Leva-LAMP: Sustainable use of levamisole through elucidation of genetic markers of resistance and molecular diagnostics in *Haemonchus contortus*

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BSc (Hons) MRes

Submitted in fulfilment of the requirements for the degree of Doctor of Philosophy

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June 2022

Abstract

Haemonchus contortus is a gastrointestinal parasitic nematode primarily infecting small ruminants. It is globally distributed, and a major cause of production losses and animal health concerns. Control largely relies on the use of broad spectrum anthelmintics, however, the effectiveness of many of these drugs is declining due to widespread anthelmintic resistance. One of the broad spectrum anthelmintics available to control *H. contortus* is levamisole (LEV), a cholinergic agonist which, when bound to the nematode acetylcholine receptor (AChR), causes paralysis in the worm. Resistance to LEV is, at the time of writing, less widespread worldwide than to other major drug classes, such as benzimidazoles (BZs) and macrocyclic lactones (MLs). However, unavailability of effective molecular diagnostics, due to the lack of fully resolved and validated molecular markers of LEV resistance in *H. contortus* and over reliance on the faecal egg count reduction test, which has inherent inaccuracies, and is poor at detecting emerging resistance, underline the urgent need to improve resistance monitoring.

Understanding of genetic markers of LEV resistance is, therefore, key to improving the diagnosis of anthelmintic resistance and maintaining the sustainability of LEV. Therefore, the goal of this PhD was to elucidate and validate genetic markers of LEV resistance, and develop proof-of-concept molecular diagnostic assays.

First, using whole genome sequencing data (WGS) from a controlled genetic cross (from a resistant and a susceptible parental line) pre- and post-LEV treatment, two quantitative trait loci (QTL) were identified. A single non-synonymous SNP variant, S168T, was identified in *acr-8*, a gene present within the major QTL, and previously shown to be essential for conferring LEV sensitivity to the *H. contortus* AChR. Extensive analysis of WGS data and single worm genotyping demonstrated that the presence of the S168T variant was predictive of a LEV resistant phenotype in all LEV resistant laboratory and field isolates examined, and absent in all susceptible isolates. Further SNP variants were also identified in LEV resistance associated genes in a second QTL on chromosome IV, which likely constitute minor markers of LEV resistance. Concurrently, putative markers of LEV resistance previously detailed in the literature were examined, but found not to constitute effective markers of LEV resistance in the isolates examined. A pilot study to validate a next-generation

amplicon sequencing panel was also undertaken, with preliminary validation complete for S168T, and several additional minor markers of LEV resistance.

Following on from these results, several molecular diagnostic assays with significant translational potential were developed and validated for the detection of the S168T variant in *H. contortus*. A two-step nested allele specific (AS)-PCR was optimised and demonstrated for the detection of the S168T variant in both laboratory and field isolates. Two emerging novel loop-mediated isothermal amplification (LAMP) technologies were then evaluated for detection of the S168T variant. Loop-primer enzymatic cleavage (LEC)-LAMP showed promising results discriminating between the resistant and susceptible alleles, with the added capacity for multiplexing. LEC-LAMP was also preliminarily adapted to point-of-care (POC) detection via a lateral flow (LF) platform. The results generated in this PhD have laid the foundation necessary to establish reliable and accurate laboratory based molecular diagnostics for the detection of LEV resistance in *H. contortus,* with potential for fast and low-cost application of these assays at the POC.

