Phenotypic and genotypic analysis of benzimidazole resistance in the ovine parasite Nematodirus battus

Alison A Morrison, Sian Mitchell, Rebecca Mearns, Iain Richards, Jacqui B Matthews, David J Bartley

To cite this version:

Alison A Morrison, Sian Mitchell, Rebecca Mearns, Iain Richards, Jacqui B Matthews, et al.. Phenotypic and genotypic analysis of benzimidazole resistance in the ovine parasite Nematodirus battus. Veterinary Research, 2014, 45 (1), pp.116. 10.1186/s13567-014-0116-5. hal-01290619

HAL Id: hal-01290619
https://hal.science/hal-01290619
Submitted on 18 Mar 2016

HAL is a multi-disciplinary open access archive for the deposit and dissemination of scientific research documents, whether they are published or not. The documents may come from teaching and research institutions in France or abroad, or from public or private research centers.

L’archive ouverte pluridisciplinaire HAL, est destinée au dépôt et à la diffusion de documents scientifiques de niveau recherche, publiés ou non, émanant des établissements d’enseignement et de recherche français ou étrangers, des laboratoires publics ou privés.
Phenotypic and genotypic analysis of benzimidazole resistance in the ovine parasite *Nematodirus battus*

Morrison *et al.*
Phenotypic and genotypic analysis of benzimidazole resistance in the ovine parasite Nematodirus battus

Alison A Morrison1*, Sian Mitchell2, Rebecca Mearns3, Iain Richards4, Jacqui B Matthews1 and David J Bartley1

Abstract

Benzimidazole resistance is common amongst many ovine trichostrongylid nematodes species globally. Although anthelmintics have been used for over half a century in some areas of the world for the control of Nematodirus battus, resistance has never been detected. Veterinary investigations conducted in 2010 demonstrated reduced efficacy in a flock that had been treated previously with fenbendazole (FBZ), suggesting probable resistance in N. battus. Infective larvae (L3; designated MNba2) were generated from the original material to conduct a controlled efficacy test (CET). Faecal egg counts showed an average of 37% reduction in the FBZ treated group 7 days post treatment compared to the untreated lambs. Average worm burden results showed no reduction after FBZ treatment compared to the untreated group (3850 and 3850 worms respectively). A molecular assay to assess the frequency of the commonly associated single nucleotide polymorphisms (SNP) in the β-tubulin isotype 1 gene, F200Y and E198A, was developed. Larval genotypes were predominantly homozygous resistant at codon 200 SNP, ranging from 56%-83% and remained stable at 70% for adult worm populations taken from treated and control lambs in the CET. Only susceptible genotypes were found at codon 198. The allele frequency for F200Y ranged between 80-83% in adult worms taken from the CET from treated and control lambs. The results confirmed initial findings and demonstrated the first report of FBZ resistance in N. battus whilst providing evidence that the P200 point mutation in the β-tubulin isotype 1 gene is a potential mechanism of resistance in the species.

Introduction

There are several species of Nematodirus that cause disease and production losses in young lambs, although most of the losses in the UK are caused by Nematodirus battus [1-3]. Nematodiosis is a disease that is normally seen in young lambs, the signs of infection are often acute and, if left unchecked, can result in a high (typically 5-20%) mortality rate [3]. Acute disease is the consequence of very heavy larval challenge and the effects of the developing larvae. As a result, lambs can present with severe scouring and other clinical signs before there are well established levels of adult worms and, hence, eggs appearing in faeces. Currently five classes of broad spectrum anthelmintics are available for use in sheep in the UK. All classes have licensed efficacy against N. battus, however, activity against certain life stages (particularly immatures) can vary by formulation and mode of application [4], particularly within the macrocyclic lactone class. Many farmers in the UK opt to use benzimidazoles due to its wide safety margin and good efficacy [5]. Reports of resistance to this class of anthelmintic are commonplace in ovine trichostrongylid nematode species such as Teladorsagia circumcincta throughout sheep flocks in the UK [6-9] and globally [10-14]; however it has never previously been reported in N. battus. The only reports of benzimidazole resistance involving Nematodirus spp. (Nematodirus spathiger) are cases of oxendazole resistance in New Zealand, Australia and Tasmania [15-17] and one case of thiabendazole resistance in a mixed species isolate of N. spathiger and Nematodirus filicollis in Australia [18].

Several genetic mechanisms have been associated with BZ resistance in parasitic nematodes of sheep; for example, loss of isotype 2 of the β-tubulin gene and single nucleotide polymorphism (SNP) within isotype 1 of the β-tubulin gene [19,20]. SNPs in the β-tubulin gene are
responsible for an amino acid transversion at each of the sites; phenylalanine to tyrosine at codon 200 (TTC → TAC, F200Y; [21,22], phenylalanine to tyrosine at 167 (TTT → TAT, F167Y; [23,24]) and glutamic acid to alanine at 198 (GAA → GCA, E198A; [25]). Not all of these mutations are found in all ovine parasitic nematode species that are resistant and the presence of one SNP is not usually accompanied by a second [26]. At present, no information relating to the mechanisms involved in benzimidazole resistance in N. battus is available.

In 2010, a clinical case of nematodirosis was submitted to Animal Health Veterinary Laboratory Agency (AHVLA) Penrith where a farmer was concerned about a group of 60 lambs showing signs of disease (diarrhoea and ill thrift); three lambs had died from the group, with a fourth being submitted for post mortem examination. Nematodirosis due to N. battus was diagnosed as the cause of death due to the high numbers of N. battus eggs detected in the faeces (1850 eggs per gram) and a small number (300) of adults present in the small intestinal contents. No other gastro-intestinal nematodes were identified in the abomasal or small intestinal contents. Treatment of the surviving lambs with FBZ resulted in little clinical improvement; however the farmer only treated the lambs to the average weight of the group. After seeking further advice, a faecal egg count reduction test (FECRT) was conducted by the attending veterinarian using FBZ at the manufacturer’s recommended dose rate (MRDR) and faecal samples were submitted to the AHVLA at the time of treatment (designated hereafter as MNba2\textsubscript{VCF}) and 10 days later [27].

