Molecular detection of benzimidazole resistance in *Haemonchus contortus* using real-time PCR and pyrosequencing

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SUMMARY

Benzimidazoles (BZ) are widely used to treat parasitic nematode infections of humans and animals, but resistance is widespread in veterinary parasites. Several polymorphisms in β-tubulin genes have been associated with BZ-resistance. In the present study, we investigated β-tubulin isotype 1 sequences of 18 *Haemonchus contortus* isolates with varying levels of resistance to thiabendazole. The only polymorphism whose frequency was significantly increased in the resistant isolates was TTC to TAC at codon 200. Real-time PCR (using DNA from 100 third-stage larvae, L3s) and pyrosequencing (from DNA from 1000–10 000 L3s) were used to measure allele frequencies at codon 200 of these isolates, producing similar results; drug sensitivity decreased with increasing TAC frequency. Pyrosequencing was also used to measure allele frequencies at positions 167 and 198. We showed that such measurements are sufficient to assess the BZ-resistance status of most *H. contortus* isolates. The concordance between real-time PCR and pyrosequencing results carried out in different laboratories indicated that these tools are suitable for the routine diagnosis of BZ-resistance in *H. contortus*. The molecular methods were more sensitive than the 'egg hatch test', and less time-consuming than current *in vivo*- or *in vitro*-anthelmintic resistance detection methods. Thus, they provide a realistic option for routine molecular resistance testing on farms.

Key words: nematode, thiabendazole, anthelmintic resistance, single nucleotide polymorphism, trichostrongyle.

INTRODUCTION

Benzimidazoles (BZ) are a major group of anthelmintics which are widely used to treat gastrointestinal nematode infections of humans and other animals, and represent an important component of plans to eliminate major parasitic diseases of humans, such as lymphatic filariasis. BZs act by inhibiting microtubule assembly (Prichard, 1994) and are highly cost efficient for worm control in geographical areas where resistance is not present. However, the emergence of widespread resistance in parasites of veterinary importance is now severely limiting the use of BZs in some countries, particularly in ruminants and horses (Kaplan, 2004; Kaplan *et al.* 2004; Wolstenholme *et al.* 2004; von Samson-Himmelstjerna and Blackhall, 2005). If similar levels of resistance were to appear in parasites of humans following mass drug administration, this could have an impact on current control strategies. An understanding of possible resistance mechanisms and the development of tests for the monitoring and spread of resistance alleles would be useful tools to support such programmes (Albonico *et al.* 2004*a*, 2008). Since BZ-resistance has become a serious problem in the veterinary field, a number of tests have been developed for assessing the BZ-resistance phenotype or genotype of such parasites. The sustainable use of anthelmintics requires a regular monitoring of drug efficacy on individual farms. The phenotypic test most often used is the egg hatch test (EHT), in which the drug concentration required to inhibit hatching of 50% of nematode eggs is determined (Coles *et al.* 2006). The sensitivity of such *in vitro* tests is relatively poor, and they are considered to permit the detection of resistance in isolates with at least 25% resistant individuals (Martin *et al.* 1989). On the other hand, molecular tests, based on the analysis of resistance-associated target gene polymorphisms through the use of PCR, are usually highly sensitive.

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In various nematode species, BZ-resistance is caused by single nucleotide polymorphisms (SNPs) in the gene encoding isotype 1 of β-tubulin. In *Haemonchus contortus*, the SNP most commonly associated with resistance is present at codon 200 (TTC to TAC), resulting in a phenylalanine to tyrosine amino acid change (F200Y) (Kwa *et al.* 1994; Elard *et al.* 1996; Elard and Humbert, 1999). However, other polymorphisms have also been identified. An SNP at codon 167 (TTC to TAC – F167Y) has been detected in different species (Silvestre and Cabaret, 2002; Drogemuller *et al.* 2004; Schwab *et al.* 2005; Melville *et al.* 2006; Hodgkinson *et al.* 2008) and another SNP at codon 198 has been reported recently in *H. contortus* (GAA to GCA – E198A) from both Switzerland and Australia (Ghisi *et al.* 2007; Mottier and Prichard, 2008). One of these 'multiple' polymorphisms is present at higher frequencies in populations of BZ-resistant *H. contortus* (see Kwa *et al.* 1994; Mottier and Prichard, 2008). This heterogeneity in resistance-associated SNPs could make it difficult to design a simple molecular test for the reliable detection of parasites resistant to BZs, and the relative importance of these polymorphisms has not been thoroughly assessed.

Since the molecular basis of BZ-resistance is relatively well characterized, this has allowed the development of PCR-based detection methods for resistance-associated polymorphisms. A number of such methods have been described to identify individual resistant trichostrongyloid parasites (Silvestre and Humbert, 2000; von Samson-Himmelstjerna *et al.* 2003; Alvarez-Sanchez *et al.* 2005). Previous studies have described methods for measuring *H. contortus* allele frequency and genotyping individual worms using real-time PCR with locked nucleic acid (LNA) (Braasch and Corey, 2001; Petersen and Wengel, 2003) Taqman probes (Walsh *et al.* 2007). These approaches allow allele frequencies to be quantified both in pooled samples, containing large numbers of nematodes, and in individual nematodes. Furthermore, a specific pyrosequencing (Ronaghi *et al.* 1996, 1998) assay has been established to detect and measure all of the previously reported BZ-resistance associated SNPs and applied to a number of laboratory maintained and recent field isolates of *H. contortus*. In the present study, we demonstrate that measuring the frequency of the resistance-associated SNPs provides a reliable prediction of the BZ-resistance status of *H. contortus* and that the SNP at position 200 was the most important in the isolates examined.

