



Genetic variants in *dyf-7* validated by droplet digital PCR are not drivers for ivermectin resistance in *Haemonchus contortus*

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ABSTRACT

Resistance to ivermectin (IVM) in the nematode *Haemonchus contortus* in small ruminants is an increasing problem throughout the world. Access to molecular diagnostics will allow early detection of IVM resistance, which in turn can limit the spread of resistant isolates. One candidate gene which has recently been suggested as a marker for IVM resistance is that for dye-filling protein (*dyf-7*). In this study, we critically investigated the suitability of A141G and G153T single nucleotide polymorphisms (SNP) of *dyf-7* as a marker in larval cultures collected from sheep farms in Sweden, involving several isolates for which resistance status had been characterised by the faecal egg count reduction test (FECRT). Initially, we designed *dyf-7* primers from a worldwide collection of adult *Haemonchus contortus* DNA. With the sequence data, we created a haplotype network. We then optimised and used the same sets of primers and probes in a droplet digital PCR (ddPCR) assay for precise quantification of *dyf-7* allele frequencies in pre- and post-anthelmintic treatment faecal larval cultures. The fractional abundance (FA) of the mutant SNP was within the range 7.8 and 31%. However, the FA was generally stable in samples collected from the same farms, even though they were obtained on different occasions up to 25 months apart. There was also no indication that the level of IVM resistance as measured by the faecal egg count reduction test was higher on farms with high FA. Furthermore, by comparing FA in samples from the same farms pre- and post-IVM treatment, we found no evidence of a correlation between *dyf-7* and level of IVM resistance. Based on these results, *dyf-7* is not a suitable marker for field testing of IVM resistance in *H. contortus*.

1. Introduction

Grazing livestock are more or less continuously exposed to strongylid gastrointestinal nematodes (GIN), which upon infection can harm host welfare, health and productivity, especially in small ruminants on grass (Sutherland and Leathwick, 2011). Gastrointestinal nematodes are arguably the most important sheep pathogens in grasslands worldwide. They include the abomasal species *Haemonchus contortus* and *Teladorsagia circumcincta*, which are regarded as the two most devastating and abundant GIN of sheep in many countries including Sweden (Waller et al., 2006). In particular, the blood-sucking *H. contortus* is considered the most harmful species, generating economic losses estimated at £84 million per annum in the UK alone (Nieuwhof and Bishop, 2005).

Anthelmintics are still the cornerstone in worm control programmes globally. A major component in the control strategy against *H. contortus* in Swedish sheep is strategic targeted deworming of ewes, which is intended to reduce parasite eggs contamination on pastures. Lambs are thereby protected from build-up and exposure to infective third-stage

larvae (L3) (Waller et al., 2006). During the past decade, ivermectin (IVM) has become the dominant class of anthelmintics used to control haemonchosis in Sweden.

Anthelmintic resistance (AR) in GIN in sheep is now a worldwide problem which threatens global food security (Morgan et al., 2013). In *H. contortus* in particular, AR has reached serious levels with global spread (Kaplan, 2004). The emergence of drug-resistant parasites is also a major concern in Swedish sheep flocks today (Höglund et al., 2015). It has been argued that early diagnosis is a crucial step to avoid selection for AR and to maintain drug efficacy (Wolstenholme et al., 2004). Although the faecal egg count reduction test (FECRT) is the method of choice for detection of clinical levels of AR in nematode parasites of veterinary interest, it is a laborious test and the results are not always easy to interpret (Cabaret and Berrag, 2004; Roeber et al., 2012; Calvete and Uriarte, 2013). Thus, there is an urgent need to identify molecular targets that can be used for detection of low frequency mutant alleles frequencies before the appearance of clinical resistance (Kaplan, 2004; Samson-Himmelstjerna, 2006; Kotze et al., 2014).

Dyf family proteins are involved in the formation of amphids during

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embryonation of *Caenorhabditis elegans* (Heiman and Shaham, 2009; Li et al., 2001). To produce a resistant model nematode for research purposes, *C. elegans* was cultured for several generations in the presence of sub-lethal concentrations of IVM (Janukavicius, 2012). This generated two IVM-resistant strains harbouring defective dye filling (*dyf*) genes (Janukavicius, 2012), and is in agreement with an earlier suggestion that the sensitivity of IVM pathways in *C. elegans* is partly modulated by *dyf* genes (Dent et al., 2000). It has also been suggested that IVM resistance is related to an altered structure of the sensory organs (amphids) in *H. contortus*. For example, comparison of IVM-susceptible with IVM-resistant strains revealed structural changes of the amphids in resistant worms, which have markedly shorter sensory cilia than their IVM-susceptible parental counterparts (Freeman et al., 2003). Furthermore, specific *dyf-7* haplotypes associated with IVM resistance have been suggested (Urdaneta-Marquez et al. 2014). According to these authors three single-nucleotide polymorphisms (SNPs) in *dyf-7* (i.e. A141G, T234C, and G438T) have been suggested as specific genetic markers for IVM resistance in *H. contortus*, although there may be additional characteristic mutations in other positions. Combined these results from *C. elegans* and *H. contortus* indicate that *dyf-7* may be involved in IVM-resistance in nematodes.

Droplet digital™ polymerase chain reaction (ddPCR) can quantify absolute copy numbers of allele frequencies by dividing the reaction into around 20,000 nano-droplets suspended in an oil emulsion (Sanders et al., 2013). As in quantitative PCR (qPCR) assays, the results often depend on fluorescence emission from hydrolysis probes binding to different targets (Whale et al., 2016). However, in ddPCR each droplet is amplified as a separate reaction where the targets disperse randomly, which increases test sensitivity (Gerdes et al., 2016). Furthermore, in contrast to qPCR there is no need for a standard curve (Huggett et al., 2015). However, to achieve optimal results, the ddPCR assay needs to be optimised properly to fit different specific targets (Miyaoaka et al., 2014). The competitive duplex ddPCR assay protocol used in this study was essentially designed for SNP assessment, as described elsewhere (Whale et al., 2016). The results format is either absolute copies quantification (ABS) of the amplified targets or the fractional abundance (FA), which is a percentage of the variant allele in the sum of background and variant alleles (Uchiyama et al., 2017; Whale et al., 2016).

