Single Nucleotide Polymorphism (SNP) in dyf7-141 of Haemonchus contortus as potential marker for ivermectin resistance

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Master Degree Project in Infection Biology, 30 credits. Spring 2017
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Abstract

*Haemonchus contortus* is the most pathogenic nematode in the sheep breeding sector, which affects animal welfare negatively and reduces production. Throughout the world *H. contortus* is treated by the anthelmintic drug ivermectin (IVM), but presence of resistant strains leads to drug failure and economic losses. There are a diversity of previous studies on drug targets of IVM and other mechanisms behind resistance. Recently it was postulated that there is a connection between IVM resistance and single-nucleotide polymorphisms in the *dyf7* gene. Ideally resistant strains can be detected before clinical outbreaks of disease to change the control strategy or before movements of resistant isolates in conjunction animal transports. In this study, we developed a diagnostic tool based on droplet digital PCR according to MIQE guidelines, which is able to measure mutant allele frequencies of *dyf7*. The developed assay was then used to measure allele frequencies in faecal larval cultures collected on sheep farms in Sweden where faecal egg count data was available. Fractional abundance (%) of the mutant allele frequencies was stable within the same farm, regardless of anthelmintic class used. By comparing tested samples from before and after treatment groups, there was a clear lack of correlation between *dyf7*-141 and IVM resistance.
What do farmers feed their sheep?

For farmers, if you could travel through time and remember when you were a kid playing with sheep in your family farm. When you filled with enjoyment to feed them from the pasture by yourself. At that moment did you imagine you were feeding them grass with parasites?

Sheep pastures are usually contaminated with different parasitic worms. Among these Haemonchus is the most harmful one for sheep. While ruminant animals as cattle and sheep have four room-stomach, it lives in their third room-stomach, sucks blood around 50μl and release 5000 to 10000 eggs daily. These eggs contaminate the pasture unless adequate parasite control is achieved. The amazing fact is that this hatched egg produces a small worm, which crawls to grass tips at dawn and dusk. Sheep eat it with grass without feeling anything. Thousands of worms can live inside the stomach and sheep become sick and emaciated.

Several years ago, sheep flocks were treated by many types of drugs and kept healthy with a lot of wool and meat production, however nowadays worms developed a resistance towards the drugs and called resistant worms. For that, few drugs become effective against them and we need to figure out if sheep has wild or resistant worms to select the suitable drug.

Scientists thought it was clear and easy to differentiate between wild and resistant worms through certain genes involved in drug action. We selected one of these genes called dyf7 to evaluate its suitability. During this master thesis, we developed a modern method to detect the proportion of resistant worms that do not respond on treatment and then we tried answer this question; whether there is any correlation between presence of this gene in resistant worms and the drug failure.

Now, we successfully can differentiate between normal and abnormal worms even you still cannot see them in the pasture when feeding your sheep. Because any abnormal changes in the genomic structure of the resistant worm mean changes in many other positions, thereby we can catch it easily.

But we could not unfortunately relate between dyf7 gene and the resistance status and do not know why drug does not effect on it?

Now, you properly argue that there should be alternative genes to be used and be more related to drug failure. Unfortunately, scientists do not know completely how worms resist drugs. Of course, we do not want to eradicate them completely but keep them under control, owing to many farmers and scientists fear from that day when all our drugs be useless against worms. But till that time, keep your eyes on your sheep and deworm them regularly.
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1. Introduction

1.1. Background

The sheep industry is an important economic sector with low maintenance requirements for farmers throughout the world. In most countries including Sweden, animals are kept on pasture, where they are grazing for varying periods of time. Thereby sheep are consistently exposed to a wide range of pasture-borne trichostrongylid gastrointestinal nematodes (GIN). There is several GIN that may infect the animals while they are grazing. In a recent study from the UK, it was shown that *Teladorsagia circumcincta* and *Haemonchus contortus* are the two dominating species, (Burgess et al. 2012). This is also the case in Sweden (Waller et al., 2006). Among all GIN of sheep, *H. contortus* is considered the most pathogenic species (Redman et al., 2008). It is responsible for a disease called haemonchosis, which is characterized by weight loss, poor production and high mortality rates especially in lambs (Deplazes et al., 2016). Worldwide haemonchosis causes huge losses in the entire sheep sector estimated to £84 million per annum in the UK (Burgess et al., 2012).

Like with any other GIN, *H. contortus* is characterized by a direct life cycle. Daily thousands of eggs are laid by the female worms and then passed with faeces into the external environment. These eventually hatch into first larval stage (L1), which gradually develop into the infective third larval stage (L3). The L3 larvae are adapted to move up on grass tips in the morning and evening to facilitate host ingestion and avoid sun radiation and dryness during the day (Li et al., 2001). Inside the ruminant host, maturation of L3 continues until adult worms in their predilection site in the abomasum. A single sheep may harbour several thousands of blood-sucking adult worms. This causes anemia, edema, severe hemorrhagic gastritis and eventually death (Deplazes et al., 2016). Due to the direct life cycle and severe consequences of infection, *H. contortus* requires a strict control strategy.

In most instances, dewormers (anthelmintic drugs) are the cornerstone in the control strategy used to control GIN of sheep based on repeated treatment with different anthelmintic classes. For example, in Sweden the control strategy against *H. contortus* is directed mainly toward ewes through deworming, to reduce pasture contamination with eggs in order to protect lambs from exposure of L3 (Waller et al., 2006).

Broad spectrum anthelmintic includes three main major groups (Deplazes et al., 2016):

1) Tetrahydropyrimidines (TH) such as levamisole (LEV)
2) Macrocyclic lactones (ML) including moxidectin (MOX) and ivermectin (IVM)
3) Benzimidazoles (BZ) such as albendazole (ALB)

TH targets nicotinic acetylcholine receptors leading to spastic paralysis, ML bind to glutamate–gated chloride channels (GluCl) causing postsynaptic membrane paralysis, whereas BZ bind β-tubulin protein which inhibits proper locomotion and reproduction (Holden-Dye and Walker 2017). Although all of these three major classes of anthelmintic have different modes of actions, they restrict parasitism adequately (Kotze et al., 2014). However selection for resistance to all major classes of anthelmintic is today a major problem and especially in *H. contortus*. It has reached serious levels throughout the world (Kaplan, 2004).

Among all anthelmintic, IVM is the preparation currently used to the greatest extent in the control of GIN in sheep. IVM belongs to ML family and was originally extracted from *Streptomyces* species in the 1970s and it is characterized by broad-spectrum activity against both nematodes and arthropods (Yates et al., 2003). In nematodes, IVM binds to GluCl receptors that are situated on motor neuron commissures within somatic muscles and pharyngeal muscle cells (Yates et al., 2003; Wolstenholme and Rogers, 2006). The binding of IVM irreversibly opens these ligand-gated ion channels and causes flaccid...
paralysis in somatic muscles at mid-body section leading to the starvation and death of the worm and/or expulsion from the host (Glendinning et al., 2011).