MATERIALS AND METHODS

Isolates of H. contortus

Eleven different laboratory-maintained isolates of *H. contortus* were used. Isolates MHco1, 3 and 4 were described by Redman *et al.* (2008). MHco5 (IRE) is a BZ-resistant derivative of MHco3 (ISE). MHco6 was kindly provided by Dr R. Kaminsky (Novartis, Switzerland) and was originally isolated in South Africa prior to 1984. MHco8 was originally isolated in Kenya and was a kind donation from Dr J. Högland (Uppsala, Sweden), who received it in 1999. MHco9 was isolated in Germany, MHco11 was originally isolated in France and kindly donated by Dr H. Hoste (INRA, Toulouse, France) and MHCo12 as well as MHco13 were recently isolated in Switzerland and provided by Dr R. Kaminsky. The SWE isolate was from Sweden and provided by Dr J. Höglund. Seven isolates (denoted HHco/x/7) were isolated from the field in Germany in 2007.

'Genotyping' of individual larvae and determination of allele frequencies using real-time PCR

Genomic DNA was isolated from *H. contortus* third-stage larvae (L3s) using the method developed by Silvestre and Humbert (2000). Single, ex-sheathed larvae were collected in $2\,\mu$l of water and digested overnight in $30\,\mu$l digestion buffer (50 mM KCl, 10 mM Tris pH 8·3, 2·5 mM MgCl$_2$, 0·45% (v/v) Nonidet P-40, 0·45% (v/v) Tween-20, 0·01% (w/v) gelatine and proteinase K ($100\,\mu$g/ml) at 60 °C, and the suspension used without purification. Also, $\sim$100 L3s (following ex-sheathment) were suspended in $75\,\mu$l of water; $200\,\mu$l of the digestion buffer were then added, and the lysates incubated at 60 °C for 12–14 h. Following the incubation, the DNA was purified using the Gene Elute genomic DNA kit (Sigma-Aldrich) and resuspended in $100\,\mu$l of resuspension buffer (10 mM Tris-HCl, 0·5 mM EDTA, pH 9·0).

LNA Taqman based real-time PCR assays were carried out as described previously (Walsh *et al.* 2007). Briefly, both probe (Proligo, Paris, France) and primer concentrations (MWG Biotech, Ebersberg, Germany) were $150\,\mu$M for the resistant allele (TAC) probe, $50\,\mu$M for the susceptible allele (TTC) probe and $100\,\mu$M of each primer. Cycling conditions were: an initial denaturation step at 95 °C for 2 min followed by 40 cycles of 95 °C for 30 s, 64 °C for 1 min. Fluorescence data were captured at the end of the annealing phase, once per cycle.

Allele frequency determination for β-tubulin from pooled larvae using pyrosequencing

To develop suitable pyrosequencing assays for the analysis of *H. contortus* β-tubulin codon 167, 198 and 200 for isotypes 1 and 2 of β-tubulin, two, isotype-specific pyrosequencing primers were designed based on an alignment of the sequences of isotype 1 from *H. contortus* (GenBank Accession number M76493) and 2 (Accession number M76491). The Pyrosequencing[TM] Assay Design Software v.1.0.6

was used for this purpose. The amplification of both β-tubulin isotypes 1 and 2 from DNA was achieved using the forward primer **HcPy2PCR For** (5′-GAC GCA TTC ACT TGG AGG AG-3′) together with the biotinylated reverse primer **HcPy2PCR Rev** (5′Biotin-CAT AGG TTG GAT TTG TGA GTT-3′). For the subsequent analysis of the β-tubulin isotype 1 at codons 167, 198 and 200 or determination of allele frequencies, the sequencing primers **Hc167PySeq1** (5′-ATA GAA TTA TGG CTT CGT-3′), **Haem.con. 198 SeqPr** (5′-GGT AGA GAA CAC CGA TG-3′) and **Hc200PySeq1** (5′-TAG AGA ACA CCG ATG AAA CAT-3′), respectively, were used. The corresponding primers used for the sequencing of the β-tubulin isotype 2 were **H.c. 167PySeq 1 Iso 2** (5′-ATA GAA TTA TGT CAT CCT-3′) and **H.c. 200PySeq 1 Iso 2** (5′-TGG AAA ATA CTG ACG AAA CGT-3′). The suitability of the universal primer set and the specificity of primers for pyrosequencing was confirmed by testing them on cloned fragments of β-tubulin isotype 1 or 2 from *H. contortus*. The isotype 1-region (899 bp) was generated using primers **H.c. Iso 1 For** (5′-CGT TCT GGA CCG TAT GGA CAG-3′) and **H.c. Iso 1 Rev** (5′-GTG TAA GCT CAG CAA CTG TCG AA-3′), whereas the isotype 2-region (640 bp) was amplified using **H.c. Iso 2 For** (5′-TCC GGA CCT TTT GGT GCT-3′) and **H.c. Iso 2 Rev** (5′-GCG TGA GCT CAG AAA CAG TTA GT-3′). These primers were designed using the Primerselect™ 5.06 software (DNASTAR). For the analysis of codon 368, the PCR primers **H.c. Codon 368 For** (5′-GGT CGT ATG AGC ATG CGA GAA GTA-3′) and **H.c. Codon 368 Rev + Biotin** (5′biotin-ATT GCT CCG AAA TAC GCT TGA AC-3′) were used. The pyrosequencing-primer was **H.c.beta-tubulin Codon368seqPr** (5′-AAA TGG CGG CTA CCT-3′).