The overall aim of this study was to develop a ddPCR protocol for absolute quantification and precise FA determination of mutant (MUT) *dyf-7* SNPs in positions A141G and G153T of *H. contortus*, which was employed on mixed-species larval cultures from sheep faeces. Larval samples were analysed before (pre) and after (post) IVM treatment on several sheep farms in Sweden, including some where IVM treatment failure had been identified with FECRT (Höglund et al., 2015). It was hypothesised that if mutations in *dyf-7* are involved in IVM resistance, then FA should increase in post-IVM treatment samples, especially in parasites from farms with documented poor IVM treatment efficacy. We also expected to find a higher frequency of mutant worms on farms with reduced treatment efficacy than on farms where the drug was fully effective. In addition, we sequenced individual adult worms from some farms with reduced treatment efficacy, in order to create a haplotype network.

2. Material and methods

2.1. DNA samples

DNA was extracted from: 1) individual adult worms of *H. contortus*, *T. circumcincta* or *Trichostrongylus* sp. and from 2) faecal larvae cultures from infected sheep with different anthelmintic treatment history. The adult worms (mainly *H. contortus*) were obtained: 1) from different farms in Sweden where the drug efficacy previously had been evaluated with FECRT and where the worms were characterised morphologically into males and females before DNA extraction; and 2) from nine

Table 1

Adult worms sequenced including country of origin and resistance status according to faecal egg count reduction test.

Country	Isolate	ML- resistance status	Sequenced worms
Argentina		Unknown	2
Australia		Unknown	9
Brazil		Unknown	18
Canada		Unknown	1
Ethiopia		Unknown	7
Germany		Unknown	12
Guadeloupe		Unknown	2
Kenya		Susceptible	19
South Africa	White River Strain	Resistant	8
Sweden	Warnewik	Resistant	10 ♀, 10 ♂
Sweden	Uhlén	Susceptible	12 ♀
Sweden	Olsson	Susceptible	8 ♀, 11 ♂
Sweden	Nordén	Resistant	9 ♀, 10 ♂
Sweden	Westberg	Resistant	23 ♀, 13 ♂
Sweden	Engh	Resistant	4 ♀

Table 2

Paired larval cultures from Swedish sheep farms collected before and after treatment with different anthelmintics. Cultures contain a mixture of nematodes, which were tested with ddPCR for frequencies of *dyf-7* A141G and G153T single nucleotide polymorphisms in *Haemonchus contortus*. The faecal egg count reductions are shown as percentages (%). For some farms (H and I) paired results were obtained on several occasions. MOX: moxidectin, IVM: ivermectin, ALB: albendazole, LEV: levamisole.

Farm	MOX	IVM	ALB	LEV
A	100		100	100
D		100	91	
F		97	100	
G	100	87	100	100
H		65 and 100	100	100
I		100 and 78	96 and 100	100 and 100
K		78	77	
M		100	91	
P		44	100	
R		100	100	
U	100	77	96	100
Z		100	100	

different countries without any background information about the sex or the resistance status (Tables 1 and 2).

Extraction was performed using a NucleoSpin Tissue kit (Macherey Nagel, Germany) and extracts were frozen at -20°C for long-term storage. The DNA concentrations in larval culture samples ranged from 0.1 to 132.5 ng/μL, while those in adult worms ranged from 2.2 to 10.5 ng/μL as measured by Quant-iT™ PicoGreen® dsDNA Kit (ThermoFisher).

2.2. Primer probe design

The homology of *dyf-7* in different species was initially investigated by amplifying a 783bp long fragment (Fig. 1). Primers P783 were forward 5'-TGGACGAGGTGTCATGCTCGT-3' and reverse 5'-17/04/2018 TATCAGGTGTCAGAGCAACGTAA-3', and were designed with Primer3Plus (<http://www.bioinformatics.nl>) using reference sequences from GenBank (Accession numbers KF927016.1, KF927017.1, KF927018.1 and KF927019.1).

New shorter primers (P262) and hydrolysis probes were then designed *in silico* with Primer3Plus according to the ddPCR Bio-Rad Applications Guide and synthesised by Eurofins-MWG Operon (Ebersberg, Germany). The primer set targeting *dyf-7* SNPs in positions 141 and 153 of *H. contortus*, were forward 5'-TCG GAT CCA GAC ATT GC-3' and reverse 5'-CAT CAC AAT GTT TCC CGT A-3'. These primers produced amplicons around 262 bp and were confirmed by sequencing

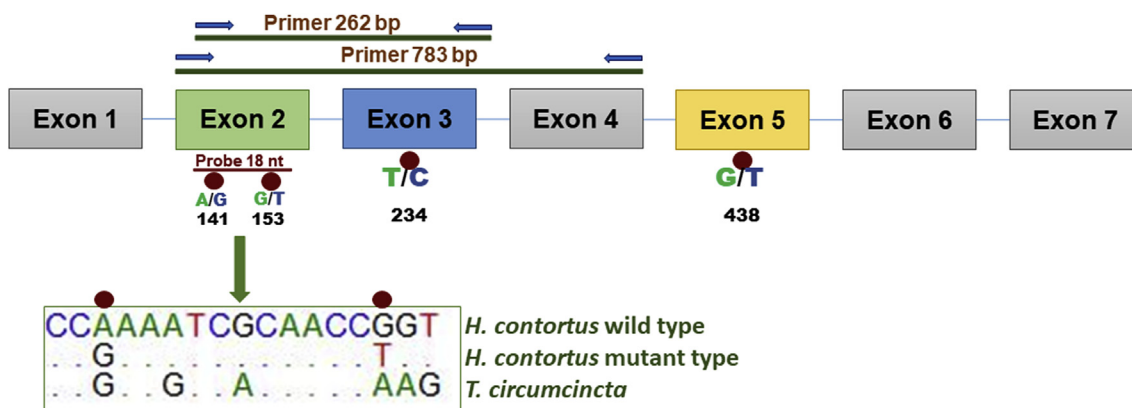


Fig. 1. *Dyf-7* gene construction. There are three single nucleotide polymorphisms (SNPs) of *dyf-7* at 141, 234 and 438 suggested to be associated with ivermectin resistance in *Haemonchus contortus*. These SNPs are located on different exons. Designed ddPCR probes detect wild type labelled with green fluorophore (HEX) and mutant type labelled with blue fluorophore (FAM) at position 141 and 153 on exon 2. (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

(see below). Hydrolysis probes were labelled on the 5' end with HEX for wild-type probe (WT probe) 5'-ACC GGT TGC GAT TTT GGC- 3' or with FAM for mutant-type probe (MUT probe) 5'-ACA GGT TGC GAT TTC GGC- 3' and both were quenched with fluorescent black hole quencher 1 on the 3' end. The binding sites for these primers and probes are described on the *dyf-7* gene construction (Fig. 1). Primers and probes were obtained in lyophilised form and then diluted in nuclease- and protease-free water (Sigma- Aldrich Chemie GmbH, Munich, Germany) and stored at -20°C . Specificity of P783 and P262 was tested using DNA templates from *T. circumcincta* and *Trichostrongylus* sp..