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Acknowledgement

First of all, I'd like to thank Valentina Busin, Roz Laing, and Eileen Devaney not only for supervising me these last four years, and for keeping me on track in the early days, and for all your excellent and invaluable feedback, advice, and support throughout, but also for trusting me to mentor undergraduate students, and to explore and develop collaborations beyond my PhD, and for mentoring me on how to move my career forward as an academic. A special thanks as well to Roz Laing for providing me with the opportunity to carry on my current, and new, work as a post-doc. I would also like to thank Victoria Gillan, Kirsty Maitland, Margaret McFadyen, and Jennifer McIntyre for their invaluable advice and help, particularly with cloning, qPCR, and DNA and RNA extraction. Thanks to James McGoldrick for teaching me the essentials of parasitology and to Kerry O'Neill for all her help with PMs and picking worms. Thanks to all the other PhD students, particularly Sam Brown, Leonidas Spathis, Paul Campbell, David Moody, and everyone in office 217 and in the parasitology department for making this a great place to work, and Maha Mansour Shalaby for helping get the lateral flow assay working. Thanks also to my yearly assessors Lubna Nasir and Paul Everest for their advice and input throughout the project. Thanks to Stephen Doyle at the Wellcome Trust Sanger Institute, Cambridge for the generation of so much of the data that was essential to this project, and thanks to Ray Kaplan for sharing field isolates with us. Thanks as well to everyone over at the Moredun Research Institute, especially David Bartley, Ali Morrison, and Lynsey Melville for their help setting up infections, and providing both parasite material and technical advice, and everyone who worked on the BUG project. I'd also like to thank all my collaborators at other institutions who've had an invaluable input into both this PhD and other projects I have had the privilege to be involved in along the way: Cedric Neveu and Claude Charvet at INRAE, France, Owen Higgins at NUI Galway, Ireland, Sinan Erez at Afyon Kocatepe University, Turkey, Livio Costa-Junior at the Federal University of Maranhao, Brazil, and Rojesh Khangembam at University of Debrecen, Hungary. Finally, I'd also like to thank my examiners Willie Weir and Jane Hodgkinson for a thoroughly enjoyable viva and for taking the time to read my work and give excellent feedback. I'd also like to thank Robert Coultous for being my convener

and for organising everything so well. A massive thanks as well to the James Herriot Scholarship (University of Glasgow Vet Fund), and the Biotechnology and Biological Sciences Research Council (BBSRC) without who's funding none of this would have been possible. Finally, thanks to my wonderful partner Betzabe Torres-Olave for all of her love and support through the past four years, and also to all of my family and friends who have always been there for me along the way.

Author's Declaration

The work presented in this thesis was performed entirely by the author unless otherwise stated. The work described herein is unique and will not be submitted elsewhere for any other degree or qualification at any other university. Some of the work detailed in Chapter 3 and Chapter 4 has been published as a pre-print paper Antonopoulos et al. (2022) included in Appendix 8 and available on pre-print server biorxiv.org. Some of the work detailed in Chapter 5 was carried out in collaboration with Dr Higgins, Molecular Diagnostics Research Group, NUI Galway. This work was funded by the James Herriot Scholarship (University of Glasgow Vet Fund) and a Biotechnology and Biological Sciences Research Council (BBSRC) Strategic Lola [BB/M003949].

Alistair Antonopoulos, June 2022

Definitions/Abbreviations

AAD	Amino acetyl derivative
ACh	Acetylcholine
AChR	Acetylcholine receptor
ALMA	Automated larval migration assay
ARMS	Amplification refractory mutation system
ART	Artemisin
AS-LAMP	Allele specific loop-mediated isothermal amplification
AS-PCR	Allele specific polymerase chain reaction
ASSURRED	Affordable, Sensitive, Specific, User-friendly, Robust and rapid, Equipment-free, Deliverable
ATP	Adenosine triphosphate
AuNP	Gold nanoparticle
BART-LAMP	Bioluminescence assay in real time LAMP
BHQ1	Black hole quencher 1
BIP	Backward inner primer
BLAST	Basic local alignment search tool
bp	Base pair
BZ	Benzimidazole
cDNA	complementary DNA
CHIKV	Chikungunya virus
CPA	Cross priming amplification
CRISPR-Cas	Clustered regularly interspaced short palindromic repeat
cRNA	complementary RNA
DD/DF/FF	Homozygous Deletion/Heterozygous/Homozygous full length

