus contortus*: the role of two beta-tubulin gene subfamilies in the resistance to benzimidazole anthelmintics. *Biochemical Pharmacology* **47**, 1705–1715.

MARTIN, P. J. (1989). Selection for thiabendazole resistance in *Ostertagia* spp. by low efficiency anthelmintic treatment. *International Journal for Parasitology* **19**, 317–325.

PRICHARD, R. K. (1990). Anthelmintic resistance in nematodes: extent, recent understanding and future directions for control and research. *International Journal for Parasitology* **20**, 515–523.

ROOS, M. H., BOERSEMA, J. H., BORGSTEEDE, F. H. M., CORNELISSEN, J., TAYLOR, M. & RUITENBERG, E. J. (1990). Molecular analysis of selection for benzimidazole resistance in the sheep parasite *Haemonchus contortus*. *Molecular and Biochemical Parasitology* **43**, 77–88.

ROSSANIGO, C. E. (1992). Rôle de l'eau et de la température sur les taux de développement des Nématodes parasites du tractus digestif des ruminants. Montpellier, Montpellier II, 133.

SAUL, A. (1995). Computer model of the maintenance and selection of genetic heterogeneity in polygamous helminths. *Parasitology* **111**, 531–536.

SILVESTRE, A., CHARTIER, C., SAUVE, C. & CABARET, J. (2000). Relationship between helminth species diversity, intensity of infection and breeding management in dairy goats. *Veterinary Parasitology* **94**, 91–105.

SMITH, G. & GALLIGAN, D. T. (1988). Mathematical models of the population biology of *Ostertagia ostertagi* and *Teladorsagia circumcincta*, and the economic evaluation of disease control strategies. *Veterinary Parasitology* **27**, 73–83.

SMITH, G., GRENFELL, B. T., ISHAM, V. & CORNELL, S. (1999). Anthelmintic resistance revisited: underdosing, chemaprophylactic strategies, and mating probabilities. *International Journal for Parasitology* **29**, 77–91.

STRONG, L. & WALL, R. (1990). The chemical control of livestock parasites: problems and alternatives. *Parasitology Today* **6**, 291–296.

WARRISS, P. D. & EDWARDS, J. E. (1995). Estimating the liveweight of sheep from chest girth measurements. *Veterinary Record* **137**, 123–124.