2.3. Sequencing

PCR reactions were performed with P783 or P262 in 22 μL reactions containing 1X PCR buffer, 0.5 mM MgCl_2 , 0.2 μM for each primer set, 200 μM dNTP and 1.25 units/50 μL PCR AmpliTaq Gold (ThermoFisher). Thermocycling conditions consisted of an enzyme activation step at 95°C for 10 min followed by 40 cycles of a denaturation step at 95°C for 30 s, annealing at 57°C for 30 s and elongation at 72°C for 1 min, then a deactivation step at 98°C for 10 min. Amplicon size was checked by gel electrophoresis with 1–2% agarose with a 100 bp ladder in a Bio-Rad Gel Doc™ system. Positive amplicons were cleaned with Illustra™ ExoProStar™ 1-step (GE Healthcare) before Sanger sequencing by Macrogen (<https://dna.macrogen.com/eng/>), and later viewed and aligned in Codon Code Aligner version 7.1. The sequences obtained have been submitted to Genbank with accession numbers MH172331-MH172361.

2.4. Haplotype network

To view the similarities between different sequences, a network was designed using Popart v 1.7 (Leigh and Bryant, 2015). This was based on sequences from the exon parts of fragments generated either by P783 or P262 from adult *H. contortus* of different origin (Table 1), but it also included sequences from a previous study (Urdaneta-Marquez et al., 2014).

2.5. ddPCR assay

Each ddPCR reaction was prepared using P262 in 22 μL reactions using 2x ddPCR supermix for probes (no dUTP) (Bio-Rad Laboratories Inc.), 900 nM for each primer set, 250 nM for each probe set (FAM) or (HEX) with either 1 μL DNA template, or 1 μL water for no-template control (NTC). DNA from sequenced individual adult *H. contortus* was used throughout method development to detect whether it had WT (SNPs 141A and 153T) or MUT (SNPs 141G and 153C) alleles. The same

samples were used as positive controls for WT and MUT, respectively. Specificity of the ddPCR primer-probe sets to *H. contortus* was also tested with DNA extracted from adult *Teladorsagia* or *Trichostrongylus*.

For each ddPCR reaction, around $20,000 \times 0.85$ nL emulsion droplets were generated using a QX100 Automated Droplet Generator (Bio-Rad Laboratories Inc.) with DG32 Cartridges (186–4108). The final thermocycling conditions were: an initial activation of the *Taq* polymerase at 95°C for 10 min, followed by 45x cycles of denaturation at 94°C for 30 s, annealing at 54.5°C for 1 min, elongation at 72°C for 2 min and a final enzyme deactivation step at 98°C for 10 min with ramping rate of $2^{\circ}\text{C}/\text{s}$, on an iCycler PCR machine (Bio-Rad Laboratories Inc.). The ddPCR amplifications were then analysed using a Droplet Digital PCR XQ200 system (Bio-Rad Laboratories, Hercules, CA, USA). Quantasoft version 1.7.4.0.917 (Bio-Rad Laboratories Inc.) was used to calculate the absolute quantification (ABS) of DNA copies/ μL in each sample and the fractional abundance FA (%) was calculated as the variant (MUT) allele divided by the sum of background (WT) and variant (MUT) alleles.

The ddPCR optimisation process was performed according to MIQE guidelines (Huggett et al., 2013) and is documented in a separate file (ddPCR optimisation file S3). In brief, we used a gradient between 52 and 59°C to find the most suitable annealing temperature in a simplex assay where we tested MUT and WT DNA separately using primer/probe set at 900/250 nM. A suitable primer/probe concentration was then selected by testing five different concentrations; 900/250 nM, 900/400 nM, 1000/400 nM, 800/350 nM and 700/500 nM, using a 1:1 (v/v) WT and MUT DNA. Finally, we investigated the optimal number of PCR cycles (45, 50 or 55) to study their effect on amplicon harvest and amplitude signal of amplified worm DNA. During the optimisation process, all samples were run in biological replicates and analysed at least in technical duplicates.

To evaluate ddPCR fidelity, the limit of detection (LoD) was defined by a series of two-fold dilutions of MUT DNA. The initial concentration of MUT was 126 copies/ μL which was diluted into a constant background WT DNA. LoD is the initial copy number needed in the sample for reliable detection. Proportion of MUT DNA in the WT background was 40, 20, 10, 5 and 2.5% respectively. We then investigated the dynamic range (i.e. the lowest amount of DNA that can be used to obtain consistent results) by using different volumes (1, 2 and 4 μL) in equal ratios of WT and MUT DNA. Finally, we evaluated FA precision in five mixtures with spiked amounts of MUT and WT DNA (100/0, 80/20, 50/50, 20/80, 0/100%). Calculations of FA were only performed for samples which met the LoD.

