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Development of a three-colour digital PCR for early and quantitative detection of benzimidazole resistance-associated single nucleotide polymorphisms in *Haemonchus contortus*

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ABSTRACT

Haemonchus contortus is the most pathogenic nematode in small ruminants and anthelmintic resistance (AR) hampers its efficient control. Early detection of AR status is required to reduce selection for AR and cannot be achieved using phenotypic tests. For benzimidazoles (BZs), the detection of AR-associated alleles characterised by single nucleotide polymorphisms (SNPs) in the isotype 1 β-tubulin gene allows early AR detection in strongyles. The F200Y, F167Y, E198A and E198L polymorphisms have been described in BZ-resistant populations with a clear variation in frequencies between regions. A novel digital PCR (dPCR) enables the detection of all of the above-described polymorphisms in *H. contortus*. Assays were validated using synthetic DNA fragments containing these SNPs. Then, larvae obtained and pooled at farm level from 26 Austrian and 10 Italian sheep farms were analysed. For all assays a detection limit of 15 copies/µl of resistance alleles and a high level of accuracy were demonstrated, allowing to detect allele frequencies of 1% in most samples. In Austrian samples, elevated frequencies of F200Y resistance alleles were detected on all farms. Polymorphisms in codon 167 and codon 198 were identified in *H. contortus* from Austria for the first time. In Italian samples, the frequency of resistance alleles was still comparatively low, but F200Y resistance alleles were traceable. In conclusion we developed for the first time dPCR assays that target all SNPs of relevance associated with BZ-resistance in *H. contortus*. Future research on AR development could benefit from an early onset of SNP-based surveillance that would include the developed assays for all SNPs of relevance. Improved surveillance in the long term will include other important, though less pathogenic, nematode genera in the analyses.

1. Background

Trichostrongyloid nematodes threaten the health and productivity of small ruminants worldwide (Roeber et al., 2013; Mavrot et al., 2015; Charlier et al., 2020). The successful control of trichostrongyloid infections is severely compromised by the development of anthelmintic resistance (AR) in populations of these gastrointestinal nematodes (Kaplan and Vidyashankar, 2012; Rose Vineer et al., 2020). In particular, *Haemonchus contortus*, a highly pathogenic species parasitising the abomasum, has the ability to develop AR rapidly. This is of great concern in relation to farm animal health and productivity, as infections with *H. contortus* frequently result in potentially fatal disease with severe anaemia, especially in young animals (Roeber et al., 2013; Besier et al., 2016). A recent meta-analysis demonstrated the worrying extent of the spread of AR in Europe and also highlighted a considerable lack of data on the prevalence of AR (Rose Vineer et al., 2020). Amongst the strategies deployed to slow down AR selection, monitoring the occurrence of AR is emphasised as an essential element (Taylor, 2012; Kotze et al., 2020; Rose Vineer et al., 2020). Early detection of AR is important to understand and control the spread of drug resistance (Kotze et al., 2020).

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The most widely used test for AR detection is the fecal egg count reduction test (FECRT), but considerable effort is needed to generate valid data (Morgan et al., 2022). In addition, the FECRT does not detect low levels of resistance (<25% resistant worms in the population), and thus does not discover AR before it is already established in a population (Martin et al., 1989).

For the anthelmintic group of the benzimidazoles (BZ) certain polymorphisms in the isotype 1 β-tubulin gene are associated with a BZ-resistant phenotype (von Samson-Himmelstjerna et al., 2009; Kotze et al., 2014). In *H. contortus and* also in other trichostrongyloids of veterinary importance, single nucleotide polymorphisms (SNPs) in codons 200, 167 and 198 are associated with AR (Gilleard and Redman, 2016; Charlier et al., 2022). For that a mutation is classified as a SNP, the variant must be found in a least 1% of the population (Johnston et al., 2019). The first described and most common SNP is found in codon 200 and results in an amino acid substitution from phenylalanine to tyrosine (TTC>TAC - F200Y) (Kwa et al., 1995; von Samson-Himmelstjerna et al., 2007). Other resistance-associated SNPs at codons 167 and 198 are widespread in *H. contortus* in certain geographical regions, while they are absent or have only been reported sporadically in other regions (Silvestre and Cabaret, 2002; Chaudhry et al., 2015; Redman et al., 2015; Ramünke et al., 2016; Zhang et al., 2016). For *H. contortus*, it has been hypothesised that the F200Y mutation has multiple independent origins, while the mutations at other codons might have evolved from a few origins and were then spread through animal movements (Chaudhry et al., 2015). For example, the F167Y (TTC>TAC) polymorphism was prevalent in *H. contortus* on some farms in the United Kingdom, while it had little or no spread in other regions of Europe (Sweden, Switzerland and Italy), India, the United States and China (Chaudhry et al., 2015, 2020; Redman et al., 2015; Ramünke et al., 2016; Zhang et al., 2016; Baltrušis et al., 2018). Similarly, the E198A (GAA>GCA) polymorphism of *H. contortus* was most dominant in China and also frequent in India, while it was detected only sporadically in other studies (Chaudhry et al., 2020; Zhang et al., 2016). The E198A polymorphism is not the only one which is relevant at codon 198 (Ghisi et al., 2007). In gDNA of *Haemonchus* derived from a Brazilian farm an E198L polymorphism (GAA>TTA) was detected (Baltrušis et al., 2018). This E198L SNP was the predominant genotype detected in BZ-resistant *H. contortus* in Sudan, where also other polymorphisms have been observed (Mohammedsalih et al., 2020). Since the importance of different SNPs varies by region and farm, the inclusion of all SNPs was advised when monitoring AR using molecular means (Avramenko et al., 2020; Kotze et al., 2020).