DENV	Dengue virus
dNTP	Deoxynucleoside triphosphate
dsDNA	Double stranded DNA
EBI	European bioinformatics institute
EC_{50}	Effective concentration at which 50% of individuals do not develop
EHA	Egg hatch assay
EPG	Eggs per gram
ER	Endoplasmic reticulum
FAM	6-Carboxyfluorescein
FAMACHA	Faffa Malan chart
FEC	Faecal egg count
FECRT	Faecal egg count reduction test
FIP	Forward inner primer
FITC	Fluorescein isothiocynate
FPKM	Fragments Per Kilobase of transcript per Million mapped reads
F_{ST}	Fixation index: proportion of total genetic variance within a sub-population relative to the overall population. Not restricted to, but can be used for, pairwise testing
gDNA	Genomic DNA
GI	Gastrointestinal
GIN	Gastrointestinal Nematode
HDA	Helicase dependent amplification
HIV	Human immunodeficiency virus
HNB	Hydroxy naphthal blue
HPV	Human papilloma virus
[I]	Deoxyinosine base
Indel	Insertion/deletion

ITS2	Internal transcribed spacer
Kbp	Kilobase pairs
L_1 L_2 L_3 L_4	Larval stages 1-4
LAMP	Loop-mediated isothermal amplification
LDA	Larval development assay
LEC-LAMP	Loop primer endonuclease cleavage loop-mediated isothermal amplification
LEV	Levamisole
LEV-R	Levamisole resistant
LEV-S	Levamisole susceptible
LFA	Lateral flow assay
LF-LAMP	Lateral flow loop-mediated isothermal amplification
LMA	Larval motility assay
LMIA	Larval migration inhibition assay
LMIC	Lower and middle income country
Mb	Megabase
ML	Macrocyclic lactone
MUSCLE	MUltiple Sequence Comparison by Log-Expectation
NMJ	Neuromuscular junction
NTC	No template control
NTD	Neglected tropical disease
O/N	Overnight
P450	Cytochrome P450 haem co-factor enzyme family
PCR	Polymerase chain reaction
PES	Polyether sulfone
Pgps	Permeability glycoprotein
POC	Point of care

PON	Point of need
qLAMP	Quantitative loop-mediated isothermal amplification
qPCR	Quantitative polymerase chain reaction
QTL	Quantitative trait locus
R/T	Room temperature
RCA	Rolling circle amplification
rDNA	Ribosomal DNA
RDT	Rapid diagnostic test
RFLP	Restriction fragment length polymorphism
RNAi	RNA interference
RNA-seq	RNA sequencing
RPA	Recombinase polymerase amplification
RR/RS/SS	Homozygous S168T/Heterozygous/Homozygous S168
RT	Reverse transcriptase
RT-qPCR	Reverse transcriptase quantitave polymerase chain reaction
rt-qPCR	Real time quantitative polymerase chain reaction
SARS-CoV-2	Severe acute respiratory syndrome coronavirus 2
SCPT	Smart connected pathogen tracer
SHERLOCK	Specific High Sensitivity Enzymatic Reporter UnLOCKing
SNP	Single nucleotide polymorphism
STH	Soil transmitted helminth
T_A	Annealing temperature
TCA Cycle	Tricarboxylic acid cycle
T_m	Melting temperature
TRACY	Basecalling, alignment, assembly and deconvolution of Sanger chromatogram trace files

TST	Targeted selective treatment
TT	Targeted treatment
UDG	Uracil DNA glycosylase
UV	Ultraviolet light
WAAVP	World Association for the Advancement of Veterinary Parasitology
WGS	Whole genome sequencing
WHO	World Health Organisation
ZIKV	Zika virus

1 INTRODUCTION

1.1 Haemonchus contortus

1.1.1 Overview

Gastrointestinal nematodes (GIN) have a major impact on the productivity of sheep farming (Perry and Randolph, 1999; McLeod, 2004; Miller et al., 2012; Kotze et al., 2016; Besier et al., 2016a; Besier et al., 2016b). There is a potential for high mortality rates, although animal losses vary between regions and seasons depending on the species of GIN, favourability of environmental conditions and effective control measures (Besier et al., 2016a). One of the most pathogenic and economically important GIN is *Haemonchus contortus*, a haematophagous parasite of small ruminants, mainly sheep and goats (Besier et al., 2016b). Estimates from Australia indicate that infection with *H. contortus* is one of the largest causes of production loss in the sheep farming sector (Lane et al., 2015). Haemonchosis is responsible for significant production loss globally due to reduced growth rates, meat yield and milk production, and mortality (Qamar and Maqbool, 2012), and is particularly pronounced in warm summer rainfall regions (Qamar et al., 2009; Salle et al., 2019).