Here, the authors report the findings from a controlled efficacy test (CET) performed at Moredun Research Institute using the field isolate of N. battus derived from the MNba2\textsubscript{VCF} generated material. Secondly, they describe a pyrosequencing assay developed to identify polymorphisms at codons 198 and 200 of the β-tubulin isotype 1 gene to investigate the potential mechanisms involved in BZ resistance in N. battus.

**Materials and methods**

**Parasite isolate**

Faecal material was collected from lambs following an on-farm FBZ FECRT which indicated the presence of benzimidazole resistance [27]. N. battus eggs were cultured and extracted from faeces using a modification of methodologies described in the Manual of Veterinary Parasitology Techniques reference book [28]. In brief, eggs were extracted from faeces by differential sieving and salt flotation. Recovered eggs were stored in tap water in 75 cm\textsuperscript{2} vented culture flasks (Corning B.V. Life Sciences, Amsterdam, The Netherlands) at around 20 °C for over 7 weeks to allow full development of larvae in the eggs. To assist hatching, eggs were sandwiched between two glass plates and gently crushed by applying downward pressure until a faint cracking sound was heard. Successful hatching was assessed by viewing the eggs down a stereo microscope (×20) before Baermannising the suspension. The resultant third stage larvae (L\textsubscript{3} n = 4300) were subsequently passaged through a parasite-naïve lamb, which was administered with FBZ at day 43 post-infection (pi) at MRDR. Faecal material was collected before and after treatment and processed as above to generate sufficient L\textsubscript{3} for the controlled efficacy trial. Eggs recovered post-treatment were artificially hatched and the L\textsubscript{3} used in the controlled efficacy trial.

**Experimental design**

Ten helminth-free, female, six month-old Texel X Greyface lambs were each artificially infected per os with 6000 L\textsubscript{3} (day 0 pi). On day 24 pi, faecal egg counts (FEC) were conducted using a modification of the salt flotation technique described by [29] and [30] with a sensitivity of up to one egg per gram. All lambs were weighed. The lambs were then allocated into one of two groups ensuring they were balanced as closely as possible for both FEC and weight. On day 25 pi, one group was orally administered fenbendazole (Panacur 2.5%, Intervet, Milton Keynes, UK; 5 mg/kg body weight (BW)), whilst the second group remained untreated. These groups were designated MNba2\textsubscript{FBZ} and MNba2\textsubscript{CON}, respectively. All anthelmintic treatment doses were calculated according to the respective manufacturer’s instructions, with doses rounded up to the nearest 0.5 mL (dosage range 5.0 - 5.3 mg/kg BW). Faecal samples were taken per rectum daily from each lamb from day 14 pi until day 32 pi and FECs conducted. All lambs were slaughtered on day 32 pi and the small intestines were conducted using a modification of the salt flotation technique described by [29] and [30] with a sensitivity of up to one egg per gram. All lambs were weighed. The lambs were then allocated into one of two groups ensuring they were balanced as closely as possible for both FEC and weight. On day 25 pi, one group was orally administered fenbendazole (Panacur 2.5%, Intervet, Milton Keynes, UK; 5 mg/kg body weight (BW)), whilst the second group remained untreated. These groups were designated MNba2\textsubscript{FBZ} and MNba2\textsubscript{CON}, respectively. All anthelmintic treatment doses were calculated according to the respective manufacturer’s instructions, with doses rounded up to the nearest 0.5 mL (dosage range 5.0 - 5.3 mg/kg BW). Faecal samples were taken per rectum daily from each lamb from day 14 pi until day 32 pi and FECs conducted. All lambs were slaughtered on day 32 pi and the small intestines were removed for saline digest to estimate total worm burdens. Methods used for necropsy and worm recovery were as described previously [31]. Total burdens of each animal were estimated from a 2% subsample (100 mL) of the small intestinal wash and digest. Worms were separated into adult males, adult females and juvenile stages using the criteria described in the Manual of Veterinary Parasitology Techniques reference book [28].

All experimental procedures conducted at Moredun Research Institute were assessed and approved by the Institute’s Experiments and Ethics Committee and were conducted under the legislation of a UK Home Office License (reference PPL 60/03899) in accordance with the Animals (Scientific Procedures) Act of 1986.

**Statistical analysis**

Nematode burdens and FECs were square-root transformed to normalize for variance. Burdens were compared using one way ANOVA (Minitab version 13, Coventry, UK), followed by Fisher’s pairwise comparisons when found to be significant (p < 0.05). Statistical
significance was accepted as \( p < 0.05 \). Fenbendazole efficacy was calculated based on group mean FECs using one of a range of standard formulae, \( (1 - [T2/C2]) \times 100 \) using arithmetic means [32], \( (1 - [T2/T1] \left[ C1/C2 \right]) \times 100 \) using geometric means [33], \( (1 - [T2/T1] \left[ C1/C2 \right]) \times 100 \) using arithmetic means [34], \( (1 - [T2/T1]) \times 100 \) using arithmetic means [35], where \( C1 \) and \( C2 \) are the FEC of untreated control animals pre- and post treatment respectively and \( T1 \) and \( T2 \) are the FEC of animals pre- and post treatment respectively. Bootstrap analysis of the data using the “BootStreat” program was also conducted, with a re-sampling number of 2000 to calculate mean treatment efficacies and upper and lower 95% confidence limits [36]. Efficacy based on total worm burdens was calculated using the WAAVP guidelines formula [32].

