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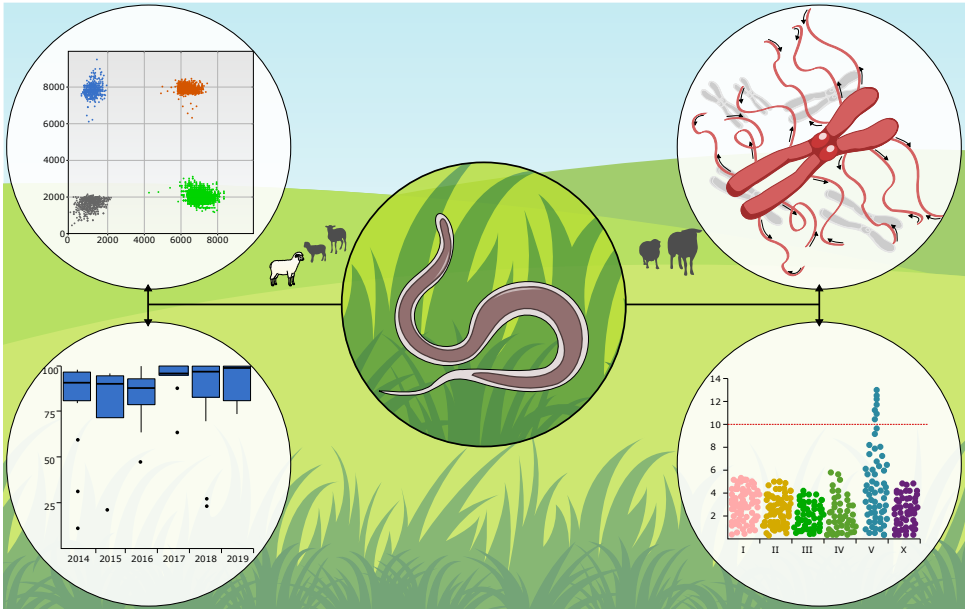
The genetic basis and monitoring of anthelmintic resistance in the pathogenic endoparasites of small ruminants

Paulius Baltrušis



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Abstract

By parasitizing livestock, gastrointestinal nematodes inflict a significant monetary burden on the producers and compromise animal health as well as the sustainability of animal farming systems. To date, the most reliable way to control parasitic infections in grazing livestock is through the use of anthelmintic drugs. However, decades of use have led to the parasites developing resistance. In small ruminants, one of the most pathogenic and prone to developing resistance is the parasite *Haemonchus contortus*. This thesis aims to describe our most recent efforts to update the current knowledge on anthelmintic resistance in sheep nematodes by studying *H. contortus* in order to i) develop molecular tools to screen for mutations associated with resistance, ii) use these tools to carry out country-wide screenings for resistance mutations on Swedish sheep farms as well as iii) investigate potential, resistance-associated candidate loci and genes through both the single-candidate and the genome-wide comparison approaches. We developed a sensitive droplet digital PCR-based tool to screen for benzimidazole resistance-associated mutations and subsequently found extremely high frequencies of the F200Y mutation but not F167Y in Swedish, field isolates of *H. contortus* collected over 6 years from 67 sheep farms. We also investigated the 63bp deletion in the *hco-acr-8* as well as its role as a possible marker for levamisole resistance and found it to correlate with (but be an unreliable predictor of) the resistant phenotype. Finally, we carried out a genome-wide comparison study between the ivermectin treated and untreated population and found a region in chromosome V to show signs of recent selection, indicating the importance of that locus in the development of ivermectin resistance in sheep nematodes.

Keywords: benzimidazole, levamisole, ivermectin, anthelmintic resistance, F200Y, hco-acr-8, droplet digital PCR, sequencing, GWAS, genome

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Santrauka

Virškinamojo trakto apvaliosios kirmėlės – nematodai – parazituojančios gyvulius, ne tik riboja gyvulių pramonės pelningumo rodiklį tačiau taip pat kenkia ir gyvulių sveikatai, gerovei bei galiausiai - aplinkai. Vienas patikimiausių būdų užkirsti kelią parazitinių kirmėlių daromai žalai (gyvuliams) - pagrįstas antihelmintinių vaistų naudojimu. Deja, dešimtmečius trukęs šių vaistų vartojimas pramonėje lėmė susidariusį atsparumą nematoduose. Vienas iš pavojingiausių, bei labiausiai linkusių išsivystyti atsparumą vaistams, parazitų priklauso rūšiai *Haemonchus contortus*. Šios disertacijos, kurioje tirtas nematodų atstovas *H. contortus*, tikslas sudarytas iš trijų esminių dalių: i) sukurti molekulinį įrankį, kuris padėtų įvertinti su vaistų atsparumu susijusių mutacijų dažnį nematoduose, ii) panaudoti šį įrankį nustatant mutacijų dažnį švediškų avių ūkiuose bei iii) panaudojant vieno-kandidato bei genomo apskrities palyginimo metodus, įvertinti tam tikrų genų arba lokusų svarbą vaistų atsparumo išsivystimui nematoduose. Mes sukūrėme itin jautrų bei patikimą mutacijų, susijusių su atsparumu benzimidazolo dariniams, nustatymo įrankį, kurį vėliau panaudojome nustatyti mutacijų F200Y (nustatyti ypatingai aukšti dažniai) ir F167Y (nustatyti labai žemi dažniai) dažnius, naudojant mėginius iš 67 avių ūkių, surinktus per šešerius metus. Taip pat įvertinome iškritos *hco-acr-8* gene, įtaką levamizolio atsparumo susidarymui ir priėjome prie išvados, jog ši mutacija, viena pati, nėra patikimas molekulinis žymuo atsparumui nustatyti. Galiausiai, ištyrėme genominės apimties pakitimus eksponuojant *H. contortus* populiaciją su vaistu ivermektinu ir radome itin svarbių pakitimų penktojoje chromosomoje, tai leidžia teigti, jog šis lokusas yra itin svarbus formuojantis atsparumui ivermektinui nematoduose.

Raktiniai žodžiai: benzimidazolai, levamizolis, ivermektinas, antihelmintinis atsparumas, F200Y, Hco-acr-8, lašalinis skaitmeninis PGR, sekvenavimas, genomas

Contents

List of publications

This thesis is based on the work contained within the following studies (i.e., scientific papers), referred to by Roman numerals in the text:

I. Baltrušis P, Halvarsson P, Höglund J (2018). Exploring benzimidazole resistance in *Haemonchus contortus* by next generation sequencing and droplet digital PCR. International Journal for Parasitology: Drugs and Drug Resistance 8 (3), 411-419.

II. Baltrušis P, Halvarsson P, Höglund J (2020). Utilization of droplet digital PCR to survey resistance associated polymorphisms in the β tubulin gene of *Haemonchus contortus* in sheep flocks in Sweden. Veterinary Parasitology 288, 109278.

III. Baltrušis P, Charvet CL, Halvarsson P, Mikko S, Höglund J. Using droplet digital PCR for the detection of hco-acr-8b levamisole resistance marker in *H. contortus* (2021). International Journal for Parasitology: Drugs and Drug Resistance 15, 168-176

IV. Baltrušis P, Doyle SR, Halvarsson P, Höglund J (2022). Genome-wide analysis of the response to ivermectin treatment by a Swedish field population of *Haemonchus contortus*. International Journal for Parasitology: Drugs and Drug Resistance 18, 12-19.

The contribution of Paulius Baltrušis to the studies included in this thesis was as follows:

I. Took major responsibility for setting up and performing experiments, analyzing data, writing the manuscript, and corresponding with the journal.

II. Took major responsibility for setting up and performing experiments, analyzing data, writing the manuscript, and corresponding with the journal.

III. Took major responsibility for setting up and performing experiments, analyzing data, writing the manuscript, and corresponding with the journal.

IV. Took major responsibility for analyzing data, writing the manuscript, and corresponding with the journal.

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Abbreviations

ABZ	Albendazole
AChR(s)	Acetylcholine receptor(s)
ANOVA	Analysis of variance
AR	Anthelmintic resistance
AVM(s)	Avermectin(s)
BZ(s)	Benzimidazole(s)
ddPCR	Droplet digital PCR
DNA	Deoxyribonucleic acid
cDNA	Copy deoxyribonucleic acid
EC_{50}	(Half-maximal) Effective concentration
FEC(s)	Fecal egg count(s)
FECRT	Fecal egg count reduction test
GABA	γ-aminobutyric acid
GIN(s)	Gastrointestinal nematode(s)
GluCl(s)	Glutamate-gated chlorine channel(s)
GWAS	Genome-wide associated study(-ies)
ITS	Internal transcribed spacer
IVM	Ivermectin
L3	Third stage/infective larvae
L-AChR(s)	Levamisole-sensitive acetylcholine receptor(s)
LD50	(Half-maximal) Lethal dose
LEV	Levamisole
LoD	Limit of detection
LMM	Linear mixed model
Mbp/kbp	Megabase pairs/kilobase pairs
ML(s)	Macrocyclic lactone(s)

mRNA	Messenger ribonucleic acid
NGS	Next generation sequencing
P167/P168/P198/P200	Codon position 167/168/198/200
PCR	Polymerase chain reaction
PCV	Packed cell volume
P-gp(s)	P-glycoprotein(s)
qPCR	Quantitative polymerase chain reaction
RS	Heterozygotes for the “resistant” and “susceptible” alleles
RT-PCR	Real-time polymerase chain reaction
SD	Standard deviation
SNP	Single nucleotide polymorphism
TBZ	Thiabendazole
QTL	Quantitative trait locus(-i)

1. Introduction

Parasitic roundworms infect and therefore exert an immense burden on the ruminant livestock across the world (Sutherland and Scott, 2009). The most recent European-wide estimates suggest that these enzootic infections in farmed ruminants could be more costly than epizootic ones (Charlier et al., 2020). In addition, not uncommonly, animals infected with GINs tend to suffer an array of subclinical as well as clinical symptoms, among which the most severe are anemia, diarrhea, and even sudden death. What is more, stunted growth and development in particular lead to increased demands for feed and water intake, which further contributes to climate change and threatens the sustainability of animal farming systems.

Increased demand for animal products and thus more intensive livestock production has led to the development and the inevitable spread (e.g., through animal trade) of AR, especially in the parasites of small ruminants, such as sheep and goats. Thus, the occurrence of worms, capable of surviving the treatment administered to the infected animals, complicates not only local but also global control of parasitic infections. Perhaps not unexpectedly, among the Swedish sheep flocks, clinical diseases as well as death, both directly tied to infections with resistant parasites, have readily been reported (Höglund et al., 2015).

In sheep, over 20 nematode species have been described, some of which are more pathogenic than others. Arguably the most important pathogen is the species *Haemonchus contortus*. This hematophagous roundworm is cosmopolitan and has been associated with significant losses in the farmed sheep industry (Besier et al., 2016). Furthermore, the adaptive potential of *H. contortus* to the adverse effects of anthelmintic compounds has been the subject of both intense research and documentation by parasitologists for multiple decades (reviewed by Kotze and Prichard, 2016). In fact, experimental data

has shown that in the mere span of three generations susceptible isolates of *H. contortus* can be driven to develop clinical resistance by reinfecting sheep with parasites exposed to gradually increasing concentrations of the anthelmintic (Coles et al., 2005).

AR detection in the field is paramount to understanding its spread, predicting sources of possible AR emergence, and making more informed choices regarding treatment strategies on individual farms. To date, diagnostic AR detection relies mostly on aged technology (Coles et al., 2006) which suffers from many drawbacks. A fundamental lack of genetics and molecular biology research within this area is a limiting factor, preventing the invention of sensitive and robust tools for the molecular detection of AR (Kotze et al., 2020). It is, therefore, necessary to improve our understanding of the genetic basis of AR in parasitic nematodes and with that knowledge develop screening assays that will provide the opportunity to study the origin and spread of AR in greater detail.

2. Background

2.1 Gastrointestinal nematodes

Clinically pronounced GIN infections result in significantly deteriorated animal health and higher mortality rates. Sub-clinical infections, on the other hand, are more common and are associated with a negative impact on animal growth, wool, milk production, and fertility rates (Fitzpatrick, 2013). It is estimated that € 1.8 billion are lost annually as a direct consequence of parasitic infections in ruminant livestock in Europe alone (Charlier et al., 2020). Out of all the GINs parasitizing livestock, those of the highest economic impact in temperate regions are generally considered to be *Ostertagia ostertagi*, *Cooperia oncophora*, *Teladorsagia circumcinta*, *Trichostrongylus* spp., and ***Haemonchus contortus***.

2.2 *Haemonchus contortus*

Haemonchus contortus is a parasitic helminth (Figure 1) belonging to the phylum Nematoda. Together with other roundworm genera such as *Trichostrongylus*, *Teladorsagia*, *Ostertagia,* and *Cooperia*, which are all part of the same family of nematodes – *Trichostrongylidae*, *H. contortus* is considered to be an evolutionary clade V parasite (Blaxter et al., 1998).

Figure 1. Multiple adult stage *Haemonchus contortus* suspended in a buffer solution (Photo: Paulius Baltrušis)

Currently, the species *H. contortus* is widely distributed across farms throughout the world. For example, in today's Swedish sheep farms, *H. contortus* is by far the most common roundworm parasite, making up ~50% of all parasitic species in lambs and an even larger 75% in adult ewes (Halvarsson and Höglund, 2021). In addition, the most recent analysis of the intestinal nemabiomes of sheep in farms struggling with AR discovered an even higher (>90%) relative abundance of this parasite (Baltrušis et al., 2022b).

Although *H. contortus* is found parasitizing both wildlife (Nagy et al., 2016) and domesticated livestock, such as alpacas (Sarre et al., 2012), cattle (Waghorn et al., 2018) as well as sheep and goats (Yin et al., 2013), small

ruminants tend to be the most extensively affected by this parasite (Waller and Chandrawathani, 2005).

Despite being somewhat susceptible to adverse weather conditions (Santos et al., 2012), especially to desiccation (Kenneth S Todd et al., 1976) and extreme temperatures (K. S. Todd et al., 1976), *H. contortus* has demonstrated unprecedented ecological and genetic plasticity. Although its origins lie in the tropical parts of the globe, this species has adopted an alternative strategy to survive the generally colder and harsher winter season in the temperate climate zone. *H. contortus* achieves this by overwintering in the infected host, as opposed to on the pasture (Troell et al., 2005), as developmentally arrested larvae (Barger et al., 1985). Perhaps the most important feature of *H. contortus* is its high genetic variability both within and between populations (Doyle et al., 2019; Sallé et al., 2019), mostly due to high fecundity and short prepatent period, which enables this species to rapidly evolve resistance to drugs. Thus, due to, among other things, its relatedness to other clade V nematodes (such as *Caenorhabditis elegans*), cosmopolitan presence, generally large population sizes, and the genetic propensity for rapid adaptation, this species has been proposed to be a good experimental model (Gilleard, 2006, 2013) to not only study the impact of parasitic infections on the host but also the development of drug resistance in parasites of veterinary interest.

2.2.1 Life cycle

H. contortus along with other species within the *Trichostrongylidae* family, have similar developmental stages within and outside the host (summarized by Angulo-Cubillán et al., 2007). Specifically, in the case of *H. contortus*, the host is infected with the infective stage larvae (or L3), which subsequently penetrate the gastric mucosal layer and molt into the L4 stage. At this stage, the still immature adult can either undergo 'hypobiosis' and remain inside the host's gastric gland or molt into its egg-laying, adult stage while simultaneously migrating to the lumen. The adult stages of the parasite undergo sexual reproduction and the females begin laying eggs (approximately 5000 to 15000 a day), which are then excreted out of the host together with feces. As the excreted eggs finish embryonation, the immature larvae emerge and begin dispersing throughout the pasture. In about a week, the immature larvae develop into the L3 stage and can be found in different strata of grass heights (Santos et al., 2012), where they are eventually ingested by

a grazing ruminant. The prepatent period, that is the time it takes for the ingested L3 to reproduce and start the egg shedding process, is approximately 3-4 weeks, irrespective of the geographical origin of the isolate (Troell et al., 2006; Herath et al., 2021).

2.2.2 Pathogenicity, immune response, and evasion

It is important to note that, while outbreaks of a particular species are not uncommon, it is usually a combination of multiple different parasitic species that together exert their burden on the host. This burden can directly result in both sub-clinical (e.g., reproductive inefficiency, reduced weight gain) as well as clinical symptoms (diarrhea, anorexia, change in coat quality, etc.) of the disease, depending on the parasitic species present, the intensity of infection as well as the nutritional state (López-Leyva et al., 2020) and genetic background (Alba-Hurtado and Muñoz-Guzmán, 2013) of the individual host.

Infections with *H. contortus* in particular can result in significant levels of anemia and edema but also lethargy, loss of weight, and reduced production of milk and wool. The parasite is especially dangerous to young lambs and peri-parturient ewes, which generally have weak or ineffective immunity. The damage to the host is primarily done by the parasite wreaking havoc on the host's mucosal layer in the abomasum, which can lead to internal hemorrhage, but also through the general hematophagous nature of the parasite (Edwards et al., 2016). It has been estimated that a single, adult *H. contortus* consumes around 0.05 ml of the host's blood per day (Clark et al., 1962). Consequently, severe infections with this parasite are known to result in significant annual death rates in farmed sheep (Kelly et al., 2010).

Natural immunity to *H. contortus* in sheep is primarily dependent on complement fixation (Meeusen and Balic, 2000) and the simultaneous, chemoattractant-molecule-mediated eosinophil activation (Reinhardt et al., 2011). In addition, it has been observed that repeatedly (with the parasite) challenged sheep develop some capacity for worm expulsion, as a consequence of mucosal mast cell, and especially intraepithelial mast cell, activation resulting in an immediate type 1 hypersensitivity reaction (Balic et al., 2002). Therefore, given the role that natural immunity plays in providing the host a degree of protection against *H. contortus,* it is unsurprising that some sheep breeds fair better than others when challenged with the parasite in natural settings (Guo et al., 2016).

Adaptive immunity to *H. contortus*, although not lifelong, can result in the delayed expulsion of the worms. This response hinges on the successful activation of CD4+, γδ T cells, and B cells but also the non-specific IgE antibodies, antibody-dependent eosinophil cytotoxicity, and the complement system activation (Balic et al., 2002).

Despite the immune defense mounted by the host, *H. contortus* utilizes multiple strategies to evade detection. First and foremost, the capacity to be motile and occupy a specific niche within the lumen affords the parasite the ability to temporarily halt detection. In addition, *H. contortus* produces various protease inhibiting cystatins (Newlands et al., 2000) and other immunomodulators such as Type C lectins (Greenhalgh et al., 1999) (to inhibit leukocyte adhesion) and calreticulin (Suchitra and Joshi, 2005) (to inhibit the complement system components) to mask its presence while inside the host.

2.3 Diagnosing *H. contortus* in the field

2.3.1 Traditional diagnosis

Haemonchosis in sheep can be suspected based on several different criteria. A typical tell-tale sign of sheep suffering high burden infections with *H. contortus* is the 'bottle-jaw' appearance, which occurs due to local swelling beneath the jaw, induced by the chronic shortage of proteins in the bloodstream. Another indirect way of diagnosing *H. contortus* infection in the host is based on the physiological changes induced by the parasite. Due to notable blood loss, measuring the hematological parameters such as PCV has been previously used to diagnose an ongoing infection (Alunda et al., 2003). Additionally, the FAMACHA system, in which a score is assigned based on the evaluation of the conjunctival mucosal membrane, has also been used to assess the severity of anemia in the infected livestock. Several other parameters, such as plasma protein, pepsinogen, and even gastric pH levels, are also indicative of, but not necessarily specific to, an ongoing *H. contortus* infection.

Non-invasive, traditional tools for the direct diagnosis of *H. contortus* typically rely on microscopically examining fecal samples from the infected hosts and demonstrating the presence of the parasite in its egg stage. Although some laboratories have demonstrated relative success distinguishing *H. contortus* eggs from other related species (Ljungström et al., 2018), it is

generally considered not possible to reliably separate the extensively overlapping morphological features of strongyle eggs. Therefore, diagnosis of particular species or genera (including *H. contortus*), more often than not, relies on hatching the eggs in laboratory conditions and microscopically examining the morphological features of the hatched L3 (Van Wyk et al., 2013). However, these examinations tend to be riddled with shortcomings, the most notable of which are observer bias, laborious (and difficult to automate for that matter) nature of such assays as well as the need for trained and experienced professionals to perform them (Johnson et al., 1996; Lichtenfels et al., 1997).

While the direct, traditional diagnostic approaches are generally straightforward and cost-effective to perform, they tend to be lacking in specificity, sensitivity, are labor-intensive, and generally have issues with repeatability.

2.3.2 Molecular diagnosis

Significant advancements in the molecular diagnosis of *H. contortus* have been made over the past three decades. The earliest studies in the 1990s identified key regions in the genomes of nematode parasites, ITS-1 and ITS-2 (Gasser et al., 1993; Hoste et al., 1995), which are found between the 18S and 28S ribosomal DNA subunit encoding genes (Figure 2). These loci are particularly fit for molecular identification of parasites due to i) low intraspecific (i.e., within species) and significantly higher interspecific (i.e., between species) variation, affording precise distinctions between closely related species (Stevenson et al., 1995) ii) multiple copies being present throughout the parasite genomes, allowing for increased sensitivity of the DNA detection based, diagnostic assays (Gasser and Newton, 2000).

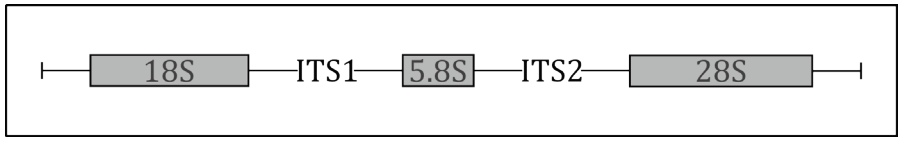

Figure 2. Ribosomal DNA gene cluster organization along with the internal transcribed spacer sequences, used for nematode parasite species identification

The very first conventional, PCR-based assays employing the amplification of the aforementioned ITS-1 or ITS-2 regions and used to detect and/or study *H. contortus* (Zarlenga et al., 1998; Heise et al., 1999) were relatively crude and inefficient but paved the way for more complex and sophisticated

detection platforms. Nearly a decade later, an RT-PCR experiment was carried out to identify six different species (among which was *H. contortus*) and compare these results directly with the morphological evaluations of the hatched larvae (Siedek et al., 2006). For the first time, a strong positive correlation was demonstrated between the results produced by the traditional and molecular diagnostic methods (84% agreement) for the identification of parasitic nematodes, thus affirming the utility of molecular diagnostic methods as not only a valuable research tool but a useful screening platform, suitable for rapid and reliable diagnosis of *H. contortus*.

Some years later, RT-PCR was put to the test in intestinal sheep parasite (including *H. contortus*) screenings in Australia (Bott et al., 2009; Roeber et al., 2011; Sweeny et al., 2011). Perhaps unsurprisingly, these RT-PCR assays demonstrated both higher sensitivities, compared to the conventional approaches, as well as increased precision in identifying closely related parasite genera (e.g., *Trichostrongylus spp* vs. *Teladorsagia circumcinta*), thus overcoming two of the most notable drawbacks with the traditional parasitological tools of diagnosis in a single stroke. While it was shown that PCR-based methodology could be successfully used to supplement (or even replace) the traditional methodology in providing more accurate nematode estimations and distinguishing morphologically similar species, these assays were still cumbersome to perform as the collected samples had to be analyzed for each species on an individual basis.

Not even three years later and multiple studies had come out addressing the aforementioned limitation (McNally et al., 2013; Bisset et al., 2014). Molecular assays began moving away from specific species identification, rather focusing on detecting multiple different species at the same time. With the adoption of the multiplex PCR, it was shown that multiple different ovine parasite species and their abundances could be estimated simultaneously. Another key point of innovation both in terms of throughput and the sensitivity as well as specificity aspect took form in the utilization of the ddPCR platform (Elmahalawy et al., 2018a). The unique nature of the ddPCR assays enabled semi-automated, rapid, precise, and extremely sensitive (Höglund et al., 2019) absolute quantification of the most important ovine parasites, such as *H. contortus*, *T. circumcinta,* and *Trichostrongylus* spp., without the need for standard curves, typically necessary for qPCR. Although both multiplex and ddPCR platforms provided sufficiently sophisticated and technologically advanced alternatives to diagnosing the presence and abundance of *H.*

contortus along with a few other major GIN species in sheep, the main weakness of these assays was exactly that: only a limited set of species, for which the assays have been designed *a priori*, could be detected.

Recently, an innovative platform to study the community structure of nematodes in ruminant livestock ('nemabiome') has been proposed as a powerful alternative to not only diagnose a single or pre-determined set of species but the entire community of parasitic nematodes within the hosts (Avramenko et al., 2015). This platform is based on the blind amplification of the nematode ITS-2 region using a universal primer pair in bulk samples and the subsequent amplicon sequencing. The sequencing data is then bioinformatically processed and the total species composition in every sample is determined. This approach has readily been applied to study the frequencies of many nematode species in sheep, including *H. contortus*, in Canada (Queiroz et al., 2020), the United Kingdom (Redman et al., 2019), and Sweden (Halvarsson and Höglund, 2021).

Thus, molecular tools for the detection of *H. contortus* have undergone drastic changes from mainly qualitative, single species focused, to precise, semi-quantitative/quantitative and focused on *H. contortus* along with a few other common parasites, to comprehensive, unbiased, and sequencing-based platforms for total species identification within hosts.

2.4 Treatment and prevention

To date, the most reliable and effective way to control GIN infections in ruminant livestock relies on the use of anthelmintic drugs. In the contemporary context of Swedish sheep flocks, the most commonly used drug classes are BZs (e.g., ABZ) along with two others - imidazothiazoles (e.g., LEV) and MLs (e.g., IVM) (Halvarsson et al., 2022).

2.4.1 Benzimidazoles

BZ class drugs, such as the very first TBZ, were introduced in the 1960s and have since then been adopted to treat a wide spectrum of nematode and trematode infections, especially in ruminants.

Collectively, BZs target the β-subunit of the dimeric tubulin protein, a key component in microtubules and thus vital for the maintenance of key cellular functions. Upon binding to the β-subunit, BZs lead to the destabilization of the microtubule polymerization and the disruption of such cellular

processes as cellular division and organelle transport. It has been further demonstrated that BZs have a much higher selective affinity for the nematode β-subunit of the tubulin protein, which helps explain little to no toxicity to the host (Lacey, 1990). Selectivity, broad-spectrum activity, and high efficacy (Bennett and Guyatt, 2000) have led to massive adoption of the drugs belonging to this class and nothing short of a breakthrough in treating helminthiases.

2.4.2 Imidazothiazoles

Imidazothiazole drug class, the most well-known member of which is LEV, is likewise a collection of broad-spectrum anthelmintics.

LEV was the first imidazothiazole to be discovered in the mid-1960s (Thienpont et al., 1966) and was henceforth adopted to treat GIN infections in livestock and companion animals. LEV was shown to selectively target and bind nematode AChRs thereby causing an influx of Ca^{2+} ions through the opened ion channels. The influx of Ca^{2+} ions into the cytoplasm then causes depolarization (Puttachary et al., 2010) and spastic muscle contractions in the worms as a consequence (Robertson et al., 2010). In the end, the paralyzed worms are eliminated from the host via normal peristaltic bowel movements. Although a relatively old drug, LEV is still considered to be one of the main anthelmintics and is occasionally used as a last resort drug on sheep farms in Sweden (Höglund et al., 2020).

2.4.3 Macrocyclic lactones

The MLs are chemical derivatives of the compounds initially discovered in the genus *Streptomyces* bacteria. Drugs belonging to this class display a potent, broad-spectrum anti-parasitic activity and are used in treating a wide range of endoparasite and ectoparasite infections (J. Nolan and B. Lok, 2012).

The most notable drug of the MLs, IVM (Campbell et al., 1983), belongs to the AVM subclass. At a molecular level, IVM is known to be an agonist of invertebrate GluCls, present in nematodes and arthropods (Forrester et al., 2004; Wolstenholme, 2012) but not in vertebrates. The binding and subsequent overactivation of GluCls leads to the paralysis of the nematodes as well as the inhibition of the pharyngeal pumping (Geary et al., 1993), which renders adult nematodes, present within the GI tract of the host, unable to move

and feed (Kotze et al., 2012). Multiple other, ligand-gated receptors are also known to be activated by MLs, such as those activated by GABA and dopamine (Feng et al., 2002; Rao et al., 2009), suggesting that MLs (and more specifically IVM) possess a range of targets, through which the drugs exert their antiparasitic effect. Currently, IVM is the most widely used drug to treat parasitic roundworm infections in Swedish sheep (Halvarsson et al., 2022).

2.5 Anthelmintic resistance

Decades of generally poorly governed anthelmintic use have led to the parasites of livestock developing AR (Figure 3). The situation regarding AR appears to be the direst in GINs of sheep, as to date parasites displaying AR to one or more drugs is a common occurrence not only on farms in the European continent (Rose et al., 2015; Rose Vineer et al., 2020) but also across the rest of the globe (Veríssimo et al., 2012; Falzon et al., 2013; Playford et al., 2014; Han et al., 2017). For both cattle and goat parasites, sporadic reports of AR (largely due to limited research and historically lower interest) seem to collectively anticipate a comparably high incidence of AR in the near future (Sutherland and Leathwick, 2011; Rose Vineer et al., 2020).

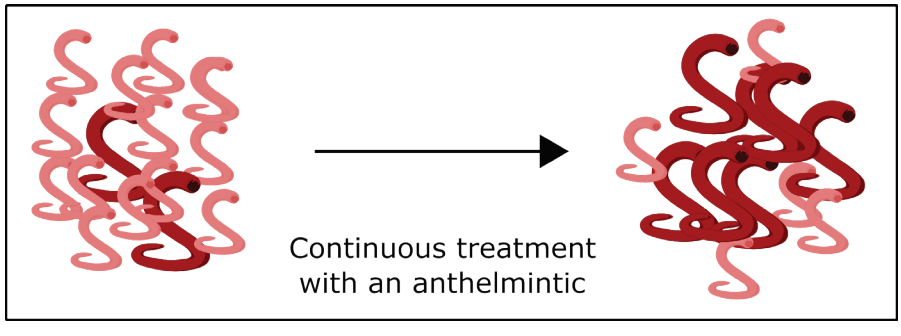

Figure 3. Poorly managed, repeated animal treatment(s) with a particular anthelmintic over time (and generations) leads to the population-wide development of drug resistance in GINs. In pink: worms susceptible to the effects of anthelmintics, in red: worms resistant to the effects of anthelmintics

AR in GINs has been by far the most extensively studied in *H. contortus*, in part because this species is cosmopolitan and has developed resistance to every single drug class available (reviewed by Kotze and Prichard, 2016). For this reason, the remaining chapters, focused on the mechanisms behind

AR to multiple drug families, will heavily rely on the findings obtained using *H. contortus* as the model organism.

2.5.1 Anthelmintic resistance in sheep parasites in Sweden

To date, only a handful of studies have reported on the situation regarding AR in ovine parasites in Sweden. However, the overall trends over the years present a serious cause for concern.

Historically, the very first study on AR in Swedish sheep parasites was conducted in the 1990s and found evidence of emerging BZ resistance in the eight flocks that were appropriately surveyed (Nilsson et al, 1993). A more comprehensive report into AR was not launched until the 2000s. In their 2009 study, Höglund et al., 2009 described the prevalence of clinical and suspected BZ resistance on randomly sampled sheep farms by employing both traditional, 'gold standard' as well as novel (at the time) molecular tools for AR diagnosis. The study found reduced drug efficacy on only four out of 45 farms, which appeared to harbor mainly BZ-resistant parasite populations. Thus it was concluded that AR levels at the time were low in comparison to other countries (Bartley et al., 2003, 2006; Besier and Love, 2003; Pomroy, 2006). A subsequent 2015 study examined AR specifically to IVM in sheep flocks, previously found to struggle with recurrent infections (Höglund et al., 2015). Not only was treatment failure recorded on six out of 11 farms, but the authors managed to trace the possible origin of resistance to a flock of sheep that had been previously imported from outside of the country. In a fairly recent case study published in 2020, the first case of monepantel (i.e., the newest anthelmintic drug, introduced in 2008 for use in sheep) (Kaminsky et al., 2008) resistance, after only a single year of use, in Sweden was reported (Höglund et al., 2020). The most recent data on the extent of AR in Sweden, albeit performed on non-randomly selected farms, appears to suggest that a continuously deteriorating situation regarding resistance in sheep parasites has gotten much worse since the initial study in 2009. Over half of the investigated farms (26/46) presented reduced efficacies to IVM, while 11/28 showed signs of treatment failure to BZs. Resistance to both drugs simultaneously was now found on six (of the 26, where both drugs were tested) farms (Höglund et al., 2022). Perhaps unsurprisingly at this point, in all five of the aforementioned studies, the presence of AR on farms (where reduced drug efficacies were recorded) was found to be intimately

linked to infections with *H. contortus*. Two other studies focused on investigating AR in *H. contortus*, in particular, were carried out to determine the extent of possible cases of BZ resistance across Sweden. Alleles carrying mutations linked to AR to BZs in *H. contortus* were found to be widely distributed across the southern part of Sweden, suggesting that various degrees of BZ-resistance can readily be found across a great number of the sheep farms in the country (Baltrušis et al., 2018, 2020). Thus, in the case of Sweden, AR and *H. contortus* appear to have been inseparably associated with one another for almost three decades.