Different methods have been applied for SNP detection, such as real-time PCR, pyrosequencing, isothermal amplification, deep amplicon sequencing and digital PCR (dPCR) (von Samson-Himmelstjerna et al., 2009; Baltrušis et al., 2018, 2020; Tuersong et al., 2020; Charlier et al., 2022). The latter technique for absolute quantification of target nucleic acids is based on the principle of template partitioning. The avoidance of sequence interaction facilitates the detection of mutations occurring at a frequency of often less than 1% (Miotke et al., 2014). Recently, dPCR assays were successfully developed for the detection of resistance-associated SNPs at codon 200 and 167 of *H. contortus* isotype-1 β-tubulin, with a detection limit of 2.6% of the resistance alleles (Baltrušis et al., 2018, 2020). For dPCR the sample is divided in several partitiones. Most dPCR systems currently applied in veterinary parasitology are droplet digital PCR systems (ddPCR) where the reaction is conducted in water droplets separated by a continuous oil phase (Baltrušis and Höglund, 2023). A second approach is the use of a chip where the droplets are physically isolated (Madic et al., 2016).

Based on field studies, the prevalence of BZ resistance at farm level in Austria was recently estimated to be high (around 85%), while in Italy it was estimated to be low (around 11%) (Rose Vineer et al., 2020). Studies investigating the occurrence of BZ-associated resistance alleles by pyrosequencing are in agreement with these observations. A very high frequency of BZ-resistance alleles in codon 200 of the isotype-1 β-tubulin gene of *Haemonchus* spp. (87%–100%) was determined in all of the farms tested in Austria with alpine transhumance management systems, while no E198A polymorphisms were detected (Hinney et al., 2020). The F167Y polymorphism was not investigated in that study. By contrast, resistance alleles in codon 200 were detected on 44% of the farms in south-western Italy with frequencies ranging from 18 to 73%, while no F167Y or E198A polymorphisms were detected (Ramünke et al., 2016).

The aim of this study was to develop and validate assays for the detection of the predominant SNPs in *H. contortus* associated with BZ resistance (F167Y, E198A, F200Y) applying three-colour dPCR with chip-technology, with a detection limit of 1% of allele frequencies. For the first time a dPCR for codon 198 was designed. For this purpose, a multiplex discrimination assay was developed that was able to detect two SNPs in one assay including the E198L polymorphism that had rarely been investigated in European *H. contortus* samples until now (Avramenko et al., 2020). These assays were further used to examine field samples from Austria and Italy to confirm the highly frequent F200Y SNP in Austria and to analyse the relevance of the other SNPs which may be present at low frequencies in both countries.

2. Materials and methods

2.1. Design and setting of the study

Three assays for three-colour dPCR targeting the polymorphisms F167Y (TTC>TAC), E198A (GAA>GCA), E198L (GAA>TTA) and F200Y (TTC>TAC) in the β-tubulin gene of *H. contortus* were developed (chapter 2.2) and validated (chapter 2.3.). The assays were then applied for the analysis of field samples from Austrian and Italian farms (chapter 2.4.).

2.2. Three-colour dPCR assay development

For the development of the dPCR assays the following steps were necessary. First, primers that amplified the target regions as well as probes that bound to the target of interest (SNP or wildtype) and were labeled with different fluorescent dyes had to be designed (subchapter 2.2.1). Second, control samples to validate the assays had to be obtained (subchapter 2.2.2 and 2.2.3) Third, the dPCR master mix was prepared and run on the naica® Prism3 crystal digital PCR system (cdPCR™) (Stilla Technologies, Villejuif, France) (subchapter 2.2.4).

2.2.1. Primer and probe designs

dPCR assays to detect the target SNPs were designed by the Application Support of Integrated DNA Technologies (Leuven, Belgium). A wildtype (WT) sequence of the isotype-1 β-tubulin gene served as a reference. It was derived from a susceptible strain of *H. contortus* that was obtained from Freie Universität Berlin (identical to GenBank: LS997562.1). Locked nucleic acid (LNA)hydrolysis probes (Affinity Plus® Table 1) were designed against the four target SNPs (F167Y (codon TAC), E198A (GCA) E198L (TTA) and F200Y (TAC)). Putative interaction between oligonucleotides and off-target amplification were evaluated using the OligoAnalyzer™ tool (Integrated DNA Technologies; and the Basic Local Alignment Search Tool (BLAST) from NCBI (Altschul et al., 1990). Special care was given that the oligonucleotide binding sites of the novel codon-200 assay were not affected by polymorphisms at the nearby codon 198 (Fig. 1).

The primers and probes were synthesized at Microsynth (Balgach, Switzerland) and Integrated DNA Technologies, respectively.

2.2.2. Synthesizing and callibration of positive controls

The target sequences, the WT and the four SNP alleles (Table 2), were synthesized as double-stranded DNA fragments (gBlocks™ Gene Fragments Integrated DNA Technologies) and used as positive controls. Additional DNA fragments of *T. circumcincta* and *T. colubriformis* were designed for the validation of species specificity.

Table 1
Oligonucleotides of digital PCR assays targeting ß-tubulin and F167Y, E198A, E198L and F200Y mutant alleles.