Molecular analysis

Due to lack of published sequences available for \( N. \) battus \( \beta \)-tubulin gene, partial \( \beta \)-tubulin isotype 1 sequences were amplified from cDNA generated from total RNA that had been extracted using TRIzol reagent (Life Technologies, California, USA) from 100, 000 \( L3 \) from a known FBZ-sensitive isolate (designated MNba1) and MNba2 \(^{CON} \) isolate \( L3 \) obtained after FBZ treatment along with a FBZ susceptible isolate of \( Haemonchus contortus \) (MHCo3; [37]), as a control. Twenty microlitres of cDNA were generated using Invitrogen superscript III reverse transcriptase kit (Invitrogen, California, USA), where 4 \( \mu L \) of RNA extract was used per sample. Generic primers (Table 1: Gen Beta-tub For1 & Gen Beta-tub Rev1) were designed to amplify an area of the isotype 1 \( \beta \)-tubulin gene that covered the three most common SNPs of interest using published sequences from other trichostrongylid nematode species; \( Trichostrongylus colubriformis \) (Accession number: L23506), \( Teladorsagia circumcincta \) (Accession number - Z69258), \( H. \) contortus (Accession number - M76491/EF198865) and \( Cooperia oncophora \) (Accession number - AY259994). PCR products were amplified using Novataq™ Hot start master mix (Novagen, Madison, USA) in a 50 \( \mu L \) reaction, with primers and \( MgCl2 \) at a final concentration of 0.3 \( \mu M \) and 3 mM, respectively, with the following cycle conditions: 94 °C for 10 min, (94 °C for 30 s, 52 °C for 30 s, 72 °C for 30 s) for 35 cycles and 72 °C for 10 min. \( \beta \)-tubulin isotype 1 sequences spanning the SNPs of interest were also generated from genomic DNA (gDNA) isolated from 10 individual adult \( N. \) battus worms (5 MNba1 and 5 MNba2 \(^{CON} \)). One microlitre of gDNA was added to a 25 \( \mu L \) PCR reaction using HcPy2 PCR For and HcPy2PCR Rev primers [38], see Table 1) at 0.3 \( \mu M \) and 3.0 mM MgCl2 final concentrations in NovaTaq™ Hotstart master mix. PCR amplicons of the appropriate size were cut from a 1.5% agarose gel (Gel Extraction Kit, Qiagen, Hilden, Germany) and the gDNA ligated into the pGEM-T vector (Promega, Madison, USA) following the manufacturer’s protocol. Transformations were carried out following the standard protocol for JM109 Escherichia coli Competent Cells (Promega) and two (for gDNA sequences) and six (for cDNA sequences) transformed colonies were picked and grown up in 10 mL LB broth overnight before DNA extraction was carried out using a Wizard SV mini prep kit (Promega). DNA concentrations for gDNA and cDNA were assessed using a Nanodrop spectrophotometer (Nanodrop Technologies Inc., Delaware, USA) and adjusted if necessary using DNA/RNA-free water according to Eurofins MWG requirements for sequencing. DNAStar (Madison, USA), Lasergene 9 EditSeq, BLAST and ClustalW were used to analyse and align sequences. Consensus \( N. \) battus sequence was obtained using the gDNA sequences and used to design primers that spanned codons 200 and 198 for a pyrosequencing assay using “Pyrosequencing assay design” software, Version 1.0 (Table 1).

Pyrosequencing

To provide gDNA for pyrosequencing, 20 ethanol-fixed adult \( N. \) battus were used from three populations; MNba1, MNba2 \(^{CON} \) and MNba2 \(^{FBZ} \). Genomic DNA was extracted using DNeasy Kit (Qiagen) as per kit instructions. For PCR, the adult gDNA extracts were diluted 1:5 in DNA/RNA-free water (Sigma, Dorset, UK). For provision of gDNA from four populations of \( L3 \) (MNba1, MNba2 \(^{VCF} \), MNba2 \(^{CON} \) and MNba2 \(^{FBZ} \)), ethanol fixed \( L3 \) were bathed in PBS for 15–30 min prior to transferring individuals into 30 \( \mu L \) of lysis buffer (50 mM KCl, 10 mM Tris (pH 8.3), 2.5 mM MgCl2, 0.45% (v/v) Nonidet P-40, 0.45% (v/v) Tween 20, 0.01% (w/v) gelatin and 0.1 mg/mL Proteinase K) in a 96-well plate (Axygen, California, USA); frozen for 30 min at −20 °C, prior to incubating at 56 °C overnight. Ethanol precipitation was carried out to clean up the gDNA and re-suspended in 25 \( \mu L \) of DNA/RNA-free water (as above). A minimum of 80 \( L3 \) were lysed for each study population. For pyrosequencing, 4 \( \mu L \) of gDNA from both adult and \( L3 \) were amplified in a mix containing 0.185 \( \mu M \) Nb B-t200 Fbio, Table 1 Primers used in this study (*invitro oligo perfect designer used; # pyrosequencing assay design software version 1.0 used; ^primer sequences obtained from [38])