2.5.2 Diagnosis of anthelmintic resistance

In nematodes of veterinary importance, resistance to a particular drug is primarily determined by performing a FECRT, however other *in vitro* tests as well as, to a degree, molecular tests can be applied to supplement the former.

FECRT is used to determine the efficacy of an anthelmintic drug by quantitatively evaluating the eggs which are shed by the flock or herd of animals before and days after the administration of treatment. The established guidelines state that a drug is considered to be effective only if the reduction in FEC after treatment is higher than 95% and the lower confidence interval limit is above 90% (Coles et al., 1992). Due to its simplicity, FECRT is considered to be the 'gold standard' method and is still heavily relied upon to this day to diagnose the presence of AR on farms. However, the methodology itself, among its other inherent flaws, is insensitive and outdated. For example, it has been shown that to be able to reliably detect resistance in a population with the FECRT, at least 25% of the individuals have to be resistant (Martin et al., 1989). On top of that, the precision of the methodology heavily relies on the chosen protocol as well as the number of eggs being shed (Levecke et al., 2012).

In vitro tests can be employed to either re-affirm the results of the FECRT or *de novo* determine the susceptibility of a population to a particular anthelmintic. These tests evaluate the capacity of the parasite to either hatch or remain motile after exposure to a drug. Usually, to test the susceptibility of the nematode population to BZs, an egg hatch test is performed since BZ derivatives are known to prevent egg embryonation (Le Jambre, 1976). Susceptibility to either LEV or IVM, i.e., drugs that induce paralysis (as previously discussed in chapters 2.4.2 and 2.4.3), can be determined by performing larvae migration/motility/paralysis assays and examining the capacity of

the individuals to remain motile after exposure to the drug (Martin and Le Jambre, 1979; Gill et al., 1991). However, the drawbacks of *in vitro* tests, mainly the lack of sensitivity and poor repeatability (Várady and Čorba, 1999), tend to limit their adoption and preference over the FECRT.

An alternative approach to the traditional diagnosis of resistance (i.e., FECR and *in vitro* tests) - the nemabiome analysis (discussed in 2.3.2) has been proposed as a viable, future diagnostic tool for AR screening (Queiroz et al., 2020). Applying this tool to study samples collected from flocks or herds before and after anthelmintic treatment yields to date the most precise and sensitive data on the species surviving treatment (Avramenko et al., 2015; Redman et al., 2019). Nevertheless, this approach is relatively new, with few studies performed thus far on, for example, ovine nematodes of veterinary importance (Redman et al., 2019; Borkowski et al., 2020; Queiroz et al., 2020; Halvarsson and Höglund, 2021). As a consequence, standardized protocols, the impact of data analysis procedures as well the translation of the ITS-2 copy numbers into clinically relevant and interpretable measurements with determined cut-off values, among other things, are yet to be established.

PCR-based diagnostic tests for AR determination are currently limited to estimating the presence certain of alleles associated with BZ-resistance (Kwa et al., 1994; Silvestre and Cabaret, 2002; Ghisi et al., 2007). Such assays based on the amplification of *β-tubulin* are, thus far, only applicable to studying BZ-resistance in GINs of sheep, such as *H. contortus*, *T.circumcinta*, *Trichostrongylus* spp., and *N. battus* (Von Samson-Himmelstjerna et al., 2007; Ramünke et al., 2016; Melville et al., 2020) and cattle, such as *Cooperia* spp., *Ostertagia* spp., *Haemonchus* spp. and *Trichostrongylus* spp. (Avramenko et al., 2020).

Despite the many advantages that molecular tools have over the traditional tests for diagnosis (discussed in 2.3.2), AR in flocks/herds of ruminants is still primarily determined by performing the FECRT, which is sometimes supplemented with other *in vitro* tests. The major drawback of using molecular (both sequencing and PCR-based) tools is that they are understudied and thus not as firmly established as the traditional tools for AR diagnosis.

2.5.3 Research and AR to Benzimidazoles

The first BZ, TBZ, was introduced in 1961. By 1964, a mere 3 years later, the first report of TBZ-resistant *H. contortus* had already come out (Drudge et al., 1964). This rapid emergence of resistance cases shortly after the introduction of a new BZ derivative has characterized much of the 20th century and in major part catalyzed the research into understanding the development of BZ resistance in nematodes of ruminants.

Much of the initial work in understanding BZs, their mode of action, and the eventual resistance development to these compounds focused on examining the effects that the drug had on carbohydrate metabolism in parasites (Prichard, 1973; MS et al., 1977). The very first study to draw the connection between BZs selectively binding to parasite tubulin examined the strength of inhibition that BZ compounds exerted on a [^{3}H]colchicine-mediated binding to parasite tubulin in comparison to mammalian tubulin (Friedman and Platzer, 1980). This finding was later confirmed by several independent studies which found that BZ compounds both controlled tubulin polymerization (Dawson et al., 1984) and showed the reduced binding capacity to resistant parasite isolate lysates (Sangster et al., 1985). Finally, experiments by Lacey and Prichard, (1986) displayed the varying association constants, and thus affinities, of ABZ binding to either BZ-susceptible or BZ-resistant isolates of *H. contortus*. At around the same time experiments by Oakley and Morris (1981) and Oakley et al. (1985) demonstrated that most mutations isolated from tubulin, occurred in the β (rather than the α) subunit of the tubulin gene and that these mutations could conditionally and selectively block some tubulin mediated functions (such as nuclear division and nuclear movement) and not others. Thereby the association between tubulin, and more precisely β-tubulin, and BZs was sealed, thus allowing further studies to focus on investigating the actual changes occurring in *β-tubulin* which discriminate the resistant parasite isolates from the susceptible.

The ultimate discovery relating to BZ-resistance in nematodes of veterinary importance was made by Kwa et al. (1994) who described the importance of the point mutation in the P200 in the isotype-1 *β-tubulin*, which further resulted in the amino acid substitution Phe → Tyr (more commonly referred to as F200Y mutation). At the time, F200Y mutation was also known to be commonly found in BZ-resistant isolates of fungi and the free-living nematode *C. elegans* (Geary et al., 1992; Jung et al., 1992; Kwa et al., 1993b). The experiments conducted by the authors of the said study for the

first time solidified the association between BZ-resistance in trichostrongylids and the F200Y mutation in the isotype-1 *β-tubulin*. Following this key piece of research, others, namely Silvestre and Cabaret (2002) and Ghisi et al. (2007), experimentally showed that point mutations in P167 and P198 in the isotype-1 *β-tubulin*, resulting in amino acid substitutions F167Y (=F167Y mutation) and E198A (=E198A mutation), were also associated with increased resilience to BZs, albeit these point mutations were much rarer in the field (Figure 4). Although some early research seemed to indicate that a deletion in the isotype-2 *β-tubulin* appeared to further modulate BZ-resistance status (Kwa et al., 1993a; Beech et al., 1994), this work was mostly overshadowed by the discovery and the immediate utility of the three (F167Y, E198A, and F200Y) mutations in the isotype-1 *β-tubulin*. For over a decade now no further clarifying evidence has been presented in support of the presence or absence of a deletion in the isotype-2 *β-tubulin* exerting an effect on BZ-resistance status in trichostrongylids (Von Samson-Himmelstjerna et al., 2009).

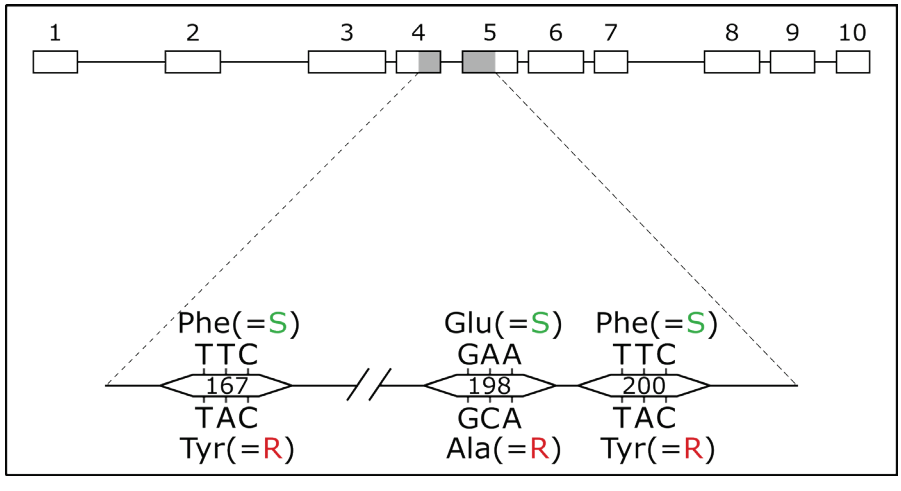

Figure 4. The organization of the isotype-1 *β-tubulin* in *H. contortus* along with the three point mutation variants in codon positions 167 (exon 4), 198, and 200 (exon 5). The phenotypic effects of the amino acid, which is incorporated in the final, translated β-tubulin subunit, are highlighted in either green (susceptible to BZs; =S) or red (resistant to BZs; =R)

Since the discovery of the point mutations in P167, P198, and P200 in the isotype-1 *β-tubulin*, researchers have started developing diagnostic tools to screen for alleles containing these mutations in various parasitic nematodes

to establish the extent and spread of BZ-resistance in the field. In the case of *H. contortus*, F200Y mutation was found to be largely present in isolates from the European continent (Von Samson-Himmelstjerna et al., 2009), in particular in countries such as Germany, Switzerland, Italy (Ramünke et al., 2016), Belgium (Claerebout et al., 2020), Hungary (Nagy et al., 2016), the United Kingdom (Redman et al., 2015), Sweden (Höglund et al., 2009; Baltrušis et al., 2020), Greece (Gallidis et al., 2012) and France (Silvestre and Cabaret, 2002), but also from parts of North America (Barrere et al., 2013) and South-east Asia (Chaudhry et al., 2015). The F167Y mutation was found to be rarer (than F200Y), but present at substantially higher frequencies in *H. contortus* isolates from South America (Lambert et al., 2017). Although generally considered to be exceptionally rare, the E198A mutation was found to be the more common BZ-resistance conferring mutation in *H. contortus* from Asia (Zhang et al., 2016), whereas a different point mutation in the same codon, leading to the E198L substitution, was found in BZ-resistant *H. contortus* isolates recovered in North Africa (Mohammedsalih et al., 2020, 2021).

As a direct result of the screening efforts to identify the prevalence of mutations F200Y, F167Y, and E198A, it was discovered that only a single mutation of the three could be found in the isotype-1 *β-tubulin* locus in an individual *H. contortus* (Mottier and Prichard, 2008; Barrère et al., 2012; Baltrušis et al., 2018). This appeared to suggest that multiple, non-synonymous mutations in the isotype-1 *β-tubulin* likely result in lethal outcomes for the individual nematode.

Recent advances in population genomics as well as the assembly of high-quality reference genomes, such as the one for *H. contortus* (Doyle et al., 2020), have allowed for an unbiased look at the genomic changes in response to selection with anthelmintics. A study by Doyle et al. (2022) not only confirmed the importance of the genetic changes in the isotype-1 *β-tubulin* (primarily the F200Y mutation) to the development of BZ-resistance but also found that the frequencies of the mutation, resulting in the E198V substitution in the isotype-2 *β-tubulin*, further correlated with increasing EC_{50} concentrations to BZ in field *H. contortus* populations from the USA. This latter finding confirms the observations from a previous, more than a decade-old study by Rufener et al. (2009) in which the authors carried out *in vitro* selection experiments with TBZ. Taken together, the evidence suggests that non-synonymous substitution(s) in different isotypes of *β-tubulin* could act as yet

another modulator of resistance to BZs in trichostrongylid parasites. In the case of *H. contortus*, four *β-tubulin* isotypes have been described so far (Laing et al., 2013) but the two most recently discovered (isotypes -3 and -4) have been considered unlikely to be involved in the development of BZ-resistance due to their homologies to the two locally expressed and specialized-functions-having genes *mec-7* and *tbb-4* in *C. elegans* (Saunders et al., 2013). Regardless, the compelling finding regarding the additional involvement of the genetic changes in the isotype-2 *β-tubulin* in BZ-resistance development is bound to further stimulate both the research and the exciting discoveries within the neglected subsection relating to molecular BZ-resistance mechanisms in parasitic nematodes.

2.5.4 Research and AR to Imidazothiazoles

One of the first cases of resistance to the imidazothiazole LEV emerged in the early 1980s (Sangster et al., 1980; Green et al., 1981), roughly 10-20 years after its initial release. Given that by that time multiple BZ compounds, such as TBZ, fenbendazole, and oxfendazole (summarized by Kotze and Prichard, 2016) had started showing signs of reduced efficacy, it was understood that to delay the emergence of widespread LEV-resistance action needed to be taken.

Early studies investigated LEV in order to determine its precise mode of action as well as the possible mechanisms of resistance. Although some evidence of inhibition of metabolic enzymes was shown (van Den Bossche and Janssen, 1969; Prichard, 1973), it was soon established that LEV acted primarily by stimulating nerve cells through cholinergic (acetylcholine dependent) muscle synapses in susceptible nematodes (Coles et al., 1975; Lewis et al., 1980; Harrow and Gration, 1985). In fact, the study by Lewis et al. (1980) was also one of the first to characterize the many uncoordinated (and therefore resistant) phenotypes exhibited by the free-living nematode isolates. A subsequent study by Sangster et al. (1991) argued that such uncoordinated phenotypes would be disadvantageous in parasitic nematodes which have to occupy specific sites within the infected host. Additionally, high doses of LEV were known to be toxic to sheep, and therefore extreme levels of resistance to LEV were unlikely to develop. Thus, the authors of the said study concluded that the LEV-resistant *H. contortus* was most likely similar to the pseudo-wild, mildly resistant *C. elegans* isolates, belonging to complementation groups unc-29, 38, 63, 74, and/or lev-8, 9. More than half a decade

later, both Sangster et al. (1998) and Moreno-Guzmán et al. (1998) performed binding experiments with radioactively labeled LEV which demonstrated that, unlike for the free-living *C. elegans* where resistance was associated with strains deficient in receptors (Lewis et al., 1980), resistance to LEV in different *H. contortus* isolates appeared to be governed by changes in the normal function of the receptor – i.e., increased or decreased binding affinity and/or binding sites for the drug. Both studies concluded that LEV was binding to a distinct population of AchRs and that the most likely route of resistance development is through the modifications of these receptors leading to altered affinities for the ligand.

Since AchRs were found to not only be the direct targets of LEV but also associated with resistance, a lot of effort was put into understanding their structure and interactions with other cellular components. In free-living nematodes, LEV-sensitive AchRs (L-AchRs) (Lewis et al., 1987) were found to be heteropentamers, composed of three α and two non-α subunits. In addition, at least three ancillary proteins have been indicated to be crucial for the expression and assembly of L-AchRs (Boulin et al., 2008). In parasitic nematodes, such as *H. contortus*, on the other hand, at least two different L-AchRs have been functionally reconstituted by assembling different sets of receptor subunits – Hco-L-AChR1 (composed of subunits Hco-unc-29.1, Hco-unc-38, Hco-unc-63a, and Hco-acr-8) and Hco-L-AChR2 (sharing the same subunit composition but lacking Hco-acr-8) (Boulin et al., 2011). Furthermore, Boulin et al. (2011) demonstrated that the presence (or absence) of a single subunit, Hco-acr-8, plays the main role in determining the sensitivity of the receptor to LEV. This observation was further supported by the findings of Fauvin et al. (2010) and later by Barrère et al. (2014), who uncovered and described a truncated *hco-acr-8* mRNA transcript (referred to as Hco-acr-8b) at the time only found in LEV resistant isolates of *H. contortus* and occurring due to a 63 bp deletion in the second intron of *hco-acr-8*. Although it is tempting to speculate, so far, no data is available on whether this truncated form of the Hco-acr-8 subunit (Hco-acr-8b) results in the dominance of the receptor Hco-L-AChR2 (lacking a full-length Hco-acr-8 subunit) over Hco-L-AChR1 in the parasite or if an altogether new type of receptor (including Hco-acr-8b) is assembled (Figure 5).

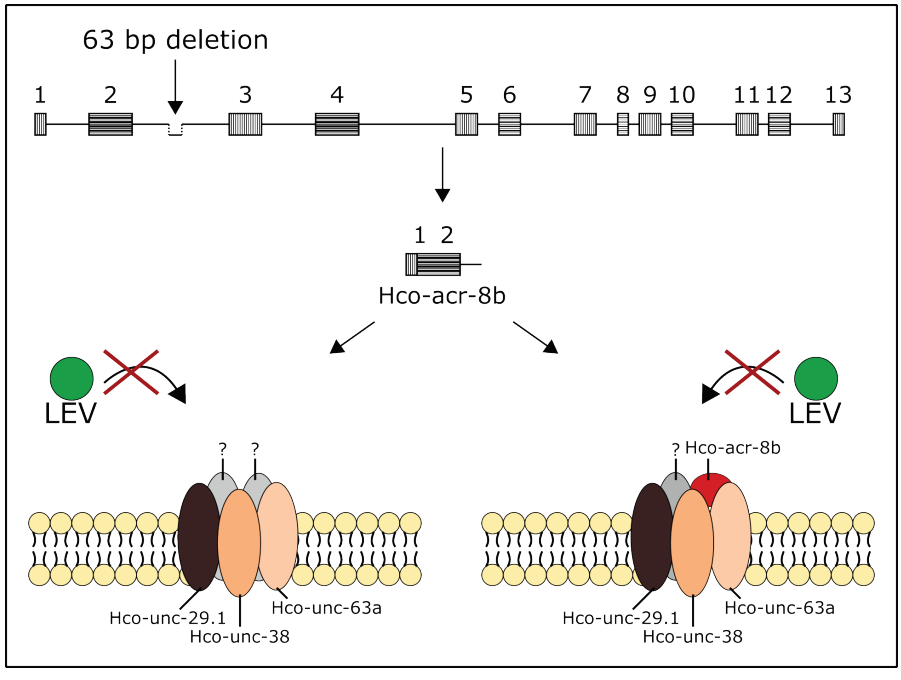

Figure 5. The currently established major contributor to LEV-resistance in *H. contortus* is thought to be a variant of the *hco-acr-8*, containing a 63 bp deletion in the intron 2 which further leads to the production of an alternatively spliced mRNA transcript Hco-acr-8b (composed of only the first two exons and some of the intron 2 sequence). The truncated form of this receptor subunit has been linked with LEV resistance in some studies and could be speculated to either lead to the dominant production of Hco-L-AChR2, shown not to be susceptible to LEV (Boulin et al., 2011), or form an altogether new, LEV insensitive receptor type

Similarly, Neveu et al. (2010) described an alternative transcript of the *hco-unc-63*, Hco-unc-63b, only found to be expressed in the resistant isolates of trichostrongylids, suggesting that LEV-resistance in parasitic nematodes could potentially be established by the parasite acquiring mutations in one (or more) of the L-AChR subunits, leading to the translation of truncated L-AChRs. Despite being a rather intuitive and sensible theory behind LEV-resistance in parasitic nematodes, especially in the case of Hco-acr-8b, it has been shown to not hold true in numerous cases (Chagas et al., 2016; Baltrušis et al., 2021; Doyle et al., 2022) as the 63 bp deletion, thought to give rise to Hco-acr-8b, was shown to be present at relatively high frequencies in both susceptible and resistant isolates of *H. contortus*. In fact, Doyle et al. (2022) suggested that rather than the 63 bp deletion in intron 2, a non-synonymous

point mutation in exon 4 (leading to the substitution Ser → Thr at P168) in *hco-acr-8* seemed to be a better predictor of the LEV-resistant phenotype. However, this finding requires further confirmation.

Several research groups attempted to investigate LEV resistance from a different angle – by identifying changes or patterns of change in gene expression upon exposing different roundworm parasite isolates to LEV. One of the first studies of this kind was carried out by Williamson et al. (2011), who found increased expression of both *hco-acr-8* and *hco-acr-8b*, whilst the expression of genes coding for the subunits Hco-unc-29.3 and Hco-unc-63 were down-regulated. A great body of work on expression level changes of AChR subunit, ancillary protein, and P-gp genes was produced by Sarai et al. In their 2013 study, Sarai et al. (2013) compared changes in gene expression levels in one susceptible and three LEV-resistant field isolates of *H. contortus*. Apart from a consistent down-regulation of *hco-unc-63a* in adult stages of the parasite, no reliable change in expression level patterns was observed for either AChR, ancillary protein, or P-gp gene groups. A year later, the authors compared expression level changes upon exposing highly resistant fractions of the *H. contortus* population to varying LEV concentrations (Sarai et al., 2014). As a consequence, a distinct pattern was observed, wherein at low LEV concentrations, expression levels of some AChR genes increased (e.g., *hco-unc-38*, *hco-unc-63b,* and *hco-unc-16*) together with non-specific P-gps (such as *hco-pgp-3*, *hco-pgp-4*, *hco-pgp-10,* and *hco-pgp-14*), whereas at intermediate and higher concentrations of the drug, down-regulated expression of primarily AChR subunits and ancillary proteins occurred. Interestingly, in the same study, the expression of *hco-acr-8b* was not detected. In 2015, Sarai et al. (2015) conducted selection (with LEV) experiments on a naïve isolate of *H. contortus* for nine generations. Throughout the generations and across the different parasite life stages, the authors found differing levels of expression for many of the previously mentioned AChR subunit as well as ancillary protein genes, which appeared to be mostly down-regulated in L1 but up-regulated in L3 and adult stages. Taken together, it appears that the mechanisms of resistance that ensure worm survival could not only be LEV concentration, but also parasite life-stage dependent.

The complicating issues with most of these studies were that the isolates used for comparison were either multi-resistant (thus making it difficult to delineate the expression pattern changes occurring specifically due to LEV-

resistance) and/or of different geographic or historic origin, which could result in considerable changes in expression or allele frequency patterns, completely unrelated to resistance. Overall, it appears that LEV-resistance is a truly polygenic trait (Sangster et al., 1998), determined by multiple mutations and/or transcriptional changes associated with, among others, AChR, ancillary protein, and perhaps even P-gp genes. Further innovative, perhaps even genomic, approaches will thus be required to untangle LEV resistance in parasitic nematodes and identify the major contributing factors.

2.5.5 Research and AR to Macrocyclic lactones

IVM was the first ML introduced for parasite control in both humans and animals in the early 1980s (reviewed by Kotze and Prichard, 2016). With the first reports of resistance to IVM emerging in sheep trichostrongilids not even a decade later (Carmichael et al., 1987; Van Wyk and Malan, 1988), significant effort was put into understanding the molecular interactions between the AVM drug subclass and the cellular components of the nematode parasites so that the possible targets and mechanisms of resistance could be uncovered.

In general, AVMs have been shown to bind to and activate a variety of membrane channels, such as mammalian glycine (Shan et al., 2001) and GABA receptors (Adelsberger et al., 2000), nicotinic AChR (Krause et al., 1998) and others (Khakh et al., 1999). In nematodes, early studies described the affinity of IVM to the membranes of parasites (Rohrer et al., 1994) and in particular to recombinant GluCl proteins (Cheeseman et al., 2001; Forrester et al., 2002). Since then, the consensus has been that the GluCls, present on the cell membranes of nematodes, are the principal targets of AVMs. The very first study that focused on characterizing the composition of the functional nematode GluCl receptor described two cDNAs of subunits GluClα1 and GluClβ, found to be related to GABA type A receptors (Cully et al., 1994). More recently, it was established that both the free-living and parasitic nematodes possess six genes, some of which are species-unique and can be alternatively spliced, coding for IVM-sensitive GluCl subunits (summarized in Yates et al., 2003; Glendinning et al., 2011). Furthermore, as pointed out by Yates et al. (2003), given that the functional nematode GluCl receptors are indeed composed of five heteromeric subunits, it could then be assumed that multiple types of GluCls are present on nematode cell membranes, thus resulting in multiple targets for AVMs.

Initial studies investigating AVM resistance were mostly focused on genotyping susceptible and resistant (phenotype exhibiting) isolates in terms of one particular property or candidate gene target. For example, Rohrer et al., 1994 measured the strength of IVM binding to a susceptible and IVM-resistant *H. contortus* isolate and, unlike the previously discussed, initial binding assays performed for BZs and LEV, found no differences in binding affinities or receptor density values between the isolates, suggesting that an alternative mechanism to the modification of the target receptor is employed by the parasite to develop IVM-resistance. In contrast to these findings, at least three investigations led by Blackhall et al. (1998b, 1998a, 2003) found increased variability and changes in the frequencies of SNPs present in genes coding for membrane receptor or transporter subunits found in GluCls, P-gps, and GABA receptors, indicating that mutational changes in the functional receptor subunits might be associated with resistance. The work conducted by Sangster et al. (1999) and Feng et al. (2002) further confirmed that heterogeneous sequences for P-gp and GABA type A receptor genes could be directly responsible for IVM resistance. The latter study, in particular, delineated the key differences between the susceptibility- and resistance-associated receptor subunit alleles (named HG1A and HG1E) in terms of the putative amino acid substitutions likely to originate (located in the extracellular and transmembrane IV domains) and thus lead to resistance. Multiple other studies have also demonstrated the importance of particular amino acid residues at either the transmembrane (Lynagh and Lynch, 2010) or the N-terminal (McCavera et al., 2009) domains of GluCls in conferring or restoring IVM susceptibility. Therefore, based on the evidence available at the time, many of the subunit genes for GluCls and GABA receptors, as well as P-gps, were assumed to be the candidate genes, involved in IVM resistance development in the parasitic nematodes.

Throughout the years the list of putative IVM resistance-conferring candidate genes continued to grow and now included variants of genes such as *avr-14* (Williamson et al., 2011), *dyf-7* (Urdaneta-Marquez et al., 2014)*, glc-1* (Dent et al., 2000), *pgp-1, pgp-2* (Raza et al., 2016) and many others (Table 1). However, in essence, genetic comparisons between geographically and historically distinct isolates or between the free-living and parasitic nematodes, coupled with high innate heterogeneity of the parasitic species, such as *H. contortus* (Sallé et al., 2019), yielded at best circumstantial evidence in most (if not all) of these candidate gene approach studies. Consequently, it

was only a matter of time before evidence to the contrary emerged - showing that the previously identified gene variants, thought to be involved in IVM resistance in parasitic nematodes, do not stand up to scrutiny (Laing et al., 2016; Elmahalawy et al., 2018b; Doyle et al., 2019; Baltrušis et al., 2022a).

Table 1. A list of proposed candidate genes, at some point associated with ivermectin resistance in either *H. contortus* and/or *C. elegans*. Adapted from Doyle et al., 2019

Gene	GeneID (PRJEB506)	Reference
avr-14b / gbr-2 or HcGlu-Clα3	HCON_00020000	(Williamson et al., 2011)
avr-15	HCON_00161180	(Dent et al., 2000)
tub8-9	HCON_00005260	(Eng et al., 2006; Mottier and Prichard, 2008)
che-3	HCON_00009080	(Dent et al., 2000)
dyf-11	HCOI01514900	(Dent et al., 2000)
dyf-7	HCON_00014570	(Urdaneta-Marquez et al., 2014)
ggr-3	HCON_00064090	(Rao et al., 2009)
glc-1	NA	(Dent et al., 2000)
glc-2 / GluClb	HCON_00001030	NA
glc-3	HCON_00148840	(Williamson et al., 2011)
glc-4	HCON_00057900	NA
glc-5/ HcGluCla	HCON_00028600	(Blackhall et al., 1998b)
lgc-36	HCON_00137830	NA
lgc-37 / HG-1	HCON_00076470	(Blackhall et al., 2003)
lgc-55	HCON_00158990	NA
osm-1	HCON_00035760	NA
osm-3	HCON_00121270	NA
osm-5	HCON_00026360	NA
pgp-1 / pgp-9	HCON_00098130/ HCON_00130050/ HCON_00130060	(Van Wyk and Malan, 1988; James and Davey, 2009; Raza et al., 2016)
pgp-2 / pgp-a	HCON_00004450	(Raza et al., 2016)
pgp-11	HCON_00162780	(Raza et al., 2016)
unc-38	HCON_00017680	NA
unc-7	HCON_00190630	(Dent et al., 2000)
unc-9	HCON_00188710	(Dent et al., 2000)
che-11	HCON_00035880	NA
osm-6	HCON_00149350	NA

Gene	GeneID (PRJEB506)	Reference
pgp-3	HCON_00042800	(Raza et al., 2016)
haf-6	HCON_00126030	(Raza et al., 2016)
pgp-13 / pgp-12	HCON_00041390	(David et al., 2018)
mrp-1	HCON_00189480	(James and Davey, 2009)

An alternative, genome-wide sequencing approach to studying complex, quantitative traits, such as resistance development, has recently started being implemented on a more frequent basis to better understand IVM resistance in parasitic nematodes. Although finding the major loci involved in IVM resistance in the genomes of parasitic nematodes has proven to be an arduous task (Luo et al., 2017; Khan et al., 2020), as a result of a combination of backcrossing, coupled with a linkage group selection approach, Doyle et al. (2019) identified a single, ~5 Mbp region on chromosome V (37-42 Mbp), which was significantly more differentiated in the backcrossed progeny of the two well-characterized, parental (F_0) isolates of *H. contortus*. Further evidence in support of this distinct chromosome V region harboring a driving locus (loci) for IVM resistance development was presented by GWAS conducted by i) Sallé et al., 2019 who sequenced over 200 single *H. contortus* belonging to 19 different isolates and compared their genomic structures based on historical resistance data ii) Baltrušis et al., 2022 who studied a wild, semi-resistant phenotype population of *H. contortus* as paired samples before and after the most recent IVM treatment. In his most recent work, Doyle et al. (2022) not only managed to narrow down the highly-differentiated chromosome V region to ~300 kbp (~37.25-37.55 Mbp) but also likely identified the major causal variant behind IVM resistance – a transcription factor encoded by *cky-1* (in *H. contortus* - HCON_00155390). Further evidence for the involvement of *cky-1* in regulating the resistance status to IVM in nematodes has also been uncovered through the analysis of transcriptomic data derived from different genetic crosses of *H. contortus* (Laing et al., 2022).

Whilst not much is known about the function of *cky-1* in parasitic nematodes, the orthologue of this protein in humans, Npas4, is known to activate the expression of genes responsible for synaptic input regulation via the inhibition of excitatory neurons and the reverse – the excitation of inhibitory neurons (Spiegel et al., 2014).

In conclusion, although AVMs and their interactions with the cellular components of parasites have been the subject of extensive investigation and have resulted in great scientific strides in terms of understanding the fundamentals of the mode of action of AVMs (Hibbs and Gouaux, 2011; Lynagh and Lynch, 2012), comparatively little is still known about the resistance development to AVMs in parasitic nematodes in field conditions. The initial studies associated resistance with mutational changes in e.g., drug receptors (GluCls and GABA receptors) or unspecific transporters (P-gps), however, the most recent data seems to suggest otherwise. Despite few consistent findings over the years, the novel genomic approaches have revolutionized the way molecular parasitologists investigate drug resistance and has already led to the identification, through robust and extensive means (using both genomic DNA and RNA sequencing), of a potential, major contributor to IVM resistance in nematode parasites.

3. Aims of the thesis

Re-infections with GINs occur and persist in small ruminants due to the parasites' ability to develop drug resistance. To date, limited data is available on the potential molecular markers of resistance as well as the spread of the resistance-encoding alleles.

The general aims of this thesis were to attempt to establish viable screening strategies for genetic markers associated with drug resistance in veterinary nematodes, determine the presence and spread of drug-resistance-encoding alleles as well as investigate the potential mechanisms underlying resistance in one of the most commonly encountered and the most pathogenic sheep parasite *H. contortus*.

The specific aims for each study were:

Study I. To develop a novel screening tool capable of estimating the frequencies of the most common, BZ-resistance-associated, mutations in field populations of *H. contortus*.

Study II. To employ the previously developed ddPCR screening tool, compare its robustness with another widely used mutation screening tool based on pyrosequencing, and then assess the frequencies of BZ-resistance-associated mutations in the previously collected and bio-banked field *H. contortus* samples from commercially owned Swedish sheep flocks.

Study III. To investigate the 63 bp deletion in the *hco-acr-8* gene as a potential resistance to LEV determinant as well as propose a molecular, ddPCR-based assay to detect the said deletion in field *H. contortus* samples.

Study IV. To identify the genomic change(s) pertaining to IVM resistance in *H. contortus* through a GWAS on a semi-resistant field population before and after the most recent IVM treatment.

4. Materials and methods

A summary of materials and methods used in all four scientific articles is outlined in the chapters and subchapters below. For more in-depth information, please refer to the published material.

4.1 Samples and parasitological evaluation

4.1.1 Sample origin

In **study I**, both single, adult stage *H. contortus* and pooled larvae cultures consisting of a mix of trichostrongylids (among which the major species was *H. contortus*) were used. The adult stage samples were previously isolated, bio-banked individuals from multiple different countries, whereas the larvae cultures were previously collected and hatched (under laboratory conditions) samples from 13 different Swedish sheep farms.

In **study II**, hatched larvae cultures from Swedish sheep were previously collected as part of a routine herd health screening service (www.gardochdjurhalsan.se) over 6 years and contained a mix of trichostrongylids. In total 174 samples from 67 farms were obtained.