Primer/probe	Mutation	Sequence (5′- 3′)
167_fwd_v3		F: **G**GA GGC ACT GGA TCT GGA AT
167_rev_v3		R: **CAC** ACG ATC TCA CCT TGG GT
198_fwd_v3		F: CCG ACA CTG TCG TGG AAC C
198_rev_v3		R: **AA**G CAG ATA TCA TAC AGA GCT TCG
200_fwd_v3		F: **T**CC ATC AAT TGG TAG AGA ACA CC
200_rev_v3		R: **G**TG GTT GAG ATC TCC ATA GGT T**G**
167 WT		P: YAK/TCG+T+T+CT+CCG+TTG/IBFQ
167 MUT	F167Y	P: 6-FAM/TCG+T+A+CT+CCG+TTG/IBFQ
198 WT – GAA		P: 6-FAM/CA+CCG+AT+G+A+AA+CA/IBFQ
198 MUT1 – GCA	E198A	P: YAK/CA+CCGAT+G+C+AAC+AT/IBFQ
198 MUT2 - TTA	E198L	P: Cy5/CA+C+CGAT+T+T+AA+CAT/IBFQ
200 WT		P: Cy5/ACA+T+T+CTGTA+TT+GA+CA/IBFQ
200 MUT	F200Y	P: 6-FAM/ACA+T+A+CTGTA+TT+GA+CA/IBFQ

F: forward primer, R-reverse primer, P: hydrolysis probe.
5′ dyes: fluorescein (6-FAM), Yakima Yellow® (YAK) and Cyanine 5 (Cy5™); 3′ non-fluorescent quencher: Iowa Black™ FQ quencher (IBFQ).
"+" sign: precedes the locked nucleic acid (LNA®) monomer incorporated in the sequence of the PrimeTime LNA® qPCR Probe to enhance stability and nuclease resistance.

To determine the input volume of DNA and to adjust for the dynamic range of dPCRs, experimental samples were first subjected to quantification by qPCR. A 15 µl reaction consisted of 1 × PCR buffer B2 (Solis BioDyne, Tartu, Estonia), 3.5 mM $MgCl_2$, 0.2 mM of each dNTP, 0.625 µM EvaGreen dye (Biotium, Fremont, CA, USA), 250 nM of each primer, 1 U HOT FIREPol® DNA Polymerase (Solis BioDyne) and 2 µl template DNA. After activation of the hot-start polymerase at 95 °C for 15 min, amplification was performed for 44 cycles (95 °C for 15 s, 60 °C for 1 min) followed by dissociation analysis from 60 °C to 95 °C using a ramp rate of 5 °C/s.

The DNA concentration was considered appropriate when yielding a quantification cycle (*Cq*) between 19.6 and 33.0. The *Cq* value determined by qPCR was used as an estimate for dPCR input volume (*Cq* of 19.6–20.4: 1 µl template; 20.5 to 20.9: 2 µl, 21 to 22.5: 4 µl, 21.6 to 23: 6 µl, and 23.1 to 33: 8 µl). More concentrated samples were diluted appropriately.

2.2.3. Biological material for assay validation

For the validation of the assays, third-stage larvae (L3) of *H. contortus*, *Trichostrongylus colubriformis* and *Teladorsagia circumcincta* were provided by Boehringer Ingelheim Vetmedica GmbH (Rohrdorf, Germany). All isolates were maintained in the laboratory without exposure to anthelmintics.

Genomic DNA was extracted with the NucleoSpin® Soil Kit (Macherey-Nagel, Düren, Germany) according to the manufacturer's instructions using the lysis buffer SL1 without the addition of Enhancer SX. Genomic DNA was eluted with 30–100 µl of Elution Buffer SE and stored at −20 °C until use.

2.2.4. Application of the dPCR

The naica® system served as an easy-to-use dPCR platform (Stilla Technologies (Madic et al., 2016)). Briefly, this system follows four steps: 1. The prepared Mastermix+sample reactions are pipetted onto a microfluidic chip that each contains four reaction chambers; 2. In a pressure cell (Geode instrument), up to 30,000 droplets are generated and distributed in a twodimensional layer in the reaction chamber, followed by thermocycling 3. The chip is read out in a specific imaging devise (naica® Prism3 fluorescence reader) 4. Data are then analysed with a specific software (Crystal MinerTM software version 2.4, Stilla Technologies).

The 25 µl dPCR consisted of 1 × PerfeCTa® Multiplex qPCR ToughMix® (Quanta Biosciences, Beverley, MA, USA), 100 nM fluorescein, 800 nM of each primer, 250 nM of each probe and 1–8 µl template. The mixture was loaded onto a Sapphire® chip (Stilla Technologies) generating up to 30,000 droplets per sample. The chip was placed in the Geode instrument for reaction partitioning and amplification. Following initial denaturation for 3 min at 95 °C, amplification was performed over 45 cycles (95 °C for 30 s and 60 °C for 30 s). Endpoint three-color multiplex fluorescence imaging was obtained using the naica® Prism3 fluorescence reader. In case of overlapping spectral characteristics of the dyes in use, fluorescence spillover can arise when signals from one channel shine into neighbouring channels and this needs to be compensated for. Spillover compensation parameters were assessed using gBlock target sequences in a forerun with software settings to mono-colour control and were then applied in Crystal Miner™ software during any data processing.

2.3. Assay validation

Sensitivity was measured using genomic DNA of *H. contortus* and synthetic double-stranded DNA (gBlocks®) covering the described WT

Table 2
Synthetic positive controls (gBlocks®) for assay validation; changes were introduced in the WT sequence (GenBank® accession number: LS997562.1

gBlock®	Length (bp)	Species	Codon 167	Codon 198	Codon 200
1	497	*H. contortus*	TTC (s)	GAA (s)	TAC (r)
2	497	*H. contortus*	TAC (r)	GAA (s)	GAA (s)
3	374	*H. contortus*	TTC (s)	GCA (r)	GAA (s)
4	374	*H. contortus*	TTC (s)	TTA (r)	GAA (s)
5	357	*T. circumcincta*	TAC (r)	GAA (r)	TAC (r)
6	357	*T. circumcincta*	TTC (s)	GCA (s)	TTC (s)
7	357	*T. circumcincta*	TTC (s)	TTA (r)	TTC (s)
8	514	*T. colubriformis*	TAC (r)	GAA (r)	TAC (r)
9	514	*T. colubriformis*	TTC (s)	GCA (s)	TTC (s)

s: susceptible, r: resistant.