For each isolate, genomic DNA, isolated from a pool of 10 000 *H. contortus* L3s, was used as the template for the PCR. The amplicons were cloned into pCR4®-TOPO® vector (Invitrogen) and their sequences determined by conventional sequencing (Seqlab, Göttingen) to confirm their isotype 1 or 2 status. The isotype status was concordant with that determined by pyrosequencing (not shown).

For the analysis of SNP allele frequencies inferred to be linked to BZ-resistance, genomic DNA samples isolated from pools of 1000 or 10 000 *H. contortus* L3s were used. One μl DNA was amplified in a mix containing 5 U HotFire polymerase (Solis Biodyne), 1·5 mM MgCl$_2$, 80 nM dNTPs and 200 μM of each forward and reverse primer. Amplification was performed, following a 15 min 95 °C polymerase activation step, for 40 cycles at 95 °C for 1 min, 53 °C for 1 min and 72 °C for 1 min, followed by a final elongation step at 72 °C for 10 min. The pyrosequencing assays were conducted according to the manufacturer's (Biotage, Hamburg) protocols using the

PSQ™ MA-96A (Biotage) instrument. In brief, following PCR amplification, the amount of amplicon was assessed by electrophoresis using Gelstar® (Lonzy) for staining, and abundant products were used for pyrosequencing (Ronaghi *et al.* 1996, 1998). Up to 40 μl of amplicon were added to 40 μl 2× binding buffer (Biotage), 3 μl Streptavidin Sepharose™ beads (Biotage) and the solution mixed for 5 min to allow the binding of the biotinylated DNA fragments to the Streptavidin Sepharose™ beads. Using a Pyrosequencing Vacuum Prep Tool (Biotage), the beads together with the DNA were aspirated to a 96 format filter tool and then passaged through wash buffers as well as a sodium hydroxide buffer to remove the non-biotinylated DNA strand. Subsequently, the beads were released on to the 96-well sequencing plate and to each sample well, 40 μl of sequencing primer (0·4 μM), dissolved in annealing buffer, were added. Initially, for each assay, 2 control samples were performed, i.e. sequencing primer only, sequencing plus biotinylated primer and biotinylated primer only samples, to demonstrate specificity. Following the completion of the pyrosequencing reaction, the software of the instrument produces a 'pyrogram' and, based on the relative peak heights for the different alleles, a percent quantification for the frequency of each can be established.

Egg hatch test (EHT)

To determine the drug susceptibility or resistance of the various nematode populations, the EHT was carried out essentially as described by Coles *et al.* (2006). This test has been standardized by 'ring testing' (von Samson-Himmelstjerna *et al.* unpublished). The modifications in the optimized protocol were: (i) thiabendazole (TBZ) was dissolved in dimethyl sulphoxide (DMSO) and (ii) tests were set up using deionized water. The calculation of the effective concentration required to inhibit 50% and 95% of the eggs from hatching (EC$_{50}$ and EC$_{95}$, respectively) was performed using the computer program GraphPad Prism 5.0 for Windows. To fit the achieved dose-response-data by non-linear regression, a 4-parameter logistic equation with a variable slope was chosen. All analyses were done following logarithmic transformation of the data ($X = \log X$) and defining the lowest value as 0.

Sequencing from pooled cDNA

PCR-primers were designed to amplify β-tubulin cDNA fragments of $\sim$500 bp, and 3 pairs of primers were used to give complete coverage and sufficient overlap in the coding sequence. RNA was isolated from pooled L3 and eggs as described previously (Laughton *et al.* 1994). Reverse transcription PCRs were performed to amplify the coding sequence of

β tubulin isotype 1 using AffinityScript™ Multiple Temperature Reverse Transcriptase (Stratagene, La Jolla, California). PCR products were purified using the QIAquick® PCR purification kit (Qiagen, Crawley, UK) and sequenced directly using the primers 5′-ATGCGTGAAATCGTTCATGTGC-3′, 5′-ATGCTGCAATTGGAAGCCCTG-3′, 5′-CTCGAGCCTGGAACGATGGACT-3′, 5′-AAC-CGGGCATGAAGAAGTGAAGAC-3′, 5′-CTG-CCTTCGATTCCCTGGACA-3′ and 5′-TCCTC-GGGATATGCCTCTTCTCC-3′ (MWG Biotech, Ebersberg, Germany).