Decades of anthelmintic invention and intensive application of sub-lethal dosage have led to the development of irreversible resistance in many livestock parasites and especially in strongyloid nematodes such as *H. contortus* (Kaplan, 2004). The resistance toward the major broad spectrum anthelmintic has theoretically evolved through four main mechanisms in the parasites: 1) reduced drug uptake through mutation of drug target, 2) increased drug efflux, 3) altered detoxification pathways, and 4) excessive production of altered target genes (Wolstenholme et al. 2004 and Kotze et al. 2014). In some instances anthelmintic resistance (AR) in nematodes has appeared as a multi-drug resistance (MDR) phenomenon with low anthelmintic efficacy determined by polygenic resistance genes (Kaplan, 2004). Therefore, it has been debated if AR can be explained by a simple single-gene resistance (Redman et al., 2008 and Kotze et al., 2014). Parasitic nematodes have high evolutionary rates in their nucleotide sequences (polymorphism) and a large population size (genetic diversity) (Redman et al. 2008). For that, parasitic nematodes are of great importance in resistance studies (Wolstenholme et al., 2004).

Correct diagnosis of AR is a crucial step to change the control strategy at the right time and by that maintaining drug efficacy (Wolstenholme et al., 2004). Today AR has increased and is spread globally to a level, which threatens animal production and global food security (Morgan et al., 2013). The faecal egg count reduction test (FECRT) is an *in vivo* method that still is used as the gold standard test for detection of resistance in nematode parasites of veterinary interest, regardless of anthelmintic class. Furthermore, there are several *in vitro* methods, but only the larval development test (LDT) and the larval migration test (LMT) can be used to detect IVM-resistance (Demeler et al., 2010). All of these tests are laborious and time-consuming to perform and not always easy to interpret (Deplazes et al., 2016). Thus there is a demand to develop an alternative test for sensitive and rapid detection of AR in most nematodes of livestock. Ideally, these should be based on a molecular detection strategy (Kaplan, 2004).

In the literature the necessity of novel molecular assays that are suitable for the routine field detection of AR has been emphasized frequently (Samson-Himmelstjerna, 2006; Kotze et al., 2014). An ideal assay will detect low mutant allele frequencies before the occurrence of clinical resistance and before a geographical spreading of resistant strains (Kaplan, 2004). But before that a better understanding of the evolution and mechanisms of AR is the fundamental first step.

*H. contortus* is often compared with the free-living model nematode *C. elegans* as both species belong to the same clade (Glendinning et al., 2011). IVM- resistance has been studied for GluCl channel subunits genes in *C. elegans*. Through comparative genomic methods, these genes were matched to the related resistant genes in *H. contortus* (Holden-Dye and Walker 2017). These genes varied a lot between nematode species, which suggests the presence of different pharmacodynamics of IVM in different nematodes with different modes of resistance (Yates et al., 2003). This illustrates the priority of basing the studies on the correct animal model. In addition to GluCl genes, there were several proposals for other mechanisms that can lead to IVM resistance in nematodes such as nucleotides polymorphisms in dyf7 (Urdaneta-Marquez et al., 2014).

All nematodes are characterized by amphids, which is a chemosensory organ formed from 12 neurons in their anterior end (Li et al., 2001). The formation of amphids is dependent on the expression of several genes including the dye filling protein (*dyf*). In *C. elegans, dyf* family genes are responsible for chemosensory responses and dauer formation (Starich et al., 1994). *Dyf7* is important for anchoring dendritic tips while cell body are migrating toward the nose tip during the embryonic stage. Mutations in *dyf7* lead to anchoring failure and defective amphid formation from larval development till adulthood.
It has been shown that resistant *H. contortus* against IVM have altered structure amphids (shorter and disorganized) compared to susceptible strains (Freeman et al., 2003). There are more than 20 genes when mutated could lead to amphid defects. Therefore mutations in these genes were thought to cause IVM resistance also in *H. contortus* (Urdaneta-Marquez et al., 2014). The idea of *dyf7* as a candidate gene for IVM resistance in nematodes appeared mainly because it is less polymorphic compared to other GluCl channel subunits genes (Dent et al., 2000 and Laing et al., 2016). Three specific SNPs (A141G, T234C, and G438T) have been found to be more related to resistance in mutated *dyf7*, where particularly A141G indicates the presence of several other mutations (Urdaneta-Marquez et al., 2014). It has been speculated that mutated *dyf7* is linked to ML resistance either by preventing IVM permeability via defected amphids (Dent et al., 2000) or due to abnormal chemosensory function, which in turn lead to stress and activation of ABC transporter which increases IVM excretion (Urdaneta-Marquez et al., 2014). However, whether these SNPs in *dyf7* are useful as molecular markers for IVM resistance, requires further testing.

Droplet digital™ PCR (ddPCR) is a very accurate, sensitive and robust technique, which can identify and quantify absolute copy numbers of allele frequencies (Sanders et al., 2013). A major advantage compared to other techniques (e.g. qPCR) is that with ddPCR quantification of alleles can be done without a calibration to a standard curve (Huggett et al., 2014). The results depend on fluorescence emission from probes based on the droplet content (Whale et al., 2016). With the ddPCR strategy, the PCR reaction is divided into around 20,000 nano-droplets suspended in an oil emulsion. Each droplet is created and amplified as a separate reaction, where the reaction content disperses randomly. This increases test fidelity and the sensitivity of the assay as it decreases competitive amplification effects (Gerdes et al., 2016). However, like any molecular diagnostic assay, the PCR amplification protocol needs to be justified starting from the design of primers and probes to other conditions related to allele frequencies and target types (Miyaoka et al., 2014).

Designing a ddPCR duplex assay enables a cheaper and more rapid tool with low technical errors in pipetting. The ddPCR duplex assay is characterized by the ability to test two different molecular targets or more within the same test. A duplex assay (Fig. 1) can either be 1) competitive and is commonly used for detection of a single nucleotide polymorphisms (SNPs) when two different probes bind to the same region, or 2) non-competitive when probes bind to different regions and this is in general used for measurements of copy number variation (Whale et al., 2016). Our ddPCR assay was designed as a competitive duplex assay, for detection of alleles frequencies (wild and mutant), where two different probes bind to the same DNA target site in the parasitic nematode *H. contortus*. An optimized competitive duplex assay is characterized by an arc shape for the double-positive populations, which links the two single positive populations (Fig. 1). In this study, a duplex assay was developed as a sensitive tool for detection of SNP of *dyf7-141* allele frequencies in complex samples containing up to several thousands of individual worms. The typical result obtained from competitive duplex assay is fractional abundance (FA), which means the ratio (%) of the variant allele to the sum of background and variant alleles (Whale et al., 2016).
Figure 1 - The ddPCR assay
(A) Non-competitive assay, where two different probes bind at different regions or (B) competitive assay, where two different probes bind to the same region. 2D plot shows results from non-competitive assay as separate populations of grey negative droplets (no template), blue FAM positive population (mutant allele), green HEX positive population (wild allele) and orange FAM and HEX double positive population (mutant and wild alleles), whereas competitive assay shows double positive population (+FAM/HEX) linked to the both single positive populations (+FAM and +HEX) (Whale et al., 2016).