In **study III**, six reference *H. contortus* isolates (n=10 per isolate) of known resistance status towards LEV as well as adult worms of Swedish origin (collected on farms where LEV was effective) were all previously harvested and bio-banked samples.

In **study IV**, mixed larvae cultures (wherein the major species was *H. contortus*) from a Swedish sheep farm were obtained by hatching the collected, paired fecal samples (before and 7-days after IVM treatment) under laboratory conditions.

4.1.2 Egg counts and FECRT

In **studies III and IV**, a modified McMaster technique (Ljungström et al., 2018) was used to evaluate the nematode egg counts both before and after treatment.

In **study IV**, the FECR estimate was calculated using *eggCounts* package for R (https://www.r-project.org/).

4.2 Molecular evaluation

4.2.1 DNA extraction and library preparation

In **all studies**, DNA was extracted from samples using a simple column extraction kit (Nucleospin Tissue kit by Macherey-Nagel), following guidelines issued by the manufacturers.

Additional library cleanup steps were taken before the samples were submitted for sequencing in the case of **studies I and III**. In the case of **study I**, AMPure XP magnetic beads were used to clean up the pooled amplicon samples, whereas Qubit dsDNA HS Assay Kit was used to determine the DNA concentrations in these samples. In the case of **study III**, amplified *hco-acr-8* intron 2 loci were cleaned up enzymatically by using Exonuclease I and FastAP Thermosensitive Alkaline Phosphatase to eliminate primer sequences and dephosphorylate DNA ends directly in the PCR mixtures.

4.2.2 Conventional, Droplet digital PCR and Pyrosequencing

Conventional PCR was utilized to amplify either the *β-tubulin* or *hco-acr-8* intron 2 amplicons in genomic *H. contortus* DNA samples in **studies I and III** before submitting these samples (either pooled or unpooled) for sequencing. The PCR protocol for the *β-tubulin* amplicon was as follows: a single cycle of initial denaturation at 95 °C for 2 min, followed by 30 cycles of denaturation at 95 °C for 30 s, annealing at 63 °C for 30 s, and extension at 72 °C for 1 min. An additional cycle of final extension at 72 °C for 10 min was included at the very end. The amplification protocol used for the *hco-acr-8* intron 2 amplicon was dependent on the primer pair used. For primers 'ForInsert' and 'RevInsert', the protocol described by Barrère et al., 2014 was employed. In the case of the custom 'ACR8F1' and 'ACR8R1' primers, the PCR protocol was as follows: 1 cycle of 95 °C for 5 min, 40 cycles of 95

°C for 45 s, 56 °C for 30 s, 72 °C for 1 min, and a single cycle of 72 °C for 10 min. In both **studies I and III**, amplification products were visualized on either 1% or 2% agarose gels.

Droplet digital PCR was utilized in **studies I, II, and III** to measure the frequencies of either the F200Y mutation in *β-tubulin* or the 63bp deletion in *hco-acr-8* intron 2 (the more in-depth information, such as the primer and probe sequence development as well as the experimental setups, is available in the published papers). The sample preparation and amplification protocol for ddPCR was as follows: the reaction mixtures were assembled in 96-well plates at a total volume of 22 µL per well. The standard protocol provided by the manufacturer of the reagents was followed (ddPCR™ Supermix for Probes, catalog # 186–3010, BioRad). In short, a single reaction mixture was prepared by introducing 11 µL of 2x ddPCR Supermix for Probes, 1.1 µL of each 20x stock solution for primers/X probe (FAM™) and primers/Y probe (HEX™) at the concentrations of 900 nM and 250 nM for primers and probes, respectively, as well as varying amounts of nuclease-free water and DNA template. Nanoliter-size water-in-oil droplets were then generated using an automated droplet generator (along with DG32 Cartridges) and dispensed into a new 96-well plate. The plate containing the droplets was heat sealed with plastic foil (PX1 PCR Plate Sealer) and transferred into the thermal cycler (MyCycler™ Thermal Cycler, BioRad). The optimized PCR conditions were as follows: a single cycle of 95 °C for 10 min, 40 cycles of 94 °C for 30 s, and then 58 °C for 1 min, followed by a final cycle of 98 °C for 10 min to deactivate the enzyme. After the amplification step, the plate containing the droplets was loaded into the droplet reader (QX200, BioRad) and further analyzed using *QuantaSoft* software, which generates DNA copy measurements and the 95% confidence interval estimates for every sample by fitting the data onto a Poisson distribution (Hindson et al., 2011). In both **studies I and III**, the developed ddPCR assays were further optimized by performing temperature gradient and either LoD and/or fractional abundance precision identification experiments, as described by Elmahalawy et al., (2018b).

Pyrosequencing was employed in **study II** to determine the frequency of the F200Y mutation and compare the data to the previously described ddPCR assay. The detailed protocol for this assay has been readily described elsewhere (Von Samson-Himmelstjerna et al., 2009).

4.2.3 Sequencing

Amplified, pooled *β-tubulin* and unpooled *hco-acr-8* intron 2 amplicons were sequenced and subsequently analyzed with either the PacBio RSII platform (**study I**) or Applied Biosystems™ 3730xl DNA Analyzer platform (**study III**). The gDNA derived from the paired larvae culture samples in **study IV** was sequenced with the Illumina platform (NovaSeq 6000, using 150 bp paired-end chemistry).

4.3 Bioinformatic analysis

4.3.1 Amplicon sequence analysis

H. contortus β-tubulin amplicon sequences, obtained in **study I**, were demultiplexed and pre-filtered with the jMHC software (Stuglik et al., 2011). Further sequence variant filtering was carried out by manually enforcing criteria to remove artifacts (the precise steps for filtering both single adult and larvae culture samples are described in detail in the materials and methods section of the published study). A phylogenetic tree, based on the filtered *β-tubulin* isotype-1 locus sequences derived from single worms, was constructed using MrBayes software (Ronquist et al., 2012). Dendroscope 3 (Huson and Linz, 2016) was used to visualize the relationships between the aligned sequences.

Sequenced *hco-acr-8* intron 2 amplicons in **study III** were analyzed with the CodonCode Aligner software. The alignments for sequences were evaluated manually, the paired-end reads were merged into a consensus sequence, primer sequences were trimmed away and the low-quality bases from the ends of the consensus reads - were removed.

4.3.2 Whole genome data analysis

Paired-end Illumina reads, obtained by sequencing the genomic DNA of *H. contortus* larvae pools (before and after IVM treatment) in **study IV**, were analyzed by first mapping the sequencing data onto a reference genome (Doyle et al., 2020) using the bwa-mem algorithm (Li and Durbin, 2009). Picard (https://github.com/broadinstitute/picard) and samtools (http://www.htslib.org/) were further used to remove duplicate reads and retain only those reads, which are properly aligned in a pair and construct pileup files. Mean nucleotide diversity and Tajima's D values were calculated per chromosome, per 100kbp genomic windows using NPstat

(https://github.com/lucaferretti/npstat). Pairwise genetic differentiation between samples from the two treatment groups was evaluated by using Popoolation2 (Kofler et al., 2011) and i) performing Cochran-Mantel-Haenszel (CMH) and Fisher's exact tests (FET) to detect consistent changes in allele frequencies after treatment as well as ii) calculating average F_{ST} values throughout the genome in a 10kbp sliding window approach in addition to every gene to detect regions in the genome which had undergone recent changes. SNP calling was carried out using bcftools (http://www.htslib.org/doc/bcftools.html) and the called SNPs were annotated using SNPEff (http://pcingola.github.io/SnpEff/).

4.4 Data visualization and statistical analysis

In **study I**, images and plots were generated with (newest at the time) Quantasoft, GraphPad, or MedCalc software. Wilcoxon matched-pairs signed rank test, used to compare the F200Y mutation frequency data between NGS and ddPCR platforms, was carried out using the GraphPad software.

In **study II**, *DescTools* package for R was used to estimate Lin's concordance correlation coefficient between the ddPCR and Pyrosequencing assay outputs. Packages *lme4*, *lmerTest*, and *emmeans* were further utilized to fit and analyze a linear mixed effect model, using the F200Y mutation frequency data as the response variable, while the time of sample collection and the drug used were both used as explanatory variables. In addition, the sample number was used as a crossed random effect.

In **study III**, frequencies of genotypes for reference isolates were analyzed with Pearson's Chi-squared test in R.

In **study IV**, nucleotide diversity and Tajima's D ratios, as well as F_{ST} measurements per genomic window, were determined to be substantially different if higher than genome-wide (or gene-wide) mean+3SD and/or genome-wide (or gene-wide) mean+5SD values. Significance ($p \leq 0.05$) in the case of FET and CMH test was determined using a genome-wide Bonferroni correction.

In **studies II**, **III,** and **IV**, images and plots were made with the ggplot2 package for R.

5. Main results and discussion

The chapters and subchapters below describe the main findings presented in studies I-IV. For more detailed information, please refer to the published studies.

5.1 Molecular analyses of samples

5.1.1 Droplet digital PCR

Study I. A novel ddPCR assay was created to provide a universal tool for the detection and quantification of the F200Y mutation in the isotype-1 *β-tubulin*. In addition, steps were taken to optimize the assay prior to comparing it to the NGS platform in terms of robustly quantifying the F200Y mutation frequencies in field larvae culture samples. Both the LoD and fractional abundance precision test experiments displayed promising results. The LoD for some ddPCR assays has been reported to be as low as 0.01–0.04% of the total mutant sequence abundance (Luo and Li, 2018). However, most of this type of research is focused on the detection of altered DNA in cancerous and/or pre-cancerous cells. Therefore, it is difficult to compare our results with those from other studies. Mutant (MT) larvae are multicellular and can be present in variable numbers in fecal cultures from infected animals. Thus, a threshold of 2.6% MT sequence abundance for our LoD is a decent starting point that could potentially be further diminished through rigorous optimal condition testing if necessary. The pilot test (i.e., fractional abundance precision test), achieved by mixing susceptibility- and resistance-encoding allele sequences at different ratios, showed a clear, linear, and reproducible pattern.

The final comparison of the frequency data (obtained with NGS and ddPCR) for the F200Y mutation from paired larvae cultures, recovered both pre- and post-treatment with ABZ from different farms around southern Sweden, showed an impressive correlation (r = 0.988, Spearman correlation). The frequencies of the MT allele (between the two methods) displayed very similar overall values, as confirmed by statistical testing (p=0.104, Wilcoxon matched-pairs signed rank test) (Figure 6).

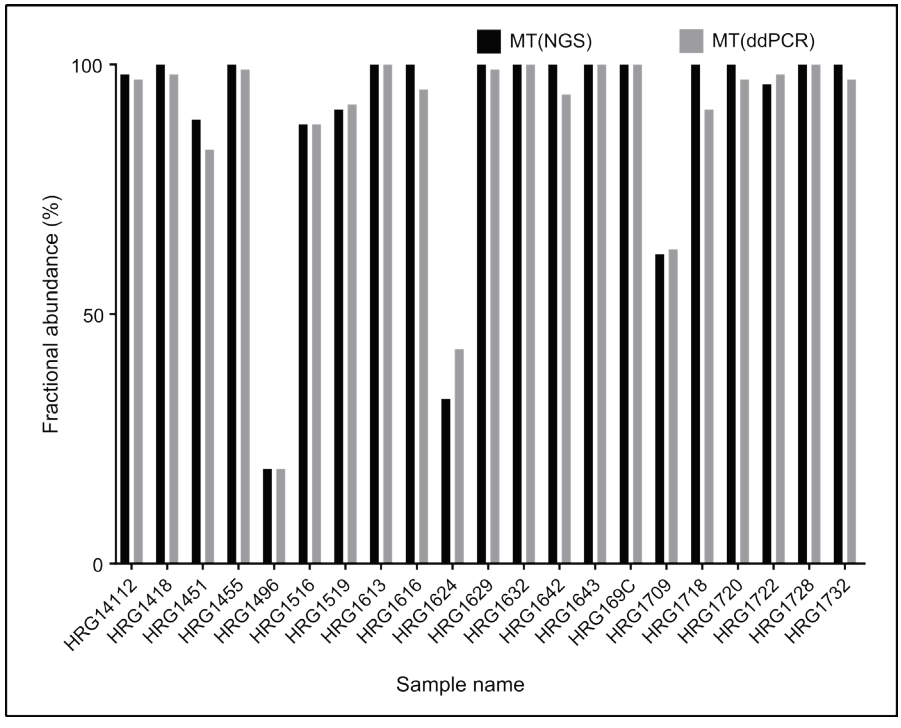

Figure 6. A comparison of identified, F200Y mutation containing (MT) allele fractional abundances (%) between NGS and ddPCR techniques. Black bars – MT DNA fractional abundance in larvae culture samples as determined by NGS, gray bars – MT DNA fractional abundance in the samples as determined by ddPCR

Study II. Previously, a study was carried out in a similar fashion (Höglund et al., 2009), however, it utilized pyrosequencing to obtain the frequencies of the F200Y mutation in the isotype-1 *β-tubulin* in the pre-treatment larvae culture samples across various *H. contortus*-positive flocks on 45 randomly selected farms. We have, thus, set out to compare the frequency

data for this mutation between the different methods to determine if our established ddPCR assay generates comparable results to those of the pyrosequencing when performed on the same samples. Data was generated by pyrosequencing and ddPCR platforms for genomic DNA extracted from seven different laboratory strains of *H. contortus* maintained in sheep. An excellent (r=0.987, Lin's concordance correlation coefficient) correlation in addition to a negligible difference in frequency estimations (1.2% on average) was obtained, indicating that the two methods display a great degree of overall similarity when measuring mutation frequencies. It is noteworthy, however, that when handling highly homozygous DNA-containing samples, unlike ddPCR, pyrosequencing assays present a limiting threshold value (≥5%) for the detection of the minor allele (Ogino et al., 2005; Spittle et al., 2007; Tsiatis et al., 2010). Furthermore, each individual sample run on the ddPCR platform is provided with an estimated 95% confidence interval, thus effectively eliminating the need for technical replicates.

The frequencies of mutations F167Y and F200Y were estimated in *H. contortus* positive, pooled larvae cultures collected from 67 sheep farms across Sweden. The frequency of the F167Y mutation was found to be comparably low in the tested samples ($0.78 \pm 2.2\%$ on average). This is in contrast to a much more diverse variation and overall higher abundance of this particular SNP in *H. contortus* isolated from sheep flocks in the United Kingdom (Redman et al., 2015). On the other hand, the F200Y mutation was found to be present at high frequencies in the samples from most farms, irrespective of the year (Fig. 7).

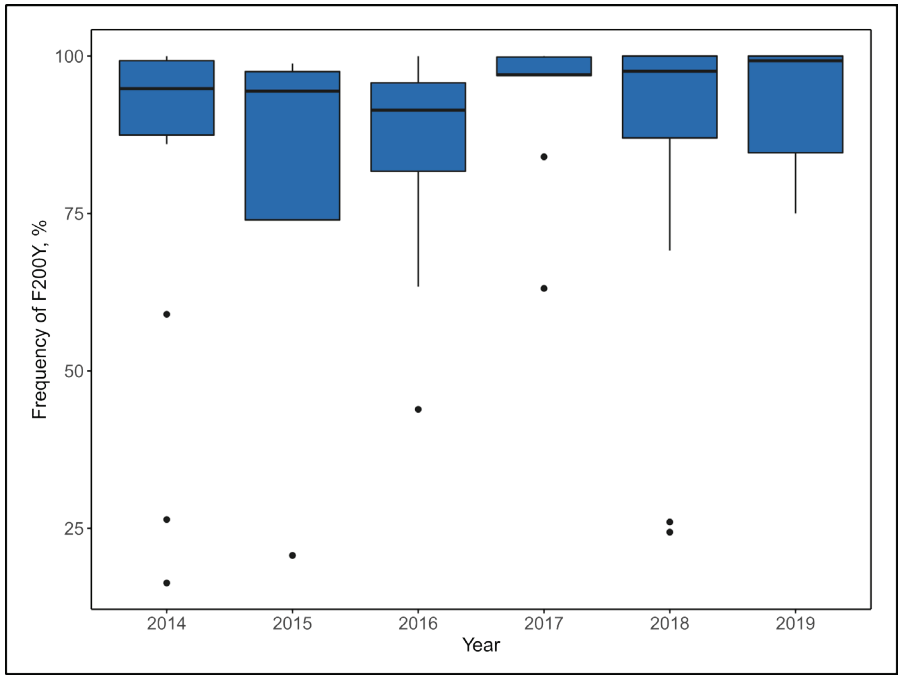

Figure 7. The frequency (%) of the F200Y mutation in the isotype-1 *β-tubulin* belonging to *H. contortus* in the pre-treatment samples collected from various farms around Sweden during the years 2014-2019. Farms were sampled 1-7 times per year. An average annual mutation frequency was calculated for farms tested more than once at any given year

This is in agreement with previous similar studies, acknowledging the F200Y mutation to be the most commonly abundant resistance to BZ drugs conferring SNP in *H. contortus* within Europe (Redman et al., 2015; Ramünke et al., 2016; Claerebout et al., 2020) and the Americas (Brasil et al., 2012; Barrere et al., 2013). When comparing the frequencies of the F200Y mutation-containing allele in the pre-treatment samples between the two Swedish studies a significant increase was observed ($p < 0.001$, t-test). This, in turn, indicates that the situation regarding the frequency of the (BZ-resistance conferring) F200Y mutation in *H. contortus* has deteriorated, compared to the findings from the study carried out a decade earlier and based on pyrosequencing (Höglund et al., 2009). We also investigated the frequency changes of the F200Y mutation on farms where both pre- and post-treatment samples were collected from naturally infected sheep treated either with ABZ or IVM. Increases in the frequencies of the F200Y mutation were observed in several of the ABZ-treated flocks, however, in most cases, there was either

a modest or no increase after treatment (with an overall increase in the F200Y containing allele frequency post-treatment of 7.1%). This is likely due to the high-frequency levels of this mutation in the populations to begin with which, makes the fractional changes, in most cases, either subtle or insignificant. Nevertheless, the LMM that we applied to our data displayed a significant increase in the frequency of the F200Y mutation between pre- and post-treatment samples ($p < 0.01$, LMM). Hence, this provides strong evidence for the selection of this mutation after treatment with ABZ. As for IVM-treated flocks, pre- and post-treatment sample comparison showed an average increase of 1.7% in the frequency of the F200Y mutation post-treatment. However, the same issue of high mutant allele frequency in pre-treatment samples was observed on most farms, which might have prevented us from detecting a more subtle selection. Although such a small increase in the frequency of the said mutation in post-treatment samples was shown to be statistically insignificant ($p = 0.15$, LMM), it has been suggested that treatment with MLs predisposes the population for the selection of individuals possessing the canonical F200Y mutation (and thus resistant to BZ drugs) (Eng et al., 2006; Mottier and Prichard, 2008; Santos et al., 2017). This, in turn, could explain the maintenance of the high frequency of the mutant (i.e., F200Y mutation containing) allele across the farms where IVM was used more extensively, although more data is needed to support this claim.

Study III. The 63bp deletion in *hco-acr-8* in *H. contortus*, previously linked with LEV-resistance, has been investigated by performing sequencing and PCR amplification (conventional and ddPCR) experiments on both single worm, reference isolates and larvae cultures from Swedish sheep farms. Despite the limitations in identifying 'RS' genotypes with both Sanger sequencing as well as conventional PCR, the results for the comparisons between the direct deletion detection (sequencing and conventional PCR) and indirect deletion detection (ddPCR) assays, in terms of identifying the genotypic status of each of the tested reference *H. contortus* isolate, were overall congruent. However, the clear advantage of ddPCR over conventional PCR is that the former is claimed to eliminate amplification bias by constraining the results of the amplification to a binary outcome (Hindson et al., 2011). In addition, by validating our ddPCR assay, we not only established that both primer and probe pairs do not cross-react with one another when used in a duplex reaction but also that the dilution of the gDNA containing only the "susceptible" allele with gDNA containing only the "resistant" allele results

in a perfectly linear pattern of decrease in the frequency of the diluted allele (and an increase in the "resistant" allele; r=0.9975, Spearman correlation). However, it is important to point out that cross-reactivity between the primers and probes in a single reaction is fundamentally different from biases, resulting from variation in primer and probe binding efficiencies. Furthermore, the calculated correlation between the sample dilution and allele frequency reflects only the technical variability and the capacity of the assay to distinguish and estimate different proportions of the "sensitive" and reference allele amplicons in a sample. Thus, while a good linear relationship across the dilution series was observed, the accuracy was not as good. Samples, containing only the "sensitive" allele did not reach 100% in the frequency of this allele (or 50% in the case of RS genotypes), likely due to differences in amplification efficiency between the two amplicons. Nevertheless, unlike in any of our previous approaches for the detection of SNPs (Baltrušis et al., 2018) or genetically variable regions for species differentiation (Baltrušis et al., 2019), this study employs a more robust design, utilizing the simultaneous absolute quantification of two distant regions within the *hco-acr-8* for the indirect determination of the frequency of the "resistant" (i.e., 63 bp deletion containing) allele. Having examined the six reference isolates (n = 10 per isolate) using the ddPCR platform, it was found that the genotypic status of individuals within the reference isolate groups (S1–S3 and R1-R3) agreed for the most part with the results obtained with conventional PCR. Notably, in the case of S1 (ISE isolate) which has been previously confirmed to be of RS genotype for the 63bp deletion (Barrère et al., 2014), conventional PCR failed to elucidate the heterozygosity due to poor amplification. However, 50% of worms (5/10) within this isolate were indeed found to be RS when analyzed with the more sensitive ddPCR assay (Figure 8).

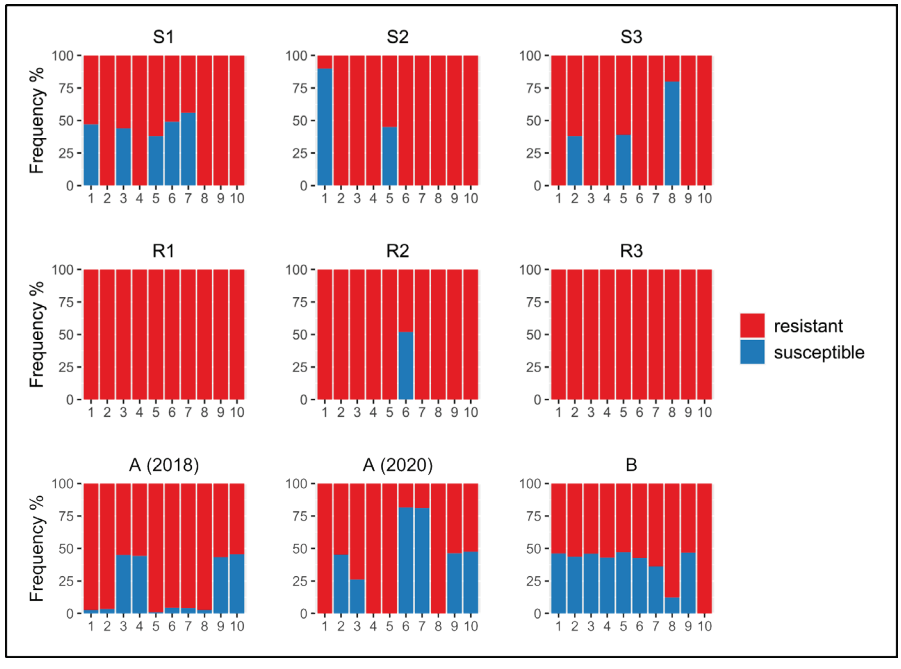

Figure 8. The relative frequencies (%) of "susceptible" (full-length second intron sequence; in blue) and "resistant" (containing 63 bp deletion in the second intron in *hco-acr-8*; in red) alleles in various individual, adult *H. contortus* isolates. S1–S3 correspond to LEV-susceptible isolates – ISE, Weybridge, and Zaïre, whereas R1-R3 to LEV-resistant – Cedara, Borgsteede, and Kokstad. A (2018), A (2020) and B represent allele frequencies in individual, adult *H. contortus* isolated from Swedish farms A (in 2018 and then 2020) and B, respectively

Even though ddPCR seemed to provide more sensitive measurements, the "susceptible" allele was, in many cases, underestimated, leading to the overestimation of the "resistant" allele. Thus, neither the 50% frequency of the "susceptible" allele, in the case of 'RS' individuals (i.e., S1-1,3,5, S2-5, and S3-2,5), nor the 100% for fully 'SS' individuals (i.e., S2-1 and S3-8) were reached. The frequency between 25% and 75% of the "susceptible" allele in a single worm sample was, therefore, considered to be indicative of the 'RS' genotype and >75% of the 'SS' genotype. A similar pattern of underestimation was also observed for adult worms derived from the Swedish farms (one of which was sampled at two different time points – 2018 and then again in 2020). Here, samples A (2020)-3 and B-8 were severely underestimated in terms of the "susceptible" allele but, at the same time, were distinguishable from what we considered to be minor contaminations, observed in A (2018)-

1, 2, 5, 6, 7 and 8. Quite unexpectedly, individuals possessing the 'SS' genotype were the rarest, even among the phenotypically susceptible isolates (2/30). Moreover, apart from populations A (2020) and B, the "resistant" allele was much more common, even in the reference isolates, confirmed to be susceptible to LEV (S1–S3). Despite the difference in the three genotypes between the susceptible (S1–S3) and resistant (R1-R3) isolates being statistically significant ($p = 0.01$, Chi-square test), which is in line with Barrère et al. (2014), in well-characterized (and susceptible to LEV) isolates half or more of the individuals were found to be of 'RR' genotype. Thus, judging by the obtained genotypes for all six isolates, it appears as the 63bp deletion in *hco-acr-8* alone is a poor predictor of the actual phenotype in individual worms.

Larvae culture samples, derived from sheep before and after treatment with levamisole, were used to quantify the presence of the deletion in the field (Figure 9).

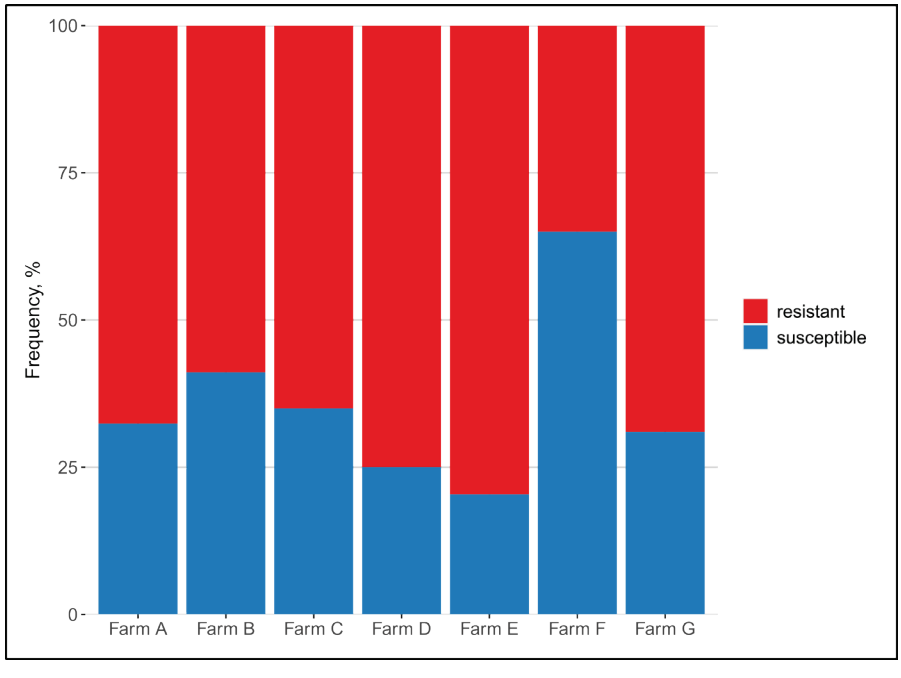

Figure 9. The frequencies (%) of "susceptible" (full-length second intron sequence; in blue) and "resistant" (containing the 63 bp deletion in the second intron in *hco-acr-8*; in red) alleles in larvae cultures, recovered from different Swedish sheep farms (A, B, C, D, E, F, G) pre-treatment with LEV. Note: Six of the post-treatment samples did not

contain *H. contortus* DNA (and are thus not shown) whereas population G did not have a post-treatment sample taken, due to low egg counts in the pre-treatment samples

Not only was the 63bp deletion found in all tested populations (Farms A-G) before treatment, but the frequency of the deletion varied to a great extent (35–80%). Interestingly, in six of those populations, no trace of amplifiable *H. contortus* DNA was found in any of the post-treatment samples, indicating that the LEV treatment had been successful. This observation was consistent with the FECRT data. Although it has been previously posited that resistance caused by the deletion is likely to be genetically recessive, i.e., 'RS' individuals might still be susceptible (Santos et al., 2019), overall, it appears that the increased frequencies of the deletion in *hco-acr-8* did not at all correspond to heightened resistance to the effect of LEV in the field isolates upon treatment. Furthermore, unlike the previous study by Santos et al., 2019, wherein the "resistant" allele frequencies were correlated with LD50 values, the variation in the frequency of the "resistant" allele among the tested Swedish single worm isolates and larvae cultures (derived from farms wherein LEV was efficacious) proved to be somewhat random.

5.2 Bioinformatic analyses

5.2.1 Amplicon sequencing

Study I. Presence and frequencies of the most common BZ-resistance conferring mutations were determined by sequencing the isotype-1 *β-tubulin* locus in the single worm and larvae culture samples containing *H. contortus* to provide an adequate reference for the creation and optimization of the aforementioned ddPCR assay. Of the total 45 identified partial isotype-1 *β-tubulin* locus sequences in single worm samples, 24% (11) had the F200Y mutation, 9% (4) – F167Y, and 4% (2) - E198? ("?" being a different combination of nucleotides at this codon, leading to the incorporation of various amino acids). This pattern of mutation frequencies and rarity level is in agreement with the previous work (Barrere et al., 2013; Chaudhry et al., 2015; Redman et al., 2015; Ramünke et al., 2016). Taken together, the data suggest that the first discovered F200Y mutation in isotype-1 *β-tubulin* of *H. contortus* is still the most relevant BZ-resistance conferring mutation to this day. As it was previously described by several authors (Silvestre and Cabaret, 2002; Ghisi et al., 2007; Von Samson-Himmelstjerna et al., 2007), all adult worm DNA

samples, in which we found mutated *β-tubulin* sequences, had only a single mutation (either F167Y, E198? or F200Y) per sequence read, meaning no double or triple mutants for amino acids at P167, P198 or P200 were uncovered. Two of those *β-tubulin* locus DNA sequences contained previously undescribed, novel mutations at the P198 codon. Sequence 347, present in one of the samples isolated from Brazil, contained GAA→TTA (Glu→Leu) mutation, whereas Sequence 394, isolated from Sweden (Troell et al., 2006), harbored GAA→ACA (Glu→Thr) nucleotide sequence change. It is not entirely clear whether these genotypes confer resistance toward BZ class drugs in *H. contortus*. However, assuming that they do would indicate that a wider array of amino acid substitutions could be tolerated at the P198, while still maintaining the resistance status of an individual. The different resistant genotypes were present between 18% to 100% in the various samples from different countries such as Australia, Brazil, Canada, Ethiopia, Germany, Guadeloupe, Greece, Kenya, and Sweden. In 9 of the 13 countries, wherein the single worm samples were collected (Australia, Brazil, Canada, Ethiopia, Germany, Guadeloupe, Greece, Kenya, and Sweden), mutant sequences were found. These findings essentially confirm the results from multiple other studies (e.g., Ghisi et al., 2007; Höglund et al., 2009; Barrere et al., 2013; Mahieu et al., 2014; Chaudhry et al., 2015; Ramünke et al., 2016; Zhang et al., 2016). Interestingly, only wild-type (i.e., BZ-susceptible) single worms were recovered from four locations - South Africa, Cambodia, Java, and Argentina. In the case of South Africa, it is highly suspicious that no mutant sequences were detected, considering that the situation regarding AR in that region has been known to be dire for almost two decades (Van Wyk et al., 1999). The same holds true for Argentina (Eddi et al., 1996; Waller et al., 1996) and Southeast Asian countries (Haryuningtyas, 2002). Thus, it is likely that no resistant isolates were found in these regions due to a limited pool of available samples.

Out of the larvae culture samples collected across southern Sweden during the 2014–2017 period – 32 unique sequences were derived. Unexpectedly, as many as 22 (73%) of these were positive for the canonical TTC→TAC transversion at the P200 in the *β-tubulin* locus, further confirming the postulation about the importance of the F200Y mutation in BZ-resistance. The other 10 sequences were wild-type at P167, P198, and P200. Paired pre- and post-treatment (with ABZ) larvae cultures were evaluated for

the presence of F200Y-containing alleles. Most of the post-treatment samples across various farms displayed either increased or readily high and thus unchanged frequencies of sequences containing the F200Y mutation. Collectively, this indicates that resistant isolates are already widespread across the southern parts of Sweden, although, the initial FECRTs performed on these samples showed efficient egg count reduction. Thus, the real situation seems to be more complicated: the resistant (F200Y mutation containing) isolates were indeed reduced in number after treatment (based on the generated read counts), but smaller proportions of the population continued to persist. This finding highlights, in part, the shortcomings of the FECRT method, mainly insensitivity (to detect resistant isolates), as reported before by others (Miller et al., 2006; Höglund et al., 2009).