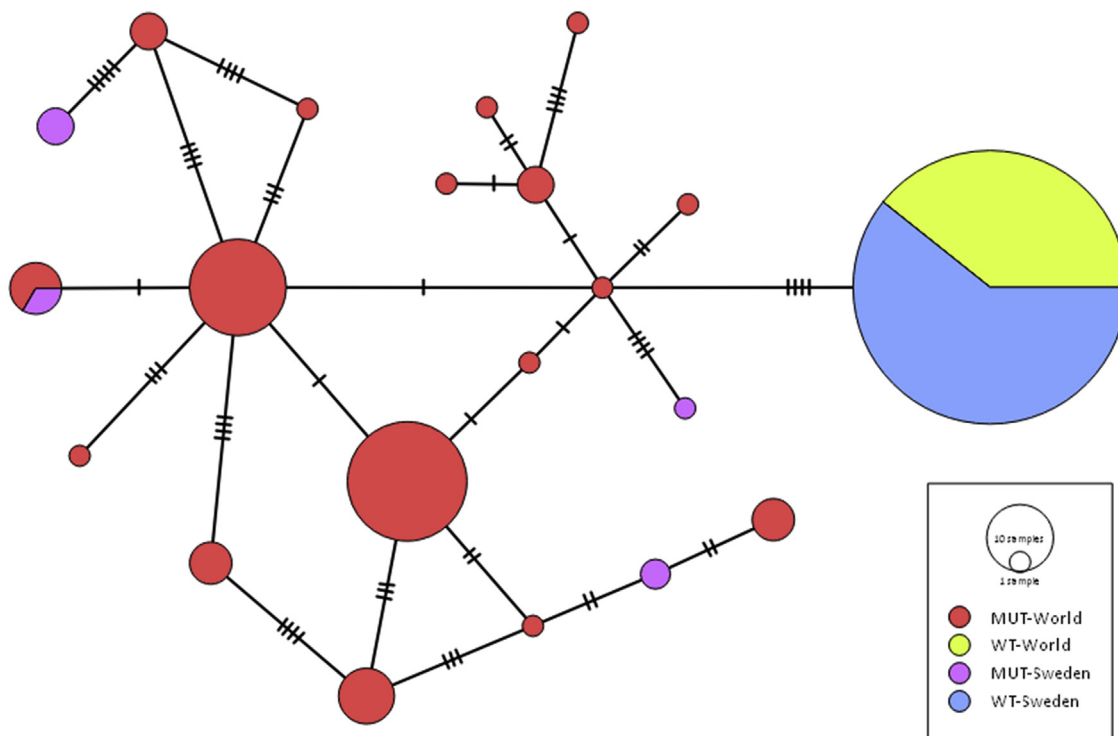


Fig. 2. Haplotype network for *Haemonchus contortus* dyf-7. The network is based on a 124 bp fragment from exons 2 and 3, amplified and sequenced with P262 (this study) or from previously published data. All ML-susceptible isolates have the same sequence and are represented by green and blue colours for Swedish and international isolates respectively. The 20 resistant haplotypes are represented by red (international) and purple (Swedish) colours. Circle sizes correspond to sample size. Vertical lines between pair of circles represent number of nucleotide differences between the isolates. (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

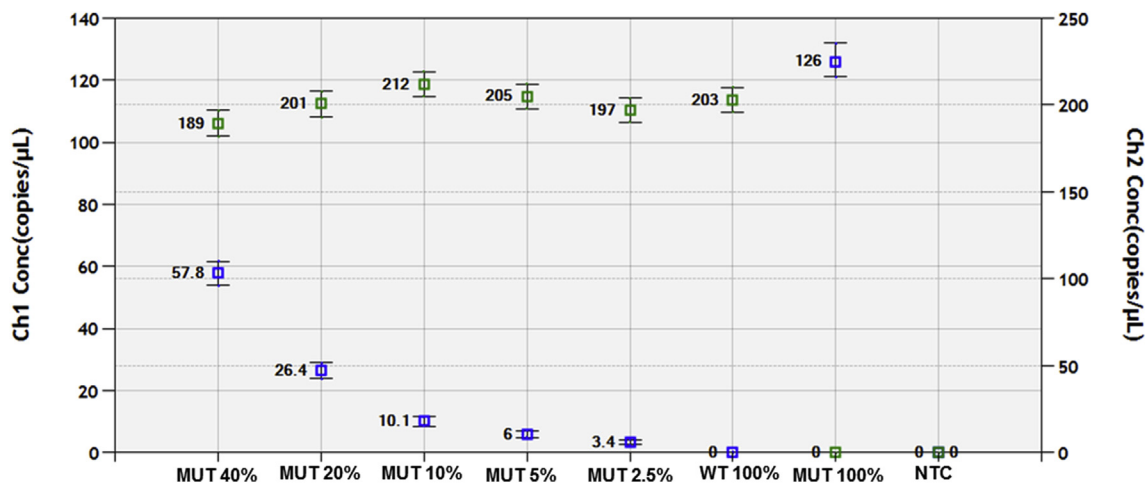


Fig. 3. Limit of detection (LoD) in the dyf-7 ddPCR assay. Mutant (MUT) worm DNA (Ch1 = blue boxes) diluted within a constant concentration of wild type (WT) DNA (Ch2 = green boxes). The concentration of WT was stable, whereas MUT decreased gradually with increasing dilution. LoD for MUT was 3.4 copies/μL and significantly higher than the non-template control (NTC) as shown in Supplementary file 3 Fig. 4. (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

2.6. Testing of larval cultures

The optimised ddPCR protocol was finally used to measure *dyf-7* A141G and G153T frequencies in pooled nematode faecal larval cultures obtained from 12 Swedish sheep farms (Table 2). The tested samples were collected both pre- and post-treatment with ivermectin (Noromectin® or Ivomec®), albendazole (Axilur®), moxidectin (Cydectin®) and levamisole (Chanaverm®), from different flocks that were dewormed according to the recommended dose (for details on doses and suppliers, see Höglund et al., 2015).

All ddPCR raw data were initially acquired and analysed by QuantaSoft, where ABS mode was selected to obtain copies/μL of PCR mixture. Threshold was usually defined automatically and occasionally adjusted manually when needed.