| Oligo                      | 5’ – 3’                        |
|----------------------------|--------------------------------|
| Gen Beta-tub For1*         | ATGCGTGARATCGTYCAY             |
| Gen Beta-tub Rev1*         | CGAGGGAARGGKACCAT              |
| HcPy2 PCR For^              | GACGCCATCTACCTTGAGGAG          |
| HcPy2PCR Rev^              | Biotin-CATAGGTGGATTGGTAGTT     |
| Nb B-t200 Fbio^             | Biotin- AGGTAGGTGTGGCCTATCAAAT|
| Nb B-t200 Rev^             | ATGTCCGGAAAACAGATCTGCAC        |
| Nb B-t200 Seq^             | TTAGTGTGCAATGCG                |
0.2 μM Nb B-t200 Rev, 4.5 mM MgCl₂, 25 μL 2 × buffer and made up to 50 μL using DNA/RNA-free water (NovaTaq™ Hot start master mix, Novagen). No template controls were included on each plate. Amplification was performed, following a 15 min 95 °C polymerase activation step, for 45 cycles at 94 °C for 30 s, 58 °C for 30 s and 72 °C for 30 s, followed by a final extension step at 72 °C for 10 min. The pyrosequencing P200/198 assay was conducted according to the manufacturer’s (Qiagen) protocols using a PyroMark ID instrument. In brief, following PCR amplification, 40 μL of the reaction was transferred into a 96-well plate and stored at 4 °C until the remaining PCR reaction (10 μL) was assessed by gel electrophoresis on 2% agarose gels stained with gel red (Biotium, California, USA). The plate was run on the Pyromark ID instrument if clear PCR amplification was observed.

Genotyping/allele frequency
Genotypes (TTC/TTC, TTC/TAC and TAC/TAC) and allele frequencies for resistant (R) and susceptible alleles (S) from each population are expressed as percentages [39]. Chi–square tests and Phi coefficient association of P200 genotypes were conducted using Minitab version 15.

Results

Faecal egg count reduction test analysis
All individual lambs were excreting N. battus eggs by day 14 pi. The FEC counts continued to rise in most animals until day 24 pi (Figure 1). Arithmetic mean FECs at day 24 pi were 265 (range 198 – 338 EPG) and 277 (range 216 – 360 EPG) EPG for the MNba2CON and MNba2FBZ groups, respectively. At post mortem the mean FEC of both groups remained similar, with 167 and 172 EPG for the FBZ treated and control groups, respectively, Table 2. No significant difference was observed between the groups.

CET
Arithmetic mean percentage establishment of N. battus in control lambs (MNba2CON) was 64% (arithmetic mean = 3850 worms), with the estimated total number of worms ranged between 2350 and 5750 (Figure 2) within the group. Arithmetic mean worm burden of the FBZ treated (MNba2FBZ) group was 3850 worms (range, 3050 – 4500 worms) and hence the treatment efficacy was 0%.

Molecular analysis

Sequencing results
A 785 bp fragment was amplified from the cDNA from the resistant (MNba2VCF) and susceptible (MNba1) isolates of N. battus. Sequences from MNba1 were subjected to a Blast search, with sequence homologies of 94% with T. circumcincta (Z69258) and 85% with C. oncophora (AY259994) isotype 1 sequences with hits also showing homology with, H. contortus, O. ostertagi and T. colubriformis isotype 1 β-tubulin sequences. Consensus sequence derived from the MNba2VCF isolate showed 87% homology to H. contortus and C. oncophora (AY259994) isotype 1 β-tubulin mRNA sequences. A smaller fragment of approximately 340 bp was amplified from genomic DNA. The consensus sequence from MNba1 gDNA blast search showed 86% and 85% homology with H. contortus (FJ981633) and T. circumcincta (Z69258) isotype 1 β-tubulin sequences respectively. Similar results were produced with MNba2FBZ consensus sequence.

Genotyping
Cross reactivity of the isotype 1 β-tubulin pyrosequencing assay was examined against other species, N. filicollis, T.
circumcincta, H. contortus and T. colubriformis. DNA from these species did not provide amplification using the Nb B-t 200 forward and reverse primers. The mean percentage genotypes (homozygous TTC/TTC, heterozygous TTC/TAC and homozygous TAC/TAC) for L3 and adult worms from the MNba1 and MNba2 populations are shown in Tables 3 and 4, respectively. For all samples tested, the MNba2 isolate was predominantly homozygous resistant genotype (TAC/TAC) at codon 200: for example, 56% of L3 (MNba2 VCF) were homozygous resistant in the population obtained from the on-farm FECRT and 83% of L3 (MNba2 CON) from the CET control population were homozygous resistant. In the case of the adult worms obtained from the CET study, 70% of all worms tested in the (MNba2 CON and MNba2 FBZ) populations were identified as homozygous resistant at codon 200.

Both L3 and adult worms derived from the MNba2 FBZ samples showed a slightly higher number of homozygous susceptible genotypes (TTC/TTC) compared to the MNba2 CON population; the Chi-square test showed that there was no significant difference (adults - $\chi^2 = 0.44$, df = 2, $p = 0.801$ and L3 - $\chi^2 = 1.025$, df = 2, $P = 0.599$) between the genotypes of these populations. The test showed a difference between the P200 genotypes identified, when worms derived from MNba2 VCF and MNba2 CON ($\chi^2 = 14.833$, df = 2, $p$-value = 0.001) were compared. No individuals from isolate MNba1 were homozygous (TAC/TAC) or heterozygous (TTC/TAC) resistant at codon 200. Only homozygous “susceptible” genotypes (GAA/GAA) were identified at codon198 for both adults and larval populations from all the N.battus isolates examined here.

The R allele frequency for 200Y ranged from 85% - 90% within the MNba2 L3 populations (MNba2 VCF, MNba2 CON and MNba2 FBZ; see Table 3). The R allele frequency was 83% and 80% for MNba2 CON and MNba2 FBZ adults, respectively (see Table 4).