5.2.2 Whole genome sequencing

Study IV. A field nematode population (dominated by *H. contortus*) was suspected to be resistant after the most recent treatment with IVM. Therefore, larvae culture samples before and after treatment were analyzed to study the genome-wide changes in response to IVM exposure. Both pre- and post-treatment L3 pools revealed very similar genome-wide profiles for nucleotide diversity and Tajima's D estimates. On average, the estimates of Tajima's D (measured in 100 kbp windows throughout the genome) were below 0 in both treatment groups, suggesting that the population had undergone selective sweep(s) in the past. This is arguably not surprising, given that the field population has undergone multiple previous anthelmintic treatments in the recent past. Nevertheless, little is known about the unique evolutionary history of this population, making it difficult to understand the patterns of nucleotide diversity as well as Tajima's D existing prior to the current treatment with IVM. Consistent with loss of diversity due to recent sweeps, we identified relatively low levels of nucleotide diversity, which was approximately 10-fold lower in both treatment groups in comparison to other field-derived but laboratory-maintained strains, MHco4[WRS] and MHco10[CAVR] (Doyle et al., 2019). Although a relatively comparable raw read coverage was sequenced (120–137 × unmapped vs. 199.65 × raw read coverage in Doyle et al., 2019), a lower proportion of reads were mapped to the genome overall, which may be a consequence of either: (i) an inefficiency of mapping divergent sequencing data to the reference, especially around variable sites, (ii) a higher number of duplicated sequencing reads, reducing the effective

mapped coverage; and/or (iii) a higher degree of homozygosity and/or reduced genetic variation in the studied population. The comparable levels of nucleotide diversity throughout the genome meant that measures of pairwise genetic differentiation by F_{ST} revealed little deviation between the two groups; although minor sporadic differentiation throughout the genome was observed (i.e., we did identify 152 10 kbp windows greater than 5SD from the genome-wide mean), the population pre- and post-treatment was broadly genetically similar. The maintenance of genetic diversity in the post-treatment group suggests that resistant alleles are likely to be present on multiple, different genetic backgrounds in the studied field population, which would reflect that the population itself has gradually evolved to be resistant over time with sufficient admixture between resistant and susceptible worms, rather than from a recently acquired *de novo* mutation which has spread quickly in the population.

Despite the genetic similarities between the groups, we attempted to find peaks of differentiation (F_{ST}) across the six chromosomes, which we defined as at least three consecutive 10 kbp windows above mean F_{ST} + 3SD/5SD. Whilst we observed multiple (n = 41) minor peaks (> mean F_{ST} + 3SD; 26 present in chromosome IV), only a single major peak (> mean F_{ST} + 5SD) was found in chromosome V beginning at around 37.4 Mbp, suggesting that the highest degree of consistent, genome-wide genetic differentiation between the groups is present within the previously suggested region containing the major QTL of IVM resistance (Doyle et al., 2019).

An analysis of genetic differentiation (post-treatment) among the top, previously proposed candidates responsible for IVM resistance did not show any of these genes to be among the statistical outliers (> 3SD from the genome-wide mean). Instead, our data suggested 636 genes with higher genetic differentiation than the most differentiated among the candidates, *lgc-37*, leading us to question the role (if any) that *lgc-37* plays in the development of IVM resistance. The three highest F_{ST} estimates were obtained for genes HCON_00141660 (orthologue to *vap-1* in *C. elegans*), HCON_00128970, and HCON_00115660, the latter two of which had no orthologues in other nematode species (according to WormBase Parasite) in addition to being located in chromosome IV (48943064–48943459 bp; F_{ST} = 0.113 and 26890603–26892649 bp; F_{ST} = 0.098); while there was not convincing evidence of a broader selection footprint around these regions in either the genome-wide differentiation (F_{ST}) or nucleotide diversity analyses, a single

SNP (pos: 44,255,647 bp) in the non-coding region of the genome and close to the location of HCON_00128970 in chromosome IV was statistically significant in the FET (but not CMH) analysis. In addition, having investigated the outlier 10 kbp windows (wherein F_{ST} value > genome-wide mean F_{ST} + 3 or 5SD) and outlier SNP frequency changes (wherein P-value > genome-wide mean P-value + 3 or 5SD in FET and CMH test) for the presence of overlapping, non-synonymous SNPs in the coding region of the genome, we found clusters of non-synonymous variants at the terminal region of chromosome IV (around 40–51 Mbp). These data suggest that the terminal end of chromosome IV and perhaps HCON_00128970, even though its function or relation to resistance is not known, require further investigation.

Pairwise comparison of nucleotide diversity did, however, identify a single outlier region around the 36.2–38.7 Mbp region (mean π value ratio + 3SD) in chromosome V which contained a 1.35 × increase in diversity in the post-(relative to pre-) treatment group (Figure 10).

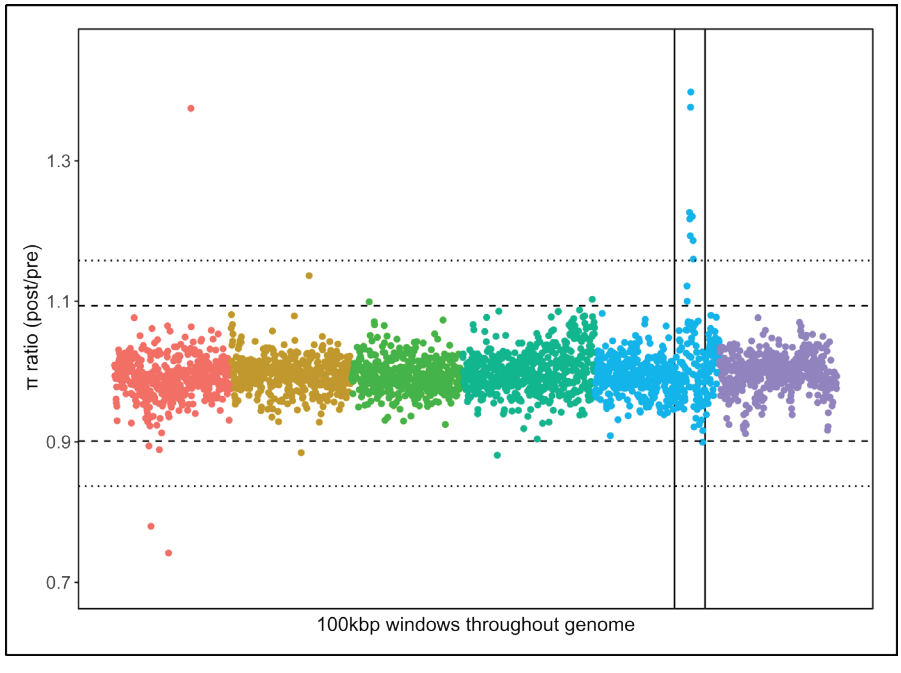

Figure 10. Genome-wide nucleotide diversity ratios were analyzed per every genomic 100 kbp window to identify subtle signs of selection in the post-treatment group. The level of significance is indicated by the dashed black line (mean ± 3SD) and the dotted black line (mean ± 5SD). Colors represent different chromosomes (I-V and chromosome

X). The peak in diversity change in the chromosome V region (36.2–38.7 Mbp) is highlighted by two vertical, solid lines

While the direction of this change was surprising, i.e., the post-treatment pool was more diverse than the pre-treatment, we hypothesize that this pattern may be associated with the improved detection of low-frequency variants (after the removal of a large proportion of the population following treatment) within a region which had previously undergone a selective sweep. Our hypothesis is based on the fact that this region coincides with the major IVM QTL previously reported in chromosome V, identified via genetic backcrossing of resistant alleles into a susceptible genetic background (Doyle et al., 2019).

6. Concluding remarks

This work, consisting of four scientific papers, contributes to our overall understanding of AR in the parasites of ruminants by putting forth appropriate protocols and knowledge to study it in addition to providing insight into the spread of resistance alleles in the field as well as possible resistance determinants. The main conclusions of the papers are:

- Droplet digital PCR is an excellent tool for conducting precise and sensitive mutation detection and quantification assays in mixed genotype populations of genetically variable parasitic organisms (such as *H. contortus*).
- The increased frequencies of the F200Y mutation, among the *H. contortus* recovered from Swedish sheep over six years, effectively suggest that the efficacy of BZ drugs is on the decline on farms in southern Sweden.
- On its own, the 63bp deletion in the intron 2 of *hco-acr-8* is a poor predictor of LEV-resistance in *H. contortus* in addition to presenting difficulties in creating reliable molecular detection assays due to the increased genetic variability within this intronic region.
- IVM resistance in field populations of *H. contortus* appears to be acquired through multiple soft-selective sweeps. However, the evidence suggests that a genomic region within chromosome V (36.2–38.7 Mbp) is distinctly differentiated in response to treatment and likely harbors important causal variant(s) behind IVM resistance development.

7. Future perspectives

The evidence of the readily widespread distribution of BZ resistance alleles in parasitic nematodes throughout Swedish sheep farms coupled with our lack of knowledge regarding the molecular determinants causing the development of LEV and IVM resistance, among our other findings, paint a grim picture for sustainable livestock farming and animal health. However, an extension of the current work could potentially address some of the issues by investigating the following topics:

- **Creating robust molecular tools for detecting resistance**. A continuous effort should be put into creating and refining cheap but robust molecular tools for reliable and rapid BZ-resistance detection on farms, replacing traditional microscopy-based methods.
- **Uncovering more causal or linked variants associated with BZ-resistance development**. Given that drug resistance is generally a quantitative trait, effort should be put into uncovering other potential determinants leading to *H. contortus* developing BZ resistance. Isotype-2 *β-tubulin*, in particular, should be investigated for possible mutations, linked with the resistance phenotype in the field.
- **Further investigating LEV resistance and the causal variants**. Both genomic, as well as subsequent genetic studies, should focus on determining and describing the molecular events leading to LEV resistance in *H. contortus*. To achieve successful monitoring and slow down LEV-resistance development, molecular tools, employing the detection and quantitation of the said molecular events leading to LEV resistance should be employed to screen parasite samples from farms.

- **Refining the chromosome V region where a major QTL for IVM was found**. The locus in chromosome V and the genes found within that region need to be investigated and the major causal variant(s) behind IVM resistance identified and exploited to create molecular screening tools capable of rapid resistance allele examination in parasite samples from sheep farms.

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Popular science summary

Parasitic roundworms are abundantly present across the globe and can infect humans, animals, and plants. Not uncommonly, species of roundworms – nematodes – infect and parasitize grazing livestock. Such species tend to produce a variety of symptoms, of different degrees, in the infected host, such as those that are subclinical (discomfort, sluggishness, reduced weight gain) or clinical (anemia, diarrhea, sudden death).

Currently, the most reliable way to control these infections with parasitic roundworms in farmed livestock is by administering drug treatment to the affected flocks or herds. Treatment with broad-spectrum drugs belonging to various anthelmintic classes, such as benzimidazole derivatives, tetrahydropyrimidines, or macrocyclic lactones is usually the most common, especially in sheep deworming procedures. However, decades of intensive use have globally led to the parasites developing resistance to all drugs belonging to the aforementioned anthelmintic classes.

The parasitic sheep nematode *Haemonchus contortus* is probably the most studied, yet enigmatic species as despite multiple decades worth of research comparably little is known about the resistance development to anthelmintic drugs in this species in the field. For instance, while three major breakthroughs took place from the mid-90s to mid-2000s when three mutations were identified as major contributors to the development of resistance to benzimidazole drug derivatives, our understanding regarding the resistance development to the other two drug classes in this species (and sheep nematodes in general) is limited in comparison. Thus, while identifying these three mutations, linked to benzimidazole drug resistance, in parasitic roundworms could potentially help inform the farmers and veterinarians on the current state of parasite resistance on an individual farm (and thereby guide

a more pragmatic treatment strategy and drug selection), it can only be achieved for a single drug class and not the other two.

The research papers presented in this thesis were therefore focused on developing a monitoring tool for benzimidazole resistance detection in the parasitic *H. contortus*, derived from Swedish sheep, as well as using this tool to establish the spread of benzimidazole drug resistance in this parasite across Sweden. In addition, we employed 'single-candidate' and 'genome-wide comparison' approaches to study the effects that different drugs have on either a specific gene or throughout the genome and in this way identify major contributors to the development of resistance.

There is an urgent need for more complementary research, particularly in identifying consistent changes in response to treatment (and thus identifying the mechanisms behind resistance) with both tetrahydropyrimidines and macrocyclic lactones in order to create novel tools to detect resistance development in parasitic nematodes on farms. As regards our contribution to the field of veterinary parasitology - we have provided a robust tool to detect benzimidazole resistance in *H. contortus*, updated the knowledge regarding the frequency of mutations predicting resistance to benzimidazole derivatives in Sweden as well as refined our understanding of the possible targets and changes in *H. contortus*, associated with tetrahydropyrimidine and macrocyclic lactone class drug treatment. Collectively this puts us a step closer to understanding anthelmintic drug resistance in the roundworm parasites of livestock, developing screening tools to halt the spread of resistance, and finally putting forth viable solutions to the problem of resistance.

Populärvetenskaplig sammanfattning

Parasitiska rundmaskar finns rikligt över hela världen och de kan infektera såväl människor, djur som växter. Det är mycket vanligt att olika rundmaskarter (nematoder) infekterar och parasiterar betande hjusdjur. De olika parasitarterna tenderar att ge en mängd olika symptom, av olika svårighetsgrad, hos den infekterade värden. Symptomen kan vara subkliniska (försämrat allmänstillstånd, nedsatt tillväxt) eller kliniska (anemi, diarré, plötslig död).

För närvarande är det mest tillförlitliga sättet att behandla infektioner av parasitiska rundmaskar hos betande husdjur att administrera läkemedel till djuren i de drabbade flockarna eller besättningarna. Behandling med bredspektrumverkande avmaskningsmedel av olika anthelmintikaklasser, såsom bensimidazolderivat, tetrahydropyrimidiner eller makrocykliska laktoner är den vanligaste, särskilt vid avmaskning av får. Decennier av global intensiv användning har dock lett till att parasiterna utvecklat resistens mot alla läkemedel som tillhör de tidigare nämnda anthelmintikaklasserna.

Den parasitära fårnematoden stor löpmagmask (*Haemonchus contortus)* är förmodligen den mest studerade, men ändå den mest gåtfulla arten. Detta, eftersom trots flera decennier av forskning är jämförelsevis lite känt om hur resistensutvecklingen utvecklas mot avmaskningsmedel hos denna art under fältmässiga förhållanden. Till exempel, det skedde tre stora genombrott från mitten av 90-talet till mitten av 2000-talet när tre mutationer identifierades som viktiga orsaker till utvecklingen av resistens mot bensimidazolderivat. Trots detta är förståelsen för hur resistensutvecklingen går till mot de andra två läkemedelsklasserna hos denna art (och fårnematoder i allmänhet) är jämförelsevis begränsade. Att identifiera dessa tre mutationer, kopplade till resistens mot bensimidazoler, hos parasitiska rundmaskar i fårbesättningar skulle potentiellt kunna hjälpa till att informera bönder och veterinärer om

det aktuella tillståndet av parasitresistens på enskilda gårdar. På så sätt kan de vägledas till en mer pragmatisk behandlingsstrategi baserat på att välja läkemedel som är effektiva. Idag kan detta bara uppnås för benzimidazoler men inte de andra två läkemedelsklasserna.

Forskningsartiklarna som presenteras i avhandlingen är därför inriktade dels på att utveckla ett övervakningsverktyg för detektion av bensimidazolresistens hos parasiten *H. contortus* från svenska får, dels att använda detta verktyg för att fastställa spridningen av bensimidazolläkemedelsresistens hos denna parasit över hela Sverige. Dessutom använde vi metoder för studier av kandidatgener och jämförelser av parasitens genom för att ta reda på de effekter som olika läkemedel har på antingen en specifik gen eller i specifika regioner i parasitens genom. På detta vis är det möjligt att utvärdera vilka gener som kan bidra till resistensutveckling.

Resultatet från forskningsartiklarna har lett till ett robust verktyg för upptäckt av bensimidazolresistens hos *H. contortus*. Det behövs dock mer kompletterande forskning, särskilt om att identifiera konsekventa förändringar som svar på behandling särskilt för såväl tetrahydropyrimidiner som makrocykliska laktoner. I avhandlingen har jag har även uppdaterat kunskapen om frekvensen av mutationer som förutsäger resistens mot bensimidazolderivat i Sverige. Fortsatt forskning är viktig, inte minst för att kunna skapa nya verktyg för att kunna spåra resistensutvecklig hos parasitiska nematoder på gårdar. Det behövs också en förfinad förståelse av de genetiska förändringar, som är associerade vid behandling med tetrahydropyrimidin och makrocyklisk laktoner. Sammantaget har vi dock kommit ett steg närmare och ökat förståelsen för de mekanismer som orsakar resistens mot anthelmintiska läkemedel. Detta är en förutsättning för att kunna utveckla rationella screeningverktyg för att kunna stoppa spridningen av resistens och slutligen lägga fram hållbara lösningar på resistensproblemet.

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Exploring benzimidazole resistance in *Haemonchus contortus* by next generation sequencing and droplet digital PCR

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ABSTRACT

Anthelmintic resistance in gastrointestinal nematode (GIN) parasites of grazing ruminants is on the rise in countries across the world. *Haemonchus contortus* is one of most frequently encountered drug-resistant GINs in small ruminants. This blood-sucking abomasal nematode contributes to massive treatment costs and poses a serious threat to farm animal health. To prevent the establishment of resistant strains of this parasite, up-to-date molecular techniques need to be proposed which would allow for quick, cheap and accurate identification of individuals infected with resistant worms. The effort has been made in the previous decade, with the development of the pyrosequencing method to detect resistance-predicting alleles. Here we propose a novel droplet digital PCR (ddPCR) assay for rapid and precise identification of *H. contortus* strains as being resistant or susceptible to benzimidazole drugs based on the presence or absence of the most common resistance-conferring mutation F200Y (TAC) in the β tubulin isotype 1 gene. The newly developed ddPCR assay was first optimized and validated utilizing DNA templates from single-worm samples, which were previously sequenced using the next generation PacBio RSII Sequencing (NGS) platform. Subsequent NGS results for faecal larval cultures were then used as a reference to compare the obtained values for fractional abundances of the resistance-determining mutant allele between ddPCR and NGS techniques in each sample. Both methods managed to produce highly similar results and ddPCR proved to be a reliable tool which, when utilized at full capacity, can be used to create a powerful mutation detection and quantification assay.

1. Introduction

The blood-sucking nematode of small ruminants *Haemonchus contortus* has become a thorn in the side for farmers across the world. Even though gastrointestinal nematode (GIN) infections in livestock are usually found to be mixed, *H. contortus* stands out as the most pathogenic and abundant species (Besier et al., 2016). In Australia, prevention costs against nematode parasites amount to the major part of the country's spending in the red meat industries (Lane et al., 2015). In the UK, total annual losses in the sheep sector due to GIN parasites constituted £84 million more than 10 years ago, and have since then likely increased (Nieuwhof and Bishop, 2005). In addition, developing countries, such as India, suffer from immense annual treatment costs which can be considerably detrimental to the countries' economies ($103 mln.) (McLeod, 2004). Apart from gross economic losses, impaired animal health and welfare are also key issues.

To date, three major drug classes are utilized to treat GIN infections in ruminants – benzimidazoles (BZ), levamisole (LEV), and macrocyclic lactones (ML), all of which are broad spectrum anthelmintics which reduce the existing worm burdens and if used persistently decrease pasture contamination and thus prevent the establishment of infective-stage larvae (L3) (Hoste et al., 2011; Sutherland and Leathwick, 2011). However, years of improper drug use have led to the development of anthelminthic resistance (AR) among these worms and somewhat halted the ever-increasing development of the animal production sector worldwide. Even in most European countries, such as the United Kingdom (Hong et al., 1996), Netherlands (Van den Brom et al., 2013), Spain (Requejo-Fernández et al., 1997), Switzerland (Schnyder et al., 2005), Germany (Bauer, 2001), Poland (Mickiewicz et al., 2017), Italy (Cringoli et al., 2007), France (Paraud et al., 2009), Denmark (Peña-Espinoza et al., 2014), Sweden (Höglund et al., 2009) and Norway (Domke et al., 2012), sheep and goat GIN parasite resistance towards benzimidazole anthelmintic drugs has become a common occurrence.

One of the most significant properties of *H. contortus*, is the propensity to develop resistance to anthelminthic drugs, which has been well documented and described by Kotze and Prichard (2016). This in part can be explained by very high gene flow between populations, remarkable within-population genetic diversity (Blouin et al., 1995;

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Troell et al., 2006) and high effective population size. This increased variability within-populations is especially true for β-tubulin genes, which account for resistance to BZ substance class of anthelmintic drugs (Beech et al., 1994).

To tackle the problem of growing resistance of *H. contortus* towards BZ drugs, alternatives to anthelminthic treatment must be considered and an end to ineffective drug use has to be implemented. To achieve this, a reliable, rapid and reasonably cost-effective screening-diagnostic tool is of paramount importance (Kenyon and Jackson, 2012). The current, gold standard for identifying clinical anthelmintic resistance in flocks of animals is the Faecal Egg Count Reduction Test (FECRT) (Coles et al., 2006). Albeit a well-described and firmly established method, it is lacking in sensitivity (Höglund et al., 2009). Furthermore, a number of elements can dramatically influence the outcome of the results, such as the level of excretion and aggregation of FEC, sample size and dilution factors, making this tool nothing more than a 'rough estimation' which is inefficient, difficult to interpret and reproduce (Levecke et al., 2012). Although a step towards developing and validating molecular tools for the detection, screening and evaluation of resistance towards anthelmintic drugs in parasitic nematodes was taken in the previous decade, mainly in the form of pyrosequencing (Von Samson-Himmelstjerna et al., 2007, 2009; Höglund et al., 2009), the search for novel, 'better' methods continues.

Droplet Digital PCR (ddPCR) is a recent modification of the third generation digital PCR. It boasts high accuracy, versatility and removes the need for standards or references in comparison to qPCR (Hindson et al., 2011). In this study, the ddPCR method was employed to create a universal protocol for the detection and quantification of the transversion occurring at the 200th codon (P200) (T**T**C→T**A**C) in the β-tubulin isotype 1 gene, resulting in the acquisition of AR to BZ drugs in different strains of *H. contortus*. For this purpose, mixed ovine faecal larval culture samples containing *H. contortus*, derived from Swedish farms pre- and post-treatment with a BZ drug (albendazole), were subjected to molecular characterization of the β-tubulin isotype 1 gene locus, first by utilizing next generation PacBio RSII Sequencing (NGS) to obtain a reference read of the samples contents' and then by the optimized ddPCR, developed by the authors. The fractional abundance (FA) values for both alleles (wild-type and P200 mutant) were compared between the two methods to determine if the newly developed protocol is reliable and precise. In addition to this, adult *H. contortus* single-worm samples, collected from 13 different countries were subjected to the same NGS of the β-tubulin isotype 1 gene locus and were further used not only in the initial development of the ddPCR protocol but also in the construction a Bayesian phylogenetic tree based on the retrieved exon sequences.

2. Material and methods

2.1. Sources of DNA

Genomic DNA was extracted from two different sources: 1) individual adult *H. contortus* worms and 2) mixed faecal larval cultures. The adult worms were previously isolated, bio-banked samples from naturally infected sheep (Troell et al., 2006), and were initially used in the ddPCR method development and optimisation stage. During the method validation stage, DNA samples, derived from a mixture of strongyles and extracted from faecal larval cultures collected from 13 sheep farms pre- and/or post-treatment around southern Sweden, were used (Supplementary Table 1) (Note: farms K-M only had a single pre- or post-treatment sample available). DNA extractions were performed according to the instructions of the supplier on thawed samples using NucleoSpin Tissue kit (Macherey Nagel).

2.2. DNA library creation and sequencing

Genomic DNA samples from *H. contortus* single-worms and faecal larval cultures of sheep were used to amplify a partial β-tubulin isotype 1 encoding gene sequence harbouring the three most common mutation sites (at codon positions (P)200, (P)198 and (P)167), changes in which confer resistance to BZ drugs. The original primer sequences were described by Redman et al. (2015). Upon confirmation of successful amplification of a partial gene product of approximately 922 base-pairs (bp) in size (using a randomly selected, readily available genomic *H. contortus* DNA template) by conventional temperature gradient PCR, unique tags were developed to be used in the library creation step (Supplementary Table 2).

The conditions for the thermal cycling reactions were selected according to the manufacturer's guidelines (Thermo Scientific DreamTaq Green DNA Polymerase #EP0712) – a single cycle of initial denaturation at 95 °C for 2 min, followed by 30 cycles of denaturation at 95 °C for 30 s, annealing at 63 °C for 30 s and extension at 72 °C for 1 min. An additional one cycle of final extension at 72 °C for 10 min was included at the very end of the reaction. The final volume of samples was 15 μL. After the amplification step, samples were run on a 1% agarose gel and visualized by a BioRad Gel Doc™ system.

Amplified adult-worm DNA samples were pooled together in categories, according to the signal strengths of visualized bands on agarose gels (larval culture samples did not undergo the initial pooling step, but were subjected to the subsequent clean-up). These were then further subjected to a clean-up step using the AMPure XP magnetic beads, following the instructions detailed by the manufacturer (Beckman Coulter Inc.). The pooled pure partial β-tubulin locus sequences present in adult-worm and larval culture DNA samples were subjected to DNA quantification. Qubit dsDNA HS Assay Kit was employed to determine DNA concentrations in each sample, strictly following the guidelines issued by the manufacturer (Life Technologies). After this step, the remaining samples were further joined together (single-worm and larval culture samples were pooled separately) in equal concentrations forming five distinct batches and stored at −20 °C before sequencing. Pooled amplicon DNA samples were sent for sequencing to Uppsala Genome Center, Science for Life Laboratory, Dept. of Immunology, Genetics and Pathology, Uppsala University, BMC, Box 815, SE-752 37 UPPSALA and further sequenced using the PacBio RSII Technology Platform.

2.3. NGS data analysis

Sequencing data for both single-worms and larval culture pools was analysed using jMHC software (version 1.6.1624) (Stuglik et al., 2011). FASTA files for each sample pool were imported and samples were demultiplexed using unique nucleotide tag combinations. Sequencing errors were filtered out using jMHC and only sequences that were found in at least 3 reads were considered. The output file for single adult worms was analysed by manually enforcing strict criteria to filter out artefacts and obtain well-characterized DNA sequence variant(s) in each sample in three steps: (1) Sequences in samples were removed if they had less than three reads, (2) Assuming only up to two allele variants should be present in each single-worm sample, a sequence, whose coverage (i.e. number of reads) was lower than 50% of the highest coverage possessing sequence in that particular sample, was removed, (3) Samples containing multiple sequences with two or more possibilities of inferring binary allele combinations after steps (1) and (2) were removed. Sequence variants found in larval culture sample pools were also manually processed using these two criteria: (1) A sequence in a particular isolate sample was filtered out if its coverage was less than 3 reads, (2) Sequences present only in a single isolate sample and having a coverage of 3 or less were removed.

2.4. Phylogenetic analysis of partial β-tubulin sequences found in single-worm samples

The β-tubulin isotype 1 locus DNA sequences found in single-worm

samples, which passed the filtering criteria, were kept and had their intron regions removed in order to construct a Bayesian phylogenetic tree using MrBayes software, version 3.2.6 (Ronquist et al., 2012). Settings used were as follows: Number of generations (Ngen; 200 mln), Number of chain swaps (Nswap; 10), Number of chains (Nchains; 10), Burnin-fraction (Burninfraq; 0.4), Number of substitution types (Nst; 6), and model for among-site variation (Rates; invgamma), whereas the other parameters were left at their default values. Phylogenetic relations between aligned sequences were then visualized using Dendroscope 3, version 3.5.9 (Huson and Linz, 2016).

2.5. Primer/probe development for droplet digital PCR

Primer sequences for ddPCR were first developed and verified by conventional PCR. CodonCode Aligner (version 7.1.1) was used to manually visualize and identify putative conserved regions, surrounding the mutation site of interest in the β-tubulin isotype 1 locus sequences from a variety of adult *H. contortus* isolates. A set of primers was then designed to anneal to those regions, whereas probes were developed separately. After successful tests were run with the primer sequences both *in silico*, using Primer3 (http://bioinfo.ut.ee/primer3/) and Sequence Manipulation Suite: PCR Primer Stats (www.bioinformatics.org/sms2/pcr_primer_stats.html), and *in vitro* using conventional PCR, two sets of primers and probes, designed specifically for ddPCR, were ordered (BioRad; Table 1).

2.6. ddPCR assay development

A ddPCR protocol was developed utilizing unique primer-probe sets and optimized for single-worm genomic (g)DNA samples with an intent on using this technique for the evaluation of the presence of mutant and/or wild-type alleles in extracted faecal larval culture gDNA isolates. The ddPCR reaction mixtures were assembled in 96-well plates at the total volume of 22 µL per well. A standard protocol provided by the manufacturer of the reagents was followed (ddPCR™ Supermix for Probes, catalog # 186–3010, BioRad). In short, a single reaction mixture was prepared by introducing 11 µL of 2x ddPCR Supermix for Probes, 1,1 µL of each 20x stock solution for primers/wild type probe (FAM™) and primers/mutant probe (HEX™) at the concentrations of 900 nM and 250 nM for primers and probes, respectively, and varying amounts of water and DNA template. Nanoliter size water-in-oil droplets were then generated using an automated droplet generator (along with DG32 Cartridges) and dispensed into a new 96-well plate. The plate containing the droplets was heat sealed with plastic foil (PX1 PCR Plate Sealer) and carefully transferred into the thermal cycler (MyCycler™ Thermal Cycler, BioRad). The thermal cycling conditions used for the PCR are readily described in the manufacturer's protocol. The temperature for the annealing step was chosen to be 58 °C, according to the results of the gradient ddPCR (data not shown).

After PCR amplification, the plate containing the droplets was loaded into the QX200 droplet reader (BioRad). As the automatic droplet generator is used to partition the 22 µL sample into approximately 20,000 water-in-oil droplets, the 2 channels for each fluorophore (FAM™ and HEX™) in the droplet reader are utilized to measure the amounts of fluorescent and non-fluorescent droplets. No-template-control (NTC), Wild-type only (WT) and Mutant only (MT) samples were included into the assays where necessary. Subsequent data was analysed using QuantaSoft software (version 1.7.4.0917). Thresholds, separating droplet clusters, were manually adjusted to obtain the best possible separation: Channel 1, which captures the fluorescence of the FAM™ dye molecule, attached to the wild type probe, had a threshold set at 7000 AU, whereas Channel 2, which respectively measures the fluorescence emitted by the HEX™ dye, attached to the mutant probe, at 5000 AU. A limit of detection (LoD) and fractional abundance (FA) precision identification in five wild-type (WT) and mutant (MT) mixtures (at 100/0, 75/25, 50/50, 25/75 and 0/100% ratios) tests (described by Elmahalawy et al., 2018) were carried out to evaluate the reliability of the designed assay.

Table 1
Primer and probe sets used for droplet digital PCR.

Primer-probe mix name:	Hc-β-tub – 200WT
Forward sequence:	TCGTGGAACCCTACAATGCT
Reverse sequence:	TCAAAGTGCGGAAGCAGATA
Probe sequence:	AACACCGATGAAACATTCTGTATTGAC
Fluorophore:	FAM™
Primer-probe mix name:	Hc-β-tub – 200MT
Forward sequence:	TCGTGGAACCCTACAATGCT
Reverse sequence:	TCAAAGTGCGGAAGCAGATA
Probe sequence:	AACACCGATGAAACATACTGTATTGAC
Fluorophore:	HEX™

2.7. Data comparison

Both NGS and ddPCR data on genomic DNA derived from larval cultures containing *H. contortus* were assembled and analysed for each sample. Sequences characterized by NGS as well as ddPCR were divided into wild-type (containing no mutation at P200) and mutant (containing an amino acid altering TTC→TAC transversion at P200) and were compared on the basis of the FA (in %): $FA(MT) = MT\left(\frac{copies}{\mu L}\right) \div \left(MT\left(\frac{copies}{\mu L}\right) + WT\left(\frac{copies}{\mu L}\right)\right)$ and $FA(WT) = 100 - FA(MT)$ for each allele between the two methods. MedCalc software (version 18.5) was utilized in the FA data processing and the visualization of the differences between the two methods (in the form of a Bland-Altman plot).

3. Results

3.1. Next generation PacBio RSII sequencing

Sequencing of the β-tubulin isotype 1 locus amplicons from single worm samples yielded 21984–27085 reads, 910–919 bp mean lengths of the products and the quality of insert varied between 98.92 and 99%. For amplicons from larval culture gDNA specimens, 31561–34677 reads, 892–898 mean bp lengths of the products and ~99% quality of inserts were achieved. Upon processing of the sequences derived from both adult single-worm and larval culture gDNA, a total of 210 final samples (out of 223) successfully passed the imposed criteria (see *NGS data analysis* in Materials and methods). A total of 13 samples for single-worm specimens were removed due to not passing the set criteria.