Statistical analyses

A key objective of the present study was to investigate the diagnostic effectiveness of the two molecular methods, i.e. the beta-tubulin codon 200 real-time PCR (Walsh *et al.* 2007) and pyrosequencing assays as methods for detection of resistance-related allele frequencies in comparison to the EHT EC$_{50}$ data. For statistical analysis, the EHT, as the current in vitro-reference method (Coles *et al.* 2006), was considered as the standard, with a cut-off point of 0·1 μg TBZ per ml for the detection of resistance (i.e. all samples with an EC50 > 0·1 μg/ml were considered as 'true positives'). The sensitivity, specificity, positive and negative predictive values were calculated using data resulting from the real-time PCR and pyrosequencing analyses, respectively, using the definitions and terms defined in the following. For the molecular assays, those with beta-tubulin isotype 1 codon 200 TTC allele frequencies of < 90% were defined as 'true-positives'.

Sensitivity is the probability that an isolate which is resistant is indeed tested as resistant:

$$\frac{\text{number of True Positives}}{\text{number of True Positives} + \text{number of False Negatives}}$$

Specificity is the probability that an isolate which is not resistant is tested as not resistant:

$$\frac{\text{number of True Negatives}}{\text{number of True Negatives} + \text{number of False Positives}}$$

The positive predictive value is the proportion of isolates with positive test results that are correctly diagnosed:

$$\frac{\text{number of True Positives}}{\text{number of True Positives} + \text{number of False Positives}}$$

The negative predictive value is the proportion of isolates with negative test results that are correctly diagnosed:

$$\frac{\text{number of True Negatives}}{\text{number of True Negatives} + \text{number of False Negatives}}$$

RESULTS

Pyrosequencing has been developed as a rapid molecular technique for the detection of polymorphisms in a variety of organisms. Here, we compared a previously described LNA-based real-time PCR procedure (Walsh *et al.* 2007) with a newly developed pyrosequencing method for the quantitative analysis of the β-tubulin isotype 1 codon 200 alleles in *H. contortus*. Various laboratory *H. contortus* isolates were examined using both methods, and some field isolates were tested using pyrosequencing alone. Pyrosequencing was also used to measure the 'susceptible' and 'resistant' allele frequencies at codons 167, 198 and 368 of the *H. contortus* β-tubulin isotype 1. Pyrosequencing requires previous PCR-amplification of the DNA fragment within which the polymorphism of interest is located. In order to test the reproducibility and accuracy of the pyrosequencing assay for β-tubulin isotype codon 200, and to compare the results obtained using this method to those from the LNA Taqman real-time PCR assay, serial mixtures of both alleles were established. For this purpose, using the universal pyrosequencing PCR primer set with genomic DNA from 1 worm of a BZ-susceptible and 1 of a BZ-resistant isolate, the 2 respective beta-tubulin codon 200 alleles were obtained and cloned into pCR4-TOPO (Invitrogen). Custom sequencing (Seqlab) confirmed the codon 200 TTC and TAC alleles, respectively. Ratios of 1:0, 9:1, 4:1, 7:3, 3:2 and 1:1 of both alleles were assessed. In three independent experiments, high regression of expected and observed allele frequencies ($R^2 = 0·996$) as well as a low standard deviation of less than 11% were recorded (Fig. 1).

The next step in the process of the validation of this allele-specific quantitation procedure included pyrosequencing-based analysis of genomic DNA isolated from different mixtures of L3s from *H. contortus* isolates with known BZ-resistance phenotype. The combinations MHco3/MHco4, MHco12/MHco3 and MHco13/MHco3 in the ratios 1:0, 9:1, 7:3 and 1:1 were prepared. Pools of either 1000 or 10000 L3s were used for each DNA extraction, and each of the combinations was tested in at least 2 independent experiments. Fig. 2 displays the regression between the expected and the observed (β-tubulin isotype 1) codon 200 TTC allele frequencies. Using the Pearson product moment correlation (SigmaStat 3.11) for the comparison of all 4 isolates, correlation coefficients of > 0·95 were calculated, showing high statistical significance ($P < 0·001$).