Accepted droplets are droplets generated from the reaction mixture with template and measured by Quantasoft version 1.7.4 software as separate reactions. Droplets containing template DNA are considered positive droplets and are amplified during the PCR cycles, while negative droplets do not contain template. Poisson distribution quantifies positive droplets in proportion to negative droplets. Dropout occur when the target fail to amplify (Whale et al., 2016). Rains are droplets expressed between the positive and negative amplitude extremes (Koch et al., 2015).

1.2. The aim of the study

The objectives of this study:
- Firstly, to develop a ddPCR assay for detection of mutant allele frequencies of dyf7-141 in H. contortus.
- Secondly, to employ this assay to measure allele frequencies in complex samples (e.g. larval cultures) collected before and after treatment with different anthelmintic (e.g. IVM, MOX, LEV, and ALB) on different sheep farms in Sweden. Some of these samples were obtained from farms where IVM treatment failure was described recently (Höglund et al., 2015).
- Thirdly, to evaluate dyf7-141 allele frequencies in relation to FECRT data and IVM resistance of these samples.

1.3. Limitation

Samples from different countries could contain different SNPs related to geographical variance and genetics accidents like bottleneck selection, which could not be evaluated in these studies and form a hidden bias. Besides absence of a biological clock to know the time point of mutation origin (Laing et al., 2016).
2. Materials and methods

2.1. Assay description

The ddPCR experiments were performed as a collective sample in a super-mix with 21µl per mix per well, excluding the DNA. The pipetting method was standardized by using automated BioRad Automated Droplet Generator with DG32 Automated Droplet Generator Cartridges (186-4108), producing 0.85 nl- sized droplets and are more efficient in droplets transferring compared to old-fashioned cartridges (Gerdes et al., 2016). These cartridges generate up to 20,000 droplets per sample, including template randomly. Poisson statistics was used to calculate the absolute quantification (ABS) multiplex of DNA molecules in the starting sample.

2.2. Samples

The sources of DNA used in this study were extracted from; 1) single adult worms of *H. contortus* and *T. circumcincta*, and 2) larval cultures that contain a mixture of parasitic nematodes including predominately *H. contortus* and *T. circumcincta*. DNA was extracted from adult worms and larval cultures by NucleoSpin® Tissue column (Macherey Nagel™) prior to this thesis.

The adult *H. contortus* were obtained from sheep 1) from different farms in Sweden where the drug efficacy had been evaluated with FECRT, or 2) from nine different countries and in general without background information about the resistance status. In addition, we used DNA from specified isolated female and male *T. circumcincta* from Swedish sheep (Table 1).

Table 1 - Adult worms DNA
Countries of Adult worms and resistance status, if known number of males are shown in brackets

<table>
<thead>
<tr>
<th>Country</th>
<th>Species</th>
<th>Resistance status</th>
<th>Number of adult worms*</th>
</tr>
</thead>
<tbody>
<tr>
<td>Argentina</td>
<td><em>H. contortus</em></td>
<td>Unknown</td>
<td>8</td>
</tr>
<tr>
<td>Australia</td>
<td><em>H. contortus</em></td>
<td>Unknown</td>
<td>14</td>
</tr>
<tr>
<td>Brazil</td>
<td><em>H. contortus</em></td>
<td>Unknown</td>
<td>24</td>
</tr>
<tr>
<td>Canada</td>
<td><em>H. contortus</em></td>
<td>Unknown</td>
<td>4</td>
</tr>
<tr>
<td>Ethiopian</td>
<td><em>H. contortus</em></td>
<td>Unknown</td>
<td>10</td>
</tr>
<tr>
<td>Germany</td>
<td><em>H. contortus</em></td>
<td>Unknown</td>
<td>15</td>
</tr>
<tr>
<td>Guadeloupe</td>
<td><em>H. contortus</em></td>
<td>Unknown</td>
<td>2</td>
</tr>
<tr>
<td>Kenya</td>
<td><em>H. contortus</em></td>
<td>Unknown</td>
<td>36</td>
</tr>
<tr>
<td>South Africa</td>
<td><em>H. contortus</em></td>
<td>Resistant</td>
<td>8</td>
</tr>
<tr>
<td>Sweden</td>
<td><em>H. contortus</em></td>
<td>Resistant</td>
<td>(7)</td>
</tr>
<tr>
<td>Sweden</td>
<td><em>H. contortus</em></td>
<td>Susceptible</td>
<td>15(10)</td>
</tr>
<tr>
<td>Sweden</td>
<td><em>T. circumcincta</em></td>
<td>Unknown</td>
<td>20 (10)</td>
</tr>
</tbody>
</table>

Swedish adult worms were characterized morphologically into males and females before DNA extraction and sequencing. They were used as wild and mutant type template during optimization process and as control during application of ddPCR on larval cultures samples.

Furthermore, we had access to larval cultures collected before and after anthelmintic treatment from several sheep farms in Sweden. The numbers of larval cultures obtained before and after treatment with different anthelmintic classes are described in (Table. 2).
Table 2 - The larval cultures
The number of larval cultures, from Swedish sheep farms, containing a mixture of different nematodes, which were tested for allele frequencies of dyf7-141 with ddPCR in relation to different anthelmintic. The FECRT efficacy is shown as % in brackets. MOX: moxidectin, IVM: ivermectin, ALB: albendazole, LEV: levamisole.

<table>
<thead>
<tr>
<th>Farm</th>
<th>MOX</th>
<th>IVM</th>
<th>ALB</th>
<th>LEV</th>
</tr>
</thead>
<tbody>
<tr>
<td>U</td>
<td>1 (100)</td>
<td>1(77)</td>
<td>1(96)</td>
<td>1(100)</td>
</tr>
<tr>
<td>D</td>
<td>2(93/65)</td>
<td>1(99)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>I</td>
<td>2(100/78)</td>
<td>2(96/100)</td>
<td>2(100/100)</td>
<td></td>
</tr>
<tr>
<td>H</td>
<td>1(65/100)</td>
<td>1(100)</td>
<td>1(100)</td>
<td></td>
</tr>
<tr>
<td>G</td>
<td>1(100)</td>
<td>1(87)</td>
<td>1(100)</td>
<td>1(100)</td>
</tr>
<tr>
<td>A</td>
<td>1(100)</td>
<td>1(78)</td>
<td>1(77)</td>
<td></td>
</tr>
<tr>
<td>R</td>
<td>1(100)</td>
<td>1(91)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>M</td>
<td>1(44)</td>
<td>1(100)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>P</td>
<td>1(97)</td>
<td>1(100)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>F</td>
<td>1(100)</td>
<td>1(100)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Z</td>
<td>1(100)</td>
<td>1(100)</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

2.3. Droplet digital PCR assay development

2.3.1. Sequencing of adult worms
This was done by using either short or long primers including the position of SNP-141(Table 3). Primers were designed by Primer3Plus from sequences in GenBank (Accession number: KF927016.1, KF927017.1, KF927018.1, KF927019.1).