Although traditionally a parasite of the tropics and subtropics (Besier et al., 2016b; Emery et al., 2016), *H. contortus* is highly adaptable, and has become established in a wide range of climatic zones (Besier et al., 2016b). Prevalence is especially high in the tropics (O'Connor et al., 2006), but its ability to take advantage of short periods of favourable climate for the free-living stages (Figure 1), which require warm and moist environmental conditions, has led to its establishment in almost all regions where small ruminants are farmed (Besier et al., 2016a). In the tropics, where temperature is permissive for larval development year-round, the seasonality of rainfall determines when infections are likely to occur; however, in wet tropics and equatorial zones, conditions are permissive all year (Barger et al., 1994; Dorny et al., 1995). In subtropical areas

the length of the dry season, and seasonal extremes of temperature and altitude have led to extensive variation in the threat of haemonchosis (Van Veen, 1978; Githigia et al., 2001). North and south of the tropics in sub-tropical and temperate zones, where survival of infective third stage larvae (L_3) for long periods is difficult due to markedly seasonal climates, fourth stage larvae (L_4) can undergo hypobiosis in the host until the return of more favourable conditions (Gibbs, 1986; Gatongi et al., 1998). Warmer temperate regions such as Southern Africa, South-Eastern Australia, mid-South America, Southern USA, and the Mediterranean experience the greatest threat of haemonchosis (Swan, 1970), whereas in cool temperate regions, like northern Europe, southern New Zealand, and southern South America, the risk diminishes when moving further north or south. Survival of *H. contortus* overwinter is associated with hypobiosis, where the larvae enter a state of facultative arrested development which allows the persistence of the L4 within the host (Gibbs, 1986), although the threat is increasing with global warming (Van Dijk et al., 2010).

Treatment of GIN infections, including *H. contortus*, relies primarily on broad spectrum anthelmintics, of which there are five main classes (Kotze et al., 2016). *H. contortus* has shown the capacity to rapidly develop resistance under drug pressure (Laing et al., 2013), and resistance to all major classes of anthelmintic has been reported (Sangster and Gill, 1999; Wolstenholme et al., 2004; Gilleard, 2013; Zhang et al., 2016b), and in many areas multi drug resistance is reported (Kaplan, 2004; Geurden et al., 2014; Kotze et al., 2016; Crook et al., 2016; Rose-Vineer et al., 2020). While many drugs remain clinically effective, the sustainability of use of many classes of anthelmintic is seriously threatened in the near future in the face of the global spread of resistance.

1.1.2 Life Cycle of *H. contortus*

H. contortus displays a remarkably high fecundity, with a single female capable of producing >15,000 eggs per day (Emery et al., 2016). The parasite has an establishment rate (i.e. the rate at which ingestion of parasites by the animal will lead to the establishment of a parasite community within the host) of ~60% (Dineen et al., 1965), and one of the shortest patency periods (i.e. the time

taken from establishment of infection to the shedding of eggs in the faeces) amongst parasitic nematodes at ~15 days, with a full life cycle from egg hatching to reproduction of ~20 days (Emery et al., 2016).

Throughout the course of its life cycle (Figure 1.1), *H. contortus* encounters a wide variety of ecosystems, which vary greatly in temperature, osmotic pressure, and redox potential. CO_2, O_2, and temperature also play a role in the hatching of eggs (Harder, 2016), with temperatures above 5^{o}C necessary for hatching and development to larvae (Troell et al., 2005). Eggs hatch in the faecal pat and develop from L_1 to L_2 (Figure 1) where both stages remain feeding on faecal bacteria until the L_2 moults to L_3. The motile and infective L_3 then migrates onto the pasture to await ingestion by a grazing ruminant. The L_3 retains the protective L_2 cuticle, which is shed (exsheathed) in the rumen after ingestion. From the rumen, the now exsheathed L_3 migrates to the abomasum and enters the lumen of the gastric glands, emerging after 2-4 days as L_4 or immature adults (Sinnathamby et al., 2018). High pH (up to 9) in the rumen constitutes a barrier that must be passed through by migrating larvae (Harder, 2016). Once developed to L_4, blood feeding begins in the abomasum and L_4 develop to mature adults, reproducing sexually, or can undergo hypobiosis to overwinter (Sinnathamby et al., 2018). The lower and more variable pH in the abomasum, where adults reside, constitutes another environmental challenge for the life cycle of the parasite (Harder, 2016).