Subsequently, the pyrosequencing approach was applied to the 11 *H. contortus* laboratory isolates to determine β-tubulin isotype 1 allele frequencies at codons 167, 198, 200 and 368 as well as the codon 167 and 200 frequencies in isotype 2 i.e. all of the polymorphisms previously suggested to be involved in
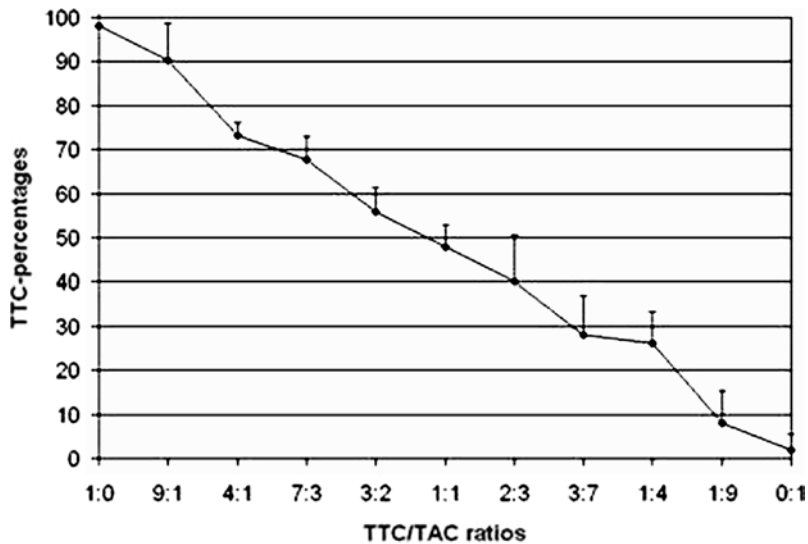
Fig. 1. Frequencies of TTC *Haemonchus contortus* β-tubulin isotype 1 codon 200 alleles using mixtures of cloned cDNA with either TTC or TAC allele determined by pyrosequencing. Results given as arithmetic means of 3 independent analyses; error bars show standard deviations.
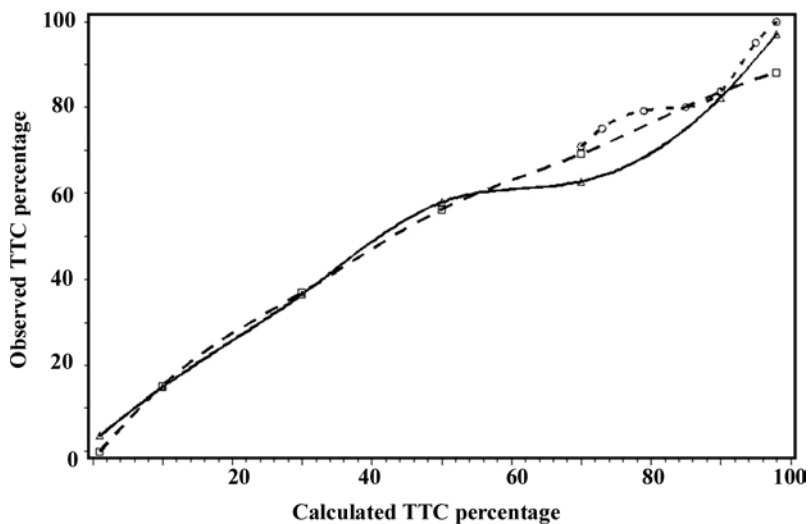

Fig. 2. Regression of observed and calculated β-tubulin isotype 1 codon 200 TTC allele frequencies using various mixtures of genomic DNA from *Haemonchus contortus* isolates with different BZ-resistance phenotype. $\bigcirc$- -$\bigcirc$ (dashed line) isolate MHco4 *versus* MHco3: Δ – Δ (solid line) isolate MHco12 *versus* MHco3: $\square$ – $\square$ (long dashed line) isolate MHco13 *versus* MHco3.

BZ-resistance (Kwa *et al*. 1994; Elard *et al*. 1996; Elard and Humbert, 1999; Silvestre and Cabaret, 2002; Drogemuller *et al*. 2004; Schwab *et al*. 2005; Melville *et al*. 2006; Hodgkinson *et al*. 2008; Ghisi *et al*. 2007; Mottier and Prichard, 2008). At codon 198, only the 'susceptible' alleles were present and no polymorphism was detected, whereas at position 368 only GTT/GTC, encoding valine, was detected. The results for codons 167 and 200 are given in Table 1. The β-tubulin isotype 1 codon 200 TTC allele frequencies were $\leqslant 20\%$ for highly BZ-resistant isolates. For isotype 2, the codon 200 TTC frequency was not reduced to the same extent in the resistant isolates and on average differed only $\sim 10\%$ between the BZ-susceptible (i.e. MHco1, 3, 6 and 9)

and highly resistant (i.e. MHco5, 12 and SWE) isolates. Interestingly, repeated attempts to amplify the β-tubulin isotype 2 from 2 of the resistant isolates (MHco11, MHco13) did not succeed. We detected the TAC allele at codon 167 of isotype 1 in the MCco4 isolate, in which it was present at low frequency (7·2%), whereas in the other phenotypically resistant isolates only the TTC allele was detected.

Since the resistance-related polymorphism at codon 200 of the β-tubulin isotype 1 was the only one that occurred at high frequencies ($>30\%$) in the resistant isolates, the allele frequency at this site was also determined in each of the 11 isolates of *H. contortus* by the LNA Taqman real-time PCR assay using genomic DNA from 100 L3s (Table 1). The

Table 1. EC$_{50}$ values for TBZ-treated *Haemonchus contortus* isolates and β tubulin allele frequencies for SNPs determined by real-time PCR and pyrosequencing

| | | EHT | | Pyrosequencing™ | | | |
| | | | | Codon 200 TTC allele | | Codon 167 TTC allele | |
Name of isolate*	Origin	EC$_{50}$ (μg TBZ/ml)	Real-time PCR Codon 200 TTC allele	Isotype 1	Isotype 2	Isotype 1	Isotype 2
MHco1	Slovakia	0·039	99·3%	92·4%	100%	100%	100%
MHco3	Netherlands	0·033	98%	93·8%	90·5%	100%	100%
MHco4*	South Africa	0·117	66·0%	69·5%	90·1%	92·8%	100%
MHco5*	Netherlands	0·401	3·0%	7·1%	81·2%	100%	100%
MHco6	Switzerland	0·038	99·5%	95·1%	100%	100%	100%
MHco8	Kenya	0·059	61·7%	71%	91·1%	100%	100%
MHco9	Germany	0·031	99·7%	93·6%	97·5%	100%	100%
MHco11*	France	0·664	0·9%	0·0%	N.D.	100%	N.D.
MHco12*	Switzerland	0·657	2·3%	2·9%	92·4%	100%	100%
MHco13*	Switzerland	0·352	2·3%	3·1%	N.D.	100%	N.D.
SWE*	Sweden	0·378	9·0%	19·1%	84·8%	100%	100%