3. Results

3.1. Genetic diversity

A total of 196 samples were sequenced from adult *H. contortus* for

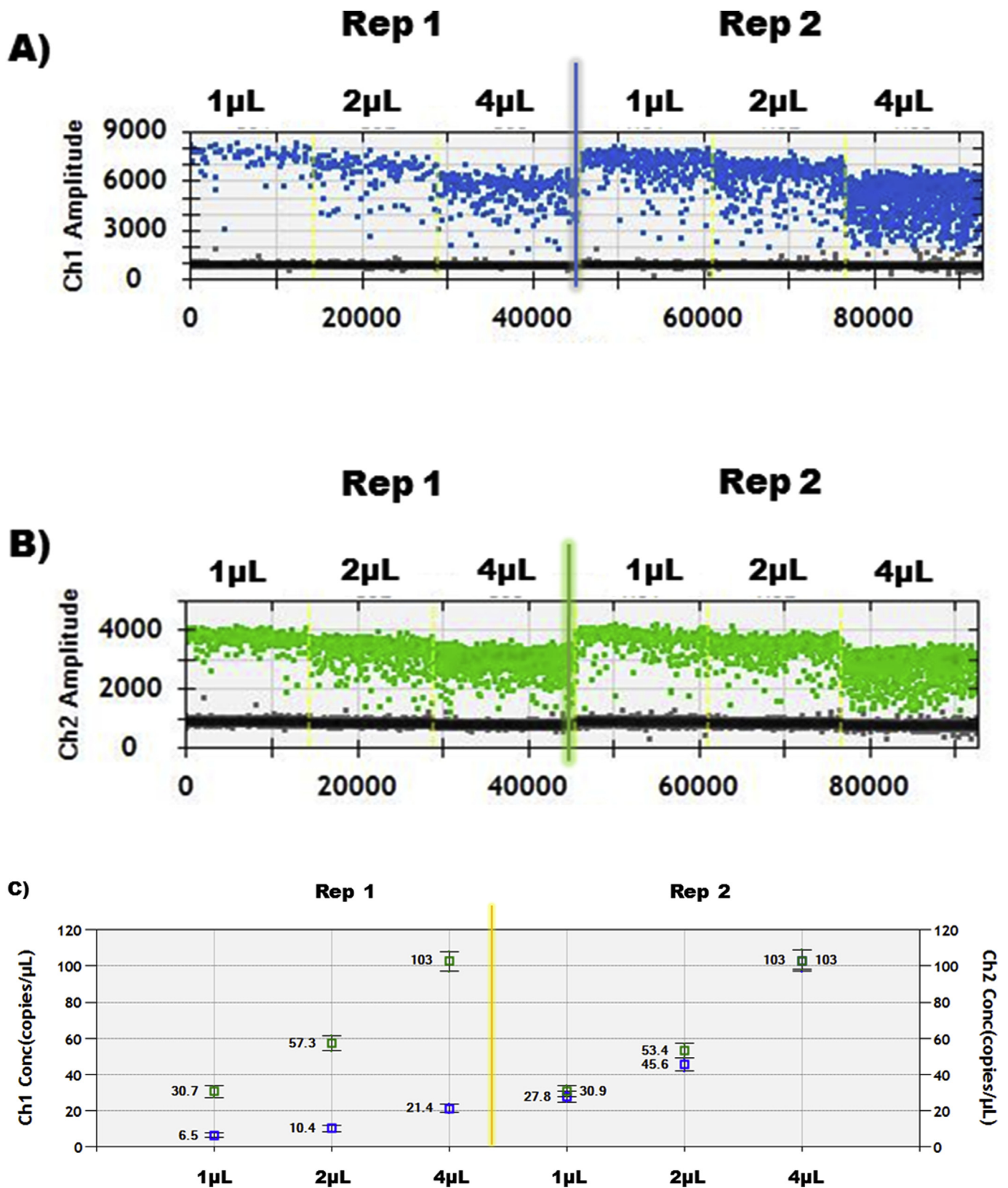


Fig. 4. Dynamic range of the *dyf-7* ddPCR assay. A and B show 1D plots for biological replicates (Rep 1 and Rep 2) with wild type (WT) and mutant (MUT) DNA respectively. To determine the dynamic range of the assay, DNA samples were added in three volumes (1, 2 and 4 μL). C show *dyf-7* concentrations in 1, 2 and 4 μL of template. Blue boxes = MUT, Green boxes = WT. (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

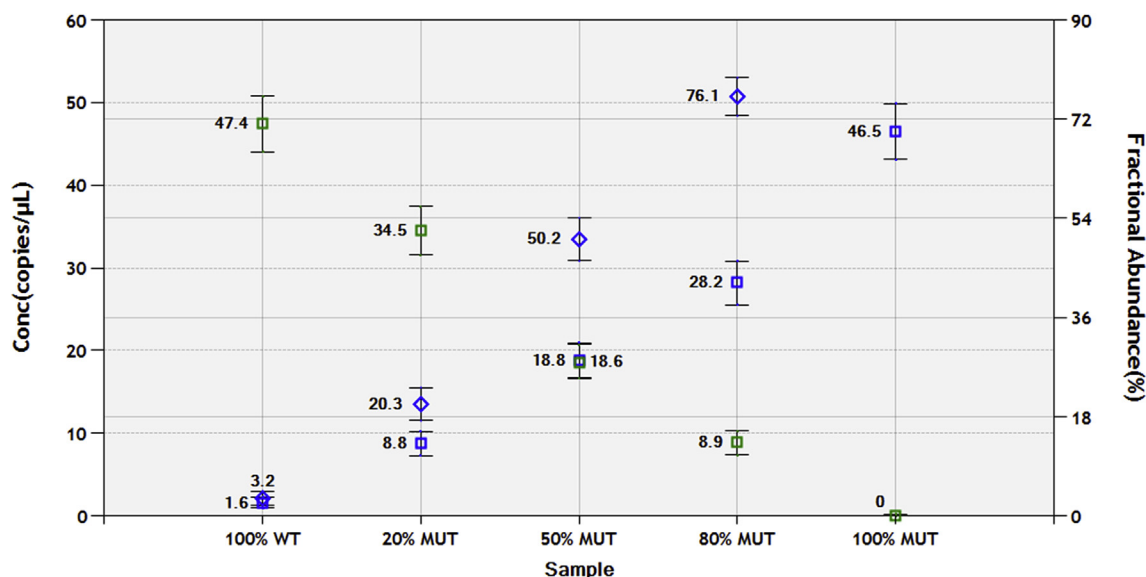


Fig. 5. Pilot *dyf-7* ddPCR assay. Different concentrations of wild (WT) and mutant (MUT) DNA in spiked samples with 100% WT, 80/20, 50/50, 20/80 and 100% MT. Each concentration was tested in duplicates. The fractional abundance FA for MUT was calculated by Quantasoft. Left Y-axis: green squares = WT copies per µL, while blue squares = MUT copies per µL. Right Y-axis: FA of MUT = blue diamonds. FAs are similar to the theoretical amount in the spiked samples and with a deviation of less than 5%. (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

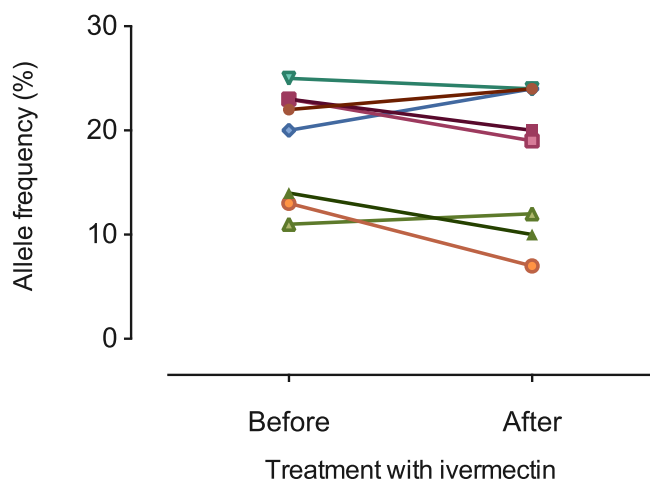


Fig. 6. Frequencies of *dyf-7* alleles on different sheep farms. Parasite DNA extracted from larval cultures collected before and after ML-treatment show nearly similar *dyf-7* mutant allele frequencies before and after ML treatment.

the P262 amplicon (Figs. S1–1 and Fig. S2-4 to S2-7) or P783 (Fig. S2-1 to S2-3). We also obtained PCR products from *Teladorsagia* ($n = 12$) using P783 (Figs. S1–2), while P262 did not amplify. With *Trichostrongylus* no amplification was observed.