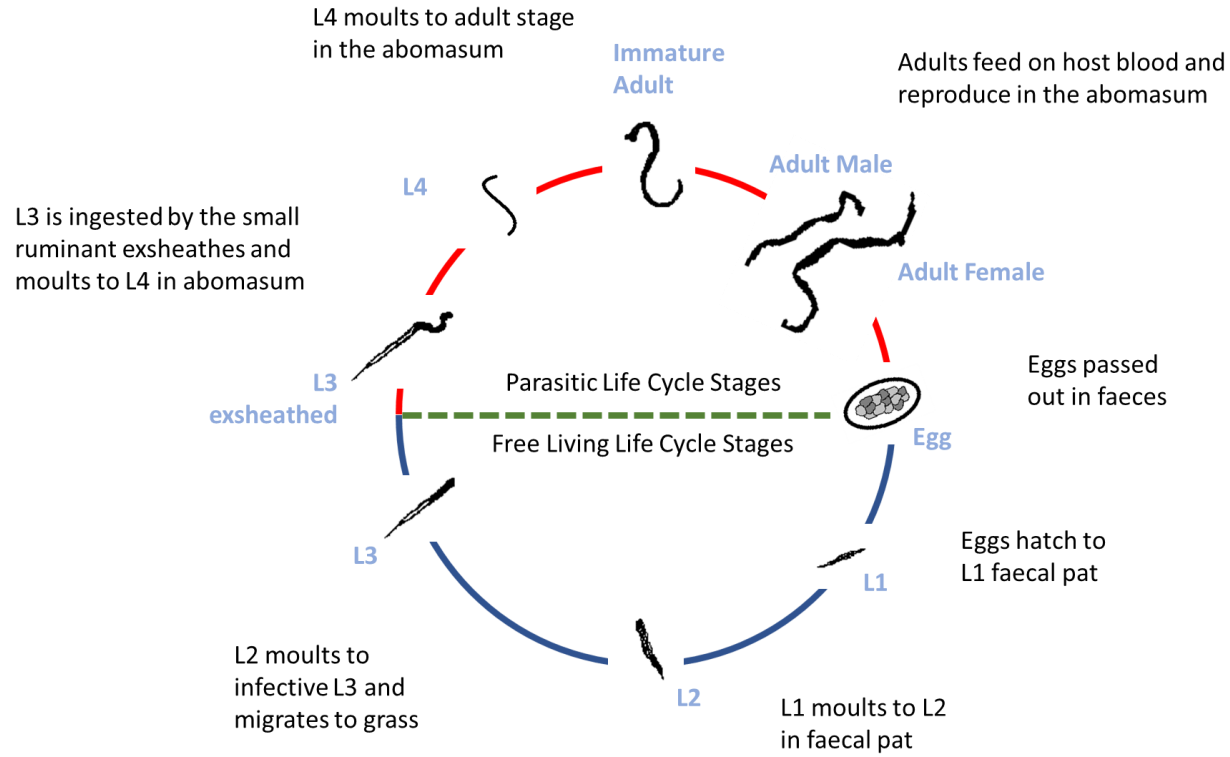

Figure 1.1: *Haemonchus contortus* life cycle. Eggs are initially passed in ruminant faeces, where if conditions are permissive, L_1 hatch, undergo growth and moult to L_2 then L_3. Once developed, L_3 migrate from the faecal pat to the surrounding pasture to be ingested by a grazing ruminant. Once ingested, L_3 exsheath and moult to L_4 in the abomasum. L_4 can undergo hypobiosis if environmental conditions are unfavourable. If environmental conditions are permissive, then L_4 will feed on blood and develop into adult male or female. The adults sexually reproduce, and eggs are shed in the faeces repeating the cycle.