**Discussion**

The results here provide confirmation, based on residual worm burden and faecal egg count analysis post benzimidazole treatment, of the preliminary results of the first reported case of BZ resistance in *N. battus* globally [27]. The zero percentage efficacy noted here, poses the question as to whether any members of the benzimidazole class of anthelmintics would be effective against

| Treatment | Faecal egg count (eggs per gram) | Percentage efficacy (%) |
|-----------|---------------------------------|-------------------------|
|           | Day 0                           | Day 7                   |
| Untreated | 287 (±39) [203–410]             | 173 (±55) [32–369]      |                            |
| Fenbendazole | 240 (±22) [162–293]          | 167 (±18) [117–221]      | 3 (0, 30)                 |

*Percentage efficacy with upper and lower confidence intervals calculated using four different methodologies 1 - [32]; 2 - [33]; 3 - [34]; 4 - [35].

![Figure 2 Individual estimated total worm burden of the two groups of lambs infected with 6000 Nematodirus battus infective larvae.](image)
this isolate. In this context, side resistance within the BZ class has been shown to occur in *T. colubriformis*, *H. contortus* [40,41] and undifferentiated *Nematodirus* species [18] and may be expected to occur with *N. battus*. Currently, the most practical method of determining anthelmintic sensitivity is the FECRT. There are a number of drawbacks to this test; it is time-consuming, requiring 10–14 days to complete [42], is relatively insensitive at low levels of resistance [43] and is potentially prone to misdiagnosis if the window of opportunity prior to reinfection is missed. With *Nematodirus*, in particular, the correlation between FEC and worm burden is particularly poor in treated [44] and untreated lambs [45,46]. *Nematodirus battus* historically was characterised as being fully developed by 14 days pi, but that maximum egg production did not occur until slightly later [47]. The results based on the isolate here suggest that significant FEC may be detectable in faeces on or before 14 days pi, even at moderate infection rates. The current findings are contrary to those found in New Zealand where in some cases of BZ resistance in *N. spathiger*, adult worms were recovered in animals that had zero FEC after BZ administration [48]. If the disparity between FEC and worm burden can occur with *N. battus* then it may explain the lack of detection of resistance in this parasite in the past and would have a bearing on detection of resistance in the field.

To implement sustainable control strategies it is essential to identify the presence of resistance at an early stage of development. In vitro detection of benzimidazole resistance in *Nematodirus* species using traditional methodologies such as the egg hatch test is complicated by the parasites development and hatching behavior as well as the egg shell morphology [16]. Therefore to better understand the acquisition and development of FBZ resistance in *N. battus* the authors here strove to identify whether any of the “commonly associated” point mutations of the β-tubulin isotype 1 gene were present in the FBZ resistant population. The results from MNba2 only yielded the F200Y SNP and found that there was no changes observed at E198A. The relationship between F200Y mutations in *N. battus* and actual anthelmintic efficacy in the field requires further examination, but numerous studies have shown that in other trichostrongylid species there is a high degree of correlation between phenotypic BZ characterisation and resistance allele frequencies of various life stages [49-52]. Researchers have found that *H. contortus* individuals which are homozygous resistant at codon 198 have a higher level of phenotypic resistance than those with SNP mutations at codon 200 [51]. Further work is required in *N. battus* to investigate other SNPs in the isotype 1 gene and also to characterize isotype 2 of the β-tubulin gene as it is possible that this is present in the populations here as indicated by amplification of some sequences with high identity to this isotype (data not shown).

The comparison of the genotyping results obtained from the MNba2 isolate over the course of the study, starting from the initial field isolate (MNba2^{VCF}) to the CET populations (MNba2^{CON} and MNba2^{FBZ}), showed that the two treatments with FBZ at the MRDR (one in the donor animal that produced the L₃ for the CET and one as part of the CET) only marginally led to a difference in the F200Y allele frequency (85, 90 and 87% respectively), which would indicate that this population is stable and highly selected. The observation of a small number of RS and SS genotypes in adult survivors following FBZ treatment, suggests either that the worms are phenotypically

| Population | Number of larvae examined | Relative proportion of codon 200 genotype (%) | Allele frequency (%) |
|------------|--------------------------|---------------------------------------------|----------------------|
| TAC/TAC    | 100                      | 0                                           | 100                  |
| TAC/TAC    | 34                       | 56                                          | 27                   |
| TAC/TAC    | 15                       | 83                                          | 10                   |
| TAC/TAC    | 20                       | 77                                          | 13                   |

| Population | Number of worms | Relative proportion of codon 200 genotype (%) | Allele frequency (%) |
|------------|-----------------|---------------------------------------------|----------------------|
| TAC/TAC    | 100             | 0                                           | 100                  |
| TAC/TAC    | 25              | 70                                          | 18                   |
| TAC/TAC    | 20              | 70                                          | 20                   |

Table 3 Beta-tubulin codon 200 genotyping results of the infective larvae of two *Nematodirus battus* isolates (MNba1 FBZ-sensitive and MNba2, FBZ-resistant phenotypes)

Table 4 Beta-tubulin codon 200 genotyping results from *Nematodirus battus* adult worms of two *Nematodirus battus* isolates (MNba1 FBZ-sensitive and MNba2, FBZ-resistant phenotypes)
sensitive but were able to “hide” from the lethal effects of treatment and then recover/resume activities as the local anthelmintic concentrations fall over time, or that there are other potential mechanisms of resistance involved in the BZ resistant phenotype. These findings are in agreement with other characterisation studies where analysis of the genotypes of first stage T.circumcincta larvae survivors from an in vitro egg hatch test, at concentrations up to 2 μg/mL thia bendazole, still had around 5% homozygous susceptible and 15% heterozygous genotypes [53].