For *H. contortus* 187 sequences were of high quality and where both forward and reverse reads could be assembled. Furthermore, based on inspection of the chromatograms there is no doubt about that all included female genotypes were homozygous. These were used in the creation of the haplotype network. More specifically amplified parts of exons 2 and 3 from *H. contortus* was used (Fig. 2). The network was based on a total of 263 MUT and WT sequences; 187 from this study and an additional 67 sequences published by Urdaneta-Marquez et al. (2014). We found 20 MUT haplotypes and one WT. Among the *H. contortus* we sequenced, four MUT haplotypes were found among Swedish isolates and two in samples from outside of Sweden. Three of these were novel to this study and only identified in Swedish worms. Among 16 unique haplotypes obtained from adult Swedish worms, seven (44%) were MUT and exclusively found in DNA from male

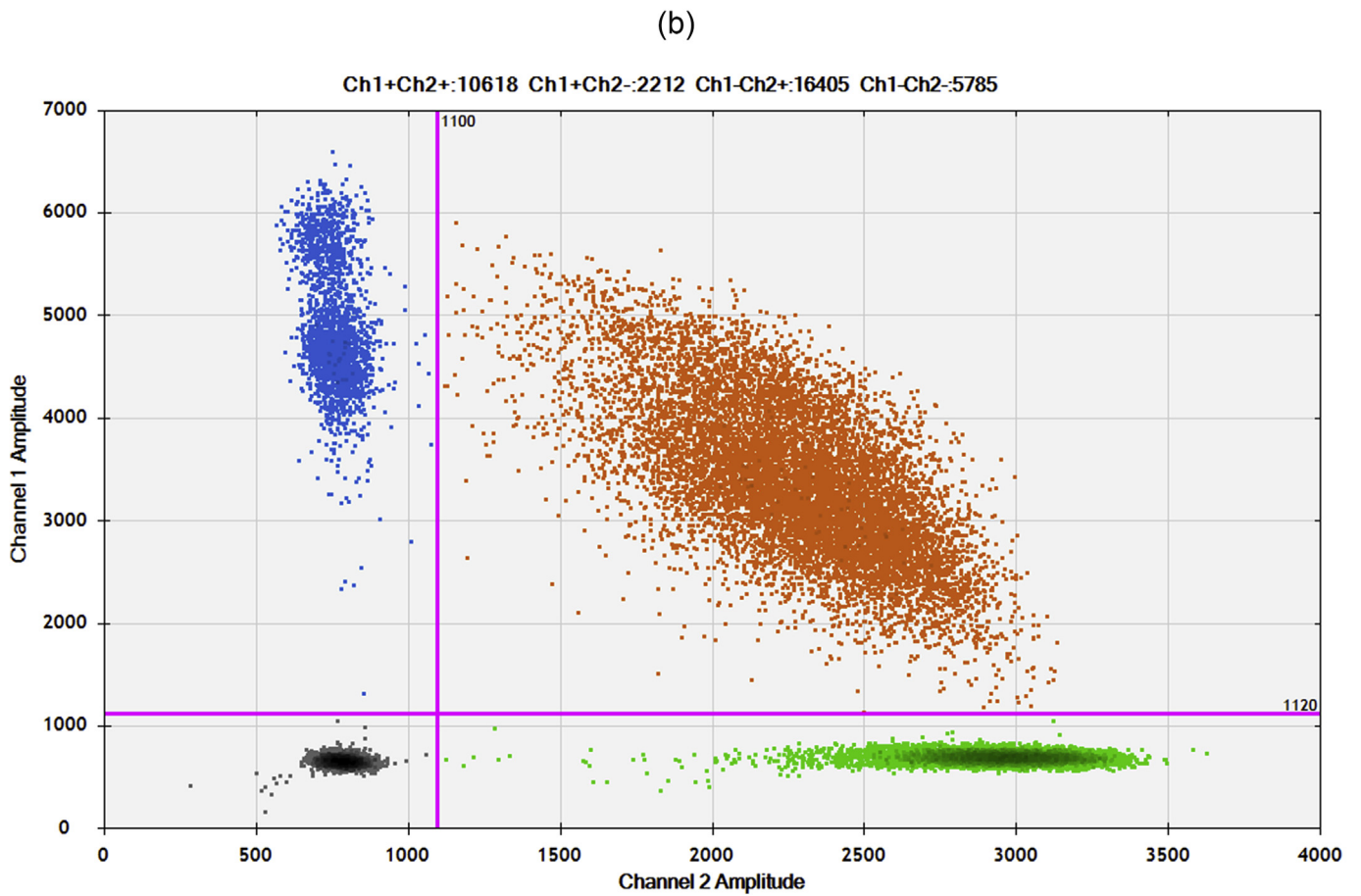
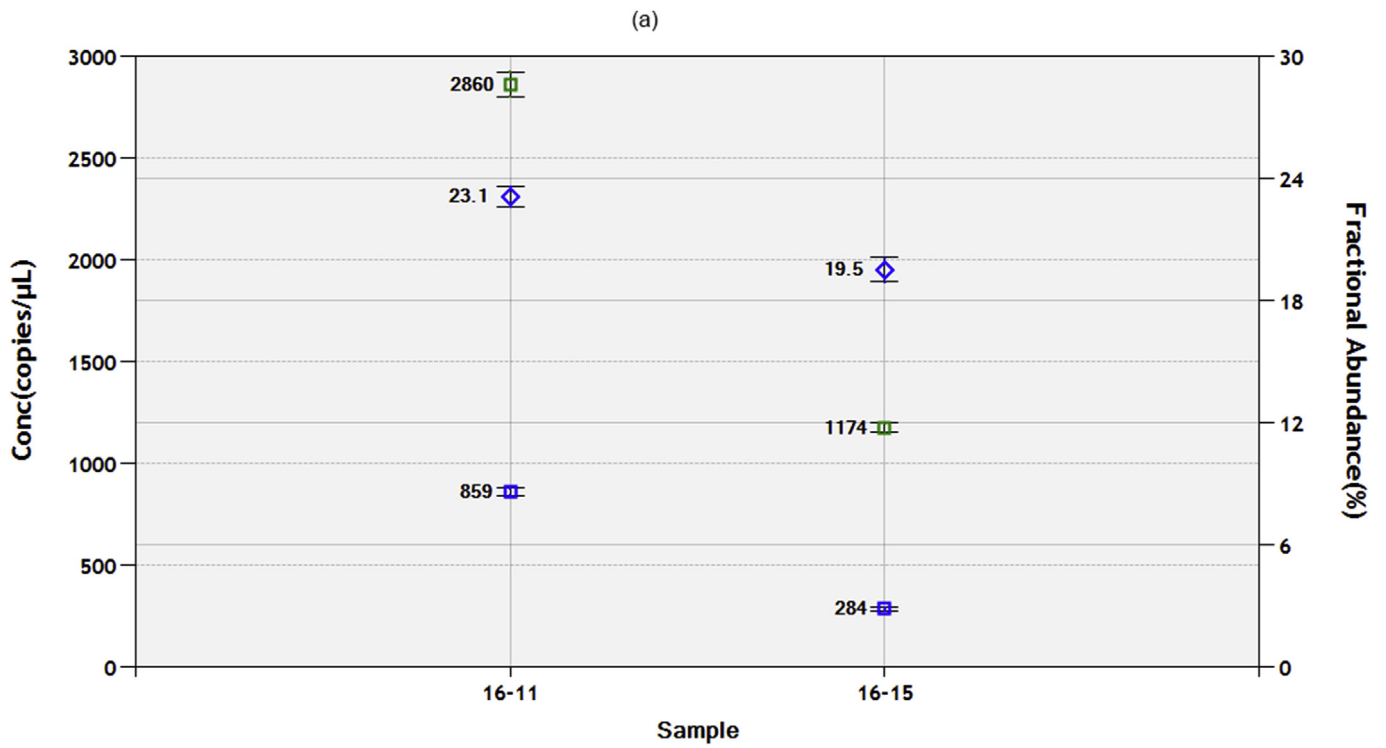
worms, whereas all female worms ($n = 60$) were WT. The 18bp region covering the ddPCR probe was identical in all MUT and WT *H. contortus* with the exception of two positions: A141G and G153T (Fig. 1). All of our MUT sequences also had SNPs in other positions (see Figs. S1–S3 and Urdaneta-Marquez et al., 2014).