* Populations that show a clinical level of resistance with an EC$_{50}$ concentration of $>0·1 \,\mu$g TBZ mL^{-1}.
N.D., Isotype not detectable.

Table 2. Phenotypic and genotypic analysis of seven field samples from Northern Germany

(The EC$_{50}$ for thiabendazole was obtained using the egg hatch test, as described, and the 'TTC' allele frequency obtained using the pyrosequencing assay.)

Population	EC$_{50}$ Thiabendazole (μg/ml)	'TTC' allele frequency at codon 200 (%)
HHco6/7	0·22$\pm$0·02	7·2$\pm$3·6
HHco9/7	0·14$\pm$0·03	6·7$\pm$3·8
HHco11/7	0·22$\pm$0·06	2·0$\pm$2·5
HHco12/7	0·1$\pm$0·01	27·4$\pm$5·3
HHco13/7	0·04$\pm$0·004	100·0$\pm$0·0
HHco14/7	0·13$\pm$0·007	2·1$\pm$3·8
HHco17/7	0·08$\pm$0·01	74·1$\pm$2·4

results obtained were very similar to those from pyrosequencing, with the exception of the SWE isolate, for which a slightly higher TTC percentage (19% instead of 9%) was obtained using the pyrosequencing approach. Overall, a statistically highly significant ($P<0·001$) correlation coefficient (Pearson product moment correlation) of 0·981 was obtained by comparing the results obtained using the 2 molecular methods. Furthermore, EC$_{50}$ (Table 1) and EC$_{95}$ values (data not shown) for TBZ in the EHT were determined for the same isolates. In this test, isolate MHco12 was most resistant, whereas isolates MHco5, MHco12, MHco13 and SWE were clearly resistant, and MHco4 and MHco8 were shown to be outside of the suggested cut-off value for 'resistance' (EC$_{50}=0·1 \,\mu$g TBZ per ml) (Coles *et al.* 1992). All other isolates were susceptible (Table 1), which was also confirmed by both the EC$_{95}$ as well as the EC$_{50}$ values (95% lower confidence limit being $<0·1$) for

the susceptible isolates and $>0·1 \,\mu$g TBZ per ml for the resistant isolates, respectively.

The studies of the laboratory-maintained isolates (several of which were recently established from the field) suggested that measuring the frequency of the beta-tubulin isotype 1 codon 200 allele frequencies would be sufficient to predict the BZ-resistance status of the parasite population. In order to confirm that this observation was true for clinical samples, 7 nematode populations, containing a substantial proportion of *H. contortus*, were isolated from infected sheep in Germany (in 2007). We then carried out EHTs and analysed the frequency of the codon 200 polymorphism using pyrosequencing (the other SNPs were not analysed due to limited availability of DNA). Table 2 shows that there was a significant relationship (determined by 2nd degree polynomial regression) between the EC$_{50}$ for TBZ, as determined in the EHT, and the frequency of the 200 TTC allele in β-tubulin isotype 1 (goodness of fit: 0·944, $P=0·0032$). Finally, to assess the diagnostic potential of the real-time PCR and pyrosequencing assays for the β-tubulin codon 200, a statistical comparison with the EHT data was performed, based on the results for all isolates. Taking the EHT as the reference and using the 0·1 μg TBZ per ml as the cut-off, the sensitivity for both assays was 1, whereas specificities of 0·57 and 0·71 for the PCR and pyrosequencing assays, respectively, were calculated. The corresponding positive predictive values were 78·57% and 84·81%, respectively, while the negative predictive value was 100% for both assays. For the opposite comparison, the pyrosequencing assay (since all isolates were tested by this assay) was regarded as standard using a β-tubulin codon 200 TTC allele frequency of <90% as the resistance threshold. In
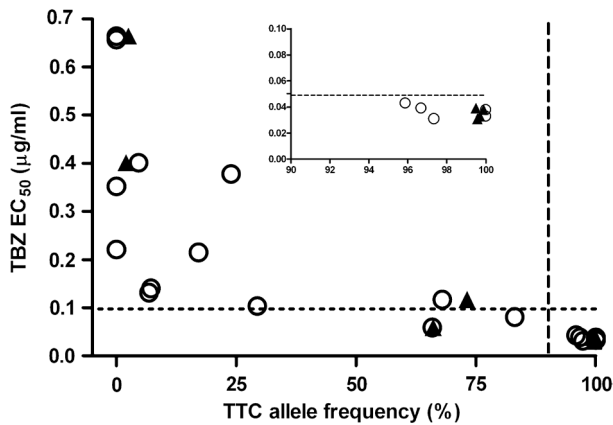
Fig. 3. Comparison of TTC allele frequency (s) at codon 200 in isotype 1 of β tubulin with EHT TBZ EC_{50}-values in *Haemonchus contortus* isolates ($n = 18$). ▲ – allele frequency of isolates determined by real-time PCR: ○ – allele frequency of isolates determined by pyrosequencing. On the main graph, lines indicate the current cut-off for resistance in the egg hatch test (EHT) ($EC_{50} \geqslant 0.1\,\mu g/ml$) and proposed allele frequency ($\leqslant 90\%$ TTC). The inset is an expanded view of the bottom right-hand corner of the graph: in this case the dashed line indicates a cut-off for resistance in the EHT of $EC_{50} \geqslant 0.05\,\mu g/ml$.