For sequences obtained from single-worm samples, a total of 45 unique sequences were extracted and evaluated for genetic variation at a nucleotide level. A total of 219 nucleotide differences were observed between the sequences and the generated consensus, most of which were prominent in the intron regions. When the obtained exon sequences were translated *in silico* and compared, only three codons were shown to be polymorphic, i.e. P167, P198 and P200. Precisely 15 of the sequences were determined to harbour non-synonymous point mutations conferring resistance to BZ drugs (typical Phe→Tyr amino-acid substitutions at P167 and P200) and two of the sequences were identified to have previously unseen nucleotide mutations for *H. contortus* at the P198 (resulting in Glu→Leu and Glu→Thr substitutions). Further information about genetic relationships between the sequences at a nucleotide level was extracted by constructing a Bayesian phylogenetic tree (Supplementary Fig. 1). Although no immediate pattern of distribution or phylogeny was recognized, the different resistant genotypes were present in between 18 and 100% in the various samples from different countries such as Australia, Brazil, Canada, Ethiopia, Germany, Guadeloupe, Greece, Kenya and Sweden.

For larval culture samples, obtained from sheep at different farms

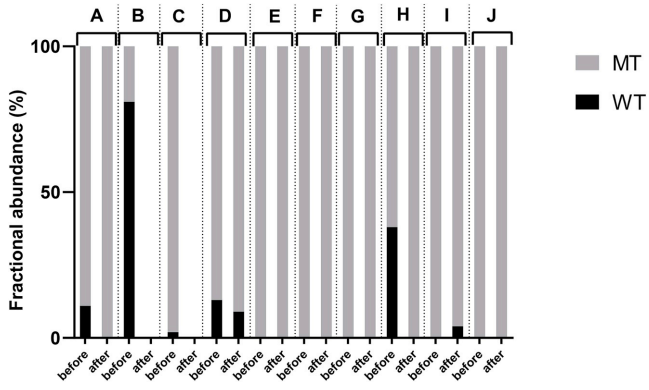

Fig. 1. Relative fractional abundance (%) of *Haemonchus contortus* β-tubulin alleles (WT or MT at the P200) in larval cultures from different sheep farms (A–J) before and after BZ treatment. MT – fraction of mutant allele in the sample, WT – fraction of Wild-type allele in the sample.

around Sweden pre- (before) and post- (after) treatment with the BZ drug, 32 unique β-tubulin isotype 1 locus sequences were retrieved. Among these, 22 had a typical F200Y (TAC) mutation conferring resistance to BZ drugs, while the other 10 were wild-type at P167, P198 and P200. The fractional abundance (FA) of wild-type and mutant *H. contortus* worms were further evaluated in pre- and post-treatment samples from ten different farms using the NGS data (referred to as A-J, Fig. 1). Farms A, D and H contrary to farm I show a decrease in the wild-type alleles post-treatment, whereas farms E, F, G and J show no change (despite G and J having reduced numbers of mutant sequence reads post-treatment) in the FA pattern pre- or post-treatment. Furthermore, post-treatment samples from farms B and C contained no amplifiable material, which was also indicated by the 100% reduction in the faecal egg count at these farms (conducted prior to this study; Supplementary Table 1).

3.2. Droplet digital PCR optimisation and reliability evaluation

A gradient ddPCR was run to establish the most optimal temperature for the annealing step during the amplification. For both sets, wild-type probe/primers and mutant probe/primers, the clearest band separation, smallest observable droplet dispersion and highest amplitude of fluorescence was achieved at 58 °C (Column D in Fig. 2).

The LoD experiment displayed the capacity to identify a mixed sample, which contains MT alleles, if the concentration of those alleles was equal to or higher than 2.6% (Fig. 3). That is to say, a single MT DNA molecule can be identified out of roughly 38 total molecules in a sample. Therefore, a FA of 2.6% was considered to be a threshold value to help indicate a clear presence of mutant DNA.

The FA precision (i.e. pilot) test demonstrated the variability in the FA between the samples to be up to ~10% (Fig. 4), the most notable difference being observed when MT and WT DNA samples are mixed at equal ratios (1:1).

3.3. Data comparison between NGS and ddPCR

The overall result of the comparison between the data extracted by these two methods yielded highly similar FA values (Fig. 5). Most data points (except for two – HRG1624 and HRG1718) in Fig. 6 fall in between 95% limits of agreement and generate a mean value of 1.1% difference in FA. The data from the two methods also displayed a linear correlation equal to $r = 0.984$ (95% confidence interval, CI = 0.971–0.995, $P < 0.001$). Wilcoxon matched-pairs signed rank test displayed no statistically significant difference between either the WT or MT FA values between the two methods ($p = 0.1041$).

4. Discussion

The relatively recent spread of BZ-resistant *H. contortus* into sheep flocks in Scandinavian countries, such as Denmark (Peña-Espinoza et al., 2014), Sweden (Höglund et al., 2009) and Norway (Domke et al., 2012) is truly worrying. In fact, most European countries are now compromised (Rose et al., 2015) as more cases of resistant strains come to light, leaving few options for European sheep farmers. It is theoretically possible, to at the very least attempt to halt the development and the establishment of resistance by treating affected individuals instead of the entire herd (Kenyon and Jackson, 2012). In order to achieve that, reliable and robust techniques must be presented, to ensure a quick, cheap and accurate screening of individual samples collected at farm level. Here, we propose droplet digital (dd)PCR as a novel platform for farm sample screening and resistance to BZ drugs identification by using β-tubulin isotype 1 gene locus as a marker in larval culture samples from animals infected with *H. contortus*.

Reports have been coming out over the last two decades heralding the worldwide distribution of drug resistant *H. contortus* strains (Waller and Chandrawathani, 2005; Kaplan and Vidyashankar, 2012; Rose et al., 2015). It was, therefore, important to look into the situation in regard to the BZ class drugs. From the 13 countries, wherein the single-worm samples were collected, in nine of them (Australia, Brazil, Canada, Ethiopia, Germany, Guadeloupe, Greece, Kenya and Sweden) mutant sequences were found. These findings essentially confirm results from several studies (e.g. Ghisi et al., 2007; Höglund et al., 2009; Mahieu et al., 2014; Barrere et al., 2013; Chaudhry et al., 2015; Ramünke et al., 2016; Zhang et al., 2016). Interestingly, single-worm samples from four locations (South Africa, Cambodia, Java and Argentina) showed only the presence of wild-type isolates. In the case of South Africa, it is highly suspicious that no mutants were detected, considering that it has been reported that the region is in dire situation in regard to anthelmintic resistance in sheep and goat parasites (Van Wyk et al., 1999). The same holds true for Argentina (Waller et al., 1996; Eddi et al., 1996) and South East Asian countries (Haryuningtyas, 2002). Thus, it is most likely that no resistant strains were found in these regions due to a limited pool of available, good-quality samples. What is more, the strains were isolated back in the early 2000s and might not be entirely representative of the present-time situations in these regions.

Of the total 45 identified partial β-tubulin isotype 1 locus sequences in single-worm samples, 24% (11) had the TTC/Phe → TAC/Tyr

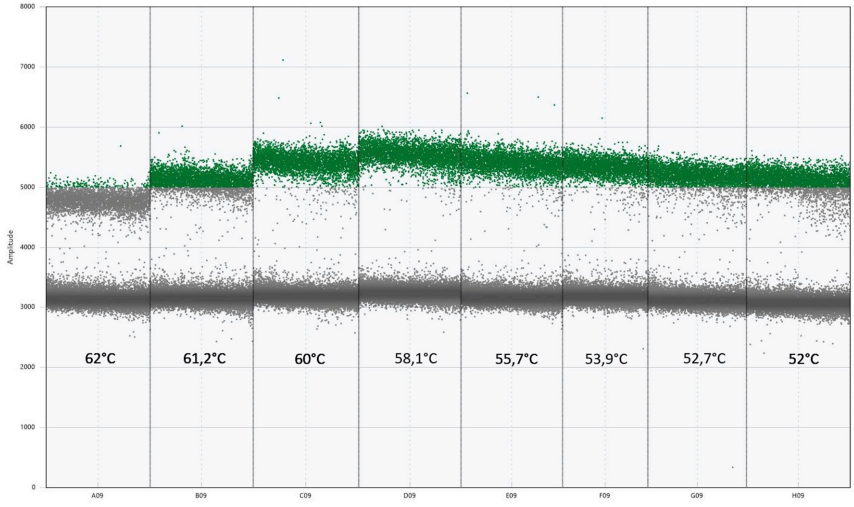

Fig. 2. Optimal primer and probe annealing temperature determination for the ddPCR assay using a temperature gradient PCR. The highest amplitude of fluorescence and the best separation between bands, positive for wild-type (not shown) or mutant (green droplets) and negative (gray lowermost threshold), was observed in column D06, wherein annealing temperature was 58,1 °C. (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

mutation at the P200, 9% (4) - TTC/Phe → TAC/Tyr at the P167 and 4% (2) - GAA/Glu → ? at the P198 ("?" being a different combination of nucleotides at this codon, leading to the incorporation of various amino acids). This pattern of mutation frequencies is in agreement with the previous work done by Barrere et al. (2013), Chaudhry et al. (2015), Ramünke et al. (2016) and Redman et al. (2015). Taken together, the data suggests that the first discovered F200Y (TAC) mutation in the β-tubulin isotype 1 gene of *H. contortus* is still the most relevant BZ resistance conferring mutation to this day. However, some discrepancy in mutation frequencies exists between different parts of the world, as a recent study conducted in China suggested the E198A (GCA) mutation in the β-tubulin isotype 1 gene to be the most widely abundant BZ resistance driving factor in *H. contortus* in 8 surveyed regions (Zhang et al., 2016). As it was previously described by several authors (Silvestre and Cabaret, 2002; Ghisi et al., 2007; Von Samson-Himmelstjerna et al., 2007), all adult worm DNA samples, in which we found mutant β-tubulin isotype 1 locus sequences, had only a single mutant codon per sequence, meaning no double or triple mutants for amino acids at P167, P198 or P200 were uncovered. Two of those β-tubulin isotype 1 locus DNA sequences contained previously undescribed, novel mutations at the P198 codon. Sequence 347, present in one of the samples isolated from Brazil, contained GAA→TTA (Glu→Leu) mutation, whereas Sequence 394, isolated from Sweden (Troell et al., 2006), harboured GAA→ACA (Glu→Thr) nucleotide sequence change. Both of these sequences were present in abundance in their respective samples and were not considered artefacts due to passing the filtering criteria. Furthermore, an analogue to Sequence 394, harbouring a Glu→Leu substitution at the P198, has been recently described but so far only for *T. circumcinta* (Redman et al., 2015 and Keegan et al., 2017). It is not entirely clear whether these genotypes

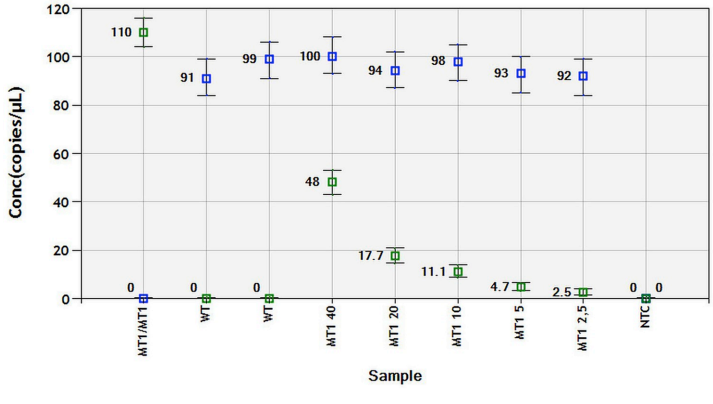

Fig. 3. Limit of detection (LoD) for a mutant (MT) β-tubulin allele. A MT-labelled sample (MT1/MT1), containing mutant allele copies (green squares) was diluted (to 40, 20, 10, 5 and 2.5% of initial concentration) in the presence of a constant concentration of a wild (WT)-type allele copies (blue squares) (WT-MT1 2,5). Two negative controls were run as 'WT' (containing the WT only DNA) and non-treatment control (NTC). The 'MT1 2,5' well displayed a significantly different mutant DNA concentration from that of the WT well. Furthermore, the fractional abundance of wild-type DNA (blue rhombi) showed that in the well 'MT1 2,5' the fractional abundance of WT was 97.4%, meaning that the fraction of the MUT was 2.6%. Fractional abundance (of MT alleles) of 2.6% was then considered to be the limit of detection for mutant alleles. (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

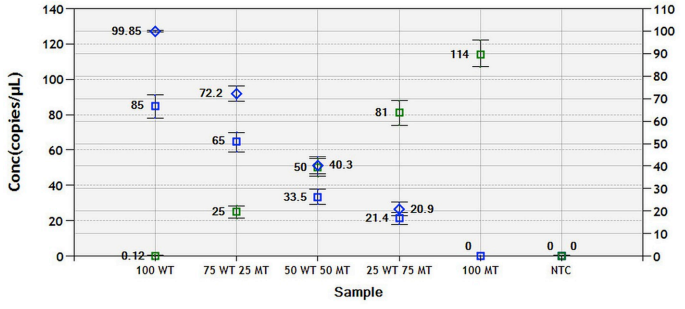

Fig. 4. Fractional abundance (FA) precision (i.e. pilot) assay. Wild type (WT) and mutant (MT) samples of similar DNA concentrations were mixed at different ratios and the outcome evaluated through fractional abundance data. Samples were mixed in equal volumes, at ratios 100:0, 75:25, 50:50, 25:75 and 0:100. A clear and anticipated pattern can be observed, confirming the validity and robustness of the assay. Blue squares indicate the number of copies of WT alleles, whereas green squares - copies of MT alleles. Blue rhombi represent the fractional abundance of WT DNA in each sample (fractional abundance of MT = 100 – fractional abundance of WT). (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

confer increased resistance towards BZ class drugs in *H. contortus*. However, assuming they do, would indicate that a wider array of amino acid substitutions can be tolerated at the P198, while still maintaining the resistance status.

Out of the larval culture samples collected across the southern Sweden during the 2014–2017 period – 32 unique sequences were derived. Unexpectedly, as many as 22 (73%) of these were positive for the TTC→TAC transversion at the P200 in the β-tubulin isotype 1 gene locus, further confirming the postulation about the importance of the F200Y (TAC) substitution in BZ-resistance. Pre- and post-treatment (with albendazole) farm samples were evaluated for the presence of MT and/or WT alleles (Fig. 1). Only in farms B and C did the post-treatment samples contain no trace of amplifiable material, indicating a 100% effective treatment, which was also confirmed by FECRT data (Supplementary Table 1). In light of this, these samples were then excluded from the comparison between ddPCR and NGS test. The rest of the post-treatment samples across various farms displayed either a reduced WT allele frequencies or an unchanged presence pattern of MT sequences. This indicates that resistant strains are already wide-spread across the southern parts of Sweden, although, the initial FECRTs performed on these samples showed an efficient egg count reduction. Thus, the real situation seems to be much more complicated: the resistant

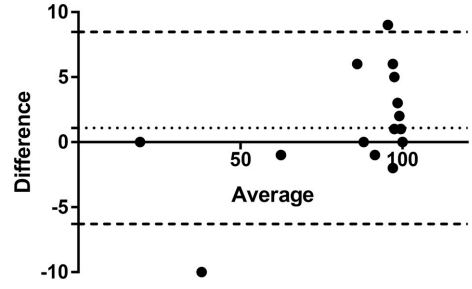

Fig. 6. Bland-Altman [difference in fractional abundance (FA) plotted against mean values of the two measurement techniques] plot for highlighting differences in FA indices for MT sequences between ddPCR and NGS techniques. Round dots represent the differences between FA between the two methods, round-dotted line parallel to the x-axis (Y = 1.1) represents bias or mean value of the differences between measurements and two horizontal, rectangle-dotted lines (Y = 8.5 and −6.3) represent 95% limits of agreement.

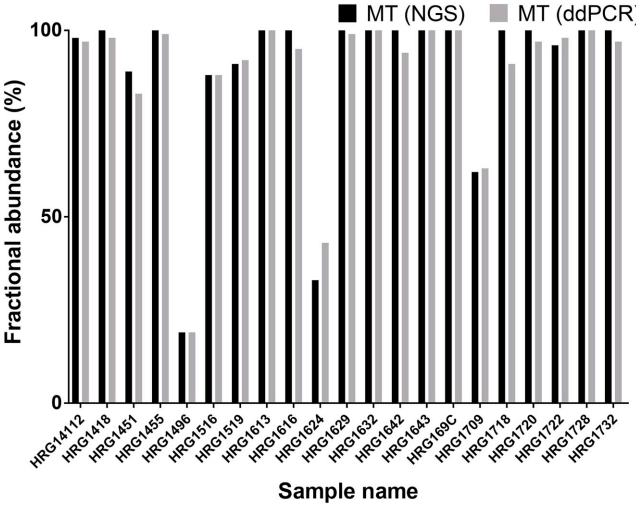

Fig. 5. A comparison of identified mutant (MT) allele fractional abundances (in %) between next generation sequencing (NGS) and ddPCR techniques. Black bars – MT DNA fractional abundance (FA) in larval culture samples as determined by NGS, gray bars – MT DNA fractional abundance in the samples as determined by ddPCR.

strains were indeed somewhat reduced in number after treatment, but some continued to persist regardless of the treatment. This finding highlights the shortcomings of the FECRT method, mainly insensitivity (to detect resistant isolates), as reported before by others (Miller et al., 2006; Höglund et al., 2009).

The major objective of this study was to compare the allele frequency (i.e. fractional abundance (FA)) results obtained for the same larval cultures by NGS with those generated by ddPCR. For that reason, a novel ddPCR assay, encompassing a combination of unique primers with two (MT and WT) probes was developed. Although little was changed in the manufacturer's (BioRad) protocol, it was noticed early on that the addition of DNA restriction enzymes before (Supplementary Fig. 2) or during (not shown) droplet generation did not yield any beneficial effects in terms of amplification efficacy or droplet 'rain' reduction, contrary to what the manufacturers of the ddPCR machinery and ingredients claim (Rare Mutation Detection, Best Practices Guidelines; BioRad). In fact, diluting the sample so that an adequate amount of the droplets remain negative proved to be the best approach when attempting to maintain a good droplet distribution and clear band separation. Diluting samples could also be beneficial for improving the PCR as any PCR inhibitors would also be diluted.

Because both probes, being nearly identical in their sequences, compete for a single target site in a DNA molecule, some probe cross-reactivity was detected (Supplementary Fig. 2). This is observable in the form of a second, lower-amplitude fluorescence band in channels 1 and 2. In channel 1, fluorescence is produced with the help of the WT probe carrying a FAM™ fluorescent dye molecule, a second band can be seen just above the double-negative droplet threshold (around 5000 AU). In channel 2, where fluorescence is emitted with the help of the MT probe carrying a HEX™ fluorescence-producing molecule, a second band is seen at ~4000 AU. Koch et al. (2016) observed a similar occurrence of the second band in channel 2 and concluded that it was likely due to the lower efficacy with which secondary dyes, such as HEX™ and VIC® are detected. However, upon further discussion with BioRad technicians, it was suggested that these lower-specificity bands represent amplified DNA being bound to by the wrong probe (i.e. amplified mutant DNA binding WT probe and amplified WT DNA binding MT probe) and thus getting dispersed into the wrong channel. This is further confirmed by the decreased fluorescence intensity for these bands, as a single-base mismatch is expected to occur between the DNA and the probe in those circumstances. Taking this knowledge into account, manual thresholds for identifying positive droplets in both channels were established. We determined 7000 AU for channel 1 and 5000 AU for channel 2 to be sufficient and provide a somewhat good separation between non-specific and target bands.

Both the LoD and pilot test experiments displayed promising results. The LoD for some ddPCR assays has been reported to be as low as 0.01–0.04% of total mutant sequence abundance (Luo and Li, 2018). However, most of this type of research is focused on the detection of altered DNA in cancerous and/or pre-cancerous cells. Therefore, it is difficult to compare our results with those of other studies. Mutant larvae are multicellular and can obviously be present in high numbers in faecal cultures from infected animals. Thus, a threshold of 2.6% mutant sequence abundance for our LoD is a decent starting point that could potentially be further diminished through rigorous optimal condition testing if necessary. The pilot test, achieved by mixing MT and WT sequences at different ratios, showed a clear and reproducible pattern (Fig. 4). Although the FA values for WT sequences are slightly below the expected ones (blue rhombi), it is evident that the initial DNA copy number is slightly lower than that of the MT, thus resulting in minor deviations. We have subsequently ruled out contamination as a potential source for this variation, as out of 16 no-template control samples, run on different occasions, not a single positive droplet was recorded.

The final comparison between the FA data of the two alleles (WT and MT at the P200) obtained by NGS and ddPCR from paired larval cultures recovered both pre- and post-treatment with the BZ drug from different farms around the southern Sweden, showed impressive correlation (Fig. 5) ($r = 0.988$). The difference in abundance of alleles between the two methods, although varied to a maximum of 10% in some cases (HRG1624, HRG1718), showed very similar overall values. This was also confirmed by the Wilcoxon matched-pairs signed rank test, which indicated no statistically significant difference between the obtained data ($p = 0.104$). However, it appears that ddPCR is somewhat more sensitive of the two: being able to pick up more rare WT sequences (in samples HRG1418, HRG1455, HRG1629, HRG1720) as seen in Fig. 5. Nevertheless, it is arguable whether such small differences in abundances are of practical importance in terms of determining the resistance status of the entire worm population.

Incidence of drug resistant *H. contortus* has risen over time both in Europe and other parts of the world (Rose et al., 2015; Tsotetsi et al., 2013; Veríssimo et al., 2012; Falzon et al., 2013; Playford et al., 2014). This not only carries a threat to the health and welfare of grazing livestock, but it will also most likely result in monumental production losses. To combat the rise in AR in *H. contortus*, vigorous attempts at surveillance and the development of novel approaches for routine screening must be undertaken at regular intervals. To date, few studies have been published, wherein authors utilize ddPCR technology for the detection of parasites (e.g. Weerakoon et al., 2017; Koepfli et al., 2016) let alone those of veterinary importance. Here we propose a novel ddPCR approach to fractional allele abundance elucidation in samples containing mixed genotype *H. contortus* (resistant or susceptible to BZ drugs) larvae using β-tubulin isotype 1 gene locus as a genetic marker. We used the newly developed assay to further characterize BZ-drug treatment outcomes for 10 Swedish farms and compare these and additional results with those obtained with the NGS. Although promising data was acquired, more work needs to be done to implement the ddPCR technique in routine faecal sample examination.

Conflicts 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.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.ijpddr.2018.09.003.

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Utilization of droplet digital PCR to survey resistance associated polymorphisms in the β tubulin gene of *Haemonchus contortus* in sheep flocks in Sweden

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ABSTRACT

Infections with gastrointestinal nematodes (GINs) in small ruminants are becoming increasingly harder to treat due to the development of anthelmintic resistance. Across Swedish sheep farms, *Haemonchus contortus* is one of the more persistent and pathogenic species encountered. Benzimidazole drugs, such as albendazole, are still widely used to control the GIN burden in small ruminants. However, the decline in efficacy of this drug has been observed across the country. In this study, we aimed to continue to investigate the presence of single nucleotide polymorphisms in the β tubulin gene associated with benzimidazole drug resistance in *H. contortus*. This was carried out for sheep flocks from 67 farms around Sweden by screening for the two most commonly encountered SNPs at codons 167 and 200 in the isotype 1 β tubulin gene utilizing the droplet digital PCR technology. We first established a good agreement (Lin's concordance correlation coefficient = 0.987) between the previously widely used pyrosequencing assay for the detection of the SNP at codon 200 (otherwise known as mutation F200Y) and our assay, as well as developed and validated primer-probe pairs for the detection of the mutation at codon 167 (mutation F167Y) in the β tubulin gene of *H. contortus*. We then screened 174 pooled larval culture samples, collected either pre- or post-treatment, for the frequencies of the mutations F167Y and F200Y. Not only did we find the latter to be present at much higher frequencies, but the overall levels of this resistance conferring mutation have stayed stable throughout the years 2014-2019 at an average value of 88.5 ± 20.3% in the pre-treatment samples across the tested farms ($p = 0.61$, Kruskal-Wallis test). Furthermore, after establishing a mixed model and fitting our data, we found a significant ($p < 0.01$) difference in the average frequency of the mutation F200Y between paired, pre- and post-treatment with albendazole, samples. Although the frequency difference in samples treated with albendazole was relatively minor (88.5% in pre- and 95.6% in post-treatment), no significant ($p = 0.15$) change in F200Y mutation frequency was observed between the samples from the flocks treated with ivermectin (90.8% and 92.6 %, respectively).

1. Introduction

Parasitic gastrointestinal nematodes (GINs) present increasingly severe problems to the sustainability of livestock production. The most commonly used method to counter the negative effects the GINs have on infected flocks is treatment with anthelmintic drugs (Kotze and Prichard, 2016). However, issue arises when treatment strategies fail due to the development of anthelmintic resistance (AR). With the current rise in demand for livestock production, increased animal transportation between farms and the shortage of newly developed and safe drugs - the problem has never been this significant (Morgan et al., 2019).

In the small ruminant production sector, three main substance classes of anthelmintics are used: macrocyclic lactones (MLs), imidazothiazoles, and benzimidazoles (BZ). Even though the knowledge regarding resistance mechanisms associated with the first two drug classes is limited, BZ drug resistance has been extensively studied - especially in *Haemonchus contortus*. As a consequence, three key single nucleotide polymorphisms (SNPs), giving rise to mutations F167Y (TTC → TAC), E198A (GAA→ GCA) and F200Y (TTC → TAC) in the isotype 1 β tubulin gene have been linked with BZ drug resistance (Kwa et al., 1994; Silvestre and Cabaret, 2002; Ghisi et al., 2007). In our previous study (Baltrušis et al., 2018), focusing on *H. contortus* recovered from sheep,

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we showed that the F200Y mutation is of major importance in both adult worms and larvae collected locally. The same can be surmised from the data available from other European countries (Gallidis et al., 2012; Redman et al., 2015; Nagy et al., 2016; Ramünke et al., 2016; Claerebout et al., 2020).

Today, in the Swedish context, ML and BZ drugs are the two most commonly used substances to deworm sheep flocks, especially to prevent further infections with the most pathogenic species *H. contortus*. A decade earlier, a study by (Höglund et al., 2009) employed pyrosequencing to investigate the frequency of the BZ-resistance conferring mutation F200Y, found in the isotype 1 β tubulin gene amplicons derived from pre-treatment larvae pools, collected from flocks on 45 farms around the country. The frequency of the mutated β tubulin allele was then found to be unexpectedly high on some of the tested farms and has, thus, prompted us to do a follow up study on the state of BZ resistance conferring mutation frequencies across Swedish sheep farms from 2014 to 2019, using the droplet digital PCR (ddPCR) approach (Baltrušis et al., 2018). Therefore, we first set out to compare the previously widely employed pyrosequencing platform and our in-house ddPCR assay for the detection of the mutation F200Y in the isotype 1 β tubulin gene of *H. contortus*. Secondly, pooled samples of mixed larvae cultures containing *H. contortus* were utilized to measure the frequencies of the two most commonly encountered mutations, F167Y and F200Y, in the isotype 1 β tubulin gene (Baltrušis et al., 2018), using our ddPCR assay across 67 farms around the south-central part of Sweden. Thirdly, samples collected pre- and post-treatment were compared in terms of changes in the frequency of the F200Y mutation in *H. contortus* across farms wherein either ivermectin (IVM) or albendazole (ABZ) was used.

2. Materials and Methods

2.1. Fecal samples and DNA extraction

Fecal samples were received from sheep flocks, usually harboring mixed nematode communities, around Sweden during a 6-year period (2014-2019), between April and October. All farms were associated with the Farm and Animal Health Service (www.gardochdjurhalsan.se) and were part of a routine herd health monitoring program. The majority of

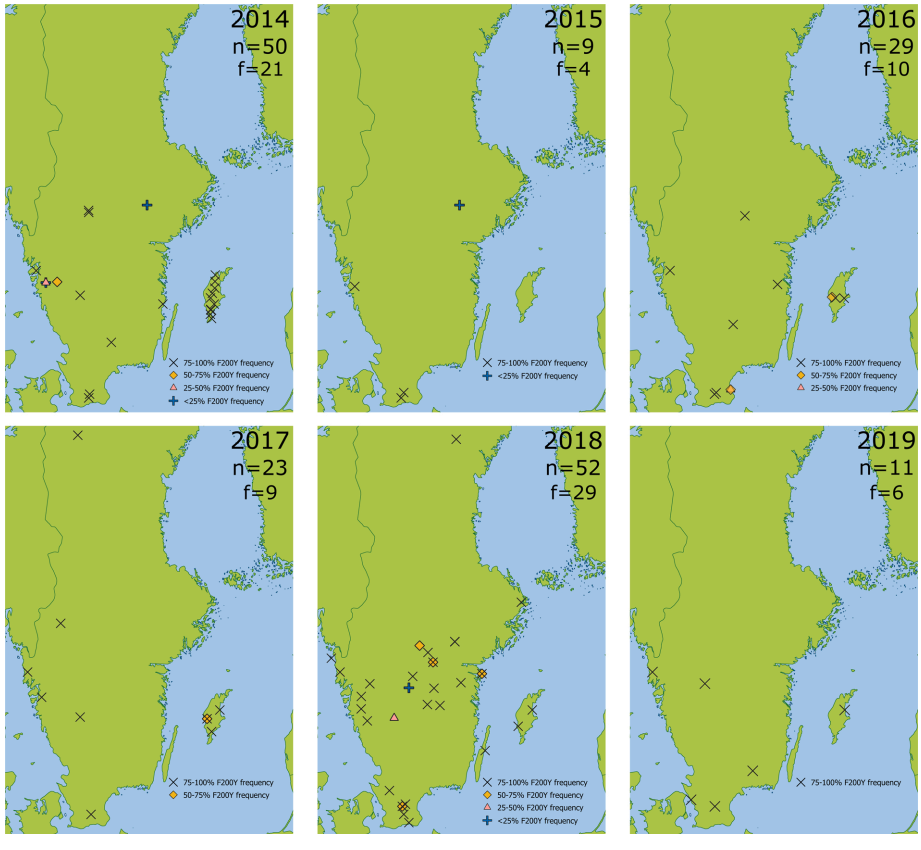

Fig. 1. The frequencies (%) and the spread of the F200Y mutation in the isotype 1 β tubulin gene in *H. contortus* across the various sampled farms during the period 2014-2019 (left to right, top to bottom). Different symbols represent different intervals in which the mutation frequency was found: <25% F200Y frequency (i.e. > 75% of alleles were wild-type for the mutation at codon 200) category was marked by a blue cross; 25-50 % F200Y mutation frequency category - by a purple triangle, 50-75% F200Y mutation frequency category - by an orange rhomboid, 75-100% F200Y mutation frequency category - by a black "X" sign. Flocks on the same farms were sampled from 1 to 7 times during a year. *n* – total number of samples collected during a given year, *f* – total number of farms sampled at a given year.

farms had the anthelmintic drug efficacy tested, mainly against IVM. Samples were collected by veterinarians or animal owners according to the established routines. Individual samples were determined to be *Haemonchus contortus* positive based on an iso-certified and validated microscopical examination, wherein egg morphology is used to distinguish *H. contortus* from other trichostrongylids (Ljungström et al., 2018). An equal amount of faeces was pooled from each of the 6 to 15 animals per flock (to approximately 20 g), cultured, harvested and stored in the freezer (−20 °C) until further use (for details see Höglund et al., 2019).

In total 174 larval culture samples from 67 farms, located in south-central Sweden, were examined using the ddPCR assay for the frequency identification of mutations F167Y and F200Y in the isotype 1 β tubulin gene (2014 – 50, 2015 – 9, 2016 – 29, 2017 – 23, 2018 – 52 and 2019 – 11 samples) (Fig. 1). Out of these, 128 samples (78 individual data points for 67 farms) were collected before treatment during the 6-year period. In the case of 46 flocks (present on 30 farms), post-treatment samples (collected 7-12 days after drug administration) were also available (ABZ, n = 16; IVM, n = 30). Different flocks were sampled between one to seven times per year and an average mutation frequency was obtained for each farm.

Genomic DNA was extracted from the pooled larval cultures usually containing a mixture of species using the NucleoSpin tissue kit (*Macherey Nagel*, Germany), following the manufacturers guidelines. Samples, which were previously identified as positive for *H. contortus* were further used in the ddPCR mutation detection assays.

2.2. Droplet digital PCR

Droplet digital PCR was run on extracted DNA samples using a previously described assay for the identification of the frequency of the F200Y mutation in the isotype 1 β tubulin gene (i.e. mutant allele frequency) (Baltrušis et al., 2018). In addition, a newly developed protocol for the evaluation of the frequency of the mutation F167Y in the same gene was employed, utilizing newly devised primers (HC167IntroF -ATTCGTGAAGAGTACCCTGATA; HC167IntroR – AAGGGTAGCATTGTAGGGTTC) and probes(probeWT167 –TTATGGCTTCGTTCTCCGTTGTTCCA and probeMT167 – TTATGGCTTCGTACTCCGTTGTTCCA). The validation for this particular set-up included a limit of detection and fractional abundance precision identification tests to evaluate reliability (Elmahalawy et al., 2018) (Supplementary Figs. 1 and 2). Furthermore, primer-probe sets used in this study were tested to assess the cross-reactivity with gDNA templates derived from adult *Teladorsagia circumcinta* and *Trichostrongylus spp*. worms. Due to the nature of similarity between the *H. contortus* and *H. placei* isotype 1 β tubulin sequences, based on *in silico* observations, it is likely that the assays would detect DNA belonging to both species. The impact of the quantity of the input material was further investigated by testing the same larvae population, recovered from sheep mono-specifically infected with *H. contortus* (previously bio-banked, stock samples) and not responding to BZ treatment, for F200Y mutation frequency using approximately 10, 100 or 1000 L3 (Supplementary Fig. 3). The original concentration of the larvae suspension was estimated to be approximately 10 L3/10 µl and was dispensed accordingly.