As to why this isolate of N. battus developed FBZ resistance when previously the species has appeared to be susceptible to treatment is unclear and may be the result of a number of factors. It is possible that detection on this farm was aided by greater awareness of anthelmintic resistance and good co-operation between the farmer and his veterinarian. Benzimidazoles had been used on the farm to control N. battus since 2007 until resistance was diagnosed in 2010. Inadvertent under-dosing could have occurred as lamb weight was estimated and they did not normally calibrate their dosing equipment. Reduced bioavailability due to rapid gut flow may also have contributed to under-dosing [54,55] either from the clinical effects of N. battus or other concurrent causes of diarrhoea, such as coccidiosis. One of the major pathogenic effects of N. battus is attributable to disruption of the intestinal mucosa and the villous atrophy associated with larval stage development. The result of this atrophy is a reduced capability for fluid exchange, acute diarrhoea and increased gut flow [56]. Under dosing has been shown to allow heterozygote resistant individuals to survive treatment and contribute genes for resistance to the subsequent populations [57,58].

In some years benzimidazole treatments had been repeated at approximately monthly intervals in the young lambs on this farm dependent on the farmer and veterinary surgeons perception of risk of disease. Prophylactic treatments three weeks apart have been recommended in high risk areas to coincide with the predicted timing of peak hatch [56]. These high treatment frequencies place a considerable selection pressure on the population and could have, over time, resulted in the development of a resistant population.

As common on many sheep farms successive lamb crops grazed the same permanent pastures year on year. This in combination with frequent treatment administrations may have resulted in the slow increase in gene frequency for FBZ resistance within the population. There was no history of dose and movement of lambs onto new pastures. Previous studies have shown that rapid selection of BZ resistance in nematode species has occurred following dose and move treatment strategies [59-61], if lambs are grazed on pastures that had been previously been “seeded” with eggs of survivors of FBZ treatment, the possibility exist that resistance could be selected and propagated [60,62]. Changes in the parasite population brought about by changes in environmental/climatic conditions leading to changes in human behaviour, land use and/or animal husbandry [63-65] could be involved. Changes in climate have been shown to lead to conditions favourable for longer grazing periods [66]. The potential for longer parasite seasons will inevitably result in subsequent changes in treatment patterns associated with controlling infections leading to possible parasite adaptation.

However, no significant risk factor(s) can be identified on this farm with respect to anthelmintic resistance development in N. battus that would not be present on a large number of other sheep farms in the UK. It may be that within N. battus, the polymorphism associated with FBZ resistance conferred a fitness cost and until the introgression of resistance gene(s) within a population was sufficiently stable, the population was unable to thrive and propagate itself leading to a sporadic appearance on the farm. Previous works conducted on other nematode species, namely H. contortus and T. colubriformis, have observed poorer development and survival of eggs in anthelmintic resistant populations at a variety of temperatures [67,68].

An understanding of whether selection for BZ resistance within this population has occurred as a result of pre-adaptation, spontaneous mutation or gene flow will provide a better opportunity to develop effective sustainable strategies for control and now requires further investigation. This study highlights that for sustainable control of N. battus, farmers need to consider monitoring treatment efficacies, to minimize treatment frequency where possible and avoid indiscriminate use of anthelmintic compounds.

Competing interests
The authors declare that they have no competing interests.

Authors’ contributions
SM, RM and IR were instrumental in initial parasite detection and collection of material for analysis. DJB and AAM participated in the design and undertaking of the controlled efficacy test, performed the statistical analysis and drafted the manuscript. AAM and JBM conducted the molecular genetic studies and participated in the sequence alignment. All authors read and approved the final manuscript.

Acknowledgements
We gratefully acknowledge The Scottish Government’s Rural and Environment Science and Analytical Services Division (RESAS) for funding this work and our colleagues in the Bovine Services Division, Moredun Research Institute, for expert care and assistance with animals. Scanning surveillance as carried out by AHVLA is funded by the Department for Environment, Food & Rural Affairs (Defra).

Author details
1Moredun Research Institute, Pentlands Science Park, Bush Loan, Penicuik EH26 0PZ, UK. 2Animal Health and Veterinary Laboratories Agency, Cramarthen, Job’s Well Road, Johnstown, Cramarthen SA13 1EZ, UK. 3Animal Health and Veterinary Laboratories Agency, Penrith, Merynought, Calthwaite, Penrith, Cumbria CA11 9RB, UK. 4Care of Westmorland Veterinary Group, Riverside Business Park, Natland Road, Kendal LA9 7SX, UK.
References