this case, the sensitivity of the EHT was 0·85, the specificity 1, the positive predictive value was 100% and the negative predictive value 71·4%. Fig. 3 shows a scatter-plot of the *in vitro* test data (i.e. the EHT EC_{50}-data) plotted against the allele frequencies for codon 200 determined using real-time PCR and pyrosequencing. Using a putative threshold for resistance in the molecular assays of <90% TTC allele frequencies, all phenotypically BZ-resistant plus 2 susceptible (i.e. MHco8 and HHco/7/7) isolates were considered to be resistant.

It was evident that the most resistant isolates had the highest resistance allele (i.e. TAC) frequencies, approaching 100%, whereas isolate MHco4 possessed a 'TTC' (susceptible) allele frequency of ~70%, as reported previously (Walsh *et al.* 2007). Isolate MHco8 had a similar 'TTC' allele frequency to that of isolate MHco4, but was more sensitive to TBZ, with an EC_{50} of less than the accepted cut-off for resistance (0·1 μg TBZ per ml). This isolate was therefore classed as 'susceptible' based on the current guidelines of the World Association for the Advancement of Veterinary Parasitology (Coles *et al.* 1992). In order to determine whether the difference in drug sensitivity between isolates MHco4 and MHco8 was due to the proportion of heterozygotes in the two isolates, we measured their individual genotype frequencies using the real-time PCR method (Walsh *et al.* 2007). There was a slight difference in the number of heterozygotes, with isolate MHco8 (45%) having more than isolate MHco4 (38%). The number of homozygous resistant (10% for MHco4; 6% for MHco8) and susceptible

individuals (52% for MHco4 and 49% for MHco8) were very similar in the two isolates.

DISCUSSION

BZ-resistance is recognized to be a serious problem in the veterinary field, and the use of these drugs for mass treatment against parasites in humans has the potential to lead to similar issues. The development of rapid and sensitive methods for detecting resistance-associated alleles would therefore be valuable for monitoring the appearance and spread of potentially BZ-resistant nematodes. In this paper, we have shown that both the LNA Taqman real-time PCR and the pyrosequencing assays are reliable for investigating resistance-associated allele frequencies in isolates and populations of *H. contortus*. Overall, there was a very good agreement between the allele frequencies of the various isolates determined using the two methods, and both assays readily identified all the populations in which TBZ had a reduced efficacy *in vitro*. Comparing the two molecular assays, our experience is that the pyrosequencing assay is quicker and easier to perform. Furthermore, it is suitable for the testing of multiple SNPs with limited extra time and effort. However, the equipment required is more expensive and less widely available than that needed for quantitative real-time PCR. The analysis and detection of drug resistance-associated alleles by pyrosequencing has been described for many different systems including viruses (Hoffmann *et al.* 2007), bacteria (Zhu *et al.* 2007) and protozoa (Zhou *et al.* 2006). Troell *et al.* (2003) showed that pyrosequencing allows an efficient analysis of the genetic make-up of nematode populations of *H. contortus* individuals. This paper is the first report of a pyrosequencing-based method for the detection and measurement of specific SNPs in pooled genomic DNA from parasitic nematodes. We show that it is a useful alternative to real-time PCR for measuring allele frequencies in *H. contortus* populations, with the potential to be applied to other species of nematodes in livestock and humans.

Using both assays, we showed that the resistance-allele frequency increased with the EC_{50} for TBZ for all of the 18 isolates tested. One susceptible isolate, MHco8, had a relatively high resistance-allele frequency (~30–40%) – almost identical to that of the resistant MHco4, yet in the EHT, it was susceptible to drug concentrations below those considered to represent a resistant phenotype. However, amongst isolates with a susceptible EHT phenotype, isolate MHco4 showed the highest EC_{50} value. Furthermore, this was the only isolate to have a small percentage (7%) of potential resistance alleles at codon 167 of β-tubulin isotype 1. It may be that the relatively small differences in the genotype frequencies between isolates MHco4 and MHco8 account for the difference and that, while, based on current