Conventional PCR was performed in 22 µl reactions containing 1X PCR buffer, 0.5 mM MgCl, 0.2 µM of each primer set, 200 µM dNTP, 1.25 units/50 µl PCR AmpliTaq Gold® (ThermoFisher) and to 22 µl water. Thermocycling PCR conditions were as following; denaturation step at 95°C (10 min), followed by 40 PCR cycles of 95 °C (30 sec), annealing 57 °C (30 sec) and elongation 72 °C (1 min) and deactivation step at 98 °C (10 min). Primers used are shown in (Table 3). Amplicons were run on 1- 2% agarose with a 100 bp ladder and checked by a Bio-Rad Gel Doc™ system. Amplicons were prepared according to Macrogen’s guidelines https://dna.macrogen.com/eng/support/ces/guide/order_guide.jsp and then sent for Sanger sequencing by Macrogen.

2.3.2. Designing of ddPCR primers and probes
All available sequences obtained for adult H. contortus, were imported and aligned with MAFFT ver. 7 in Mesquite ver. 3.2 (Maddison and Maddison 2017). Primers and probes for ddPCR were then designed in silico based on multiple sequences obtained as described above with help of Primer3Plus, according to the Droplet Digital PCR Applications Guide (Bio-Rad 2014). We picked (5´ TCGGATCCAGACATTGC 3´) as the forward primer sequence and (5´ CATCACAATGTTTCCCGTA 3´) as the reverse primer sequence. The lengths of the amplicons produced with the short and long primers 262 bp and 783 bp respectively.

Amplicons containing SNP-141 (Fig. 2), were generated by the short primer set through ddPCR (Table. 3). The two tested hydrolysis probes were labeled either with a Black Hole Quencher (new probe) or Iowa Quencher (old probe) on the 3´ end, and a FAM or HEX fluorophore at the 5´ end. Amplicon
lengths for both targets (MT and WT) were 262 bp fragment (Table. 3). Primers and probes were ordered from Eurofins (primers and new probe) or Integrated DNA Technologies (old probe).

![Genetic map of dyf7](image)

**Figure 2 - Genetic map of dyf7**

There are three single nucleotide polymorphisms (SNPs) of dyf7 at 141, 234 and 438 suggested to be associated with ivermectin resistance in *H. contortus*. As seen in the figure SNPs are located on different exons. **F**: a forward primer. **R**: a reverse primer. **P**: short amplicon of interest cover a 262 nt stretch from exons 2 to 3 of *dyf7*. **P**: long amplicon of interest cover a 783 nt stretch from exons 2 to 4 of *dyf7*.

<table>
<thead>
<tr>
<th>Nucleotides</th>
<th>Sequences (5’ to 3’)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Long PCR primers amplifying a</td>
<td>(forward) TGG ACG AGG TGT CAT GCT CGT</td>
</tr>
<tr>
<td>783-bp fragment</td>
<td>(reverse) TAT CAG GTG TCA GAG CAA CGT AA</td>
</tr>
<tr>
<td>Short PCR primers amplifying a</td>
<td>(forward) TCG GAT CCA GAC ATT GC</td>
</tr>
<tr>
<td>262-bp fragment</td>
<td>(reverse) CAT CAC AAT GTT TCC GT GT</td>
</tr>
<tr>
<td>Old probes [G vs C =5/6] = 20 nt</td>
<td>(FAM)(mutant type) <strong>CG</strong> AAA TCG CAA <strong>CCT</strong> GTG GT</td>
</tr>
<tr>
<td></td>
<td>(HEX)(wild type) <strong>CA</strong> AAA TCG CAA <strong>CG</strong> GTG GT</td>
</tr>
<tr>
<td>New probes for the complement</td>
<td>(FAM)(mutant type) <strong>AC</strong> GGT TGC GAT <strong>TTC</strong> GGC</td>
</tr>
<tr>
<td>strand [G vs C =6/4] = 18 nt</td>
<td>(HEX)(wild type) <strong>AC</strong> GGT TGC GAT <strong>TTT</strong> GG C</td>
</tr>
</tbody>
</table>
2.3.3. ddPCR optimisation

During ddPCR optimization process, samples were tested in biological replicates and analyzed in technical duplicates or triplicates. Every assay was also designed to include a non-template control (NTC), which was prepared from the 21µl master mix without DNA template, to avoid any pre-reaction contamination.

Annealing temperature

In the annealing temperature experiment, DNA templates from adult worms were separated to mutant (MT) and wild (WT) based on sequence information and then tested in biological replicates. Short primers/old probe set concentration was 900/250 nM added to the reaction mixture and amplified by the basic PCR protocol as following; initial activation of the Taq polymerase at 95°C (10 min), followed by 45 PCR cycles of denaturation step at 94°C (30 s), annealing 57°C (1 min) and elongation 72°C (2 min) with ramping rate 2°Cs⁻¹ and enzyme deactivation step at 98°C (10 min). The temperature gradient ranged down from 59°C to 52°C, to analyse the effect of the annealing temperature within a narrow range.

Probes

Two types of probes (Old and New) with a different G/C content (Table 3) were compared. The performance of both probes was analyzed by three biological samples from adult mutant worm DNA and with technical triplicates. Probes were evaluated according to the magnitude of the relative fluorescence units (rfu).

Primer/probes concentration

According to MIQE guidelines for ddPCR, five different primer/probe concentrations were tested as following (900/250 nM, 900/400 nM, 1000/400 nM, 800/350 nM and 700/500 nM), using sample mixture formed from single (1MT: 1WT) adult worm DNA and amplified with the basic PCR protocol.

Dynamic range

The dynamic range specifies the lowest concentration of the template at which ddPCR assay could detect the SNP of interest. To decide the suitable DNA template concentration, the dynamic range experiment was held. The experiment was designed in duplicates to test equal ratio of mutant and wild types DNA in different volumes (1, 2 and 4µl) after amplification by the basic PCR protocol.

Restriction enzymatic digestion

HaeIII was the only restriction enzyme recommended by Bio-Rad that does not interfere amplicon sequence according to BLAST by NBCI. Restriction enzyme was added as 1µl to the ddPCR reaction mixture while the same amount was subtracted from water volume. The experiment was designed as technical duplicates from two different biological replicates representing either the MT or WT template. Samples were incubated during different times (0, 30 and 60 min) at room temperature 37°C, before the droplets generation step. The reaction was amplified by the basic PCR amplification protocol.