3.2. Validation of ddPCR assay

The lowest detection limit (LoD) in our assay was 2.5% of MUT *H. contortus* DNA (126 copies/µL), corresponding to 3.4 copies/µL in a WT DNA background with ~ 200 copies/µL. The non-template control (NTC) only contained an average of 0.005 false positive copies per µL (Figs. S3–5). Although LoD was dependent on the initial number of MUT copies per µL in the undiluted starting sample, the FA of MUT was unaffected by WT DNA concentration (Fig. 3). Dynamic range, i.e. the lowest number of MUT copies that could be detected in the reaction volume, was higher than the LoD (3.4 copies/µL) and lower than the detection limit of the Droplet Digital PCR XQ200 system (100,000 copies/µL) (Fig. 4). The pilot test showed that the variation in FA between the same samples was $\leq 5\%$, irrespective of the MUT/WT ratio (Fig. 5). DNA samples from all individual *H. contortus* tested with ddPCR proved to be homozygous either for the MUT or WT allele. Furthermore, no signal of cross reactivity was observed in the ddPCR assay with DNA template extracted from adult *Teladorsagia* or *Trichostrongylus*.

3.3. Allele frequencies in larval cultures

First, we compared *dyf-7* allele frequencies in samples collected before treatment on different occasions from 12 different farms, of which half were tested between two and four times up to several months apart. The FA of MUT varied between 7.8 and 31.2% in pre-treatment samples, irrespective of the treatment history of the farm. We then tested *dyf-7* allele frequencies post-IVM treatment on a total of 10 farms where FECRT varied between 44 and 100% (data not shown). We found that, while FA varied significantly ($F_{(9,16)} = 11.94$, $p < 0.001$) between different farms, it was stable within samples from the same farm (Fig. 6). However, there were no differences in FA between samples collected pre- and post-IVM treatment (paired *t*-test, $t = 1.123$, $p = 0.2985$). An example of how FA data were generated for the different farms is shown in Fig. 7. Furthermore, there was no correlation



(caption on next page)

Fig. 7. Application of ddPCR *dyf-7* assay on larval cultures. An example of *dyf-7* allele frequencies of mutants (MUT) and wild type (WT) in paired samples collected on a Swedish sheep farm. Samples were collected before and 13 days after ivermectin treatment on a farm (P) with 44% faecal egg count reduction. (A) shows the fractional abundance (FA) stability of *dyf-7* before (16–11) and after (16–15) treatment. FA = blue diamonds, WT = green boxes and MUT = blue boxes. (B) 2D plot show the separation between droplets in the optimised assay. Dots in black are droplets negative for both WT and MUT, whereas blue and green dots contain MUT and WT only. Orange dots contain both MUT and WT. The x-axis shows the intensity of for HEX labelled WT probe, whereas the y-axis shows the intensity for the FAM labelled MUT probe. (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

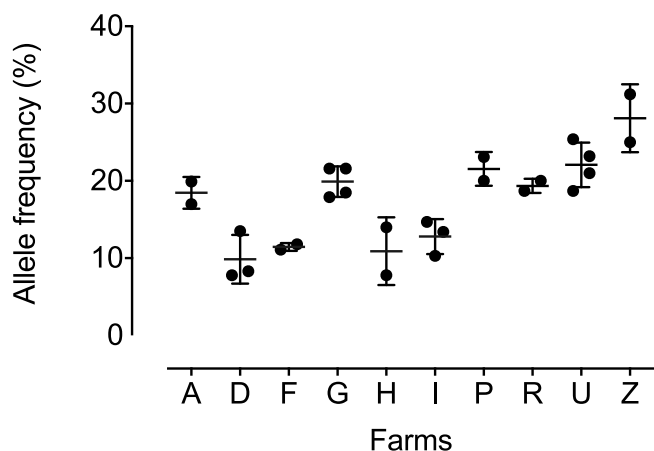


Fig. 8. *Dyf-7* alleles frequencies in pre-treatment larval cultures. Variations in fractional (FA) abundance in the mutant (MUT) allele. Samples were amplified from faecal larval cultures from different sheep farms. MUT *dyf-7* allele frequencies ranged from 7.8 to 31.2% on the different farms. However, FA were nearly identical in samples collected on different occasions from the same farm.

between IVM FERCT reductions and *dyf-7* allele frequencies ($z = 0.754$, $p = 0.451$; Kendall's rank correlation).

4. Discussion

This study tested the suitability of *dyf-7* SNPs as a molecular marker for IVM resistance in different isolates of *H. contortus*. To do this, we first developed and validated a ddPCR assay, which was then used to measure the mutant allele frequencies of *dyf-7* in faecal larval cultures containing a mixture of strongly lipid nematode species from sheep. Larvae samples were collected both before (pre-) and after (post-) IVM treatment on 10 Swedish sheep farms, but no significant shift in FA of MUT alleles suggested to be associated with IVM resistance was observed in the surviving parasites collected after IVM treatment (Fig. 6). Rather, we found evidence that most of the variation in FA was related to individual farms ($n = 12$), rather than to anthelmintic treatment (Fig. 8).