1.1.3 Pathogenesis of haemonchosis

Pathogenesis of *H. contortus* infection is linked to its haematophagy and the consequent anaemia that develops in the host (Dunn, 1978; Urquhart et al., 1996). Following infection with L_3 and moulting to L_4 (Figure 1.1) blood loss begins once L_4 begin feeding (Monnig, 1950). Each individual adult worm can consume up to a maximum of ~30-50 µl of blood per day (Clark et al., 1962), leading to a daily blood loss of up to 30 ml in infections with a heavy worm burden (Albers and Le Jambre, 1983). There is a strong correlation between severity of disease and number of adults worms (Le Jambre, 1993). The degree of anaemia is responsible for the presentation of clinical signs, and generally defines the boundary between chronic and acute haemonchosis (Besier et al., 2016a), although the delineation between chronic and acute haemonchosis is not

clear cut, and is better thought of as running through a spectrum. Lambs, not yet having acquired immunity, and pregnant and lactating ewes (due to relaxation of immunity around the time of lambing), are most at risk (O'Sullivan and Donald, 1973). Immunologically naïve adult sheep imported from non-endemic regions can also become infected when introduced to endemic regions. Variation in resistance to *H. contortus* exists between breeds, with selective breeding (Toscano et al., 2019) being practiced as part of integrated worm control (Preston and Allonby, 1979; Woolaston and Baker, 1996; Arif et al., 2018; Toscano et al., 2019). The outcome of haemonchosis depends on the rate of intake of infective L_3 and the capacity of the host to expel worms and/or replace blood loss (Besier et al., 2016a). Haemonchosis has been classified into three general syndromes that exist in a continuum with some overlap between (Besier et al., 2016a). Hyperacute haemonchosis occurs with loss of a large volume of blood resulting from worm burdens of ~30,000+, leading to haemorrhagic gastritis and severe anaemia (Dunn, 1978), with sudden deaths prior to clinical signs of disease being readily apparent (Besier et al., 2016a) and before eggs can be detected in the faeces. Acute haemonchosis is characterised by the development of significant anaemia over 4-6 weeks from the start of infection, with worm burdens of 2,000-20,000 (Urquhart et al., 1996). Chronic haemonchosis occurs with smaller persistent worm burdens, presented sub-clinically, or clinically apparent only when worm intake increases, or when nutritional conditions of the host deteriorate (Urquhart et al., 1996). Nutrition has an important effect on the degree of pathology experienced in an infection, with animals on low protein feed showing much lower tolerance of an equivalent worm burden in sheep with sufficient nutrition (Wallace et al., 1996; Nnadi et al., 2009).

1.1.4 The *H. contortus* genome

H. contortus has one of the larger genomes of any nematode sequenced to date, at 283.4 Mb (Doyle et al., 2020), although high heterozygosity can potentially inflate size estimates for parasitic nematode genome size, which causes alternative haplotypes to feature within assemblies (Coghlan et al., 2019).