Number of PCR cycles

According to MIQE guidelines, increasing cycles number usually improves amplicon harvest and amplitude signal. Cycles may range between 40 cycles to 60 cycles. This experiment was designed to examine separate worm DNA (MT and WT) in duplicates that were amplified for three different cycles (45, 50 and 55cycles) within the basic PCR protocol.
Modified PCR protocol
The final optimized PCR protocol, which was used for testing samples as the following: initial activation of the Taq polymerase at 95°C (10 min), followed by 45 PCR cycles of denaturation step at 94°C (30 s), annealing 57°C (1 min) and elongation 72°C (2 min) with ramping rate 2°C s\(^{-1}\) and enzyme deactivation step at 98°C (10 min) according to (Uchiyama et al., 2016).

Limit of detection
To specify the limit of detection for the mutant allele frequency, serial two-fold dilutions of MT template DNA (either from a single mutant worm or containing a mixture of 3 mutant worms) were prepared by diluting the MT template in supermix dispensed into a constant background of WT template. The final proportions (volume to volume) of MT/WT were 40, 20, 10, 5 and 2.5 %, respectively.

Pilot assay
The fractional abundance (%) from the optimized ddPCR assay was compared to the actual prepared mixtures containing both WT and MT. Mixtures were formed from WT and MT mixed at different proportions (eg. WT/MT; 100/0, 80/20, 50/50, 20/80, 0/100). All mixtures were tested in technical duplicates.

Application of ddPCR assay
The optimized ddPCR protocol was finally used to measure dyf7-141 alleles frequencies in nematode larval cultures obtained from sheep faeces. Samples (i.e. larval cultures) were tested both before and after anthelmintic treatment and were classified into two groups; 1) IVM treated group or 2) by another anthelmintic (MOX, LEV, and ALB) treated group. All samples were tested at least in duplicates with mutant adult worm DNA control and NTC as positive and negative controls, respectively.

Statistical analysis
All raw data was initially analyzed and graphs were produced by QuantaSoft\textsuperscript{TM} ver. 1.7.4 software (BioRad). Data output was evaluated and collected in different formats as; copies per µl, accepted droplets and fractional abundance (%). Thresholds in all experiments were mostly defined automatically by the software. The manual threshold was only used in specific situations (e.g. No Call) to set a middle line between positive and negatives populations in both channels via the 1D plot graph. Additional graphs were designed by GraphPad Prism v 7 and the haplotype network was designed by Popart v 1.7.

3. Results

3.1. Conventional PCR
Sheep in Sweden is predominately infected with *H. contortus*, *T. circumcincta* and *Trichostrongylus*, so it was important to detect presence of any cross reactivity between these important species during developing primers and probes. To get additional information on the nucleotide variation in dyf7-141, necessary for the ddPCR primer and probe design, samples from 163 adult worms (including both *H. contortus*, *T. circumcincta* and *Trichostrongylus*), were amplified by conventional polymerase chain reaction (PCR). Short primers were used to sequence specific region of dyf7-141 and detect any repetitive pattern for SNPs, while long primers were used to investigate a wide region in case presence of common nematodes similarities.
Amplicons of conventional PCR were amplified with the long or short primer sets using either *Haemonchus*, *Teladorsagia* or *Trichostrongylus* DNA as templates generated high-quality sequences for both species tested. Firstly, samples from different sources were tested in a preliminary PCR experiment with ddPCR short primers to evaluate primers suitability. In this experiment all samples produced amplicons as shown in (Fig 3), which indicated primers suitability besides possibility of cross reactivity with *T. circumcincta*. long primers were used in following conventional PCR to test twenty samples of *T. circumcincta and Trichostrongylus spp.* *T. circumcincta* amplicons indicates primers cross reactivity, while *Trichostrongylus* was not as shown in (Fig 4) and for that positive samples were sent for sequencing. Foreign adult *H. contortus* DNA were tested with long primers to build a SNPs map, which shows relationship between SNPs pattern and geographical sources. Results are shown in (Figs. 5 to 7) indicate that the amplicons with the long primers were between 600 and 700 nt.

![Figure 3 - Preliminary PCR](image)

The first PCR was done for all samples type to detect suitability of ddPCR short primers. All samples produced amplicons. **T.cir** for sample from *Teladorsagia*, **N.H5**, **N.H10**, **H8** and **H13** are samples from adult *H. contortus* DNA, while **H15**, **H41**, **H59** and **H64** are samples from larval cultures and **NTC** for negative control containing loading dye only. The amplicon fragment length is around 300 bp. One-hundred bp ladder was used.
Figure 4 - Primer cross reactivity
Twenty samples of *Teladorsagia* were tested by *H. contortus* long primers. The amplified fragments with 783 nucleotides length from some samples of *Teladorsagia* DNA were sent for sequencing. Only one *Trichostrongylus* was involved in a preliminary way and was negative. One-hundred bp ladder was used.

Figure 5 - PCR amplification with long primers for adult *H. contortus* DNA from Brazil and Kenya
Foreign adult *H. contortus* DNA were amplified with long primers and sent for sequencing. Amplicon fragment length is around 783 bp. B for samples from Brazil, K for samples from Kenya. One-hundred bp ladder was used.
Figure 6 - PCR amplification with long primers for adult *H. contortus* DNA from Ethiopian, Canada and South Africa

Foreign adult *H. contortus* DNA were amplified with long primers and sent for sequencing. Amplicon fragment length is around 783 bp. E for samples from Ethiopian, C for samples from Canada and S samples from South Africa. One-hundred bp ladder was used. Negative samples were repeated again in next PCR.
Figure 7 - PCR amplification with long primers for adult *H. contortus* DNA from Argentina, Guadeloupe and South Africa

Foreign adult *H. contortus* DNA were amplified with long primers and positive amplicons were sent for sequencing. Amplicon fragment length is around 783 bp. Ag for samples from Argentina, G for samples from Guadeloupe, -VE sample for negative control containing a loading dye only and S samples from South Africa, which some were negative in previous PCR. One-hundred bp ladder was used.

Then short primers were used to test more of foreign adult *H. contortus* DNA to help in building a haplotype map, generated amplicons were around ≈ 262 nt. Negative samples in previous PCR with long primers were repeated with short primers to assure from they are negative samples.
Figure 8 - PCR short amplicons of foreign adult H. contortus DNA (group 1)
Foreign samples were tested with short primers and positive amplicons were sent for sequencing to investigate SNPs in details. Amplicon length is around 262 bp. K for samples from Kenya, K, K for samples from Kenya kesima and Aus for samples from Australia. One-hundred bp ladder was used.

Figure 9 - PCR short amplicons of foreign adult H. contortus DNA (group 2)
Different foreign adult H. contortus DNA were amplified with short primers for sequencing. Amplicon length is around 262 bp. K for samples from Kenya, B for samples from Brasil and Ag samples from Argentina. One-hundred bp ladder was used.
Figure 10 - PCR short amplicons of foreign adult H. contortus DNA (group 3)
Foreign adult H. contortus DNA samples were tested with short primers. Positive amplicons of foreign adult H. contortus DNA were sent for sequencing. Amplicon length is around 262 bp. Aus for samples from Australia, G for samples from Germany and GH for samples from Germany (Heise). One-hundred bp ladder was used.