The detailed process of analyzing the samples using ddPCR technology has already been described (Baltrušis et al., 2018). In short, sample reactions were assembled in 96-well plates (final volume 22 µL), following the guidelines issued by the manufacturer (BioRad). Droplets were generated and dispensed into a new 96-well plate using an automated droplet generator (QX200, BioRad). The new plate was heat sealed and transferred into a thermal cycler (MyCycler™ Thermal Cycler). The PCR conditions were as follows: a single cycle of 95 °C for 10 min., 40 cycles of 94 °C for 30 sec. and then either 58 °C (for mutation F200Y detection) or 60 °C (for mutation F167Y detection) for 1 min., followed by a single cycle of 98 °C for 10 min. to deactivate the enzyme. After the amplification step, the plate containing the droplets was loaded into the droplet reader (QX200, BioRad) and further analyzed using QuantaSoft (v1.7.4.0917) software, which generates DNA copy measurements and error bars based on Poisson statistics (Droplet Digital™ Applications guide http://www.bio-rad.com/webroot/web/pdf/lsr/literature/Bulletin_6407.pdf). The output from QuantaSoft was then visualized using the *ggplot2* package (v3.2.1) for R software (v3.6.2).

2.3. Pyrosequencing

The pyrosequencing assay for the determination of frequency of the mutation F200Y in the isotype 1 β tubulin gene was carried out on seven different *H. contortus* laboratory maintained isolates at the Freie university, Institute of Parasitology and Tropical Veterinary Medicine, following the previously developed, detailed protocol (Von Samson-Himmelstjerna et al., 2009).

2.4. Statistical analysis and model fit

Lin's concordance correlation coefficient was estimated using the package *DescTools* (R;version 0.99.34). Linear mixed effect model was adapted to our data set, following logit transformation to improve the fit. The R command *lmer* was utilized and allele frequencies were taken as response variable. Time of sample collection (pre- or post-treatment) and the drug used (ABZ or IVM) were both explanatory variables. Sample number (i.e. pairing pre- and post-treatment samples together) was used as a crossed random effect. The command *emmeans* was used to extract transformed mean values for pre- and post-treatment samples with each drug and compare in pairs. The analysis was conducted using R software (version 3.6.2) and packages *lme4* (version 1.1-21), *lmerTest* (version 3.1-1) and *emmeans* (for pair-wise analysis; version 1.4.3.01).

3. Results

3.1. Mutation detection assays and input material

Frequencies of the mutation F200Y in the isotype 1 β tubulin gene were compared for seven *H. contortus* laboratory strains maintained in sheep between the pyrosequencing and ddPCR assays. Lin's concordance correlation coefficient value of 0.987 and an average difference of 1.2% were obtained between the two assays (Supplementary Table 1).

The importance of the number of larvae tested was evaluated by running each of the five replicated samples containing either 10, 100 or 1000 L3, harvested from the same population with the F200Y primer/probe set. The same allele frequency (100%) was found, irrespective of the amount of the input material (Supplementary Fig. 3). Furthermore, no-cross reactivity between F167Y and F200Y mutation frequency detection assays and gDNA derived from *Teladorsagia circumcinta* or *Trichostrongylus spp.* was observed.

3.2. Allele frequencies in field samples

The frequency of the mutation F200Y across all pre-treatment samples (n = 128) varied between 14.1% and 100% with an overall mean value of 88.5 ± 20.3% (Table 1). The variation in values during the 2014-2019 period is displayed in Fig. 2. Whilst no significant difference was found between the frequencies from different years ($p = 0.61$, Kruskal-Wallis test), the mean frequency of the F200Y mutation from the 2014-2019 period was significantly higher than the one previously reported in Sweden (Höglund et al., 2009) (66 %; $p < 0.001$, t-test). Unfortunately, both studies had only four investigated farms in common, two of which were found negative for *H. contortus* DNA by the previously mentioned study. Thus, no direct comparison was feasible.

A total of 46 post-treatment samples, collected from different flocks following anthelmintic treatment with either ABZ (n = 16) or IVM (n = 30), were further analyzed for changes in the frequency of the mutation F200Y (Fig. 3 and Supplementary fig.4). The frequency of this

Table 1
Summarized descriptive statistics regarding the presence and frequencies of mutations F167Y and F200Y in all collected samples (column "All samples"), all pre-treatment samples (column "All pre-treatment samples") and in samples before (pre) and after (post) treatment with a particular drug – albendazole (ABZ) or ivermectin (IVM). The information presented in the table includes: the number of samples the F200Y and F167Y mutations were found in (in total 174 samples were analyzed from 67 farms), minimum and maximum frequencies (*Min. freq.* and *Max. freq.*) (%) of the said mutations, the average frequencies (%) and standard deviation (*Stdev*) (%). *n* – number of samples in a given category.

All samples (n = 174; total 67 farms):	
F200Y observed on	67 farms
Min. freq. F200Y %	14.1
Max. freq. F200Y %	100
Average F200Y %	88.52
Stdev F200Y %	20.38
F167Y observed on	30 farms
Min. freq. F167Y %	0
Max. freq. F167Y %	11
Average F167Y %	0.74
Stdev F167Y %	2.1
All pre-treatment samples (n = 128; total 67 farms):	
Min. freq. F200Y %	14.1
Max. freq. F200Y %	100
Average F200Y %	87.7
Stdev F200Y %	19.3
Min. freq. F167Y %	0
Max. freq. F167Y %	10.9
Average F167Y %	0.79
Stdev F167Y %	2.19
Samples pre- and post-treatment with ABZ (n = 16):	
Average F200Y pre %	88.47
Stdev F200Y pre %	17.23
Average F200Y post %	95.62
Stdev F200Y post %	9.9
Average F167Y pre %	0.4
Stdev F167Y pre %	0.67
Average F167Y post %	0.14
Stdev F167Y post %	0.29
Samples pre- and post-treatment with IVM (n = 30):	
Average F200Y pre %	90.83
Stdev F200Y pre %	12.31
Average F200Y post %	92.58
Stdev F200Y post %	9.26
Average F167Y pre %	1.12
Stdev F167Y pre %	2.31
Average F167Y post %	0.86
Stdev F167Y post %	2.12

point mutation in pre-treatment samples for ABZ ranged from 43% to 100%, whereas for IVM - between 45% and 100%. Overall, an average increase of 7.1% in the frequency of the point mutation in post-treatment samples, following exposure to ABZ, was observed, in contrast to an average increase of 1.7% in flock samples treated with IVM.

According to the mixed model we applied, the interaction between the time the samples were collected (i.e. pre- or post- anthelmintic treatment) and the utilized drug (IVM or ABZ) was significant ($p = 0.02$). Furthermore, a significant difference between the frequency values for the mutation F200Y in pre- and post-treatment samples was found for the samples taken from flocks treated with ABZ ($p < 0.01$), but not with IVM ($p = 0.15$).

Mutation F167Y was found to occur at much lower levels: 0-11% ($0.74 \pm 2.1\%$ on average; Table 1). Pre- and post- treatment frequency comparisons for ABZ as well as IVM drugs did not display significant changes ($p = 0{,}16$ and $p = 0.64$ respectively; t-test).

4. Discussion

Due to the continuous growth of AR among GINs of livestock in European countries, it is vital to maintain rigorous monitoring and screen for the presence of AR on a local scale. Herein we investigated the situation regarding two mutations (F167Y and F200Y), linked to AR against BZ drugs in *H. contortus*, in pooled larval culture samples, retrieved from sheep in Sweden during the past 6 years.

Previously, a study was carried out in a similar fashion (Höglund et al., 2009), however, it utilized pyrosequencing to obtain the frequencies of the mutation F200Y in the isotype 1 β tubulin gene in pre-treatment larval culture samples across various *H. contortus*-positive flocks on 45 randomly selected farms. We have, thus, set out to compare the frequency data for this mutation in order to determine if our established ddPCR assay generates comparable results to those of the pyrosequencing, when run on the same samples. Data was generated by pyrosequencing and ddPCR platforms for genomic DNA extracted from seven different laboratory strains of *H. contortus* maintained in sheep. An excellent ($= 0.987$) correlation coefficient (Lin's concordance correlation coefficient) was obtained, indicating that the mutation frequencies obtained by the two methods display a great degree of overall similarity. The average value for the difference in the frequency was only 1.2% between the two techniques, demonstrating that ddPCR produces similar results when compared to pyrosequencing. It is noteworthy, however, that when handling highly homozygous DNA containing samples, unlike ddPCR, pyrosequencing assays present a limiting threshold value (≥5%) for the detection of the minor allele (Ogino et al., 2005; Spittle et al., 2007; Tsiatis et al., 2010). What is more, each individual sample run on the ddPCR platform is provided with an estimated 95% Poisson confidence interval, thus effectively eliminating the need for technical replicates.

In the present study we analyzed the frequency of mutations F167Y and F200Y in *H. contortus* positive, pooled larval cultures collected from 67 sheep farms across Sweden. The frequency of the F167Y mutation was found to be comparably low in the tested samples ($0.78 \pm 2.19\%$ on average). This is in contrast to a much more diverse variation and overall higher abundance of this particular SNP in *H. contortus* isolated from sheep flocks in the UK (Redman et al., 2015). On the other hand, mutation F200Y was found to be present at high frequencies in the samples from most farms, irrespective of the year (Fig. 1). This is in agreement with previous similar studies, acknowledging the mutation F200Y to be the most commonly abundant resistance to BZ drugs conferring SNP in *H. contortus* within Europe (Redman et al., 2015; Ramünke et al., 2016; Claerebout et al., 2020) and the Americas (Barrere et al., 2013; Brasil et al., 2012). When comparing the frequencies of the mutated allele (containing the F200Y mutation) in the pre-treatment samples between the two Swedish studies a significant increase is observed ($p < 0.001$, t-test). This, in turn, indicates that the situation, described in this paper, regarding the frequency of the BZ-resistance conferring mutation F200Y in *H. contortus* has deteriorated, compared to the findings from the study carried out a decade earlier based on pyrosequencing (Höglund et al., 2009).

Although over the last decade IVM has become the most widely used drug for sheep producers across Sweden (Höglund et al., 2019), the high frequencies of the mutation F200Y at the investigated farms where IVM was used would suggest that the selection pressure appears to not have declined over the years. This indicates that the maintenance of resistance alleles occurs at a relatively low fitness cost. This is line with the previously published work (Prichard, 1990; Jackson and Coop, 2000), likely suggesting F200Y mutation fixation in most tested flocks, where the frequencies of the alleles carrying this mutation are approaching 100%.

Based on the findings in our previous study (Baltrušis et al., 2018), where we subjected both single-adult individuals as well as larval culture pools containing *H. contortus*, collected from different farms around Sweden, to the PCR amplification of the isotype 1 β tubulin amplicon and then PacBio sequencing, we decided not to include the screening for the canonical mutation E198A (GAA→GCA) in the present paper as no such SNP was previously found apart from two adult individuals possessing atypical changes at that codon. Interestingly, in that study we did not identify any SNPs at codon 167 in the tested larval culture samples

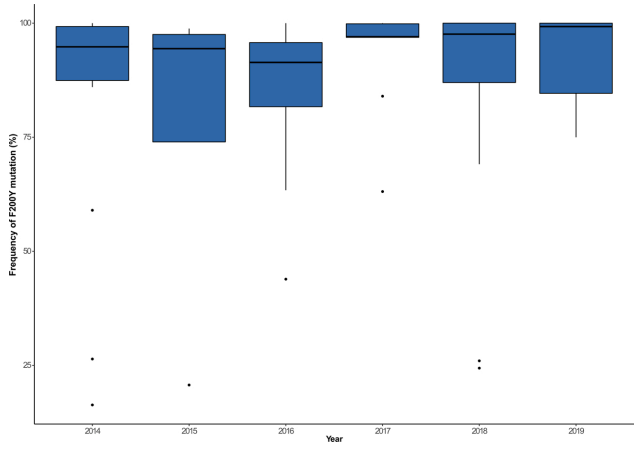

Fig. 2. The frequency (%) of the F200Y mutation in the isotype 1 β tubulin gene belonging to *H. contortus* in pre-treatment samples collected from various farms around Sweden during the years 2014-2019. Farms were sampled 1-7 times per year. An average annual mutation frequency was calculated for farms tested more than once at any given year.

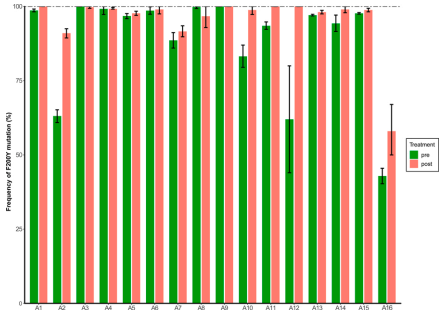

Fig. 3. The frequency (%) of the mutation F200Y in the isotype 1 β tubulin gene in paired pre- and post-treatment with albendazole samples from 16 different flocks. Flocks are denoted as A1, A2, … etc. Data was generated by Quantasoft software (v. 1.7.4).

either, suggesting that it's likely low frequencies, at which the mutation F167Y is present, are either lost when sequencing or filtered out as possible artefacts upon sequence analysis.

We also investigated the fractional changes of the mutation F200Y on farms where both pre- and post-treatment samples were collected from naturally infected sheep treated either with ABZ or IVM (Fig. 3 and Supplementary fig. 4). Increases in the frequencies of the mutation F200Y were observed in several of the ABZ treated flocks (namely A2, A10, A12, A16; Fig. 3), however, in most cases, there was either a modest or no increase after treatment (with an overall increase in the mutated allele frequency post-treatment of 7.1%). This is likely due to the high levels of the frequency of this mutation in the populations to begin with and, thus, making the fractional changes, in most cases, either subtle or insignificant. Nevertheless, the linear mixed model that we applied to our data displayed a significant increase in the frequency of the mutation F200Y between pre- and post-treatment samples ($p < 0.01$). Hence, this provides strong evidence for the selection of this mutation after treatment with ABZ.

As for IVM treated flocks, pre- and post-treatment sample comparison showed an average increase of 1.7% in the frequency of the mutation F200Y post-treatment. However, the same issue of high mutant allele frequency in pre-treatment samples was observed on most farms, which might prevent from detecting a more subtle selection. Nevertheless, although such a small increase in the frequency of the said mutation in post-treatment samples was shown to be insignificant ($p = 0.15$), it has been suggested that treatment with MLs predisposes the population for the selection of individuals possessing the canonical F200Y mutation and resistant to BZ drugs (Eng et al., 2006; Mottier and Prichard, 2008; Santos et al., 2017). This, in turn, could explain the maintenance of high frequency of the mutant allele across farms where IVM was used more extensively, although more data is needed to support this claim.

Certain limitations exist with our current approach. Namely, gDNA derived from the closely related *H. placei* (although the species itself has not been found in Swedish sheep) could potentially be detected by the described F167Y and F200Y allele frequency estimation assays, due to genetic similarities shared within the primer/probe binding regions in the isotype 1 β tubulin gene. Furthermore, our approach included using a fixed amount of pooled fecal material, previously identified as positive for *H. contortus* eggs, for subsequent larvae culturing and DNA extraction steps, instead of a fixed number of characterized L3. The main drawback of this aspect being that the relative abundance of *H. contortus* in the pooled samples remains unknown. Nevertheless, as demonstrated herein, the quantity of the input material did not have a major influence on the estimation of F200Y DNA copy number or mutation frequency (Supplementary Fig. 3). What is more, we ruled out the possibility of sample contamination by the inclusion of negative control samples as well as testing for cross-reactivity with closely related species (*T. circumcinta* and *Trichostrongylus spp.*). In addition, the key inherent advantages of the ddPCR technology are greater sensitivity and increased tolerance to inhibitors compared to other conventional PCR methods (Dingle et al., 2013; Uchiyama et al., 2016).

To conclude, here, we describe the use of droplet digital PCR to determine the frequencies of the two most commonly encountered SNPs in the isotype 1 β tubulin gene, conferring BZ-resistance in *H. contortus*, in field samples collected from 67 sheep farms around Sweden from 2014 to 2019. The subsequent discovery of the high F200Y mutation frequencies (both before and after the most recent anthelmintic treatment with either ABZ or IVM) points towards limited efficacy of the BZ

drugs on most tested farms.

CRediT authorship contribution statement

Paulius Baltrušis: Investigation, Writing - original draft, Formal analysis, Methodology. **Peter Halvarsson:** Supervision, Visualization, Writing - review & editing. **Johan Höglund:** Supervision, Writing - review & editing, Project administration, Funding acquisition.

Declaration of Competing Interest

The authors report no declarations of interest.

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Appendix A. Supplementary data

Supplementary material related to this article can be found, in the online version, at doi:https://doi.org/10.1016/j.vetpar.2020.109278.

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Using droplet digital PCR for the detection of *hco-acr-8b* levamisole resistance marker in *H. contortus*

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ABSTRACT

The nematode *Haemonchus contortus* is one of the most prevalent and pathogenic parasites in small ruminants. Although usually controlled using anthelmintics, the development of drug resistance by the parasite has become a major issue in livestock production. While the molecular detection of benzimidazole resistance in *H. contortus* is well developed, the molecular tools and protocols are far less advanced for the detection of levamisole resistance. The *hco-acr-8* gene encodes a critical acetylcholine susceptible subunit that confers levamisole-sensitivity to the receptor. Here, we report the development of a droplet digital PCR assay as a molecular tool to detect a 63 bp deletion in the *hco-acr*-8 that has been previously associated with levamisole resistance. Sanger sequencing of single adult *H. contortus* yielded 56 high-quality consensus sequences surrounding the region containing the deletion. Based on the sequencing data, new primers and probes were designed and validated with a novel droplet digital PCR assay for the quantification of the deletion containing "resistant" allele in genomic DNA samples. Single adult worms from six phenotypically described isolates (n = 60) and from two Swedish sheep farms (n = 30) where levamisole was effective were tested. Even though a significant difference in genotype frequencies between the resistant and susceptible reference isolates was found (p = 0.01), the homozygous "resistant" genotype was observed to be abundantly present in both the susceptible isolates as well as in some Swedish *H. contortus* samples. Furthermore, field larval culture samples, collected pre- (n = 7) and post- (n = 6) levamisole treatment on seven Swedish sheep farms where levamisole was fully efficacious according to Fecal Egg Count Reduction Test results, were tested to evaluate the frequency of the "resistant" allele in each. Frequencies of the deletion ranged from 35 to 80% in the pre-treatment samples, whereas no amplifiable *H. contortus* genomic DNA was detected in the post-treatment samples. Together, these data reveal relatively high frequencies of the 63 bp deletion in the *hco-acr-8* both on individual *H. contortus* and field larval culture scales, and cast doubt on the utility of the deletion in the *hco-acr-8* as a molecular marker for levamisole resistance detection on sheep farms.

1. Introduction

Haemonchus contortus is one of the most pathogenic and commonly encountered haematophagous, parasitic gastrointestinal nematode (GIN) species, responsible for contributing to productivity and profitability setbacks in the small-ruminant farming sector across the world (Charlier et al., 2014, 2020). To date, the most effective and reliable measure to control *H. contortus* (as well as other GIN) infections is through the use of broad-range anthelmintic drugs. However, there is a limited number of drugs available and widespread anthelmintic resistance to all major drug classes used to treat sheep has been reported worldwide (Kaplan, 2004; Rose et al., 2015; Kotze and Prichard, 2016).

Levamisole is a broad-spectrum anthelmintic drug, used for the treatment of GIN-infected sheep beginning in the 1960s. It exerts its effect by targeting the nicotinic acetylcholine receptors (nAChRs) present in nematode body-wall muscles, where activation of the receptor by levamisole causes spastic muscle paralysis that, subsequently incapacitates the worm, resulting in its expulsion from the host (Martin et al., 2012). Although generally considered as a last-line drug in terms of efficiency, reports describing levamisole-resistant field *H. contortus* range from multiple-decades old (van Wyk et al., 1989; Waruiru, 1997) to quite recent (Almeida et al., 2010; Chaparro et al., 2017).

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Nevertheless, the development of levamisole resistance in the field appears to be somewhat slower in comparison to other drug classes (Rose Vineer et al., 2020). Therefore, in some regions, levamisole remains the last efficient solution when the other drugs are no longer effective (Cristel et al., 2017; Kelleher et al., 2020).

Within the context of Sweden, levamisole has only been rarely used (relative to benzimidazoles – BZs and macrocyclic lactones – MLs), and only on farms, where treatment failure with BZ and ML compounds has been confirmed by the Fecal Egg Count Reduction Test (FECRT; Farm and Animal Health Service, www.gardochdjurhalsan.se). Since the number of cases of resistance to the BZ and ML drug classes has been increasingby pathogenic species such as *H. contortus* (Höglund et al., 2009, 2015), the use of levamisole is almost certain to increase with time. Therefore, in an attempt to anticipate the inevitable rise in levamisole resistance in *H. contortus*, the identification of reliable molecular markers associated with resistance and the subsequent development of resistance-detection assays, capable of screening field populations, would be of major interest.

Initial studies, performed in *Caenorhabditis elegans*, identified the role of acetylcholine receptors in mediating levamisole resistance (Lewis et al., 1980), which has prompted further investigation in parasitic nematodes. In *H. contortus*, levamisole resistance was shown to result either from changes in the binding characteristics of the levamisole-sensitive nAChRs (L-AChRs) or in the reduction of the number of channels (Sangster et al., 1988, 1998). More recently, the molecular composition of the *H. contortus* L-AChRs was deciphered (Boulin et al., 2011). A candidate gene approach allowed for the identification of the four subunits (Hco-UNC-29.1, Hco-UNC-38, Hco-UNC-63 and Hco-ACR-8) that make up the L-AChRs (Neveu et al., 2010). Furthermore, the reconstitution of functional L-AChRs required three ancillary proteins and demonstrated the key role of the Hco-acr-8 subunit in the sensitivity to levamisole *in vitro* and *in vivo* (Boulin et al., 2011; Blanchard et al., 2018). However, as pointed out in the review by Kotze and Prichard (2016), evidence seems to suggest at least three different pathways by which resistance to levamisole can develop – truncation of nAChR genes, reduced transcription of nAChR genes and reduced transcription of ancillary protein genes.

Fauvin et al. (2010) described an alternatively spliced transcript for the *hco-acr-8*, which was specifically expressed in three *H. contortus* isolates resistant to levamisole. This finding was subsequently confirmed in other studies using *H. contortus* isolates from different geographical origins (Williamson et al., 2011; Sarai et al., 2013). A few years later, Barrère et al. (2014) not only confirmed the link between the production of the *hco-acr-8b* transcript and levamisole resistance, but also established that the transcript arises due to a 63 bp deletion in the second intronic region. It is worth noting, that, although the deletion was found to be significantly associated with phenotypic resistance in *H. contortus* ($p < 0.01$), the authors observed that the presence or lack of the 63bp deletion does not account for phenotypic resistance or susceptibility in each and every case. Nevertheless, this led to another recent study (Santos et al., 2019), which looked into the frequency of the truncated, deletion containing *hco-acr-8b* gene in field populations of *H. contortus* in Brazil. The authors of the stated study found a significant positive association ($p < 0.05$) between the approximated frequency of the resistance-causing (i.e. deletion containing) allele and EC50 as well as EC95 values, determined through larvae development tests, performed on five farm populations. Due to the limited number of samples and sequences, upon which the qPCR assay was based, further investigation is necessary.

There is a clear need to develop molecular tools for levamisole resistance detection (Kotze et al., 2020) and further validate the association between *hco-acr-8b* and levamisole resistance in the field. We have previously successfully utilized droplet digital PCR (ddPCR) to screen for BZ-resistance associated mutations in *H. contortus* (Baltrušis et al., 2018,2020). In this study, we have developed and optimized a ddPCR assay to discriminate between the "susceptibility" and "resistance"-associated 63 bp deletion. The primers and probes utilized in our ddPCR assay were designed based on the sequence information retrieved from the second intron region of the *hco-acr-8* (spanning the 63 bp deletion region) in single adult *H. contortus* genomic DNA (gDNA) samples, derived from levamisole-susceptible or -resistant reference isolates as well as from Swedish sheep farms. Our optimized assay was subsequently used for the quantification of the deletion in i) a collection of phenotypically characterized adult worm isolates and ii) adult worm and larval culture samples from farms, wherein levamisole was still effective, according to the FECRT.

2. Materials and methods

2.1. Sample origin and DNA extraction

Worms of Swedish origin and reference isolates were all previously collected, bio-banked samples of single adult *H. contortus*. As references, we used gDNA from male worms belonging to the levamisole-susceptible isolates - *ISE* (Inbred-susceptible-Edinburgh; henceforth referred to as S1), HcoWEY (Weybridge; S2), HcoZA (Zaïre; S3), and three levamisole-resistant isolates HcoCE (Cedara; R1), HcoRHS6 (Borgsteede; R2), HcoKOK (Kokstad; R3), which all have been phenotypically characterized and described previously (Hoekstra et al., 1997; Fauvin et al., 2010; Neveu et al., 2010; Barrère et al., 2014). The male and female *H. contortus* of Swedish origin were opportunistically collected in the field from the abomasa of culled sheep either by the farmer or a veterinarian. Some of these adult-stage individuals were recovered from animals on two farms (A and B), where levamisole has been proven to be effective, according to the FECRT. Farm A was sampled both in 2018 and 2020, whereas farm B only in 2020.

Paired, pooled larval cultures, each from between 10 and 15 animals (~2g feces/animal), were obtained pre- ($n = 7$) and 7–10 days post- ($n = 6$) treatment with levamisole from seven farms around Sweden. These samples were prepared and harvested using the Petri-dish method, as described earlier (Elmahalawy et al., 2018). For farms A, B, C, D, E and F, pre- and post-treatment samples were utilized, whereas for farm G only the pre-treatment sample was available.

gDNA of *H. contortus* single-adult worms or larval cultures of Swedish origin was extracted using the NucleoSpin tissue kit (*Macherey Nagel*, Germany), whereas gDNA from reference isolate worms – was isolated using the DNeasy blood and tissue kit (Qiagen, Germany), according to the manufacturer's recommendations. Finally, either 1 μl (for ddPCR) or 5 μl (for conventional PCR) of the extracted DNA were used in the following analyses.

2.2. Fecal egg count reduction test

Two tablespoons of fresh feces were collected from each animal for every tested farm. Generally, 15 sheep were sampled pre-levamisole treatment and 10 sheep with the highest pre-treatment egg counts 7–10 days post-treatment. The fresh individual samples were then immediately placed in separate marked, airtight (zip-locked) plastic bags and shipped overnight by the national post service to the diagnostic laboratory (Vidilab AB). Subsequently, 3 g of feces were screened for strongyle eggs using a modified McMaster method with a minimum diagnostic sensitivity of 50 eggs per gram (EPG) as described earlier (Ljungström et al., 2018).

2.3. Amplicon sequencing and primer design

Single worm genomic DNA was used to amplify (AmpliTaq Gold™ DNA Polymerase, ThermoFisher) the partial second intronic region of the *hco-acr-8* using previously suggested primers ("ForInsert" - 5′ACCTTACCTATACACCCGTC3′ and "RevInsert" - 5′CTTGCCGTTATTACACCCTCG3′) and protocol (Barrère et al., 2014). In short, the amplification protocol included a single cycle of 94 °C for 3

min, 40 cycles of 94 °C for 30 s, 55 °C for 30 s, and 68 °C for 30 s, as well as a final extension cycle of 68 °C for 5 min (MyCycler™ Thermal Cycler). Amplicon DNA was quantified (Qubit™ dsDNA HS), cleaned up enzymatically (Exonuclease I [20 U/μL; Thermofisher Scientific] and FastAP Thermosensitive Alkaline Phosphatase [1 U/μL; Thermofisher Scientific], according to the manufacturers guidelines) and submitted for Sanger dideoxy sequencing in both directions to Macrogen Europe.

The obtained sequenced amplicon data was evaluated for quality and trimmed. Upon unsuccessful attempts to split heterozygous indels, consensus sequences were derived from forward and reverse reads (where possible) and aligned using the *CodonCode Aligner* software (v.9.0.1; CodonCode Corporation, Massachusetts, USA). Subsequent evaluation was performed by manually examining the alignments.

The sequenced amplicon data has been deposited to GenBank (accession numbers: MT679733- MT679792).

The assembled sequences were used to manually create a new pair of primers to be used in the ddPCR assay - ACR8F1 (5′CTCCATATTC GAGTTGTGTCTT3′) and ACR8R1 (5′GTATCCAACATTGAATTAAAG GC3′), which create amplicons of either 182 bp (full-length sequence) and/or 119 bp (containing the 63 bp deletion). The optimal annealing temperature was determined via a gradient PCR (Supplementary figure 1). After optimization, the amplification steps for using the pair of primers were as follows: 1 cycle of 95 °C for 5 min, 40 cycles of 95 °C for 45 s, 56 °C for 30 s, 72 °C for 1 min, and a single cycle of 72 °C for 10 min. The products were visualized using GelRed® dye on a 2% agarose gel.

The primers for the reference amplicon in the exon 1 of the *Hco-acr-8* gene were developed *in silico* using the available sequence information for the *hco-acr-8* (HCON_00151270) on the WormBase ParaSite database.

2.4. Droplet digital PCR (ddPCR)

2.4.1. Assay setup

The ddPCR assay was designed to use two primer and probe pairs (Fig. 1). The first pair was designed to quantify the presence of the "susceptible" allele, i.e. to estimate the number of amplicon copies/μl for the *hco-acr-8* allele, containing the full-length intronic sequence (forward – "ACR8F1" 5′CTCCATATTCGAGTTGTGTCTT3'; reverse –"ACR8R1" 5′GTATCCAACATTGAATTAAAGGC3'; probe – "ACR8P1" 5'/56-FAM/ATCGCCGCAGTACGCGTAAGGCTGATTA/3IABkFQ/3′). Therefore, in samples, containing the "susceptible" allele (i.e. no deletion is present), the probe will bind and, upon cleavage, produce a fluorescence-emitting molecule, whose signal is measured. However, in samples where the deletion containing "resistant" allele is present, the probe does not bind and, therefore, no detection and quantification measurements are generated. Thus, the ratio of fluorescence forms the basis of the genotyping assay – homozygous susceptible individuals (SS) will emit fluorescence, heterozygous individuals (RS) will generate a fluorescence signal equal to approximately half that produced by the SS, while the homozygous resistant individuals (RR) will not produce any fluorescence. The second primer and probe pair was designed to anneal and estimate the copy number of a short reference amplicon (152 bp) in exon 1 of the *hco-acr-8*.As an independent amplicon, the product should be amplified regardless of whether the downstream intron region contains the deletion or not and, thus, provides a robust reference measurement for the sum of both alleles in a single sample (forward – "Exon1F1" 5′GTCTATGATACGGATAAGCG3'; reverse – "Exon1R1" 5′CAATCGTCGTATACATAGTGG3'; probe – "Exon1P1" 5'/5HEX/CGTTCTTTACCGGTCGCACA/3IABkFQ/3′). By running the primer and probe pairs simultaneously, we established the frequency (%) of the "resistant" allele (*a*) for each sample according to the formula:

$$a = \frac{c - b}{c} \times 100(\%)$$

Where *b* is the average obtained copy number for the "susceptible" allele and *c* is the average copy number for the reference amplicon in exon 1.

2.4.2. Assay validation and reaction conditions

The optimal annealing temperature for the primers and probes was determined by performing a gradient ddPCR (Supplementary figure 2). The primer and probe pairs were then used to quantify both amplicons separately, as well as together in a single reaction mix, in order to establish whether or not either of the primer and probe pair mediated amplifications significantly influence the quantification of the other amplicon. To test this, five, individual worm gDNA samples (previously analyzed using Sanger sequencing and with conventional PCR) were evaluated in terms of copy number measurements for both the reference and "sensitive" allele amplicons in *hco-acr-8*. To ensure that technical differences in allele ratios ("susceptible" vs. "resistant") could be reliably estimated in a mixed population setting, a linear association between the dilution factor and the frequency of the "resistant" allele was estimated by mixing the gDNA derived from a phenotypically resistant isolate sample (Cedara3) with gDNA from a previously sequenced susceptible (i.e. not containing the 63bp deletion) Swedish field isolate sampled at various ratios of the two DNA pools (100:0, 80:20, 60:40, 50:50, 40:60, 20:80 and 0:100).

The sample reactions were assembled in 96-well plates (final volume 22 μl), following the guidelines issued by the manufacturer (BioRad). Droplets were generated and dispensed into a new 96-well plate using an automated droplet generator (QX200, BioRad). The new plate was heat sealed and transferred into a thermal cycler (MyCycler™ Thermal Cycler). The optimized PCR conditions were as follows: a single cycle of 95 °C for 10 min, 40 cycles of 94 °C for 30 s and then 58 °C for 1 min, followed by a final cycle of 98 °C for 10 min to deactivate the enzyme. After the amplification step, the plate containing the droplets was loaded into the droplet reader (QX200, BioRad) and further analyzed using QuantaSoft (v1.April 7, 0917) software, which generates DNA copy measurements, fractional abundance data, and error bars based on Poisson statistics (Hindson et al., 2011). No-template control samples were included in every run to monitor for possible contamination.