The workflow in ddPCR is similar to that used for most other TaqMan probe-based assays. However, a key aspect of ddPCR is sample partitioning into several thousands of droplets, which after separation into positive and negative droplets allows for absolute quantification of rare alleles (Huggett et al., 2013). A complication that can interfere with analyses of allele frequencies using ddPCR is the presence of droplets between positive and negative clusters (rain) due to incomplete reaction conditions (Dingle et al., 2013). Presence of rain is affected by several factors, such as annealing temperature, primer/probe concentration and cycle numbers (Huggett et al., 2013). To minimise the risk of rain, we assessed the optimal annealing temperature through a gradient PCR, different primer/probe concentrations and number of PCR cycles (ddPCR optimisation file S3). After optimisation of the ddPCR conditions, our assay proved to be very sensitive to measuring FA of MUT/WT alleles in faecal larval cultures from infected sheep, irrespective of the DNA concentrations (0.1–132.5 ng/ μ L). This is basically in agreement with findings in other studies quantifying the frequencies of rare alleles (Taylor et al., 2015; Koch et al., 2016; Uchiyama et al., 2017).

For any quantitative DNA-based tests intended for routine diagnostic use, it is crucial that it can measure allele frequencies with the same specificity and sensitivity irrespective of target species DNA concentration. According to ddPCR terminology, LoD is the lowest threshold where the mutant allele can be distinguished in a sample (Uchiyama et al., 2017). With our ddPCR test, 126 MUT copies/ μ L were detected in samples that were diluted down to 2.5%, corresponding to ≈ 3 copies/ μ L. This is within the same range or better than in other studies (e.g. Uchiyama et al., 2017). It also proved to be sufficient for our purposes, since we were able to detect MUT FA in every larval culture. Furthermore, we demonstrated that LoD for the mutant allele was independent of WT allele frequency (Fig. 4). The test fidelity of our ddPCR was verified by repeated testing, which showed similar FA results. The consistency of the analyses is in agreement with that in other ddPCR studies for other applications (e.g. Dobnik et al., 2016; Koch et al., 2016; Uchiyama et al., 2017). Taken together, these results show that our ddPCR *dyf-7* test is robust and can be used for analysing the FA of *dyf-7* in ovine faecal larval cultures.

The optimised ddPCR assay was used here to investigate the FA of *dyf-7* alleles in larval cultures from 12 sheep farms, some of which harbour IVM-resistant *H. contortus* according to FECRT data (some of which are included in Höglund et al., 2015). Our working hypothesis was that if *dyf-7* is involved in clinical IVM resistance on different farms, then MUT allele frequency will increase after IVM treatment, in accordance with previous observations (Urdaneta-Marquez et al., 2014). We also expected to find poor anthelmintic treatment efficacy against *H. contortus*, especially on farms with high MUT allele frequency before IVM treatment. Furthermore, in a survey conducted between 2006 and 2007, none out of 90 sheep farms showed signs of IVM resistance (Höglund et al., 2009). Accordingly, IVM-resistant *H. contortus* was most likely recently imported to Sweden along with sheep harbouring IVM-resistant *H. contortus*, and thus low MUT *dyf-7* allele frequency was expected on most farms (Höglund et al., 2015).

Analysis of MUT *dyf-7* frequencies in DNA from larval cultures revealed three major findings. First, the FA of the MUT *dyf-7* allele was similar in cultures obtained pre- and post-IVM treatment. The variation observed was $\leq 5\%$, which has previously been shown to be within the acceptable level for false positive and negative rates (Dobnik et al., 2016). Second, MUT frequencies were often high (up to 25%) in samples obtained pre-treatment, despite IVM being 100% efficient according to FECRT. Third, there was rarely any significant variation in FA of *dyf-7* in pre-treatment larval cultures, which were sometimes collected up to 25 months apart on the same farm. In fact, most of the variation in FA observed was between, rather than within, different farms.

In essence, this is in agreement with results in a previous study (Laing et al., 2016), which found no differences in *dyf-7* between IVM-treated and non-treated UK sheep farms. Thus, based on the combined observations, the most likely explanation is that the SNPs in *dyf-7* are unrelated to IVM resistance or treatment frequency. This contradicts previous claims of a correlation between IVM resistance in *H. contortus* and mutations in *dyf-7* (Dent et al., 2000; Urdaneta-Marquez et al., 2014).

These results were further supported by the analyses of *dyf-7* sequences obtained from individual adult *H. contortus* included in the haplotype network (Fig. 2). Interestingly, all WT samples were identical homozygous genotypes, irrespective of the geographical origin of the worms. In contrast, MUT worms with the A141G and G153T mutations

also had several other divergent SNPs, including those observed by Urdaneta-Marquez et al. (2014). As shown in our haplotype network (Fig. 2), 20 different MUT haplotypes were observed, of which three are unique for Swedish *H. contortus*. In agreement with previous studies (Urdaneta-Marquez et al., 2014), we found evidence that SNPs at positions A141G and G153T are linked to other SNPs, such as T234C. Furthermore, all of our MUT samples were exclusively male, but they could not be linked to the clinical IVM resistance status of the sheep farm of origin. Thus, our results provide support for that *dyf-7* is sex linked and just not that it is under IVM selection. This is partially in contrast to Urdaneta-Marquez et al. (2014), who suggested that *dyf-7* is a sex-linked gene and increases in IVM treated populations. However, our findings are in agreement with a recent study where *dyf-7*, along with a number of other candidate IVM resistance loci, was found not to be correlated with IVM resistance after serial backcrossing (Rezansoff et al., 2016).

5. Conclusions

The ddPCR approach proved to be a suitable and robust method for measuring MUT *dyf-7* allele frequencies in mixed larval culture ovine samples. However, in analyses of DNA from adult worms and from larvae, we were unable to verify that *dyf-7* is involved in IVM resistance in *H. contortus*. Thus, our data confirms other results that have been published recently, indicating that the *dyf-7* SNP (A141G) is not under selection in field isolates of resistant *H. contortus*. More investigations are clearly required to identify other more suitable candidate genes linked to IVM resistance.

Conflict of interest

The authors of this manuscript certify that they have NO affiliations with or involvement in any organization or entity with any financial interest, or non-financial interest in the subject matter discussed in this manuscript.

Appendix A. Supplementary data

Supplementary data related to this article can be found at <http://dx.doi.org/10.1016/j.ijppdr.2018.04.005>.

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