Figure 11 - PCR short amplicons of foreign adult H. contortus DNA (group 4)
Different foreign adult H. contortus DNA were amplified with short primers. Amplicon length is around 262 bp. These PCR experiment included negative samples from previous long primers PCR. Ag for samples from Argentina, B for samples from Brazil, C for samples from Canada, E for samples from Ethiopian, G for samples from Guadeloupe, K for samples from Kenya and SA for samples from South Africa. One-hundred bp ladder was used.
Figure 12 - The second PCR with short primers for negative adult H. contortus DNA
This PCR experiment was for negative amplicons in figures 8, 9 and 10 with short primers. All positive amplicons were sent for sequencing. The amplicon fragment length around 262 bp. \( \text{Ag} \) for samples from Argentina, \( \text{C} \) for samples from Canada, \( \text{K} \) for samples from Kenya, \( \text{B} \) for samples from Brasil, \( \text{K} \) for samples from Kenya kesima and \( \text{GH} \) for samples from Germany (Heise). One-hundred bp ladder was used.

Sequencing generated 181 and 14 high-quality sequences for \( H. \) contortus and \( T. \) circumcincta, respectively. When viewed in Mesquite, it was evident that the sequences for each species were highly different and according to \textit{in silico} analysis, there are no opportunities for cross-reactions between species with the probes used in the ddPCR as shown in (Fig 13).
Figure 13 - Probe binding site in adult *H. contortus* DNA
The figure shows examples of sequences for some amplicons of wild and mutant adult *H. contortus* DNA from different countries. Wild type samples had an identical sequence as marked by red box, while mutant type samples had divergent sequences. Probe alignment with sequences showed binding suitability for both wild and mutant DNA.

Figure 14 - Probe unspecificity to adult *T. circumcincta*
Probe binding sites related to SNPs presence were marked with (x) to compare between sequenced amplicons of adult *T. circumcincta*, wild and mutant type *H. contortus*.
The sequence diversity within *dyf7-141* for all (263) samples based on a 124 bp fragment spanning exon 2-3 was displayed together with previously published sequences (Urdaneta et al. 2014), in a haplotype network (Fig.14). As shown in this figure all wild-type sequences were alike irrespective of origin of *H. contortus* isolates. In contrast, the mutant isolates were much more various and they contained 20 haplotypes. The Swedish samples contained four mutant haplotypes and involved one pre-identified haplotype (Urdaneta et al. 2014). They differed at most in 5 positions, which makes no specific sequence for resistant allele. Among a total of 16 high quality sequences obtained from adult Swedish worms all mutants (7) were males.

### Figure 15 - Dyf7 haplotypes network of Haemonchus contortus

The network based on a 124 bp exon 2-3 fragment amplified and sequenced by short primers described in (Table.3). Susceptible group has the same sequence and represented by blue colour for (Swedish samples) and green colour for (foreign samples). Resistant groups have different sequences and divided according to mutations number of nucleotides. Red resistant groups represent foreign resistant samples, while purple resistant groups represent Swedish resistant samples. The number of samples in every group is represented by the size of the group circle.

**3.2. Optimization of ddPCR assay**

To develop a specific ddPCR assay toward *dyf7-141* gene, there were a series of required experiments according to MIQE guidelines.

**3.2.1. Annealing temperature**

Specification of annealing temperature was a necessary to ensure the suitable temperature for amplification of assay mixture. The results of the most suitable annealing temperature for primer/probe set was investigated through a uniplex assay of thermal gradient PCR, which ranged from 59°C to 52°C. The optimal band separation and the highest amplitude for both probes was observed at 54.5°C. At this temperature, we noticed few rains i.e. intermediate droplets between positive and negative droplets (Fig. 7).
Figure 16 - Temperature gradients PCR for optimizing annealing temperature
In a single assay mutant (A) and wild-type DNA (B) were tested at different temperatures ranging from 59°C to 52°C. The figure shows the highest FAM and HEX amplitude at 54.5°C.

3.2.2. Selection of probes
Old probes were designed from previous pilot study, while new probes were designed herein. The comparison between two different probes (Table 3) with different G/C contents was done. Although the number of copies per µl was detected steadily irrespective to the probe type, the old probe showed a stronger rfu compared to the new probe (Fig. 8). Thus the old probe was selected for the following optimization process as it produced a distinct separation between positive and negative droplets.

Figure 17 - Probe selection
Two types of probes were tested with three different mutant adult worm DNA as a target in a uniplex assay. (A) FAM amplitude was much higher with the old probe than with the new probe. (B) Both probes detected the same copies/µl per sample. No template control (NTC) was used as negative control.

3.2.3. Primer/probe concentration
For the optimization process, the most suitable concentration of primer/probe set is specified through the comparison between different concentrations. Different mixtures of primer/probe sets were compared in a duplex assay with equal proportion mixture of mutant and wild adult worm DNA or NTC as template and NTC. Obtained results presented the optimal concentrations of primer/probe was 700/500nM, and as shown in (Fig. 9), this ration induced the highest amplitude for the positive population (mutant target).
Figure 18 - Primer/probe concentration
The duplex assay was designed to compare different primer/probe concentrations in triplicates with no template control (NTC). The selected concentration was 700/500nM with clear separation between bands and the highest amplitude strength (the black band represents negative droplets and the thicker blue band is droplets containing mutant DNA.

3.2.4. Dynamic range
Sample concentration affect copies detection in the reaction, so the dynamic range of ddPCR should be assessed to detect the suitable concentration to detect the target. The dynamic range of the ddPCR assay was determined through using different targets volumes (i.e. increasing concentrations) in the ddPCR reactions (i.e. 1, 2 and 4 µl of a 1:1 mixture of mutant and wild-type). The 1D plot graphs present the ability to detect low and high sample concentration (Fig. 10A and 10B), whereas the 2D plot shows the separation between droplets for all wells of Mix1 (Fig. 10C). Results are also shown in the concentration graph (Fig.10D), which demonstrates that copy numbers increased in accordance with the sample concentration both for the mutant and wild-type targets, but the FA was stable irrespective of the concentration tested. This was regardless of the used target mixture concentration.
Figure 19 - The dynamic range of the assay

Three concentrations (1, 2 and 4 µl) of a mixture containing mutant and wild targets (v/v 1:1) were tested in a duplex assay. Two biological replicates (Mix1) and (Mix2) were tested to determine the dynamic range of the assay. The 1D plot in (A) shows detection of mutant DNA and (B) detection of wild DNA, whereas the 2D plot (C) shows all results combined for Mix1, which demonstrates the excellent separation between the different populations irrespective of the sample dilution. The concentration graph in (D) shows that the fractional abundance (%) (Blue diamonds) was constant whatever the concentration of added targets.