2.4.3. Analysis

The output from QuantaSoft was visualized using the *ggplot2* package (v3.2.1) for R software (v3.6.3). The frequencies of the genotypes for reference isolates (SS; RS; RR) were analyzed with Pearson's Chi-squared test in R (v3.6.3).

3. Results

3.1. Amplification and sequencing

To design primers and probes to assay the 63 bp deletion site in the *hco-acr*-8, we first amplified and sequenced the second intron region containing the 63 bp deletion site in the *hco-acr-8* from 90 samples of individual adult *H. contortus* (42 of Swedish origin, 48 – reference isolates) using the previously described PCR protocol by Barrère et al. (2014). Among these, 56 individual samples (21 of Swedish origin, 35 - reference isolates) produced high-quality, full-sequence length sequences and were retained (Supplementary figure 3), whereas 34 samples yielded either only partially resolved or were low-quality, i.e. noisy (especially surrounding the area of the deletion), and were removed from further analysis. No heterozygous individuals were found upon attempting to 'split heterozygotes indels' within the *CodonCode Aligner* software and, therefore, only the major allele was recovered for each sample. Out of the 56 sequences, 14 (25%) did not possess the deletion (11 sequences of Swedish origin and 3 - reference isolates), whereas the other 42 (75%) presented the expected 63 bp deletion (10 of Swedish origin, 32 - reference isolates).

The reference isolate samples were further genotyped using primers ACR8F1 and ACR8R1 (10 worms per isolate: ISE, Weybridge, Zaïre,

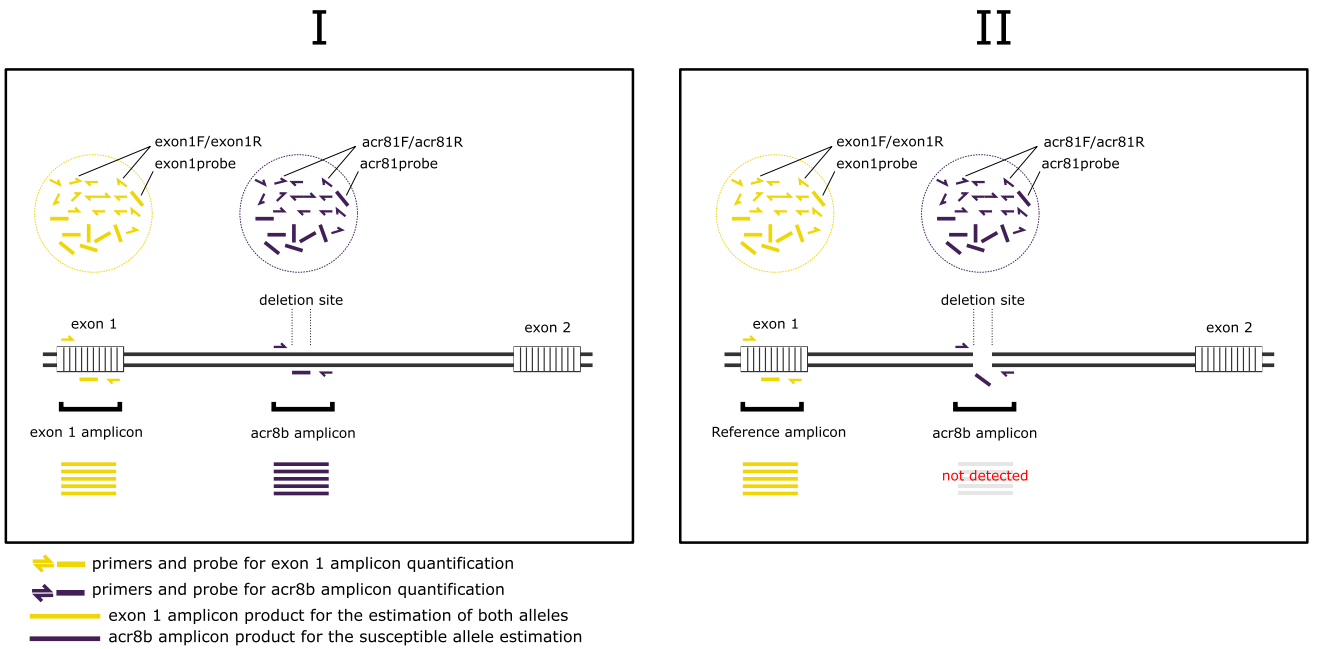

Fig. 1. The setup of the ddPCR assay for the estimation of the alleles containing the 63 bp deletion in the *hco-acr-8* in *H. contortus*. (I) and (II) depict the quantification outcomes for both allele types, i.e. when the 63 bp deletion is not present (full-length intron sequence – "susceptible" allele; amplification occurs) and present (amplification occurs, but the probe does not bind, and thus, no detection takes place), respectively. (I) Should the "susceptible" allele be encountered, the amplification of exon 1 and the deletion-containing region occur, resulting in equal amounts of amplicon copies and fluorescence signals, produced by the cleavage of both probes (II) If the "resistant" (i.e. deletion containing). allele is encountered, the fluorescence of only the probe binding to exon 1 is recorded. Primers-probe and exon 1 amplicon are in yellow; Primers/probe and acr8b amplicon are in dark purple; Genomic DNA is in black; Exons 1 and 2 are white, striped rectangles. (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

Cedara, Borgsteede, Kokstad) (Supplementary figures 4 and 5). Out of the 35 sequenced reference isolate samples, four individuals (11%) were incorrectly genotyped as RR by sequencing, where further analysis with conventional PCR found these to be RS (Supplementary table 1). The genotypes of the remaining worms were concordant between the two independent assays.

3.2. Droplet digital PCR

3.2.1. Adult worms

The newly developed droplet digital PCR approach was first evaluated for potential cross-reactivity between the two primer and probe sets in a single reaction mix as well as for the technical consistency of the "susceptible" and "resistant" allele frequency estimation via increasing dilutions.

Optimally, both primer and probe pairs for the detection of the target and reference amplicons would be used in the same reaction. Due to the possibility of primer and/or probe interactions (i.e. cross-reactivity) resulting in a reduced efficiency of the assay, we tested for this bias by performing both amplicon quantifications in single-plex (one primer and probe pair) and duplex reactions (both primer and probe pairs) for comparison. Quantifications of the amplicons were not influenced by the presence of the other primer and probe pair in the mix, as measurements between the single-plex and duplex reactions were highly similar across the five individual gDNA samples (Fig. 2). Moreover, the previously established genotypes (Fig. 2B) for these five samples were confirmed with our ddPCR assay – adult1 and adult2 were SS, adult3-RS, whereas adult4 and adult5 were of RR genotype. Linear increase in the ratio of the "resistant" allele was observed as the proportion of "susceptible" allele containing gDNA was reduced through increasing dilutions (Spearman correlation = 0.9975; Supplementary figure 6).

The previously examined, reference isolate genomic DNA samples as well as single, adult worms derived from two Swedish farms (wherein levamisole was determined to be efficacious according to the FECRT; Table 1) were evaluated with the ddPCR assay in order to estimate the frequencies of the "resistant" allele (Fig. 3). After the comparison of reference isolate genotype data (obtained with both conventional and ddPCR) and due to the observed difference in amplification efficiency between the two primer-probe sets, cut-off values were set for SS- (<25% frequency of the "resistant" allele), RS- (between 25 and 75% frequency of the "resistant" allele) and RR- (>75% frequency of the "resistant" allele) genotype-possessing individuals.

Table 1
Strongylid eggs per gram of feces counts obtained from sheep (n = 10–15) on farms (A-G), from which the before and after (7–10 days) levamisole treatment, *H. contortus*-dominated, field larvae populations were derived.

Farm	Average pre-treatment EPG count	Standard deviation	Average post-treatment EPG count	Efficacy of treatment (%)
A	6340	4510	0	100
B	5356	3787	0	100
C	394	308	0	100
D	6340	4510	0	100
E	419	827	0	100
F	195	152	0	100
G	292	228	NOT TESTED	–

Among the 60 ddPCR-genotyped reference isolates, five (8%; samples ISE1,3,5,6,7) were found to be RS, instead of either RR or SS (as determined previously by conventional PCR). Furthermore, in comparison to the sequencing data, four samples (6%; ISE5, Zaire2, Zaire5, Borgsteede6) were identified as RS, instead of either RR or SS (Supplementary table 1).

In total, eight RS and two SS samples were identified within the phenotypically susceptible isolate category (S1–S3; n = 30), whilst all but one worm (Borgsteede6 - RS) were found to be RR in the phenotypically resistant isolate group (R1-R3; n = 30) (Fig. 3). Although Pearson's Chi-squared test showed significant difference (p = 0.01) in the genotype (SS, RS, RR) frequencies between the susceptible and resistant isolates, the RR genotype was the most common in both categories, irrespective of phenotypic status.

In addition, 30 individual adult worm gDNA samples from the two Swedish farms (SW.A1, SW.A2 and SW.B; Fig. 3) were tested for the frequencies of the "resistant" allele. Overall, 16 gDNA samples were deemed to be of RS (the most common genotype) and 12 of RR genotype, whilst only two were found to be SS.

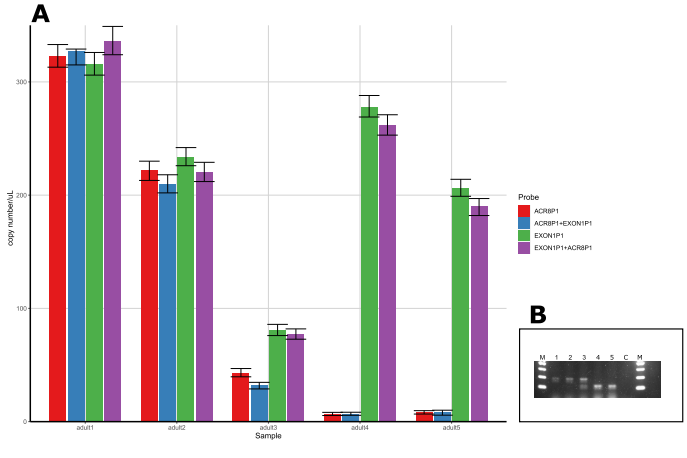

Fig. 2. (A) Cross-reactivity examination between primers and probe sets for the quantification of the 63 bp deletion as well as exon 1 amplicon in five adult *H. contortus* of distinct genotypes (two homozygous "susceptible" (SS), one heterozygous (RS) and two homozygous "resistant" (RR)). Amplicon DNA copy number was estimated for the "susceptible" full-length *hco-acr-8* fragment as well as exon 1 (in separate fluorescence detection channels) using individual primer and probe sets ("ACR8P1" and "EXON1P1" in the figure) and both sets together ("ACR8P1+EXON1P1", "EXON1P1+ACR8P1" in the figure). In red: DNA copy number for the "susceptible", full-length *hco-acr-8*, obtained by analyzing the specific primers and probe in a single-plex reaction setup. In blue: DNA copy number for the "susceptible", full-length *hco-acr-8*, obtained by analyzing both primer and probe sets in a single duplex reaction. In green: DNA copy number for the amplicon in exon 1, obtained by analyzing the specific primers and probe in a single-plex reaction setup. In purple: DNA copy number for the amplicon in exon 1, obtained by analyzing both primer and probe sets in a single duplex reaction. Error bars represent 95% Poisson confidence interval values. (B) shows the genotype of each individual (adults (1–5), as confirmed by conventional PCR using primers ACR8F1 and ACR8R1. M – 100bp DNA ladder (ThermoFisher Scientific). C – negative template control. (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

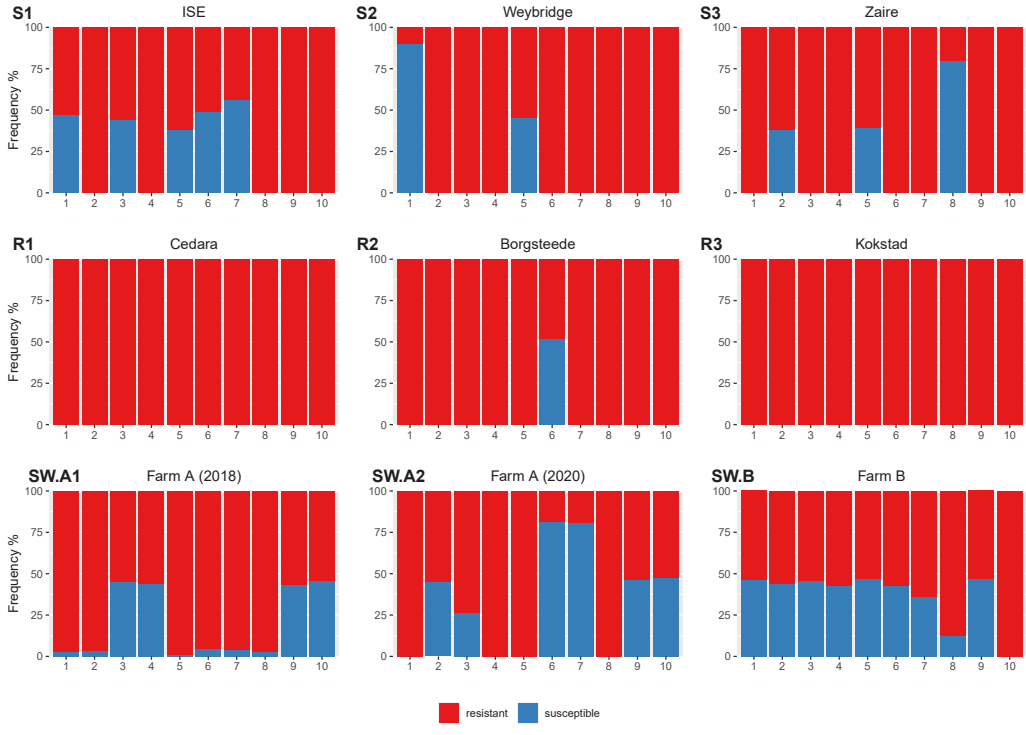

Fig. 3. The frequencies (%) of "susceptible" (full-length second intron sequence; in blue) and "resistant" (63 bp deletion in the second intron in the *hco-acr-8*; in red) alleles in various individual, adult *H. contortus* isolates. S1–S3 correspond to levamisole susceptible isolates – ISE, Weybridge and Zaïre, whereas R1-R3 to levamisole resistant – Cedara, Borgsteede and Kokstad. SW.A1, SW.A2 and SW.B represent allele frequencies in individual, adult *H. contortus* isolated from farms A (in 2018 and then 2020) and B, respectively. (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

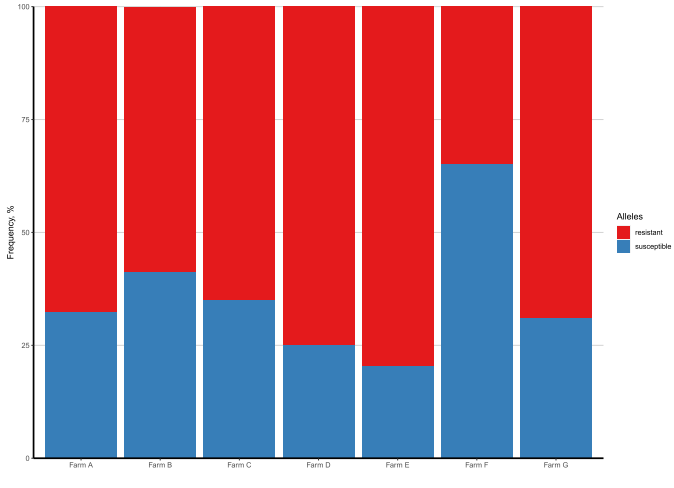

Fig. 4. The frequencies (%) of "susceptible" (full-length second intron sequence; in blue) and "resistant" (63 bp deletion in the second intron of the *hco-acr-8*; in red) alleles in larvae cultures, recovered from different farms (A, B, C, D, E, F, G) pre-treatment with levamisole. Note: All six post-treatment samples did not contain *H.contortus* DNA (thus are not shown) whereas population G did not have a post-treatment sample taken, due to low egg counts in the pre-treatment samples. (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

3.2.2. Larval cultures

The pre-treatment FECs varied between 195 ± 152 (on farm F) to 6340 ± 4510 EPG (on farm A), whereas all post-treatment samples were negative. The reduction on all six farms was 100% (Table 1).

The ddPCR assay was subsequently used to screen all six paired (A-F) and one unpaired (G) field-derived larval culture samples (producing on average a total of 11,5–9717 copies/μl) (Fig. 4). The "resistant" allele was detected in all pre-treatment larvae culture samples at ratios ranging 35–80%. In contrast, all post-treatment samples contained no trace of *H. contortus* amplicon DNA (data not shown).

4. Discussion

Due to the worrying state of highly pathogenic GINs, such as *H. contortus*, found exhibiting elevated resistance to benzimidazoles, macrocyclic lactones and monepantel (Höglund et al., 2009, 2015, 2020), it is vitally important to maintain the efficacy of levamisole – currently the "last line of defense" drug against GIN infections in small ruminants in Sweden. In order to monitor the development of levamisole resistance by *H. contortus* on Swedish sheep farms, a molecular screening tool for the rapid detection and estimation of resistance-encoding alleles in field samples would be of immense use. Here, we have described the development of an assay to detect a 63 bp deletion in the *hco-acr-8* L-AChR subunit gene (Barrère et al., 2014) previously identified to be associated with levamisole resistance.

Using the obtained sequencing data for intron 2 amplicon in *hco-acr-8* from 56 adult *H. contortus*, a primer pair was developed to be used in further genotyping (with conventional PCR and ddPCR) of individual worm gDNA samples. Although amplification was observed for all samples, varying degrees of amplification efficiency were found not only between the different individuals but also between the two alleles belonging to the same worm (Supplementary figures 4 and 5). This is in agreement with previous work (Barrère et al., 2014) and possibly explains why only the major allele was possible to retrieve from each sequenced single worm sample. Whilst DNA concentration could have played a role, considering the normally high mutation frequencies within the intronic regions in eukaryotic organisms, this can most likely be attributed to nucleotide variation within the primer binding sites.

Despite the limitations in identifying RS genotypes with both Sanger sequencing as well as conventional PCR, the results for the comparisons between the direct deletion detection (sequencing and conventional PCR) and indirect deletion detection (ddPCR) assays in terms of identifying the genotypic status of each of the tested reference *H. contortus* isolate are overall congruent (Supplementary table 1). However, the clear advantage of ddPCR over conventional PCR is that the former is claimed to eliminate amplification bias by constraining the results of the amplification to a binary outcome (Hindson et al., 2011). In addition, by validating our ddPCR assay, we not only established that both primer and probe pairs do not cross-react with one another when used in a duplex reaction (Fig. 2) but also that the dilution of the gDNA containing only the "susceptible" allele with gDNA containing only the "resistant" allele results in a perfect linear pattern of decrease in the frequency of the diluted allele (and an increase in the "resistant" allele; Supplementary figure 6). However, it is important to point out that cross-reactivity between the primers and probes in a single reaction is fundamentally different from biases, resulting from variation in primer and probe binding efficiencies. Furthermore, the calculated correlation between the sample dilution and allele frequency reflects only the technical variability and the capacity of the assay to distinguish and estimate different proportions of the "sensitive" and reference allele amplicons in a sample. Thus, while a good linear relationship across the dilution series was observed (Supplementary figure 6), the accuracy is not as good. Samples, containing only the "sensitive" allele did not reach a 100% in the frequency of this allele (or 50% in the case of RS genotypes), likely due to differences in amplification efficiency between the two amplicons. Fundamentally, ddPCR estimates the intensity of fluorescence produced by the amplification of a distinct amplicon (and converts that intensity into target copies/μl) and, therefore, the subsequent copy measurements depend not only on the DNA concentration in the sample, but also the degree of nucleotide variability. Therefore, we believe this inconsistency between the copy numbers for two amplicons to be a direct consequence of the reference amplicon being situated in a more conserved exonic region, whereas the "sensitive" allele amplicon was in an inherently more variable intron. Nevertheless, unlike in any of our previous approaches for the detection of SNPs (Baltrušis et al., 2018) or genetically variable regions for species differentiation (Baltrušis et al., 2019), this study employs a more robust design, utilizing the simultaneous absolute quantification of two distant regions within the *hco-acr-8* for the indirect determination of the frequency of the "resistant" allele.

Having examined the six reference isolates (n = 10 per isolate) using the ddPCR platform, it was found that the genotypic status of individuals within the reference isolate groups (S1–S3 and R1-R3) agreed for the most part with the results obtained with conventional PCR. Notably, in the case of S1 (ISE isolate) which has been previously confirmed to be of RS genotype for the 63bp deletion (Barrère et al., 2014), conventional PCR failed to elucidate the heterozygosity due to poor amplification (Supplementary figure 4). However, 50% of worms (5/10) within this isolate were indeed found to be RS when analyzed with the more sensitive ddPCR assay (Fig. 3). Even though ddPCR seemed to provide more sensitive measurements, the "susceptible" allele was, in many cases, underestimated, leading to the overestimation of the "resistant" allele. Thus, neither the 50% frequency of the "susceptible" allele, in the case of RS individuals (i.e. ISE-1,3,5, Weybridge-5 and S3-2,5), nor the 100% for fully SS individuals (i.e. Weybridge-1 and Zaire-8) were reached. The frequency between 25 and 75% of the "susceptible" allele in a single worm sample was, therefore, considered to be indicative of the RS genotype and >75% of SS genotype. A similar pattern of underestimation was also observed for adult worms derived from the Swedish farms (one of which was sampled at two different time points – 2018 and then again in 2020). Here, samples SW.B-8 and SW.A2-3 were severely underestimated in terms of the "susceptible" allele, but were distinguishable from what we considered to be minor contaminations, observed in SW. A1-1, 2, 5, 6, 7 and 8. Quite unexpectedly, individuals possessing the SS genotype were the rarest, even among the phenotypically susceptible isolates (2/30). Moreover, apart from populations SW.B and SW.A2, the "resistant" allele was much more common, even in the reference isolates, confirmed to be susceptible to levamisole (S1–S3). Despite the fact that the difference in the three genotypes (i.e. SS, SR, RR) between the susceptible (S1–S3) and resistant (R1-R3) isolates was statistically significant ($p = 0.01$), which is in line with Barrère et al. (2014), in well-characterized (and susceptible to levamisole), isolates, such as ISE, Weybridge and Zaïre, half or more of the individuals were found to be of RR genotype. Thus, judging by the obtained genotypes for all six isolates, it appears as the 63bp deletion in *hco-acr-8* is a poor predictor of the actual phenotype in individual worms.

Larvae culture samples, derived from sheep before and after treatment with levamisole, were used to quantify the presence of the deletion in the field (Fig. 4). Not only was the 63bp deletion found in all tested populations (Farms A-G) before treatment, but the frequency of the deletion varied to a great extent (35–80%). Interestingly, in six of those populations no trace of amplifiable *H. contortus* DNA was found in any of the post-treatment samples, indicating that the levamisole treatment had been successful. This observation was consistent with the FECRT data (Table 1). Although it has been previously posited that resistance caused by the deletion is likely to be genetically recessive, i.e. RS individuals might still be susceptible (Santos et al., 2019), overall it appears that the increased frequencies of the deletion in the *hco-acr-8* did not at all correspond to heightened resistance to the effect of levamisole in the field isolates upon treatment. Furthermore, unlike the previously discussed study (Santos et al., 2019), wherein the "resistant" allele frequencies were correlated with LD50 values, the variation in the frequency of the "resistant" allele among the tested Swedish single worm

isolates and larval cultures (derived from farms wherein levamisole was efficacious) proved to be somewhat random.

Judging from the results obtained for single worm and larvae culture samples, we conclude that the ddPCR assay cannot be reliably used for the estimation of the deletion frequency in larvae pools. While certain limitations, present for single worm gDNA samples (mainly higher overall efficiency of amplification of the reference allele in comparison to the "sensitive" allele, leading to the overestimation of the frequency of the deletion) can be mitigated by employing cut-off thresholds to define genotypes, the amplification efficiency bias cannot be easily addressed in pools of multiple individuals.

From a practical standpoint, our results demonstrate the 63 bp deletion is not a predictive marker for levamisole resistance status determination in a field population context, as it is apparent that the deletion is not associated with the resistant phenotype in every case. A similar conclusion was drawn by Chagas et al. (2016), who observed the presence of the 63 bp deletion in individual larvae belonging to a susceptible *H. contortus* strain and, thus, determined that the absence of the 63 bp fragment could not always be linked to levamisole resistance in *H. contortus*.

In addition to *hco-acr-8*, other genes of subunits within the L-AChRs, such as *hco-unc-63* (Neveu et al., 2010; Boulin et al., 2011), as well differences in the expression patterns of the auxiliary and P-glycoprotein genes (Sarai et al., 2014), are involved in the determination of the phenotypic status and, thus, could be important contributing forces in the development of resistance. Sarai et al. (2014 and 2015) have demonstrated that not only does the expression of putative candidate genes, thought to be involved in the development of resistance to levamisole, vary considerably depending on the concentration of levamisole administered to the infected animals, but that the expression of those genes (including *hco-acr-8b*) appeared to also fluctuate in different life cycle stages of *H. contortus* (L1, L3 and adults). Interestingly, the latter study also found the adult stages to be persistently susceptible according to the drench efficacy test results (FECRT), even after nine generations of selective propagation and extreme, subsequent increases in resistance, as measured by the larval development assays. Thus, the determination of which key cellular changes in *H. contortus* are essential in order to develop resistance to levamisole as well as to what degree each of these changes contribute to the phenotypic differences in field isolates, remains somewhat elusive.

A significant challenge in this study is related to the heightened nucleotide variation within the intron region where the 63 bp deletion was first discovered. This can adversely affect the precision and overall applicability of the quantitative, diagnostic measurements via biased primer binding and uneven allele amplification, especially when the species under the investigation, such as *H. contortus*, is exceptionally genetically diverse (Yin et al., 2013; Sallé et al., 2019; Doyle et al., 2020). Yet another disadvantage of the current approach is that this assay does not directly detect and estimate the proportion of the deletion containing allele. The alternative approach would be to design a probe, flanking the deleted region, thus, resulting in detection and quantification of only the truncated form. Yet another approach would consist of using degenerate primers and probes. However, such approach would require a more in-depth knowledge of the nucleotide variation present within the deletion surrounding region. Although the current assay setup was chosen to mitigate the observed nucleotide variation, surrounding the 63 bp intronic deletion based on Sanger sequencing, overall, the data presented here, suggests that genetically variable sites, such as intronic regions, are not an ideal target in creating PCR based amplification assays for anthelmintic marker detection and quantification.

In conclusion, we have further evaluated the suitability of the 63 bp deletion in the *hco-acr-8* as a potential marker to track levamisole resistance in *H. contortus* field isolates. The presence of the deletion (i.e. "resistant" allele) in reference isolates as well as Swedish single adult worms and field populations was identified by sequencing, conventional and droplet digital PCRs. However, neither reduced levamisole efficacy, nor an increased proportion of surviving individuals in the field populations, subjected to levamisole treatment were observed, despite the high frequency of the "resistant" allele. Furthermore, although a significant difference ($p = 0.01$) was found between the reference isolates (susceptible vs. resistant) in terms of the genotype frequencies, the deletion containing "resistant" allele was more prominent, even in the susceptible isolate category. Although ddPCR proved to be a valid molecular tool for mutation detection and quantification (despite some inherent challenges with the current approach), it is important to keep in mind that resistance to levamisole has been reported to be a polygenic trait (Kotze and Prichard, 2016), associated with multiple, perhaps even simultaneous, cellular changes. Thus, further genome-wide approaches, as discussed previously by (Gilleard, 2006) and Doyle and Cotton (2019) are necessary to provide new insights into potentially better molecular markers for levamisole resistance detection in *H. contortus*.

Declaration of competing interest

The authors have no conflicts of interest to declare.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.ijpddr.2021.03.002.

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Genome-wide analysis of the response to ivermectin treatment by a Swedish field population of *Haemonchus contortus*

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ABSTRACT

Haemonchus contortus is a pathogenic gastrointestinal nematode of small ruminants and, in part due to its capacity to develop resistance to drugs, contributes to significant losses in the animal production sector worldwide. Despite decades of research, comparatively little is known about the specific mechanism(s) driving resistance to drugs such as ivermectin in this species. Here we describe a genome-wide approach to detect evidence of selection by ivermectin treatment in a field population of *H. contortus* from Sweden, using parasites sampled from the same animals before and seven days after ivermectin exposure followed by whole-genome sequencing. Despite an 89% reduction in parasites recovered after treatment measured by the fecal egg count reduction test, the surviving population was highly genetically similar to the population before treatment, suggesting that resistance has likely evolved over time and that resistance alleles are present on diverse haplotypes. Pairwise gene and SNP frequency comparisons indicated the highest degree of differentiation was found at the terminal end of chromosome 4, whereas the most striking difference in nucleotide diversity was observed in a region on chromosome 5 previously reported to harbor a major quantitative trait locus involved in ivermectin resistance. These data provide novel insight into the genome-wide effect of ivermectin selection in a field population as well as confirm the importance of the previously established quantitative trait locus in the development of resistance to ivermectin.

1. Introduction

The nematode *Haemonchus contortus* is one of the most pathogenic helminth species of small ruminants across the world. Together with other commonly found gastrointestinal nematodes (GINs), *H. contortus* contributes to reduced weight gain in infected individuals, the consequences of which often result in the decreased production of meat and dairy products. Severe cases of infection with *H. contortus* lead to anemia and can even result in sudden death of the host. Infection by GINs has a staggering impact on animal husbandry, estimated at approximately €686 million in treatment and prevention costs annually in Europe alone (Charlier et al., 2020). *H. contortus* is a cosmopolitan GIN, partly due to the species capacity to adapt to both colder and warmer climates, to infect a number of different host species, and to rapidly develop resistance to every drug class currently used to control it (Van den Brom et al., 2013; Geurden et al., 2014; Cazajous et al., 2018; Höglund et al., 2020).

Despite the broad impact on animal health and production, our understanding of the mechanisms by which resistance evolves, especially to certain drugs such as ivermectin (IVM), is generally lacking. Although numerous candidate genes have been proposed over the years as putative drivers of resistance to IVM in *H. contortus* (summarized in Doyle et al., 2019), the evidence for the involvement in IVM resistance for some of these candidate genes remains inconclusive. For example, some genes initially proposed to be associated with resistance, for example, *dyf-7, lgc-37, glc-5, avr-14,* have subsequently been shown not to be significantly associated with the resistant phenotype in follow-up studies (Laing et al., 2016; Rezansoff et al., 2016; Elmahalawy et al., 2018). The many genes proposed to be associated with resistance has led to a widely accepted view that IVM resistance may be caused by one of a number of changes throughout the genome, including genetic changes in both coding and non-coding sequences. The high genetic diversity among GINs such as *H. contortus*, together with the fact that many studies focus on only a few individuals among limited, yet genetically and phenotypically diverse strains, suggests that some candidates may be falsely associated with resistance. Similarly, approaches to identify

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genetic markers associated with resistance can also produce false associations; even if a genetic marker is linked to a causal variant, a lack of understanding of linkage disequilibrium (LD) patterns among variation in genomes of individuals in the studied populations (Doyle and Cotton, 2019) and the potential changes in these patterns over time and in different populations, may result in differences in the degree of linkage, and in some cases, the associated variants becoming unlinked from the causal variants. Candidate gene approach studies which focus only on the frequency changes of single target genes and/or mutations (Blackhall et al., 1998; Eng et al., 2006; Urdaneta-Marquez et al., 2014), without taking into account the genome-wide genetic variation, are limited. Thus, the current gaps in the understanding of the mechanism of IVM resistance cannot simply be addressed through conventional candidate gene approaches alone.

Genome-wide studies of genetic variation have begun to provide a more comprehensive and unbiased framework towards identifying genomic regions associated with quantitative, phenotypic traits of interest (i.e. Quantitative trait loci, QTL), including genes associated with anthelmintic resistance in veterinary nematodes. These advances, made possible by technological improvements and rapid decrease in the cost of sequencing, have already provided important insight in the genetics of trait variation in other fields, including the study of important human pathogens (Chevalier et al., 2014; Abkallo et al., 2017; Anderson et al., 2018). Several studies, performed on other parasitic worm species (Choi et al., 2017; Doyle et al., 2017) as well as protozoans (Cheeseman et al., 2015), have similarly shown the capacity of genome-wide approaches in combination with relevant statistical analyses to identify genomic regions under selection as well as propose genes associated with drug resistance. For *H. contortus*, the availability of the high-quality reference genome (Doyle et al., 2020) and genetic tractability via the ability to perform genetic crosses (Redman et al., 2012), has accelerated research to refine the large list of candidate genes to identify specific discrete regions and, in some cases, individual genes and alleles responsible for driving resistance to several anthelmintics. For example, genetic crossing together with whole-genome sequencing has been used to map monepantel resistance (Niciura et al., 2019), which identified a single major locus in chromosome 2 (7.2–8.7 Mbp) containing the previously reported *mnptl-1, deg-3* and *des-2* genes thought to be involved in monepantel resistance development (Kaminsky et al., 2008; Rufener et al., 2009). For IVM, a backcross experiment followed by sequencing identified a single QTL in chromosome 5 (37–42 Mbp) associated with a resistance phenotype in two geographically distinct *H. contortus* strains (Doyle et al., 2019) which has since been refined to approximately 300 kbp with a putative driver identified among 24 genes within the QTL (Doyle et al., 2021). One concern of the use of the genetic crosses is that evidence of selection (i.e. the identified QTL, linked to the resistance phenotype) may not be representative of selection acting on the parasites in the field setting, and thus evidence outside of the laboratory is necessary. Although the chromosome 5 QTL has been replicated in field populations in the US (Doyle et al., 2021) and other field populations phenotypically defined for IVM resistance (Sallé et al., 2019), a study of *H. contortus* from China has identified multiple genomic regions in each chromosome between the phenotypically resistant and susceptible to IVM field isolates (Khan et al., 2020). Even though genome-wide scans suffer from many of the same complications as candidate gene studies (Doyle and Cotton, 2019), i.e. direct comparison of resistant and susceptible strains will identify many regions of the genome which differ due to their unique evolutionary histories and not due to resistance (Doyle et al., 2019), field validation of QTL identified in genetic crosses will strengthen the association between genetic variants and resistance. Together, these data will provide greater confidence in prioritizing variants for the development of molecular diagnostics used to monitor the evolution and spread of resistant alleles in the field (Kotze et al., 2020).