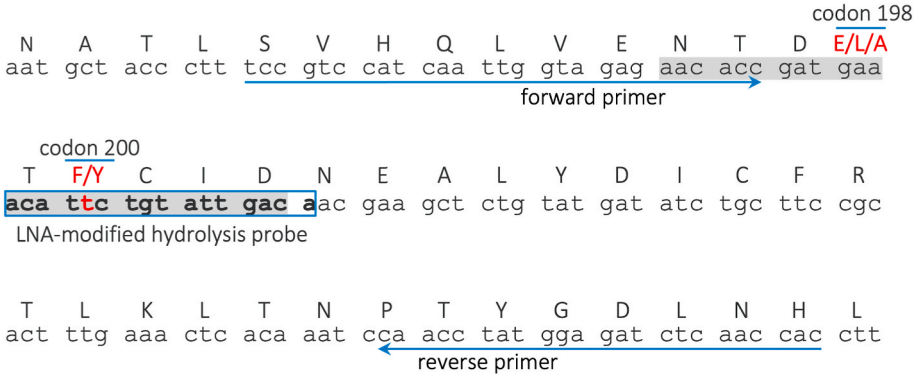

Fig. 1. Oligonucleotide binding sites of the novel codon-200 assay are not affected by polymorphisms at the nearby codon 198. In contrast, the allele-specific probes designed by a former assay against codon-200 mutations (Baltrušis et al., 2018) would be affected by a mutated codon 198 (shaded sequence). The location of the two LNA-modified hydrolysis probes targeting the two codon-200 alleles of the *Haemonchus contortus* β-tubulin gene is depicted by the box. Oligonucleotide sequences are depicted by arrows (primers) or a box (allele-specific probes). Nucleotide or amino-acid substitution(s) at codons 198 and 200 are highlighted in red.

and polymorphic sequences.

For assay evaluation, serial dilutions of all fragments containing SNPs and WT sequences were prepared using the synthetic positive controls. Thus, two serial dilutions (one for the WT and one for the mutation) were prepared for the assays investigating codons 200 and 167, whereas three serial dilutions were prepared for the multiplex assays against the codon-198 mutations E198A and E198L. For the serial dilutions of mutant variants and wildtype sequences at codons 200 and 167, the corresponding WT/mutant was added as a constant background.

To evaluate the codon-198 assay, the WT was used as a constant background for the serial dilutions of the two mutations, while controls without target DNA of the respective other mutant were added (no-template-control (NTC); see Fig. 2). For the serial dilutions of the WT, both mutants were used as a constant background.

Exact copy number of stock concentration was determined by measuring the concentration in the optimal range of the dPCR. This standard-free absolute quantification method without the need of a reference is the gold standard for copy number evaluation and more precise than qPCR or spectro-/fluoro-photometric methods. At the level of 3000 copies/µl, the optimal range for the most precise measurement is reached. For the serial dilutions, the synthetic positive control was added with a final concentration of approximately 3000 copies/µl in a reaction volume of 25 µl (~75,000 copies/reaction = stock concentration) and four ten-fold serial dilutions from this stock concentration (approximately 300, 30, 3, and 0.3 copies/µl corresponding to 7,500, 750, 75 and 7.5 copies/reaction) and with triple-diluted stock concentration (approximately 1000 copies/µl or 25,000 copies/reaction) with the same serial dilution (approximately 10, 1, 0.1 copies/µl or 250, 25, 2.5 copies/reaction) (Supplementary files 1 and 2). The dilutions containing less than 30 copies/µl were tested in triplicate and higher concentrations in duplicate, thus allowing to test for repeatability. The synthetic positive control of the corresponding WT/mutant for background was used at a constant level of approximately 3000 copies/µl.

The false-positive estimate was determined for each WT/mutant combination by using only a single mono-colour control of the corresponding mutant/WT at approximately 3000 copies/µl in six replicates. Species specificity was also assessed using genomic DNA and synthetic positive controls of *T. colubriformis* and *T. circumcincta*.

To estimate repeatability, the proportion of resistance alleles of the replicates from the test validation were compared. Furthermore, 29 field samples collected at Austrian farms from study 1 were measured in duplicates for all assays.

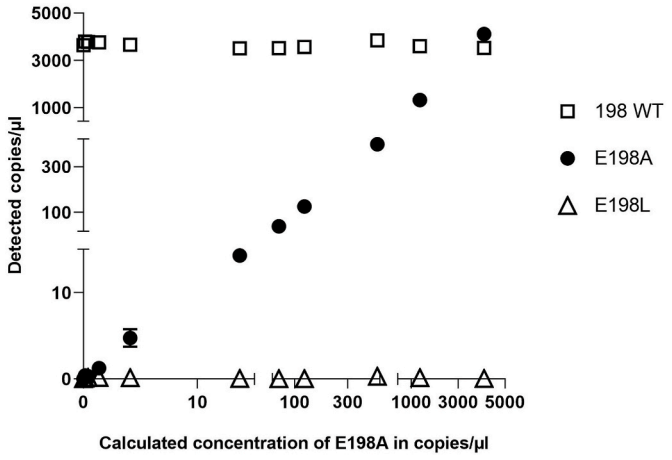

Fig. 2. Assay validation with the E198A-assay as an example. The E198A-SNP was applied in serial dilutions while a no-template-control was used for the E198L SNP for each measurement. The wildtype of codon 198 was applied with around 3000 copies/µl as a constant background. For clarity, axes breaks are inserted with different scales of the three segments.