3.2.5. Feasibility of the restriction enzyme

The restriction enzyme is suggested by BioRad to decrease rainy droplets but the type of enzyme, duration of incubation and feasibility of the enzyme is assessed individually according to each assay. So,
the usefulness of restriction enzyme (HaeIII) in the ddPCR reaction was assessed through different incubation times (i.e. 0 min, 30 min, 60 min) at room temperature before droplet generation. The outcome from this experiment was not affected by the restriction enzyme (Fig. 11). Therefore the restriction enzyme was not included in the final optimized ddPCR assay.

Figure 20 - The feasibility of the restriction enzyme
In a uniplex assay wild and mutant worm DNA were tested separately with a restriction enzyme (HaeIII) and incubated at room temperature for three different time points (0min, 30min, and 60min) before droplet generation step. (A) Mutant DNA and (B) Wild DNA had the same clear band separation between different time points. Restriction enzyme had not any effect on our amplicon length or rainy droplets.

3.2.6. Cycle numbers
PCR cycles can be ranged from 40 cycles according to basic Bio-Rad recommendation to 60 cycles according to (Huggett et al. 2013). By increasing PCR cycles number, increases the amplicon amount and facilitates detection. The experiment was performed for three different PCR cycle numbers (45, 50 and 55) and the results are displayed in 1D plot graphs. By increasing the cycle numbers, the amplitude was gradually increased (Fig. 12). Tested adult worm DNA did not show any nonspecific amplification in wild allele wells or variation in FA (%) more than 5%. 
3.2.7. Limit of detection

For our test validation, we investigated the lowest detectable mutant allele frequencies that could be detected. The results were obtained from two biological replicates through two-fold serial dilutions of mutant adult worm DNA (i.e. 40, 20, 10, 5 and 2.5%) dispensed in a constant background of wild adult DNA. Mutant DNA from a source that contained 126 copies/µl was detectable down to 2.5% dilution, while another mutant DNA sample with only 23 copies/µl was detected only down to 5% dilution (Fig. 13). Limit of detection was not affected by the presence of DNA background.
Figure 22 - Limit of detection
Two-fold serial dilutions of mutant adult worm DNA (blue) were prepared with constant wild adult worm DNA concentration (green) and compared to 100% mutant and wild adult worm DNA. Concentration graph shows copies/µl in (A) Contains three mixed mutant DNA and the limit of detection was 2.5%, as mutant sample 100% contained 126 copies/µl. (B) Contains single mutant DNA and detection limit was 5%, as mutant sample 100% contained 23 copies/µl.

3.2.8. Pilot assay
To evaluate the optimized duplex ddPCR assay test fidelity, a pilot assay containing different known concentrations of WT/MT adult worm DNA (i.e. (v/v) 100/0, 80/20, 50/50, 20/80 and 0/100) was performed. The fractional abundance (%) was calculated by Quantasoft. As can be seen from the results mutant type DNA increased in accordance with the sample dilutions, whereas the wild type showed the opposite pattern (Fig.14). Thus, the optimized ddPCR assay proved the ability to detect known mixed samples.
Figure 23 - Pilot assay
Different concentrations of mixed wild and mutant type DNA (100% WT, 80/20 MT, 50/50, 20/80 and 100% MT) were tested in duplicates. The concentration graph presents the fractional abundance (%) for mutant allele frequencies was calculated by Quantasoft. Numbers next to the green square shows copy number of wild type, while numbers next to the blue squares show the copy number of the mutant allele. The fractional abundance (%) represents by numbers next to the blue diamonds. FA % expresses the percentage of mutant allele in the template mixture, which is nearly similar to the actual content.

3.2.9. Application of ddPCR assay
After excluding all channels with No Call or low accepted droplets below 10,000 or mutant allele < 2 copies/µl from the results, results were compared with FECRT results.

*Dyf-7 allele frequencies in larval cultures*
After pilot assay validation, the duplex ddPCR assay was ready to test mutant allele frequencies in larval cultures obtained from different sheep farms and with different treatment histories. An example of *dyf-7* frequencies from the same farm compared before and after treatment is shown in (Fig.15). These samples had FECRT 44% reduction after IVM treatment, according to previous FECRT results. Fractional abundance (%) results are shown in (Fig. 15D) beside blue diamonds and as can be seen in the graph they were nearly similar both before and 13 days after treatment.
Figure 24 - Comparison between dyf7 allele frequencies before and after treatment

The selected samples from farm P were before treatment sample with concentration (49 ng/µl), while after treatment sample with concentration (19 ng/µl). The 1D plot in (A) and (B) shows a very high droplets density of mutant and wild targets in both before and after samples. (C) The 2D plot shows a good separation between different populations in the optimized assay. (D) Concentration graph shows the FA (%) stability of dyf7 between before and after treatment.
**Dyf-7 allele frequencies pattern**

All data for larval culture obtained by Quantasoft and then figured by Graphad Prism 7. Larval cultures tested from different farms showed a range for *dyf-7* allele frequency on 6 different farms on different sampling occasions (Fig. 16A). On all IVM treated farms FA(%) was nearly stable before and after treatment (Fig. 16B).

![Figure 25 - Allele frequencies of dyf7-141 in Haemonchus contortus](image)

(A) Different farms show nearly identical *dyf7*-141 allele frequencies irrespective of anthelmintic class used. (B) Farms show similar *dyf7* allele frequencies before and after treatment with ivermectin (except two outliers).

### 4. Discussion

The suitability of *dyf7-141* as a molecular marker for IVM resistance in *H. contortus* was recently suggested by (Urdaneta et al. 2014). In this study, we first developed and validated a ddPCR assay that then was utilized to measure the mutant allele frequencies of *dyf7-141* in faecal larval cultures containing a mixture of strongylid nematode species. Although, larval samples were collected from Swedish sheep farms both before and after anthelmintic treatment, no significant shift in the factional abundance (%) of mutant allele was observed in surviving parasites collected after IVM treatment.

The first aim of this study was to develop and validate a technique for quantification of SNP at *dyf7-141*. The workflow in ddPCR is similar to those used for most standard TaqMan probe-based assays. However, a key aspect of ddPCR is sample partitioning into several thousand droplets, which allows for absolute quantification of rare alleles (Huggett et al., 2013). Presence of rain (droplets between positive and negative populations) may hinder droplets analysis. Thus one method to decrease this mess through specifying a suitable manual threshold to definitely separate populations (Gerdes et al., 2016). The manual threshold is applied by detecting the midpoint between positive and negative amplitudes (Pinheiro et al., 2012), we had no problem to observe differences between positive and negative droplets with the conditions set for the present *dyf7-141* ddPCR assay. The major differences between the two tested probes were their G/C content and their length of nucleotides, but the results were not severely affected except for the strength of amplitude signal (as shown in Fig.8). According to the Droplet Digital PCR Applications Guide, the most suitable primer/probes concentration is 900/250 nM (Bio-Rad 2014). In the present study, we assessed 5 different primer/probe concentrations in an attempt to further improve band separation, but in this case it seemed to be a factor of limited importance. Another option to decrease rain is to subject the template DNA to enzymatic digestion. While this enhanced droplets signal...
for example in an experiment on mating types genes in rotifers (Koch et al., 2015). However, for others it did not work at all (e.g. Gerdes et al., 2016), which is in agreement with our results. As restriction enzyme probably did not affect amplicon length to any major extent, rainy droplets remain constant. According to Bio-Rad the recommendation is to use 40 cycles, but through MIQE guidelines, the user can select another suitable number of cycles for the specified assay under development (Huggett et al., 2013). When the number of cycles in the current study was increased from 40 to 55 cycles, this affected the amplitude signal of the present dyf7-141 assay. From our results it was safe to select the highest numbers of cycles, as the template was worm DNA with lower tendency for non-specific amplification compared to genomic DNA or gblocks (Whale et al., 2013). Also wild type DNA has not shown any unacceptable non-specific amplification, as FA (%) variations remain \( \leq 5\% \).