Compared to the model nematode *Caenorhabditis elegans*, the *H. contortus* genome is predicted to contain a similar number of protein coding genes, however, gene density is significantly lower, at around 59 per Mb, with 7% of the genome being protein coding, vs 30% for *C. elegans*. On average, *H. contortus* genes are longer, with more introns per gene, and longer intronic sequences than *C. elegans* (Laing et al., 2013). *Pristionchus pacificus,* another Clade V nematode related to both *C. elegans* and *H. contortus,* also shows an expansion of intronic sequences relative to *C. elegans*, although to a lesser degree than that seen in *H. contortus*. Repetitive sequences make up more than a third of the genome and are enriched towards the centre and ends of chromosomes, with particularly striking enrichment seen in sub-telomeric regions of the X chromosome (Doyle et al., 2020). This could be due to a higher level of recombination amongst parasitic compared to free-living nematodes, as a higher degree of polymorphism is seen in these species, however, this is not fully understood at the time of writing. A high degree of synteny (genes present on the same chromosome in each species) exists between *C. elegans* and *H. contortus*, although gene order is not well conserved (Laing et al., 2013; Doyle et al., 2020). Microsynteny disintegrates beyond the gene pair level, ~50% of shared orthologue pairs are adjacent, whereas only a single group of 10 orthologues are colinear in *C. elegans* and *H. contortus* (Doyle et al., 2020). Intrachromosomal rearrangements are a common occurrence in nematodes, and serve to disrupt local synteny across species, whereas interchromosomal rearrangements are much rarer, and most genes remain associated with a chromosome for long evolutionary periods (Lee and Sommer, 2003; Foster et al., 2020). The structure of operons (groups of genes clustered together driven from the same promoter) is partially conserved in relation to *C. elegans*, although intergenic distance is much longer (Laing et al., 2013). *H. contortus* retains a wide range of one-to-one orthologues with *C. elegans*, although there are a significant number of gene family expansions with potential roles in parasitism. Genes for carbohydrate metabolism, proteolysis, secreted proteins, cuticle proteins, and neurotransmission are enriched in the *H. contortus* genome relative to the *C. elegans* genome (Laing et al., 2013). In addition, several ligand gated ion channel subunits show marked expansion in *H. contortus* (Duguet et al., 2016), some implicated as anthelmintic drug targets or involved in

conferring resistance (Neveu et al., 2010; Boulin et al., 2011; Doyle et al., 2021).

H. contortus is diploid, with five autosomal chromosomes (Bremner 1955; Redman et al., 2008), and an XX/XO sex determination system, with two X chromosomes present in the female, whereas a single X chromosome is present in the male (Redman et al., 2008). There is no evidence of asexual reproduction in *H. contortus*. In *C. elegans*, sex–dependent genes are concentrated in certain regions of the genome (Albritton et al., 2014). Consistent with this, in *H. contortus*, a greater proportion (~24%) of female biased genes are found on the X chromosome versus only ~5% of male biased genes (Doyle et al., 2020). It is likely this aspect of apportioning of male and female dependant genes is shared between the two species. *H. contortus* is obligate dioecious (male and female sexual organs present in discrete sexes) and reproduces sexually (Redman et al., 2008). Chromosomes in *C. elegans* are holocentric (Albertson and Thomson, 1993), where spindle microtubules do not attach to a defined kinetochore but attach along the whole length of the chromosome (Zedek and Bures, 2012). Areas of little or no recombination in the termini of chromosomes have been proposed to be indicative of holocentric chromosomes, as this may serve as the site of segregation during meiosis (Cutter et al., 2009). Areas of low or no recombination have been identified in the chromosomal termini of both *C. elegans* and *H. contortus*, suggesting that *H. contortus* chromosomes are also holocentric, although no data to directly support this are available at present (Doyle et al., 2018).

The presence of repetitive elements has also been negatively correlated with recombination rates in *H. contortus* and *C. elegans* (Rockman et al., 2009; Doyle et al., 2018). Recombination rates of each chromosome are also distinct, with chromosomes III and V showing different recombination rates compared to chromosomes I, II, and IV, and to all chromosomes of *C. elegans* (Doyle et al., 2018). Recombination is driven by homologous crossover during meiosis, and *H. contortus, P. pacificus,* and *C. elegans* tend to undergo only one crossover per homologous chromosome pair during meiosis (Brenner, 1974; Barnes et al., 1995; Zalevsky et al., 1999).

1.1.5 Transcriptome and metabolism of *H. contortus*

During the course of its life cycle, *H. contortus* expresses genes that facilitate its adaption to the diverse environments encountered, from the initial free-living larval stages in the faecal pat and pasture, to its transition to parasitism and maturation in the ruminant gut. Sex-specific genes and genes related to reproduction are also highly differentially expressed in the adult life cycle stages. This transcriptomic plasticity has also been implicated in drug resistance, leading to alterations in gene expression following selective pressure with levamisole (LEV) (Sarai et al., 2013; Sarai et al., 2014; Sarai et al., 2015; Raza et al., 2016), and ivermectin (IVM) (Raza et al., 2016; Matouskova et al., 2016; Kellerova et al., 2019).