2.4. Field samples

For the analysis of field samples, pooled L3 were subjected to DNA extraction as described in 2.3. The samples were derived from 26 Austrian (Tyrol, study 1, 11 farms; Salzburg, study 2, 15 farms) and 10 Italian sheep and one Austrian goat farm. On these farms FECRTs with different compounds (BZ, macrocyclic lactones (ML), monepantel; Tables 3–5) had been conducted (published in (Bosco et al., 2020; Untersweg et al., 2021; Hinney et al., 2022)). All available samples before treatment ($n = 47$) and after treatment ($n = 26$) were included. Data on the proportion of *Haemonchus* spp. was not available for some samples from Austria. The mean frequencies of *Haemonchus* spp. before treatment in the pooled samples were 70% (range: 25–97%) and 36% (range: 3–87%) in studies 1 and 2, respectively (Tables 3 and 4) (Untersweg et al., 2021). Larvae counts were not available for the Austrian samples but were available for all Italian samples. The proportions of *Haemonchus* spp. from the Italian samples are shown in Table 5 (Bosco et al., 2020). On the goat farm, the proportion of *Haemonchus* spp. was 79% (Hinney et al., 2022).

Field samples were analysed using the three assays as described above. As no larvae counts were available, *Cq* values were determined for the Austrian samples. For the Italian samples, the amount of template input for dPCR (Supplementary file 4) was determined by quantification of the DNA concentration on a NanoDrop spectrophotometer (Thermo Fisher Scientific, Waltham, MA USA). Samples were analysed once except for the Austrian samples collected during study 1, which were measured in duplicate.

2.5. Statistical analyses

Confidence intervals and relative uncertainties were calculated using Crystal Miner™ software. The relative uncertainty is a ratio of the uncertainty to the measured quantity and expressed in percent (Supplementary file 1 H).

To evaluate repeatability, the mean and maximum deviation of variant allele fraction (VAF) between replicates were calculated.

For the calculation of resistance-allele frequencies the VAF-calculator provided by Stilla Technologies was used.

IBM® SPSS® version 28.0.1.0 was used for the analyses described below. For the validation of the assays, the number of copies to be added to the assay and the number of copies detected between zero and the first (threefold) dilution of the stock (corresponding to a calculated number of 27,620 to 34,275 copies/reaction) were first analysed by a scatter plot to identify extreme outliers. To analyse linear correlation, the Pearson correlation coefficient (r) was calculated for all assays. A two-sided *p*-value of less than 0.05 was considered statistically significant.

For the alteration of SNP frequency resulting from treatment, a paired Wilcoxon rank-sum test was performed for all samples for which data were available before and after treatment. A *p*-value below 0.05 was considered significant. This was calculated for each anthelmintic and each study separately, but also for all studies combined.

3. Results

3.1. Assay design and validation

The project aimed to develop assays to detect F167Y (TTC>TAC), E198A (GAA>GCA), E198L (GAA>TTA) and F200Y (TTC>TAC) in the β-tubulin gene of *H. contortus* at an allele frequency of ≥1% using dPCR with chip-technology and to validate these assays.

A high level of agreement between the detected and calculated copies of SNPs was observed for all assays (Supplementary Files 1 and 2). No outliers were evident in the scatter plots (Supplementary File 3). Pearson correlation coefficient (r) values showed strong significant correlations (range: 0.998 to 1.000), indicating a high accuracy of the dPCR assays (Supplementary File 3).

Table 3
Single nucleotide polymorphism frequencies for *Haemonchus contortus*, Austria Study 1 (Untersweg et al., 2021) (BT = before treatment; PT = post treatment; <1% = below background threshold, *adjusted detection limit due to low background; excl. = excluded as not enough copies of the targeted *Haemonchus*-DNA were amplified; ND = no data [not analysed or no material available]; bold numbers = resistance alleles detected), R = resistant; SR = suspected resistance; S = susceptible, 95% confidence intervals and relative uncertainty are shown in the supplementary file 4.

Farm	Drug	% of *Haemonchus* larvae BT/PT	% F200Y BT/PT	% F167Y BT/PT	% E198A BT/PT	% egg count reduction (95% CI)
1	FBZ	92/88	**90/93**	excl./**1**	**6/3**	95 (87–99) SR
2	MOX	88/ND	**83**/ND	**4**/ND	**12**/ND	98 (87–100) SR
3	MOX	36/86	**92**/excl.	**1**/excl.	**3**/excl.	79 (53–97) R
4a	FBZ	94/90	**93/81**	**2**/excl.	**4/14**	only one animal treated; 30% reduction
4b	MOX	94/99	**93/94**	**2**/excl.	**4**/excl.	96 (87–99) SR
5	FBZ	ND/ND	**96/96**	<1/excl.	**4/3**	96 (92–98) S only 9 animals treated
6	MOX	97/ND	**97/94**	**1**/excl.	**2/3**	97 (87–100) SR only 9 animals treated
7	MOX	25/100	**95/94**	**1/1**	**2/5**	99 (97–100) S
8	no treatment	ND/ND	**97**/ND	**1**/ND	**2**/ND	not done
9	MOX	ND/ND	**89**/ND	excl./ND	**3**/ND	100 (99–100) S
10	MOX	60/ND	**96/97**	<6*/**1**	excl./**1**	98 (92–100) S
12a	FBZ	47/99	**96/96**	excl./excl.	**3**/excl.	48 (23–66) R
12b	MOX	47/99	**96/95**	excl./<4*	**3/5**	84 (61–96) R

Table 4
Single nucleotide polymorphism frequencies for *Haemonchus contortus*, Austria Study 2 (Untersweg et al., 2021) (BT = before treatment; PT = post treatment; <1% = below background threshold; *adjusted detection limit due to low background; excl. = excluded as not enough copies of the targeted Haemonchus-DNA were amplified; ND = no data [not analysed or no material available]; bold numbers = resistance alleles detected), R = resistant; SR = suspected resistance; S = susceptible, 95% confidence intervals and relative uncertainty are shown in the supplementary file 4.