Overall we found out that the dynamic range of our ddPCR assay is very sensitive enough to measure larval cultures samples at different concentrations without prior justification, which in contrast to a previous study where it was important to test three sample concentrations from each allele (Koch et al., 2015).

It is crucial for a routine test that it can employed with great accuracy on different occasions. It is also important that it can measure FA (%) allele frequencies with the same sensitivity in different samples. For natural reasons the limit of detection in a sample depends upon sample concentrations of the mutant allele (Uchiyama et al., 2016). In this study we showed that a sample with 30 copies/µl of mutant allele in the concentrated sample could be detected when it was diluted down to 5% but not when diluted down to 2.5%. Furthermore, we demonstrated that the limit of detection for the mutant allele was independent on the wild-type allele frequency (Fig. 14). Also test fidelity of ddPCR was proven after repetition of experiments generating the same FA (%) results following re-analysis of the same samples.

The second aim of this study was to measure mutant allele frequencies in faecal larval cultures collected on sheep farms in Sweden both before and then again in cultures collected 7-13 days after treatment with different anthelmintics. A total of 42 of the samples were obtained from farms where IVM treatment failures have been described recently (Höglund et al., 2015). The working hypothesis was that in case dyf-7 is involved in the resistance then the mutant allele frequency should increase after treatment in accordance with the observations by (Urdaneta-Marquez et al., 2014). Secondly, we also expected a fairly low mutant allele frequency as none of the ninety Swedish surveyed sheep farms showed signs of harbouring IVM resistant *H. contortus* between 2006 and 2007 (Höglund et al., 2009). Analysis of mutant dyf7-141 allele frequencies in DNA from larval cultures revealed three major findings. Firstly, all mutant allele frequencies were sometimes high and also in samples obtained pre-treatment. They had a FA between 3 and 43%. Secondly, on sample level FA (%) of dyf7-141 was nearly similar in cultures obtained before and after IVM treatment with variations in dyf7-141 FA (%) \( \leq 5\% \), which are within the acceptable level for both the false positive and negative rates (Dobnik et al., 2016). Thirdly, dyf7-141 FA (%) in larval cultures collected on different occasions at the same farms were within a certain range for each farm whatever anthelmintic class was used for treatment. In essence these results are in agreement with (Laing et al., 2016), who demonstrated no differences even between IVM treated and non-treated UK farms. Thus, based on these observation the most likely explanation is that SNP in dyf7-141 is unrelated to IVM resistance, which is in contrast to the main postulation of a correlation between IVM resistance in *H. contortus* and mutations in the dyf gene (Dent et al. 2000; Urdaneta-Marquez et al., 2014).

These results were further supported by the analyses of dyf7 sequences obtained from continental adult *H. contortus* and designing the haplotype network. Our final explanation for repeated negative samples in conventional PCR with short and long primers, was may be due to damaging of samples content by time.
All susceptible samples (e.g. wild type) were identical irrespective of their geographical origin of the worms. As the mutant samples were had several divergent alleles, they do not have a unique resistant sequence, which could be related to geographical origin (Laing et al., 2016). In agreement with (Urdaneta-Marquez et al., 2014), we also found that SNPs at position A141G indicates the presence of other substitutions in other positions such as T234C and G438T. Furthermore, all the mutant samples from Swedish farms were male worms and they could not be linked to the IVM resistance status of the sheep farms of origin. This is in contrast to (Urdaneta-Marquez et al., 2014) who proved that dyf7 is a sex-linked gene and increased in treated populations. However, it is partly in agreement with a recent study, where dyf7 along with a number of other candidate IVM resistance loci could not be correlated to IVM resistance after serial backcrossing (Rezansoff, Laing and Gilleard, 2016). Similarly, several other studies had proved no direct relationship between dyf7 mutation and the IVM resistance status (Laing et al., 2016; Rezansoff, Laing and Gilleard, 2016).

Conclusion
The results show that the ddPCR assay provides a sensitive diagnostic tool with our molecular marker dyf7-141. Thus there is no doubt about that ddPCR is suitable to measure mutant allele frequencies in mixed larval culture samples generating high fidelity results. However, after analysis of larvae in faecal cultures, the results could not prove any relevance between dyf7-141 mutation frequency and IVM treatment history. More investigations are required to identify another and more suitable candidate gene linked to IVM resistance in H. contortus.
Abbreviations

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<th>Abbreviation</th>
<th>Definition</th>
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<tr>
<td>BLAST</td>
<td>Basic Local Alignment Search Tool</td>
</tr>
<tr>
<td>Dyf gene</td>
<td>Dye filling gene</td>
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<tr>
<td>ddPCR</td>
<td>Droplet digital polymerase chain reaction</td>
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<tr>
<td>Droplets</td>
<td>The divisions of the reaction and could reach to 20,000 droplets</td>
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<tr>
<td>Dropout</td>
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<td>The relevance between mutant to both wild and mutant allele frequencies</td>
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<td>MIQE</td>
<td>Minimum Information for publication of Quantitative real-time PCR Experiments</td>
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<td>Population</td>
<td>Group of similar droplets</td>
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<tr>
<td>Negative population</td>
<td>Droplets do not contain DNA</td>
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<tr>
<td>Positive population</td>
<td>Droplets contain either wild or mutant DNA</td>
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<td>Double positive population</td>
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<td>Rains</td>
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<td>The single nucleotide polymorphisms</td>
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<td>Threshold</td>
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</table>

Acknowledgement

Johan Höglund is thanked due to encouraging me to depend on myself from designing the experiment to change the protocol completely, he usually opens to all possibilities. Several informative discussions about *Haemonchus*, the main aim of research in this area and intensive debates ended by yes or no answer to stop wasting time and resources in a chronological manner, which I can see. Moa Skarin learned me from beginning and solved a lot of technical issues. Peter Halvarsson is thanked for designing the haplotype network. Behdad Tarbiat is thanked for sequences alignment instructions by using Bioedit software. Genetic department members for their informative discussion about utilizing ddPCR machines.

Special thanks for my small family, my son presence, supported and pushed me toward my goals.
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