1. Thomas RJ (1959) Field studies on the seasonal incidence of Nematodirus battus and N. filicollis in sheep. Parasitology 49:387–410
2. Kingsbury PA (1953) Nematodirus infestation: a probable cause of loss amongst lambs. Vet Rec 65:167–169
3. Thomas RJ, Stevens AJ (1956) Some observations on Nematodirus disease in Northumberland and Durham. Vet Rec 68:471–475
4. NOAA Compendium. [http://www.noahcompendium.co.uk/Compendium/Overview/217893.html]
5. Abbott KA, Taylor MA, Stubbings LA (2012) A technical manual for veterinary surgeons and advisors. Sustainable Control of Parasites in Sheep. 4th edition. http://www.scops.org.uk/vets-manual.html
6. Britt DP (1982) Benznidazole-resistant nematodes in Britain. Vet Rec 110:343–344
7. Cawthorne RJ, Whitehead JD (1983) Isolation of benzimidazole resistant strains of Ostertagia circumcincta from British sheep. Vet Rec 112:224–227
8. Scott DW, Duncan JL, McKellar QA, Coop RL, Jackson F, Mitchell GB (1991) Benzimidazole resistance in sheep nematodes. Vet Rec 128:618–619
9. Bartley DJ, Jackson E, Johnston K, Coop RL, Mitchell GGB, Sales J, et al. (2003) A survey of anthelminthic resistant nematode parasites in Scottish sheep flocks. Aust Parasitol 117:61–71
10. Vlassof A, Kettle PR (1988) Benzimidazole resistance in Haemonchus contortus. N Z Vet J 28:23–24
11. McKenna PB (1995) The identity of nematode genera involved in cases of ovine anthelmintic resistance in the southern north island of New Zealand. N Z Vet J 43:225–227
12. Waller PJ (1997) Anthelmintic resistance. Vet Parasitol 72:391–405
13. Howell SB, Burke JM, Miller JE, Terrill TH, Valencia E, Williams MI, et al. (2008) Prevalence of anthelmintic resistance on sheep and goat farms in the southeastern United States. J Am Vet Med Assoc 233:1913–1919
14. Wolstenholme AJ, Fairweather I, Prichard R, van Son-Matthijasma G, Sangster NC (2004) Drug resistance in veterinary helminths. Trends Parasitol 20:469–476
15. Middelberg A, McKenna PB (1983) Oxendazole resistance in Nematodirus battus. N Z Vet J 31:65–66
16. Obendorf DL, Parsons J, Nicholls J (1986) An egg development test for the evaluation of benzimidazole resistance in Nematodirus battus spathiger. Aust Vet J 63:382–383
17. Obendorf DL, Nicholls J, Koen T, Lacy E (1991) Benzimidazole resistant Nematodirus sp in Tasmania. Aust J 68:72–73
18. Martin PJ, Anderson N, Jaret RG (1985) Resistance to benzimidazole anthelmintics in field strains of Ostertagia and Nematodirus in sheep. Aust Vet J 63:238–43
19. Kwa MSG, Veenstra JG, Roos MH (1993) Molecular characterization of beta-tubulin genes present in benzimidazole resistant populations of Haemonchus contortus. Mol Biochem Parasitol 60:133–144
20. Kwa MSG, Kooyman FN, Boersena JH, Roos MH (1993) Effect of selection for benzimidazole resistance in Haemonchus contortus on beta-tubulin isotype-1 and isotype-2 genes. Biochem Biophys Res Commun 191:413–419
21. Kwa MS, Veenstra JG, Roos MH (1994) Benzimidazole resistance in Haemonchus contortus is correlated with a conserved mutation at amino acid 200 in beta-tubulin isotype 1. Mol Biochem Parasitol 63:299–303
22. Elard L, Humbert JF (1999) Importance of the mutation of amino acid 200 of the isotype 1 beta-tubulin gene in the benzimidazole resistance of the small rumen parasite Teladorsagia circumcincta. Parasitology Res 85:452–456
23. Silvestre A, Cabaret J (2002) Mutation in position 167 of isotype 1 beta-tubulin gene of trichostrongylid nematodes: role in benzimidazole resistance? Mol Biochem Parasitol 120:297–300
24. Prichard RK (2001) Genetic variability following selection of Haemonchus contortus with anthelmintics. Trends Parasitol 17:445–453
25. Ghisii K, Hengartner J, Naser F (2007) Phenotyping and genotyping of Haemonchus contortus isolates reveals a new putative candidate mutation for benzimidazole resistance in nematodes. Vet Parasitol 144:313–320
26. Silvestre A, Humbert JF (2002) Diversity of benzimidazole resistance alleles in populations of small ruminant parasites. Int J Parasitol 32:921–928
27. Mitchell S, Meams R, Richards I, Donnan AA, Bartley DJ (2011) Benzimidazole resistance in Nematodirus battus. Vet Rec 168:623–624
28. MAFF (1986) Ministry of Agriculture, Fisheries and Food, Manual of Veterinary Parasitological Laboratory Techniques, Reference Book 418. Her Majesty’s Stationary Office (HMSO), London
29. Christie M, Jackson F (1982) Specific identification of strongyle eggs in small samples of sheep faeces. Res Vet Sci 32:113–117
30. Jackson F (1974) New technique for obtaining nematode ova from sheep faeces. Lab Pract 23:655–66
31. Patterson DM, Jackson F, Huntley JF, Stevenson LM, Jones DG, Jackson E, et al. (1996) Studies on caprine responsiveness to nematodiasis: segregation of male goats into responders and non-responders. Int J Parasitol 26:187–194
32. Coles GC, Baurer C, Borgsteede FHM, Geerts S, Klei TR, Taylor MA, Waller PJ (1992) World association for the advancement of veterinary parasitology (WAAVP) methods for the detection of anthelmintic resistance in nematodes of veterinary importance. Vet Parasitol 44:35–44
33. Presidente PJA (1985) Methods for detection of resistance to anthelmintics. In: Anderson N, Waller PJ (ed) Resistance in Nematodes to Anthelmintic Drugs. CSIRO Division of Animal Health, and Australian Wool Corporation, Gebe, NSW, pp 13–27
34. Dash WM, Hall E, Barger IA (1988) The role of arithmetic and geometric mean worm egg counts in faecal egg count reduction tests and in monitoring strategic druching programs in sheep. Aust Vet J 65:66–68
35. McKenna PB (1990) The detection of anthelmintic resistance by the fecal egg count reduction test - an examination of some of the factors affecting performance and interpretation. N Z Vet J 38:142–147
36. Cabaret J (2014) Reliable Phenotypic Evaluation of Anthelmintic Resistance in Herbivores: how and When Should They Be Done? In: Quick W (ed) Anthelmintics, Clinical Pharmacology, Uses in Veterinary Medicine and Efficacy. Nova science publisher, Nova, New York, pp 1–26
37. Roos MH, Otten M, Hoekstra R, Veenstra JG, Lenstra JA (2004) Genetic analysis of introbreeding of two strains of the parasitic nematode Haemonchus contortus. Int J Parasitol 34:109–113
38. von Samson-Himmelstjerna G, Walsh TK, Donnan AA, Carriere S, Jackson F, Stuce PJ, et al. (2009) Molecular detection of benzimidazole resistance in Haemonchus contortus using real-time PCR and pyrosequencing. Parasitology 136:349–358
39. Falconer DS, MacKay TFC (1996) Introduction to quantitative genetics, 4th edition. Pearson Education Ltd, Harlow
40. Hall CA, Kelly JD, Campbell NJ, Whitlock HV, Martin IC (1978) The dose response of several benzimidazole anthelmintics against resistant strains of Haemonchus contortus and Trichostrongylus colubriformis selected with thiabendazole. Res Vet Sci 25:364–367
41. Hall CA, Campbell NJ, Richardson NJ (1978) Levels of benzimidazole resistance in Haemonchus contortus and Trichostrongylus colubriformis recorded from an egg hatch test procedure. Res Vet Sci 25:360–363
42. Coles GC, Jackson F, Pomroy WE, Prichard RK, van Son-Matthijasma G, Silvestre A, et al. (2006) The detection of anthelmintic resistance in nematodes of veterinary importance. Vet Parasitol 136:167–185
43. Martin PJ, Anderson N, Jaret RG (1989) Detecting benzimidazole resistance with fecal egg count reduction tests and in vitro assays. Aust Vet J 66:236–240
44. Black WJM (1964) The development of a preventative routine against Nematodirus disease of lambs I. Administration of bephenium compounds at 21-day intervals. Br Vet J 130:301
45. Kingsbury PA (1965) Relationship between egg counts and worm burdens of young sheep. Vet Rec 77:900–901
46. McKenna PB (1981) The diagnosis value and interpretation of faecal egg counts in sheep. N Z Vet J 29:129–132
47. Thomas RJ (1959) A comparative study of the life histories of Nematodirus battus and N. filicollis, nematode parasites of sheep. Parasitology 49:374–386
48. Chalmers K (1985) Detection of benzimidazole resistant Nematodirus spathiger. N Z Vet J 33:53
49. von Samson-Himmelstjerna G, von Wittendorff C, Sievers G, Schneider T (2002) Comparative use of faecal egg count reduction test, egg hatch assay and beta-tubulin codon 200 genotyping in small strongyles (cyathostominae) before and after benzimidazole treatment. Vet Parasitol 108:227–235
50. Droegemuller M, Failing K, Schneider T, von Samson-Himmelstjerna G (2004) Effect of repeated benzimidazole treatments with increasing dosages on the phenotype of resistance and the beta-tubulin codon 200 genotype distribution in a benzimidazole resistant cyathostomin population. Vet Parasitol 123:201–213
51. Kotze AC, Cowling K, Bagnall NH, Hines BM, Ruffell AP, Hunt PW, et al. (2012) Relative level of thiabendazole resistance associated with the E198A and F200Y SNPs in larvae of a multi-drug resistant isolate of *Haemonchus contortus*. Int J Parasitol Drugs Drug Resist 2:92–97

