Sensitive PCR for detecting Benzimidazole resistant sub populations of ovine nematodes in the Waikato

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ABSTRACT

Repeated sheep treatment with the anthelmintic drug benzimidazole (BZ) resulted in selection of genetically benzimidazole-resistant (BZ-R) nematodes, which code for a specific allele of isotype 1 ß-tubulin. The presence of this allele was tested in the Waikato ovine nematode, using an allele specific PCR. Two isotype allele specific oligomers for *O. circumcincta* (*O.c.*) ß-tubulin gene were designed. The last base at the 3' end of each primer, corresponding to amino acid 200, confers the specificity. PCR with those 2 upstream primers, in combination with a common downstream primer at the reverse side, distinguished between BZ-R and BZ-sensitive *O.c.* nematodes. To detect <1% resistant *O.c.* subpopulations which are undetectable with allele specific PCR, Amplification Created Restriction Site (ACRS) PCR was developed. An upstream primer was designed which is cleavable by the restriction enzyme *Sca* 1 in resistant but not sensitive *O.c.* The test using individual larva DNA proves this approach is potentially sensitive for widespread use under field conditions.

Keywords : Benzimidazole; ß-tubulin; *O. circumcincta*; benzimidalole resistance; allele specific PCR.

INTRODUCTION

Widespread use of benzimidazole chemotherapy against ovine nematodes has led to the emergence of benzimidazole resistant (BZ-R) nematode populations (Waller, 1986). These BZ-R nematodes were characterised as coding for a specific allele of isotype 1 ß-tubulin (Roos, 1990). A mutation changing the amino acid at position 200 from Phe to Tyr in this protein has been implicated in the resistance (Kwa *et al.*, 1994). Kwa has developed an allele specific PCR assay which detects this mutation for *Haemonchus contortus* (*H.c.*) and *Trichostrongylus colubriformis*. In this work we adopted the test to survey nematodes in the Waikato region in New Zealand (Lehrer *et al.*, 1994). Kwa's allele specific primers enabled us to identify BZ-R/BZ-S *H.c.* larvae. However, little PCR product was obtained for *Ostertagia circumcincta* (*O.c.*) suggesting slight interspecies differences in the DNA sequence of ß-tubulin in the region where the primer anneals.

Efficient management of BZ resistance depends on early detection of BZ-R subpopulations while they still constitute <1% of the nematodes. The aim of this study was to improve PCR assay for detecting BZ-R in *O.c.* by, first, sequencing the ß-tubulin region and designing new allele specific primers which better amplify the *O.c.* DNA and, second, developing an improved assay capable of detecting small fractions of resistant *O.c.* in samples of nematodes.

MATERIALS AND METHODS

Sequencing of the DNA of the ß-tubulin region responsible for BZ resistance has been described elsewhere (Lehrer *et al.*, 1994). Allele specific primers for *O.c.* were designed from the sequence (Figure 1). PCR for detecting the BZ-R *O.c.* population was carried in a total reaction mix of 20 µl containing 50 mM KCl, 10 mM Tris HCl (pH 8.3), 4 mM $MgCl_2$, 0.01% gelatin, 0.12 µM of each primer, 100 µM of each dNTPs, 1 U of Taq Polymerase and ~10 ng of *O.c.* genomic DNA. The reaction mix was subjected to 94°C for 3 minutes to denature DNA and to 30 cycles at 94°C for 15 seconds, 67°C for 15 seconds, 72°C for 20 seconds. For detecting 1% or less resistant sub populations of *O.c.* a primer of 30 bases was designed by changing one base at position 28 from C to A, which gives rise to an amplification created restriction site (ACRS) in resistant but not sensitive strains of *O.c.*

Genomic DNA from individual larva was extracted in 35 µl of lysis buffer containing 50 mM KCl, 10 mM tris/HCl pH 8.3, 2.5 mM $MgCl_2$, 0.45% NP-40, 0.45% tween 20, 0.01% gelatin, 60 µg/ml Proteinase K. The reaction was incubated 10 minutes at -80°C and overnight at 60°C. To inactivate Proteinase K, the mixture was heated for 15 min. at 90°C. 6µl of the extracted DNA was used for PCR (Roos, 1994).

The PCR protocol was as described above, except for decreasing the annealing temperature to 58°C. The PCR product was digested with 10 U of the enzyme *Sca* 1 for 2 hours at 37°C and the fragments analysed on 10% acrylamide gel.