Farm	Drug	% of *Haemonchus* larvae BT/PT	% F200Y BT/PT	% F167Y BT/PT	% E198A BT/PT	% egg count reduction
14	BZ	3/ND	excl./ND	excl./ND	excl./ND	69 (35–98) R only 5 animals treated
15		16/5	**98/99**	<4*/excl.	<3*/<6*	57 (30–90) R only 9 animals treated
17		ND/ND	excl./excl.	<1/excl.	excl./excl.	52 (25–87) R only 6 animals treated
18		22/56	**80/60**	**1**/excl.	**15**/excl.	89 (83–94) R
19		ND/ND	ND/excl.	ND/excl.	ND/excl.	61 (40–77) R
27		ND/ND	excl./ND	excl./ND	ND/ND	97 (93–99) S
20	ML	40/ND	**83**/excl.	excl./excl.	excl./excl.	53 (30–79) R
21		ND/ND	excl./ND	excl./ND	excl./ND	86 (62–98) R
22		ND/ND	excl./ND	excl./ND	excl./ND	99 (92–100) S
23		30/15	**93/98**	excl./excl.	excl./excl.	100 (100-100) S
24		87/ND	**98**/ND	excl./ND	excl./ND	100 (97–100) S
25		ND/ND	**94**/ND	excl./ND	<8*/ND	100 (100-100) S
26		ND/ND	excl./ND	excl./ND	excl./ND	100 (100-100) S
28	MON	ND/ND	excl./ND	excl./ND	excl./ND	100 (100-100) S
29		12/2	**82**/excl.	<1/excl.	excl./excl.	87 (59–99) R

For the NTC, the highest number of false positive results in all assays (upper confidence interval) was 1.09 copies/µl (Supplementary File 2). Furthermore, in all tests for species specificity no amplification of the *H. contortus* isotype 1 β-tubulin fragment was observed (maximum number of false positive copies/µl <1 using *T. circumcincta* or *T. colubriformis* as template, data not shown). In the assays, no overlap of confidence intervals with the NTC values was observed at detected concentrations between 1.3 and 11 copies/µl. With a background of the counterpart of 3000 copies/µl, this corresponds to VAF of 0.03–0.4% and a relative uncertainty between 25 and 57% (Supplementary File 1). Based on these values, the minimum number of copies/µl that had to be positive to assign a sample as positive for the polymorphism and calculate an allele frequency of the polymorphism in a sample was set to 15 copies/µl. With a background of 3000 copies/µl, a VAF of 0.5% was detectable. The intended detection limit of a VAF of 1% could thus be achieved with a background of 1500 WT copies/µl. Based on these values the thresholds for field samples were set. Samples with a VAF >1 were only considered, when at least 15 copies/µl of the SNP were detected. Samples with a VAF <1 were only considered, when at least 100 copies/µl of the wildtype were detected. Otherwise samples were excluded. With a background of 100 copies/µl the detection limit was thus a VAF of 15%. The detection limit was adjusted for each field sample based on the background level.

To estimate repeatability, the mean differences and maximal differences of VAF between the six replicates (NTC), triplicates and duplicates at assay validation of the F200Y, F167Y, E198A and E198L ranged between 0.06 and 0.14 (mean) and 0.16–0.84 (max). In field samples measured in duplicates, VAF-variation for all assays ranged between 0.000 and 0.008% (mean) and 0.001–0.087% (max), indicating a high precision.

3.2. Application of dPCR to field samples

We next applied the newly developed assays to field samples from Austrian and Italian farms.

73 samples were analysed three times. As 75 results had to be excluded due to an insufficient amount of *Haemonchus*-DNA, 144 results were available. The average copy number/µl of *Haemonchus*-DNA (mutant+wildtype) of samples before treatment was 2479 for study 1, 808 for study 2 and 1051 for Italy. In these samples, the amount of wildtype DNA was not always sufficient to reach a sensitivity for the detection of allele frequencies of 1%, increasing the detection limit to up to 14% (Tables 3–5 and Supplementary Table 4).

3.2.1. Evaluation of the presence of resistance on austrian farms

In Austrian farms (study 1 and study 2), elevated frequencies of F200Y resistance alleles were detected in all 18 samples before treatment (mean 92%, median 94% range 80–98%). F167Y was present in 7 out of 11 samples (mean 1%; median 1% range 1–4%). E198A was detected in 12 out of 14 farms (mean 4%; median 3%; range 2–12%) while E198L was not detected.

Table 5
Single nucleotide polymorphism frequencies for *Haemonchus contortus*, Italy (Bosco et al., 2020) (BT = before treatment, no post treatment data were available; <1% = below background threshold; *adjusted detection limit due to low background; excl. = excluded as not enough copies of the targeted *Haemonchus*-DNA were amplified; ND = no data [not analysed or no material available]; bold numbers = resistance alleles detected), 95% confidence intervals and relative uncertainty are shown in the supplementary file 4.