Motivated by the recent progress made in QTL mapping using genetic crosses and need for further validation, here we describe an approach to detect and characterize IVM-mediated selection in a field population of *H. contortus* from Sweden. By performing pooled whole-genome sequencing on infective stage larvae of the same population pre- and post-IVM treatment, we measured changes in nucleotide diversity and allele frequencies in response to treatment throughout the genome.

2. Materials and methods

2.1. Sample collection and parasitological analysis of phenotypic resistance using the FECRT

A commercial sheep farm in the south-eastern part of Sweden was chosen for this study, due to the presence of suspected but unconfirmed IVM treatment failure as well as high egg counts both pre- and post-treatment of which ≥90% belonged to *H. contortus*. Macrocyclic lactones (which included ivermectin) had been used extensively since at least 2012 to treat GIN infections in animals.

Field *H. contortus* samples were obtained pre-treatment and seven days post-treatment with IVM (200 μg/kg) from the same flock of sheep ($n = 11$) in connection with fecal egg count reduction testing (FECRT). Oral drenching of the sheep and the collection of fecal samples was carried out by the animal owner under the supervision of a veterinarian. Fecal samples were sealed in plastic bags and sent to a local diagnostic laboratory (*Vidilab* AB) where they were processed to purify the eggs, after which egg counts were performed using a modified McMaster method in accordance with Ljungström et al. (2018). Fecal egg count reduction from pre-to post-treatment was then calculated using the R package *eggCounts* (v. 2.3.).

The remaining fecal material not used in the FECRT was pooled into either pre- or post-treatment categories and cultured to facilitate egg hatching, after which the infective third stage larvae (L3) were harvested as previously described (Halvarsson and Höglund, 2021).

2.2. DNA extraction, sequencing library preparation, and whole-genome sequencing

The pre- and post-treatment pools of L3 were each subdivided into four microcentrifuge tubes, containing approximately the same packed volume representing thousands of individual larvae (>1000) per tube. This generated eight samples in total, consisting of four technical replicates for each of the pre-treatment and post-treatment categories. Genomic DNA was extracted from the eight samples using the Nucleospin Tissue kit (Macherey-Nagel), following the guidelines issued by the manufacturer. The DNA concentration for each replicate for the pre-treatment category was - 2.3 ng/μl, 9.46 ng/μl, 2.64 ng/μl and 2.78 ng/μl; whereas for the post-treatment – 53 ng/μl, 12,1 ng/μl, 65 ng/μl and 17.9 ng/μl.

The genomic DNA samples for each replicate were sent to *Annoroad Gene Technology* (AGT) (Beijing, China) for sequencing. The sequencing library was prepared using the Illumina DNA Prep kit and was sequenced using one lane of an Illumina NovaSeq 6000 using 150 bp paired-end sequencing chemistry. Sequencing yielded 231.9 Gbp of data in 1.546 billion reads. Prior to data delivery, AGT prefiltered the data to remove adapter sequences, low-quality reads where more than 50% of the bases had a $Q \leq 19$ and reads containing more than 5% missing bases (N).

2.3. Sequencing data analysis

The FASTQ files for the eight samples were mapped onto the *H. contortus* reference genome (available at: https://parasite.wormbase.org/Haemonchus_contortus_prjeb506/Info/Index/; Doyle et al., 2020) using *bwa-mem (v.0.7.17)* (Li and Durbin, 2009). *Picard (v. 2.23.4;* https://github.com/broadinstitute/picard) was used to remove duplicate reads and only perfectly mapped reads pairs (i.e. reads that are properly aligned in a pair, within 1000 bp of one another) were retained for

further analysis (*samtools* (v.1.10; http://www.htslib.org/) *view -f 2*). Comparisons within and between treatment groups involving the mitochondrial genome were not considered.

For subsequent nucleotide diversity (π), Tajima's D statistic, pairwise genetic differentiation comparisons (F_{ST} and Fisher's exact test) analyses, the BAM files from the four replicates per each treatment category were merged into a single pre- or post-treatment group using *samtools merge*. For the CMH test, the replicates were kept separate (as described below).

Pileup file generation for the sequence data analysis with either *NPstat* (Ferretti et al., 2013) or *Popoolation2* (Kofler et al., 2011) tools has been described previously (Doyle et al., 2019). In short, pileup files were generated using *samtools mpileup* (*-d 500 –min-MQ 30 –min-BQ 30 –adjust-MQ 50*).

2.4. Analyses of within group genetic diversity and between group genetic differentiation

Within group genetic diversity was determined using *NPstat (v.1),* from which nucleotide diversities as well as Tajima's D statistic within the two treatment groups was determined in 100 kb windows throughout the genome (*-n 200 -l 100000 -maxcov 500 -minqual 20*). This analysis was performed per chromosome per sample by splitting the mpileup and running the analysis on each respective part of the data separately, before merging the output.

Pairwise genetic differentiation between the pre-treatment and post-treatment sample groups was determined using *Popoolation2*. The previously generated pileup files were converted into synchronized files (*popoolation2 mpileup2sync.pl –min-qual 20*) and sequence around indels +5 bp (*popoolation2 identify-indel-regions.pl –min-count 2 –indel-window 5)* was removed (*popoolation2 filter-sync-by-gtf.pl*). The synchronized file containing the data for each of the eight samples was used to perform the Cochran-Mantel-Haenszel (CMH) test in order to investigate for the presence of any consistent allele frequency changes between the replicates belonging to each of the two treatment groups (*cmh-test.pl –min-count 4 –min-coverage 20 –max-coverage 2%*). However, since the replicates were previously pooled, they do not represent true, distinct pairs (from the same animals) and were, therefore, compared in an arbitrary fashion. In a similar way (but using a synchronized file containing the merged replicates), per nucleotide allele frequency differences between the two treatment groups were estimated using Fisher's exact test (FET) (*fisher-test.pl –min-count 4 –min-coverage 20 –max-coverage 2% –suppress-noninformative*). Pairwise F_{ST} values were also determined throughout the genome in 10,000 bp windows using a step size of 5000 bp (*popoolation2 fst-sliding.pl –pool-size 1000 –min-count 4 –min-coverage 20 –max-coverage 2% –window-size 10000 –step-size 5000*) and per genes (*popoolation2 fst-sliding.pl –pool-size 1000 –min-count 4 –min-coverage 20 –max-coverage 2% –window-size 1000000 –step-size 1000000*) after having created a synchronized file using *H. contortus* genome annotation (available at: https://parasite.wormbase.org/Haemonchus_contortus_prjeb506/Info/Index/; WBP15) as an input (*popoolation2 create-genewise-sync.pl*).

SNP calling was performed separately using *bcftools* (v. 1.12; http://www.htslib.org/doc/bcftools.html; *bcftools mpileup -d 500 –min-MQ 30 –min-BQ 30 –adjust-MQ 50 -a FORMAT/DP | bcftools call -mv | bcftools view -i '%QUAL>=20 & FORMAT/DP>=20'*) and the called SNPs were annotated using SNPEff (Cingolani et al., 2012) (v.4.3t; http://pcingola.github.io/SnpEff/).

2.5. Data and statistical analysis

Data was visualized using the R package ggplot2 (v. 3.3.3; https://github.com/tidyverse/ggplot2) in Rstudio (v. 1.2.5033). Nucleotide diversity and Tajima's D ratios as well as F_{ST} measurements per genomic window were determined to be substantially different if higher than genome-wide (or gene-wide) mean + 3 standard deviations (SD) and/or genome-wide (or gene-wide) mean + 5 SD values. Significance ($p \leq 0.05$) in the case of FET and CMH test was determined using a genome-wide Bonferroni's correction ($= \frac{0.05}{number\ of\ genome-wide\ SNPs}$).

Full code used in the different steps of the analyses together with explanations is available at https://github.com/pauliusbaltrusis/WGanalysis.

3. Results

3.1. Measurement of resistance by FECRT and whole-genome sequencing of pre- and post-treatment populations

We first set out to determine the efficacy of IVM on the treated farm by performing a FECRT. This was important to confirm the presence of resistance on this farm for which resistance was suspected. The average egg counts per gram of feces were 21568 ± 16313 before treatment and 2327 ± 3094 after treatment, resulting in an egg count reduction of 89% (approximate 95% confidence interval = 71.7–95.8%), consistent with the presence of resistance (Coles et al., 1992).

We sequenced pools of larvae collected pre- and post- IVM treatment, represented by four technical replicates at each time point for a total of eight pools. In total, we obtained 1.5×10^9 raw reads which on average 89.5% mapped to the genome; after processing and deduplication, an approximate 52.5 × coverage per chromosome (excluding mtDNA) per replicate pool was achieved. Analysis of genetic variation within the pooled mapped sequencing data identified up to approximately 2.7 million (in the case of FET) biallelic SNPs across all eight replicates.

3.2. Genome-wide genetic diversity within the pre- and post-treatment populations

To understand the effect of treatment on genetic diversity within each group, we calculated nucleotide diversity (π) as well as Tajima's D for each group (Fig. 1a and b and Fig. 1c and d). No significant differences between the π value distributions for all chromosomes were observed between both treatment groups (*p*-value = 1; one-sample Kolmogorov-Smirnov test). We did note that the average diversities for the X chromosome were approximately half ($\pi = 0.001 \pm 0.001$ for both pre- and post-treatment) of those observed for the autosomes (for both pre- and post-treatment $\pi = 0.003 \pm 0.001$), consistent with a previous study (Doyle et al., 2020). Mean Tajima's D values for the autosomes (−0.742 ± 0.514 pre-treatment and −0.756 ± 0.513 post-treatment) as well as chromosome X (−0.880 ± 0.816 pre-treatment and −0.872 ± 0.816 post-treatment) were also similar between the two treatment groups (*p*-value = 0.8; one-sample Kolmogorov-Smirnov test) and no large-scale differences between the two treatment group pools were observed for chromosome 5 (Fig. 1e and f).

3.3. Window-based measures of genetic differentiation in response to ivermectin treatment

To identify treatment-induced genetic changes throughout the genome, pairwise genetic differences (F_{ST}) between the two treatment group pools were estimated in 10 kbp windows throughout the genome, as well as for every single gene. Pairwise F_{ST} analysis revealed a low degree of differentiation between the two treatment groups (mean genome-wide $F_{ST} = 0.0083$; Fig. 2a; Supplementary Table 1). A total of 770 and 152 outlier 10 kbp windows were identified above 3 ($F_{st} > 0.024$) and 5 ($F_{ST} > 0.035$) SD from the genome-wide mean, respectively, most of which were present in chromosome 4 (n = 306 or n = 51, respectively). Of those 770 outlier windows, instances of at least 3 consecutive 10 kbp windows were recorded in 41 cases (26 of which were in chromosome 4), whereas only one such instance of at least 3 consecutive 10 kbp windows was observed above the mean F_{ST} + 5 SD - in chromosome 5, starting at 37.4 Mbp. Analyses of differentiation per

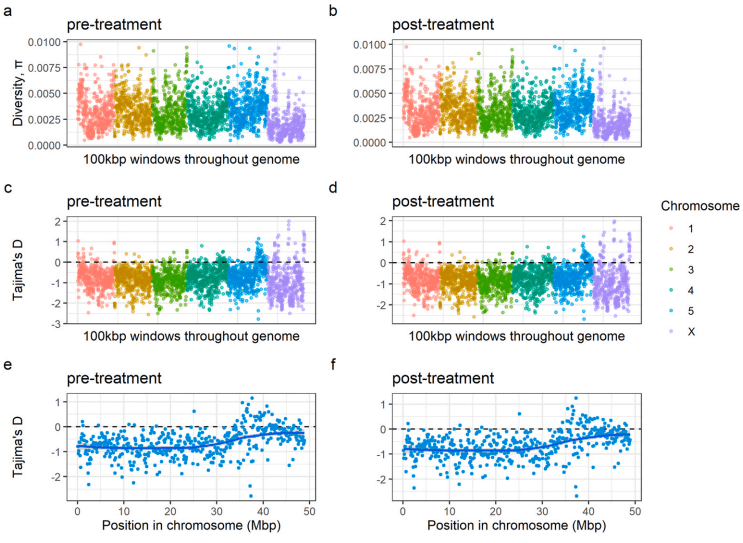

Fig. 1. Genetic diversity within pre- and post-treatment groups. Within treatment group nucleotide diversity (a and b) and Tajima's D (c and d) comparisons per 100 kbp genomic windows per chromosome based on pooled sequencing of *H. contortus* L3, recovered before and seven days after IVM treatment from the same flock of sheep. (e and f) Tajima's D estimates per 100 kbp genomic window were evaluated specifically for chromosome 5. The blue line represents a LOESS function drawn through the data points. The dashed line in (c and d) and (e and f) is drawn through value 0 which indicates neutrality and otherwise serves to separate windows wherein Tajima's D is either positive or negative. (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

gene also displayed a low degree of differentiation between the groups (mean F_{ST} = 0.007; Supplementary Table 2). We analyzed all genes in the genome to provide context for the analysis of F_{ST} from previously described candidate genes thought to be associated with IVM resistance (taken from Doyle et al., 2019); all candidates were within 3 SD of the genome-wide mean, of which the highest degree of differentiation was for *lgc-37* (F_{ST} = 0.022; Supplementary Table 3) (Supplementary Fig. 3). A total of 636 genes had an F_{ST} value at least as great as *lgc-37*, and a total of 92 genes showed substantial differentiation (F_{ST} > 5 SDs from genome-wide mean, i.e. F_{ST} > 0.043), of which the highest differentiation was observed for genes *HCON_00141660* (chromosome 5, ~15 Mbp; F_{ST} = 0.122; Tajima's D_{pre} −1.17, Tajima's D_{post} −1.15), HCON_00128970 (chromosome 4, ~48 Mbp; F_{ST} = 0.113; Tajima's D_{pre} −0.4, Tajima's D_{post} −0.5) and HCON_00115660 (chromosome 4, ~26 Mbp; F_{ST} = 0.098; Tajima's D_{pre} −0.41, Tajima's D_{post} −0.4).

In order to observe any subtle differences between the treatment groups, ratio comparisons per genomic 100 kbp windows between the π values (i.e. post-treatment/pre-treatment) as well as Tajima's D were made. π ratio comparison revealed a sudden increase in nucleotide diversity in the post-treatment group (up to roughly 1.35 ×) between 36.2 and 38.7 Mbp (mean π value ratio + 3SDs; cut-off = 1.09; 217 overlapping genes) or 37.2–38.7 Mbp (mean π value ratio + 5 SDs; cut-off = 1.15; 124 overlapping genes) in chromosome 5 (Fig. 3a). Further exploration of Tajima's D value ratio per every 100 kbp window in chromosome 5 showed that the differentiation of the values between the groups was observed to be highest at the terminal end of the chromosome (roughly 35–45 Mbp; 810 overlapping genes) wherein four outlier values (above mean Tajima D ratio + 5 SDs or below mean Tajima D ratio - 5 SDs) were found (Fig. 3b).

3.4. Analysis of single nucleotide variants in response to ivermectin treatment

We further analyzed allele frequency changes for every SNP by performing Fisher's exact tests (FET) on the pooled technical replicates (ie. pre-vs post) and Cochran–Mantel–Haenszel (CMH) tests on pairs of technical replicates (ie. 4 × pre-vs post) (Supplementary Figs. 1a and 1b). FET yielded roughly 2.7 million SNPs in the merged pre- and post-treatment groups, whereas the CMH test included 1.3 million SNPs throughout the replicate sample comparisons. Comparing the SNPs shared between the two tests (n = ~1.3 million; Supplementary Fig. 1c) revealed a high level of concordance between FET and CMH (Pearson's correlation; r = 0.97), but none of the changes in frequency were significant for both tests (*p*-value < 0.05; Bonferroni correction). A single SNP (in the non-coding part of the genome) in chromosome 4 (44,255,647 bp) showed significant changes in frequency above the genome-wide correction in the FET, but not in the CMH test.

Bcftools mediated SNP calling yielded roughly 1.3 million SNPs (roughly 1 variant every 213 bp), most of which were located in the non-coding regions of the genome (94%). In addition, around 73% of all SNPs occurring in the coding regions of the genome were synonymous. 436 out of the 770 (56%) and 60 out of the 152 (39%) outlier 10 kbp windows from the previous genetic differentiation analysis (in Fig. 2a) were found to harbor at least one non-synonymous mutation in the coding region of the genome, the majority of which (170 above mean + 3SD and 24 above mean + 5 SD; 39%) were clustered around the terminal region (40–50 Mbp) in chromosome 4 (Supplementary Fig. 2a). Outlier frequency changes for SNPs (with values above 3 or 5 SD of the genome-wide P-value mean) retrieved from the previous FET and CMH tests and resulting in missense mutations in the coding regions of the genome showed a cluster of these outlier values in chromosome 4 (29 values in CMH, i.e. 30% or 49 in FET, i.e. 28% > genome-wide mean + 5SD in the 40–51 Mbp region), again displaying an accumulation of non-synonymous SNPs in the terminal region of this chromosome (Supplementary Fig. 2). In contrast, frequency changes for non-synonymous SNPs present in the coding regions were few (5 in CMH test - 5%/9 in FET in region - 5% > genome-wide mean + 5SD) in the nucleotide diversity rich chromosome 5 terminal region (between 35 and 45 Mbp).

4. Discussion

In order to understand IVM resistance in *H. contortus*, relevant regions in the genome (QTL) as well as the underlying genetic changes within them which are responsible for the development of the resistant phenotype need to be defined. Here, we assessed the impact of IVM treatment on *H. contortus* from a Swedish farm population by sampling

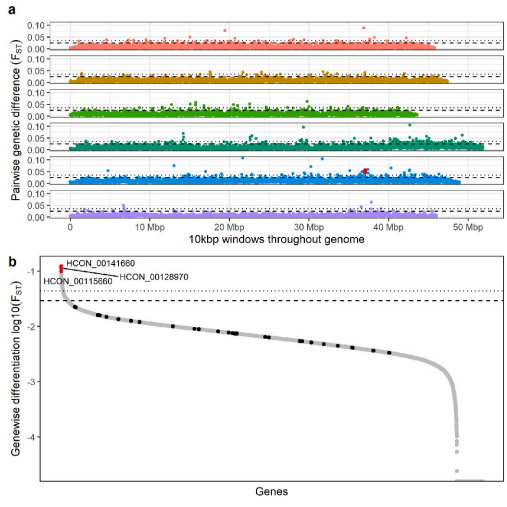

Fig. 2. Genetic differentiation between pre- and post-treatment groups. Pairwise genetic differentiation between the two treatment groups was calculated as F_{ST} values per (a) 10 kbp windows throughout the genome or (b) for entire genes. In both (a) and (b), the level of significance is indicated by dashed black line (mean F_{ST} + 3 SDs) and dotted black line (mean F_{ST} + 5 SDs). (a) Three consecutive 10kbp windows above the mean F_{ST} + 5 SDs present in chromosome 5 are displayed in red. (b) F_{ST} values (left to right; in a decreasing manner) for genes *lgc-37, haf-6, osm-3, osm-5, lgc-55, pgp-9.1, pgp-9, avr-15, glc-1, avr-14, che-11, dyf-11, pgp-1, che-2, osm-1, lgc-36, mrp-1, che-3, pgp-12, che-12, glc-3, glc-2, che-13, glc-5, ggr-3, osm-6, pgp-3, unc-9, unc-38, dyf-7* are shown as black dots (standalone figure with gene names is shown as Supplementary Fig. 3), whereas the points in red represents the three top most F_{ST} value having genes (located in chromosomes 5 - HCON_00141660 and 4 - HCON_00128970 and HCON_00115660). Colours in (a) represent different chromosomes as indicated in the legend of Fig. 1. (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

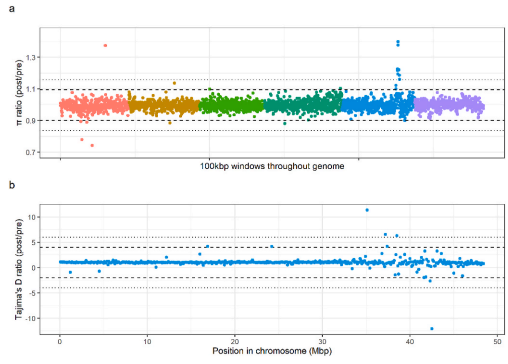

Fig. 3. Ratios of nucleotide diversity and Tajima's D highlight outlier variation in response to treatment in chromosome 5. Genome-wide nucleotide diversity (a) and chromosome 5 Tajima's D (b) ratios were analyzed per every genomic 100 kbp window to identify subtle signs of selection in the post-treatment group. In both (a) and (b), the level of significance is indicated by dashed black line (mean ± 3 SDs) and dotted black line (mean ± 5 SDs). Colours represent different chromosomes as indicated in the legend of Fig. 1. (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

infective stage larvae (L3), recovered from the same animals, before and after the most recent IVM treatment and characterizing changes in genetic diversity genome-wide by pooled whole-genome sequencing. A key finding of this analysis was that a region around 36.2–38.7 Mbp on chromosome 5, consistent with a QTL previously implicated in IVM resistance (Doyle et al., 2019), was associated with IVM resistance in a new, geographically distinct population. Here we discuss our rationale, experiment and subsequent analyses in order to build upon the methodology used and make progress towards increasing our understanding of the mechanisms underpinning the development of IVM resistance.

A sheep farm on which reduced IVM efficacy had been previously suspected and the dominant species was determined to be *H. contortus* (≥90% of all recovered eggs) was selected for this study. We confirmed the resistance of the population by the FECRT (89% reduction estimate) and, therefore, hypothesized that a substantial change in the worm population after IVM treatment would correspond to large changes in the allele frequencies between the pre- and post-treatment populations, particularly in regions of the genome associated with resistance. Surprisingly, little genome-wide differentiation between the two groups was observed. Both pre- and post-treatment L3 pools revealed very similar genome-wide profiles for nucleotide diversity and Tajima's D estimates (Fig. 1a and b). On average, the estimates of Tajima's D (measured in 100 kbp windows throughout the genome) were below 0 in both treatment groups, suggesting that the population has undergone selective sweep(s) in the past. This is arguably not surprising, given that the field population had undergone multiple previous anthelmintic treatments in the recent past. Nevertheless, little is known about the unique evolutionary history of this population, making it difficult to understand the patterns of nucleotide diversity as well as Tajima's D existing prior to the current treatment with IVM. Consistent with loss of diversity due to recent sweeps, we identified relatively low levels of nucleotide diversity, which was approximately 10-fold lower in both treatment groups in comparison to other field-derived but laboratory-maintained strains, MHco4[WRS] and MHco10[CAVR] (Doyle et al., 2019). Although a relatively comparable raw read coverage was sequenced (120–137 × unmapped vs. 199.65 × raw read coverage in Doyle et al., 2019), a lower proportion of reads were mapped to the genome overall, which may be a consequence of either: (i) an inefficiency of mapping divergent sequencing data to the reference, especially around variable sites, (ii) a higher number of duplicated sequencing reads, reducing the effective mapped coverage; and/or (iii) a higher degree of homozygosity and/or reduced genetic variation in the studied population. The comparable levels of nucleotide diversity throughout the genome meant that measures of pairwise genetic differentiation by F_{ST} revealed little deviation between the two groups; although minor sporadic differentiation throughout the genome was observed (i.e., we did identify 152 10 kbp windows greater than 5 SDs from the genome-wide mean), the population pre- and post-treatment was broadly genetically similar. The maintenance of genetic diversity in the post-treatment group suggests that resistant alleles are likely to be present on multiple, different genetic backgrounds in the studied field population, which would reflect that the population itself has gradually evolved to be resistant over time with sufficient admixture between resistant and susceptible worms, rather than from a recently acquired *de novo* mutation which has spread quickly in the population. In addition, since the post-treatment samples were collected 7 days after ivermectin exposure, the residual effects of the drug could have affected egg deposition rather than worm survival. This in turn could help explain the unexpected genetic similarities between the two treatment groups. Nevertheless, studies seem to indicate only a short-term (up to 3–5 days) inhibition of nematode egg production upon ivermectin treatment (Scott et al., 1991; McKenna, 1997; Sutherland et al., 1999), effectively

suggesting that temporary egg deposition suppression after treatment is unlikely to be of much significance here. Despite the genetic similarities between the groups, we attempted to find peaks of differentiation (F_{ST}) across the six chromosomes, which we defined as at least three consecutive 10 kbp windows above mean F_{ST} + 3 SD/5 SD. Whilst we observed multiple (n = 41) minor peaks (>mean F_{ST} + 3 SD; 26 present in chromosome 4), only a single major peak (>mean F_{ST} + 5 SD) was found in chromosome 5 beginning at around 37.4 Mbp (Fig. 2a; red dots in the chromosome 5 part of the panel), suggesting that the highest degree of consistent, genome-wide genetic differentiation between the groups is present within the previously suggested region containing the major QTL of IVM resistance (Doyle et al., 2019).

Many genes have been proposed to be associated with ivermectin resistance. However, high levels of genetic diversity as a consequence of large effective population sizes and the high fecundity of *H. contortus*, particularly in field populations, complicates the genetic association between resistance phenotype and discovery of causal mutations. This is particularly evident in recent genome-wide analyses of anthelmintic resistance (Doyle et al., 2019; Niciura et al., 2019; Sallé et al., 2019; Khan et al., 2020), and is consistent with the data presented here. An analysis of genetic differentiation among the top proposed candidates between pre- and post-treatment did not show any of these genes to be among the statistical outliers (>3 SD from the genome-wide mean). Our data suggests 636 genes with higher genetic differentiation than the most differentiated among the candidates, *lgc-37*, leading us to question the role (if any) of *lgc-37* and resistance here. The three highest F_{ST} estimates were obtained for genes *HCON_00141660* (orthologue to *vap-1* in *C. elegans*), *HCON_00128970* and *HCON_00115660*, the latter two of which had no orthologues in other nematode species (according to WormBase Parasite) in addition to being located in chromosome 4 (48943064–48943459 bp; F_{ST} = 0.113 and 26890603–26892649 bp; F_{ST} = 0.098); while there was not convincing evidence of a broader selection footprint around these regions in either the genome-wide differentiation (F_{ST}) or nucleotide diversity analyses, a single SNP (pos: 44, 255,647 bp) in the non-coding region of the genome and close to the location of *HCON_00128970* in chromosome 4 was statistically significant in the FET (but not CMH) analysis. In addition, having investigated the outlier 10 kbp windows (wherein F_{ST} value > genome-wide mean F_{ST} + 3 or 5SD) and outlier SNP frequency changes (wherein P-value > genome-wide mean P-value + 3 or 5SD in FET and CMH test) for the presence of overlapping non-synonymous SNPs in the coding region of the genome, we found clusters of non-synonymous variants at the terminal region of chromosome 4 (Supplementary Fig. 2; around 40–51 Mbp). These data suggest that the terminal end of chromosome 4 and perhaps *HCON_00128970*, even though its function or relation to resistance is not known, require further investigation.

Pairwise comparison of nucleotide diversity did, however, identify a single outlier region around the 36.2–38.7 Mbp region (mean π value ratio + 3 SDs) in chromosome 5 which contained a 1.35 × increase in diversity in the post-relative to pre-treatment group. While the direction of this change was surprising, i.e. the post-treatment pool was more diverse than the pre-treatment, we hypothesize that this pattern may be associated with the improved detection of low-frequency variants after removal of a large proportion of the population after treatment within a region which had previously undergone a selective sweep. Our hypothesis is based on the fact that this region coincides with the major IVM QTL previously reported in chromosome 5, identified via genetic backcrossing of resistant alleles into a susceptible genetic background (Doyle et al., 2019). Comparison of the Tajima's D values between the two groups showed no substantial pattern of change. Nevertheless, the most extreme variation (window values above/below mean ± 5 SDs) in the ratio of Tajima's D was observed in the downstream terminal region of chromosome 5 (around 35–45 Mbp), suggesting that this region is somewhat more genetically diverse or undergoing some degree of change (Fig. 3b). If our hypothesis is correct, it would further support our hypothesis that resistant alleles are on diverse genetic backgrounds, and that they have been present in this population of parasites for some time.

While significant progress is being made toward mapping drug resistant-associated variation using genetic crosses (Choi et al., 2017; Doyle et al., 2019, 2021; Niciura et al., 2019), the validation of these data in field populations is critically important towards the development of molecular markers of resistance (Kotze et al., 2020). However, the analysis of variation in the field, even from a genome-wide perspective, still presents significant challenges as we have demonstrated here. Although our approach - to sequence pools of larvae pre- and post-treatment from a single farm - was simple in theory, there was still substantial biological and technical variation within the experiment resulting in increased variation within the sequencing data. Unlike experiments involving genetic crosses where genetic variation for a particular trait segregates in a controlled environment, studies of field populations are more heavily influenced by the larger effective population sizes and variation in the underlying genetic structure of parasite populations. *H. contortus* is highly genetically variable (Sallé et al., 2019) and, therefore, it can be reasonably expected that some changes between populations, even between two different time points from the same population (in the case of this study), can arise due to reasons unrelated to resistance (Gilleard and Beech, 2007). Although we expected to account for this variation to some degree by studying a single population, recovered as paired samples from the same animals before and seven days after IVM treatment, our approach could be improved by using time-matched but drug-naive controls (i.e. sampling an untreated group at the same time as the treated group, before and after treatment), as well as studying larvae recovered from individual hosts (as opposed to larvae pooled across hosts, as was performed here) in order to account for genetic differences not involved in resistance (i.e. time and host effects). Although a clear reduction in the egg counts was observed upon performing the FECRT (89% average reduction), the obtained value is only indicative of the efficacy of the treatment and, therefore, it would be sensible to employ *in vitro* tests (such as egg hatch or larvae development tests) to determine if the surviving fraction is truly resistant to the effects of the drug. In addition, another viable approach would be to sample multiple farms with different response phenotypes and correlate the genetic and phenotypic responses. In this way, stochastic noise (i.e. genetic variability arising due to reasons other than resistance) could be addressed, thereby reducing the frequency of false positive signals and amplifying the real signal behind resistance. Finally, while we were able to identify a moderate level of differentiation specifically in a previously identified IVM QTL, the overall genetic similarity may also reflect selection occurring at multiple independent genetic loci and that the experimental approach together with a pool-sequencing based analysis is underpowered to detect very small shifts in genetic variation at multiple loci. While increasing evidence suggests this is not the case (Doyle et al., 2019, 2021), we expect that significant variation in field populations would likely provide the necessary variation upon which selection can act to overcome the negative effects of drug exposure and may do so through multiple mechanisms. We note that we have only examined genomic and not transcriptomic variation in our data, which may provide additional insight into the direct and/or indirect consequences of drug exposure and subsequent development of resistance (Laing et al., 2021). As technologies become cheaper and more accessible, analysis of populations of individual phenotypically well-defined parasites using whole-genome and/or transcriptome sequencing will help to resolve the question of the contribution of major and minor loci toward resistance.

Collectively, the data obtained here suggests that, despite a significant reduction in egg counts post-treatment, the genetic diversity of a single field population of *H. contortus* sampled before and after treatment is maintained throughout the genome. Importantly, we do find evidence of genetic differentiation in chromosome 5, which lends further support for the previously identified IVM QTL (Doyle et al., 2019) in a new genetically and geographically distinct population of

H. contortus. While we have not identified the causal variant, the validation of this QTL in a new field population continues to refine our understanding of IVM resistance, as well as identify a number of key areas of focus and improvement which future research should consider for mapping resistance alleles in the field.

Declaration of competing 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.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.ijpddr.2021.12.002.

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Gastrointestinal nematodes, such as *Haemonchus contortus*, are quick to develop resistance to therapeutics, which threatens animal health, welfare, and the sustainability of the modern livestock production industry. The work presented in this thesis primarily investigated the potential of using mutations, associated with drug resistance, as molecular markers to create novel screening assays and survey sheep farms for resistant parasites in addition to undertaking experiments to evaluate putative resistance mechanisms and the loci in the genome responsible for resistance development.

Paulius Baltrušis received his doctoral education at the Department of Biomedical Sciences and Veterinary Public Health. His master's degree in Infection biology at Uppsala University, Sweden was awarded to him in 2018.

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