52. Cudekova P, Varady M, Dolinska M, Konigova A (2010) Phenotypic and genotypic characterisation of benzimidazole susceptible and resistant isolates of *Haemonchus contortus*. Vet Parasitol 172:155–159

53. Stenhouse LJ (2007) Characterisation of Anthelmintic Resistance in a Multiple Drug Resistant Teladorsagia circumcincta Isolate. PhD Thesis. University of Glasgow, Glasgow

54. Sargison ND (2012) Pharmaceutical treatments of gastrointestinal nematode infections of sheep—future of anthelmintic drugs. Vet Parasitol 189:79–84

55. Lanusse CE, Prichard RK (1998) Relationship between pharmacological properties and clinical efficacy of ruminant anthelmintics. Vet Parasitol 49:123–158

56. Taylor MA, Coop RL, Wall RL. (2007) Veterinary Parasitology, 3rd edition. Blackwell Publishers, Oxford

57. Coles GC, Roush RT (1992) Slowing the spread of anthelmintic resistant nematodes of sheep and goats in the United Kingdom. Vet Rec 130:505–510

58. Roush RT, McKenzie JA (1987) Ecological genetics of insecticide and acaricide resistance. Annu Rev Entomol 32:361–380

59. Waghorn TS, Miller CM, Oliver AM, Leathwick DM (2009) Drench and shift is a high-risk practice in the absence of refugia. N Z Vet J 57:359–363

60. Martin PJ, Le Jambre LF, Claxton JH (1981) The impact of refugia on the development of thiabendazole resistance in *Haemonchus contortus*. Int J Parasitol 11:35–41

61. Martin PJ (1989) Selection for thiabendazole resistance in *Ostertagia* spp. by low efficiency anthelmintic treatment. Int J Parasitol 19:317–325

62. Van Wyk JA (2001) Refugia – overlooked as perhaps the most potent factor concerning the development of anthelmintic resistance. Onderstepoort J Vet Res 68:55–67

63. van Dijk J, Morgan ER (2012) The influence of water and humidity on the hatching of *Nematodirus battus* eggs. J Helminthol 86:287–292

64. van Dijk J, Morgan ER (2010) Variation in the hatching behaviour of *Nematodirus battus*: polymorphic bet hedging? Int J Parasitol 40:675–681

65. van Dijk J, Morgan ER (2008) The influence of temperature on the development, hatching and survival of *Nematodirus battus* larvae. Parasitology 135:269–283

66. Barnett C, Hossell J, Perry M, Procter C, Hughes G (2006) A handbook of Climate Trends Across Scotland. Scotland & Northern Ireland Forum for Environmental Research, Scotland, Northern Ireland

67. Scott EW, Baxter P, Armour J (1991) Fecundity of anthelmintic resistant adult *Haemonchus contortus* after exposure to ivermectin or benzimidazoles in vivo. Res Vet Sci 50:247–249

68. Echevarria FA, Armour J, Bairden K, Duncan JL. (1993) Laboratory selection for ivermectin resistance in *Haemonchus contortus*. Vet Parasitol 49:265–270