guidelines for the interpretation of the *in vitro* tests (Coles *et al.* 1992), isolate MHco8 is currently considered 'susceptible', high-level resistance might develop very rapidly under further selection pressure. Measuring the resistance-allele frequency was shown to be a reliable means of identifying isolates that would be considered 'resistant' in the EHT, but the resistance allele needs to be present at a relatively high frequency ($>30\%$) for the population as a whole to infer resistance to a normal therapeutic dose of TBZ, which might indicate some problems with the use of the *in vitro* test to detect low-level resistance in the field. This finding is in agreement with the findings of Martin *et al.* (1989), who concluded that BZ-resistance would only be detected using *in vitro* tests if $\geqslant 25\%$ of the population carried resistance genotypes. However, the findings in Fig. 3 suggest that the EHT would perform better, at least for *H. contortus*, if the threshold for resistance was lowered to $0\cdot05\ \mu$g of TBZ per ml, rather than $0\cdot1\ \mu$g TBZ per ml. The ability to detect the presence of a significant proportion of resistance-alleles in the phenotypically susceptible isolates MHc08 and HHco17/7 confirms that molecular tests, such as real-time PCR and pyrosequencing, make it possible to genetically identify parasite populations in which resistance is emerging but has not yet manifested itself in treatment failures. Based on the EHT and molecular data obtained in this study for a total of 18 *H. contortus* isolates, an allele frequency of less than 90% at β-tubulin for codon 200 TTC appears to suggest that BZ-resistance selection has occurred. The lowest TTC frequency seen in an isolate with an EC$_{50}$ $<0\cdot05\ \mu$g TBZ/ml was 92%; 10% is a conservative estimate for the sensitivity of the molecular techniques' ability to detect the resistance allele, an important consideration if the assays were to become automated and 'high-throughput'. However, this speculative resistance threshold requires further evaluation using the molecular assays on a larger set of isolates with known BZ-resistance phenotypes and, ideally, including additional field isolates containing multiple species of nematodes.

The F200Y SNP is the major determinant of BZ-resistance in *H. contortus*; however, this and other organisms possess multiple SNPs elsewhere in the coding sequence that contribute to the resistance phenotype. For example, in a number of fungal species, SNPs at codon positions 6, 50, 134, 165, 167, 198, 200 and 241 have been associated with BZ-resistance (Koenraadt *et al.* 1992; Ma *et al.* 2003), whereas for parasitic nematodes, codons 167 and 198 have also been implicated (Silvestre and Cabaret, 2002; Ghisi *et al.* 2007). Recent studies of some isolates of *H. contortus* has shown that the BZ-resistance-related polymorphism was E198A and not F200Y (Ghisi *et al.* 2007) or both in combination (Mottier and Prichard, 2008). We found no clear evidence of such variation, or of any other coding

SNP in any resistant isolate examined, except for a small (7%) percentage of the resistance-associated sequence at codon 167 of MHc04, and the absence of significant levels of these alleles in the isolates we studied makes it difficult for us to assess their relative contribution to global resistance. Since we made no attempt to choose isolates carrying any specific SNPs for our study, one interpretation is that the SNPs at codons 167 and 198 are less common in *H. contortus* than that at codon 200. However, a much more extensive survey than undertaken here would be required to support any such interpretation. It is possible that the small number of resistance-alleles at codon 167 of isolate MHc04 accounts for its slightly higher EC$_{50}$ than MHc08, but we have no direct evidence to support this proposal. Another interesting observation was that the attempts to amplify β-tubulin isotype 2 regions in 2 of the resistant isolates (MHco11 and MHco13) failed. This finding may reflect the finding/s of an earlier report on the elimination of this isotype following intensive BZ-selection in *H. contortus* (see Kwa *et al.* 1993). However, this observation was not repeated in 3 additional highly resistant isolates (SWE, MHco12 and MHco5). Recently, it was reported that the treatment of parasitic nematodes with macrocyclic lactone anthelmintics selects for the BZ-'resistance' alleles at positions 167, 198, 200 and 368 (Eng *et al.* 2006; Mottier and Prichard, 2008). Some of the isolates used in our study, such as isolate MHc04 (Van Wyk and Malan, 1988), are also resistant to macrocyclic lactones. The relationship between resistance to the two classes of anthelmintic will clearly be of great interest, but the treatment history of the isolates does not seem to impact on our main conclusion, that the BZ-resistance status of *H. contortus* isolates can be predicted by measuring the allele frequency of 3 SNPs. In our experience, the SNP at position 200 is likely to be that most frequently encountered; nonetheless, all 3 positions should be included where possible. The pyrosequencing assay is well suited to this and would increase in cost by ~60% if all 3 positions were included in it.

In conclusion, we have demonstrated that the benzimidazole-susceptibility of *H. contortus* can be determined by measuring a relatively small number of SNPs. If the mechanism/s of resistance present in human filarial or soil-transmitted helminths is/are similar, then such methods might be useful in ensuring that resistance does not become as great a problem in humans as is the case in parasites of ruminants. Previous attempts to detect these specific polymorphisms in possible BZ-resistant human hookworms were not successful (Albonico *et al.* 2004*b*; Schwenkenbecher *et al.* 2007), although they have been inferred for *Wucheria bancrofti* (see Schwab *et al.* 2005) and may reflect the difficulty of identifying true anthelmintic resistance in human parasites. Irrespective, the molecular approaches

evaluated herein should be able to be adapted for the detection of resistance in a range of nematodes of animal and/or human health importance.

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