Farm	Drug	N larvae in pooled samples BT/PT	% of *Haemonchus* larvae BT/PT	% F200Y BT	% F167Y BT	% E198A BT	% egg count reduction
1	ML	43067/2600	78/7	<1	<1	<2*	97.9 (97.1–98.5) S
2		33333/33	77/24	<1	<1	<1	99.9 (9.4–100) S
3		8733/83	67/50	**7**	<1	<6*	99.1 (98.3–99.6) S
4		4700/17	67/0	<3*	excl.	excl.	99.7 (99.3–99.9) S
5		11533/683	73/73	<4*	<4*	4*	97.8 (97.2–98.3) S
6		55133/2050	93/11	**5**	<1	<1	97.8 (97.4–98.2) S
7		2250/0	28/ND	excl.	excl.	excl.	99.5 (97.3–100) S
8		5267/0	65/ND	<2*	<2*	<2*	99.8 (99.2–100) S
9		12533/0	83/ND	<1	<1	<1	100 (99.5–100) S
10		4000/0	38/ND	excl.	<7*	<6*	96.7 (95.8–97.5) S
1	BZ	14000/300	79/29	<1	<1	<1	**73.7 (64.3–81.0) R**
2		17800/367	78/6	<1	<1	<1	**92.6 (89.9–94.7) SR**
3		28133/483	72/59	**8**	<1	<1	97.0 (96.2–97.7) S
4		8133/17	68/82	<1	<1	<1	100 (99.6–100) S
5		1400/50	21/11	excl.	excl.	excl.	99.6 (98.9–99.9) S
6		28800/533	32/30	**6**	<4*	<4*	99.6 (99.5–99.8) S
7		5267/0	37/ND	excl.	<13*	<3*	99.8 (98.8–100) S
8		6533/0	61/ND	<2*	<1	<2*	100 (99.3–100) S
9		7800/0	81/ND	**84**	<8*	<8*	97.7 (96.6–98.5) S
10		2333/0	29/ND	excl.	<6*	<6*	100 (99.6–100) S

Altough the changes in SNP frequencies varied from −20 to +10 after BZ treatment and −4 to +7 for ML treatment, no significant trend in allele shifts was observed after treatment for any codon.

When F167Y or E198A polymorphisms increased above 10%, F200Y were more than 10% below the median (farm 2, 4; study 1 and 18; study 2 in Tables 3 and 4).

A FECRT using BZ was performed on eight farms where SNP-frequency data were available (Untersweg et al., 2021). For these farms high frequencies of the F200Y polymorphism (>80%) were observed by SNP-analysis. On six of the farms (farms 12, 14, 15, 17, 18, 19, Tables 3 and 4) BZ resistance was found by FECRT, and on one farm (farm 1) suspected resistance was observed. For one farm (farm 5) the FECRT indicated susceptibility. For another farm (4a), pre- and post-treatment data for BZ was available for a single animal and a treatment failure was observed (Untersweg et al., 2021).

The pre- and post-moxidectin treatment samples from the goat farm contained 77 and 82% of F200Y, 2 and 3% of F167Y, as well as 10 and 12% of E198A respectively.

3.2.2. Occurrence of BZ resistance alleles on Italian farms

In three out of nine Italian farms with five out of 15 samples, F200Y was detected (mean 22%, median 5%, range 5–84%). All resistance alleles on the other codons examined were below the threshold. No post-treatment SNP-data were available, as samples had to be excluded due to an insufficient amount of Haemonchus-DNA. (Table 5 and Supplementary File 4 and 5).

Data on FECRT with BZ was available for all farms. Resistance was detected only on farms 1 and 2, where no SNPs were detected in *Haemonchus*. In these farms, the proportion of *Haemonchus* in the pool decreased after treatment, while the proportion of *Trichostrongylus* increased from 21 to 71% on farm 1 and from 17 to 94% on farm 2 (Bosco et al., 2020).

4. Discussion

In the present study, dPCR assays were developed for four *H. contortus* β-tubulin SNPs associated with BZ-resistance. They showed high degrees of sensitivity, specificity, accuracy and precision. With the ability to reliably detect and quantify resistance alleles at levels as low as 0.5%, these assays thus enable early detection of BZ-resistance alleles in a parasite population, long before a resistant phenotype becomes apparent. However, a prerequesite for this low detection limit is a sufficient amount of *H. contortus*-DNA, which will not always be reached in field samples with mixed strongyle species.

It is still unclear what level of resistance alleles would result in a population with a resistant phenotype. This value has previously been set arbitrarily at ≥ 10% (Barrere et al., 2013; Chaudhry et al., 2020), but more studies are required to verify this. Early (i.e. pre-phenotypic) detection of resistance alleles is important because it allows to identify the risk of AR development at a stage when action can still be taken to prevent its manifestation in a population (Kotze et al., 2020).

The detection limit of our dPCR assays is lower than that of most pyrosequencing assays, which detect resistance at a level of 10% or more (Ramünke et al., 2016). Deep amplicon sequencing based on next generation sequencing technology, on the other hand, can detect allele frequency as low as 0.1% (Avramenko et al., 2020) and allows for large-scale screening of multiple SNPs in several worm populations. However, they are only available in a few laboratories and are more difficult to interpret than dPCR or pyrosequencing results (Charlier et al., 2022). They are also the most expensive techniques at present. Whereas the dPCR assay can be easily used in routine diagnostics.

Our newly developed assay for codon 200 has the advantage that the primers and probe do not target codon 198. Thus, amplification is also possible when polymorphisms at codon 198 occur in the population, which might otherwise cause problems since the existing codon 200 assays all use a sequencing primer or probe spanning over codon 198 (von Samson-Himmelstjerna et al., 2009; Baltrušis et al., 2018). Also, our separate assay for codon 198 is the first that detects two SNPs in one assay. Thus, with our assays we were able to screen field samples for early detection of all relevant SNPs associated with BZ-resistance in *H. contortus*.