RESULTS AND DISCUSSION

Sequencing the relevant part of the *O.c.* ß-tubulin gene showed that two bases differed from the *H.c.* sequence in the allele specific primers. These differences are underlined in Fig. 1 (primers 1, 2). Allele specific primers for *O.c.* were designed with these bases (Fig. 1 Primers 2, 3).

FIGURE 1: Allele specific primers used for PCR

1. *H.c.* BZ-R allele specific primer	5' GAG AAC ACC GAT GAA ACA TA 3'
2. *O.c.* BZ-R allele specific primer	5' GAA AAC ACC GAT GAA ACG TA 3'
3. *O.c.* BZ-S allele specific primer	5' GAA AAC ACC GAT GAA ACG TT 3'
4. *O.c.* Common downstream primer	5' CAC CAG ACA TTG TGA CAG A 3'

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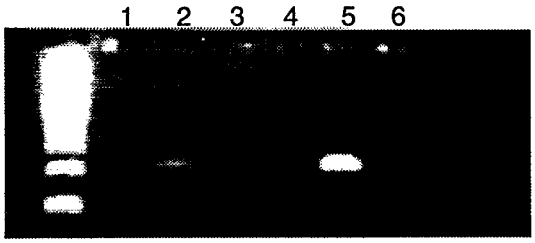
Allele specific PCR was carried out using primers 2 and 3, in combination with a common downstream primer, 4. This primer is ß-tubulin isotype 1 specific but does not distinguish between BZ-R and BZ-sensitive larvae. The last base at primers 2 and 3 confers the specificity in case of mismatch on the DNA tested extension will not occur. This is shown in Fig. 2, in which two populations of *O.c.* larvae were tested.

FIGURE 2: Analysis of larvae genomic DNA from benzimidazole-resistant and benzimidazole sensitive *O.c.* populations.

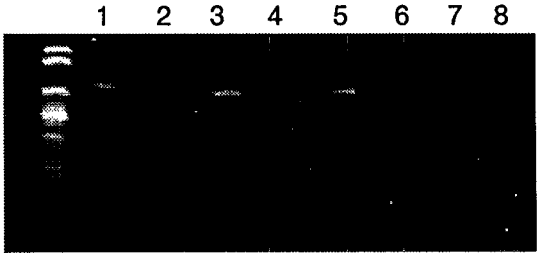


Genomic DNA from *O.c.* BZ-R larvae was used as a template in lanes 1 & 2 and from *O.c.* BZ-S larvae at lanes 5 & 6. Lanes 3 & 4 served as negative controls with no template DNA. Each DNA was amplified with the two allele specific primers. Analysis of PCR product amplified with primers recognizing BZ-R allele in combination with a downstream primer (primers 2 & 4) shows an amplification product in lane 2 and no amplification product in lane 6. In contrast, PCR product of the primer recognising BZ-S allele (primers 3 & 4) is shown only in lane 5 and not in lane 1, which means that PCR can distinguish between BZ-R (lanes 1 & 2) and BZ-S (lanes 5 & 6). *O.c.* larvae.

Amplification Created Restriction Site (ACRS) PCR

For detecting small numbers of a resistant subpopulation, DNA from individual larvae was amplified by PCR using the primer that is cleavable by the enzyme *Sca* 1 (5'ACC AAT TGG TAG AAA CAC CGA TGA AAA GTac....3') in resistant but not sensitive larva (Figure 3).

FIGURE 3: ACRS-PRC comparison between sensitive and resistant larvae.



Genomic DNA from single BZ-R larva was used as a template in lanes 1 & 2, and 3 & 4, while DNA from BZ-S larva was used as a template in lanes 5 & 6, 7 & 8. PCR product from each larva was divided in two, one part serving as a control (lanes 1, 3, 5 & 7), whereas the second part was cut with the restriction enzyme *Sca* 1, resulting in a 30 base difference between resistant cut (lanes 2, 4) and uncut product (lanes 1, 3). Lanes 6, 8 (very weak band) showed no difference in size in sensitive larvae between the control and the cut product.

CONCLUSION

Allele specific PCR can be used to distinguish between BZ-R and BZ-S *O.c.* populations in Waikato sheep. The same results have been achieved by using the modified (non allele specific) PCR (Figure 3). Such PCR using radiolabelled primers might enable us to quantify the percentage of resistant individuals in a population.

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