On the Austrian farms, the F200Y SNP was shown to be the most frequently observed. In contrast to our previous studies, where we did not detect E198A (Hinney et al., 2020), the E198A SNP was observed on several farms, albeit at low levels. This could be due to the higher sensitivity of our dPCR assay compared to previously conducted pyrosequencing. The F167Y SNP occurred sporadically and seemed to be of low relevance, while the E198L SNP was not detected at all. The low-level occurrence of E198A on all farms from study 1 could be explained by the hypothesis that the simultaneous occurrence of BZ-resistance associated SNPs is lethal (Ghisi et al., 2007; Kotze et al., 2012). A high homozygous level of F200Y would thus exclude the

presence of high levels of E198A. The observation that an increase in SNPs in codon 167 or 198 was often accompanied by a proportional decrease in SNP in codon 200, so that the sum of both never exceeded 100%, is in line with this explanation.

Thus, the F200Y SNP might have been the one that spread first in Austria, while the E198A polymorphism was only later introduced into the population due to animal movements from regions where it was more prevalent. It would only increase in proportion if it provided a selection advantage over the F200Y SNP. The analysis of a resistant *H. contortus* strain showed that the E198A SNP conferred higher levels of BZ resistance than the F200Y SNP (Kotze et al., 2012). Under these circumstances, the E198A SNP would slowly replace the F200Y SNP if the population remains under selection pressure as a result of BZ treatment. The increasing occurrence of the E198A SNP would thus show the advanced level of BZ resistance in the region of study 1. We observed a decrease of the F200Y in favour of an increase of E198A in a single animal of study 1 (farm 4) after BZ treatment, which could suggest such a selection. In contrast, recent observations in *C. elegans* showed that the E198A SNP and the F200Y SNP conferred similar levels of BZ resistance and that none of them were dominantly inherited (Dilks et al., 2020, 2021). In addition, no fitness disadvantage was observed for any of these SNPs in the absence of selection with BZs.

On the Italian farms, the SNP frequency was low, which was also reflected in the lower prevalence of BZ resistance compared to most European countries (Rose et al., 2015). In Italy, the F200Y polymorphism was also the only SNP detected, while all other SNPs where absent or below the detection limit. These values are lower than those obtained in a previous study, where also only F200Y was of relevance but occurred in frequencies above 10% in 40% of the farms (Ramünke et al., 2016). However, because no representative sampling was conducted in the same regions, the two studies are not directly comparable. In our study, only farm 9 reached SNP frequencies of >10% with 84% of F200Y alleles. This level of resistance alleles indicates a resistant phenotype, but the FECRT did not detect BZ resistance (Bosco et al., 2020). Also in Austria, where the level of F200Y was almost 100% in one out of 10 farms where pre- and post-treatment data was available, susceptibility was nevertheless indicated by FECRT. It has already been discussed that although BZ resistance is clearly associated with polymorphisms in the isotype 1 β-tubulin gene, a direct correlation with the level of resistance alleles cannot be expected (Kotze et al., 2020). Furthermore, we only looked at polymorphisms in *H. contortus*, and other species could mask the development of resistance in one species in the FECRT (Morgan et al., 2022).

Conversely, we were also unable to detect resistance alleles on two farms in Italy where the development of BZ resistance was actually observed using the FECRT (Bosco et al., 2020). Again, this can most likely be explained by the fact that samples of mixed gastrointestinal nematode species were tested. Indeed, on both farms a significant increase in *Trichostrongylus* spp. was observed after treatment, while the proportion of *Haemonchus* decreased, so it can be assumed that *Trichostrongylus* was the resistant species leading to the low FECR. To improve the monitoring of resistance development, assays for *Trichostrongylus* spp. (and maybe also other strongylids) also need to be developed and applied in the future.

In addition, it is important to standardise the sampling procedure when pooled samples are examined, so that the pooled sample represents the true allele frequency of the species community. A standardization would be especially relevant in cases where the proportion of *Haemonchus* is different between individuals, e.g. in different age groups. Furthermore, other resistance mechanisms that cannot be detected by SNP analysis on the three codons of the β-tubulin gene are most likely to occur (Gilleard and Redman, 2016; Charlier et al., 2022).

Nevertheless, the development of BZ resistance in southern Italy still seems to be at a rather low level. At this stage, refugia-based management strategies to slow resistance selection have better chances of success than in Austria, where almost no susceptible *H. contortus* populations seem to be left. Constant monitoring of SNP development of all relevant trichostrongyloid species in Italy could help to evaluate the sustainability of these strategies.

No significant changes in allele frequency were observed before and after treatment with BZ. However, if the allele frequency is already close to 100%, no further increase is possible. In addition, at these extreme levels of allele frequencies (close to zero in Italy and close to 100% in Austria), the power of such a test is presumably very low and would require a larger sample size to detect changes that cannot be explained by chance.

5. Conclusions

For the first time a dPCR assay targeting two SNP in parallel in the isotype 1 β-tubulin gene of *H. contortus* on codon 198 was designed. Through the use of LNA-modified hydrolysis probes, it was possible to develop an assay for codon 200 that was not affected by mutations on codon 198. The dPCR assays developed in this study facilitate very sensitive and accurate detection of the major SNPs associated with BZ resistance in *H. contortus*. Naturally, the performance of the assays depends on the quality of DNA received from field samples and mixed species samples challenge the sensitivity of the test.

Ethics approval and consent to participate

Not applicable (approvals for the field studies can be found in the respective publications).

Consent for publication

Not applicable.

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Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Appendix A. Supplementary data

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