RNA sequencing of different life cycle stages of *H. contortus* identified two distinct over-arching clusters of differentially expressed genes between free-living and parasitic stages: those with higher expression in the free-living stages and lower expression in parasitic stages and the vice versa (Doyle et al., 2020). In addition, there are distinct clusters of genes expressed in each specific life cycle stage, and a host of sex linked differentially expressed gene clusters in adults (Laing et al., 2013; Doyle et al., 2020). Throughout the life cycle, genes related to metabolic function are some of the most differentially expressed, presumably due to the diverse environments and nutritional requirements experienced by the parasite. During development from egg to L_1 and L_2, genes involved in muscle development, larval morphogenesis, motor function, and a variety of enzymatic functions are up regulated. During the transition to L_3, the gene expression profile dramatically changes, with waste processing genes such as oxidoreductases and cytochrome P450s upregulated, along with gluconeogenesis and cobalamin binding genes (Laing et al., 2013). Whilst eggs initially rely on endogenous energy stores that have already been obtained prior to shedding from the ruminant (Harder, 2016), energy metabolism in the free-living larval stages (L_1-L_3) is generally dependent on O_2, and larvae are able to use the tricarboxylic acid (TCA) cycle to degrade carbohydrates ingested from the host faeces (Harder, 2016). The L_3 are capable of synthesising glycogen from lipid stores as they develop (Kapur and Sood, 1987; Kapur and Sood, 1991), and

rely on energy reserves to complete its transition from free-living to parasitic stages of the life cycle, as L_3 are a non-feeding stage (Sinnathamby et al., 2018). Metabolism in the *H. contortus* L_3 is consistent with expression profiles seen in *C. elegans* following reduced food intake (Laing et al., 2013) and mirrors *C. elegans dauer* larvae, where reduced resources lead to larvae entering a quiescent state (Fuchs et al., 2010). Transition from quiescent L_3 to motile and blood feeding L_4 in the ruminant gut (Sinnathamby et al., 2018) leads to an increase in expression of genes involved in motor activity, growth and morphogenesis, and haem, cobalamin, lipid, sugar, and carbohydrate binding reflecting motility and active feeding. The L_4 to adult stage transition leads to expression of a wide range of sex specific genes. In females, these are related to egg and oocyte development, meiosis regulation, and vulval development, whereas in the male spermatogenesis genes are highly up-regulated, as expected (Laing et al., 2013). Adults also utilise carbohydrates as their preferred energy source, obtained from the blood of the host (Harder and Wunderlich, 1991), but can also make use of glycogen stores in times of shortage (Harder, 2016). Adult *H. contortus* cannot however, completely degrade carbohydrates via the TCA cycle (Harder and Wunderlich, 1991), but rather makes use of the glycolytic pathway due to the high CO_2 tension in the mammalian gut (Harder, 2016). A wide variety of nematode intestinal specific genes are also upregulated in the adult stages, related to digestion and detoxification (Laing et al., 2013).

1.1.6 Genetic diversity and lineages of *H. contortus*

High levels of genetic variation exist both amongst and between isolates (Doyle et al., 2018; Salle et al., 2019). A high degree of polymorphism is present, with estimates from whole genome sequencing of 19 global isolates indicating one SNP per 9.94 bp of the genome relative to the reference MHco3(ISE) isolate (Salle et al., 2019). In addition, many indels are expected to be present across the genome (Rufener et al., 2009a; Rufener et al., 2009b; Gilleard and Redman, 2016; Doyle et al., 2020). High levels of genetic diversity in *H. contortus* could be attributed to both high mutation rates, and large population sizes (Gilleard and Redman, 2016). *H. contortus* has been shown to engage in polyandrous

mating behaviour (Redman et al., 2008; Doyle et al., 2018), with single females mating with multiple males. This is believed to come at some cost, including increased energy expenditure in location of a mate, along with disruption of mucosal attachment and feeding (Redman et al., 2008). As mentioned above, the adult female produces thousands of eggs per day, and this entails a high level of zygote production necessitating a constant supply of sperm (Redman et al., 2008). In addition, the genetic diversity of offspring produced by a single female mating with multiple males leads to a higher level of genetic variation than would be seen with single pair mating (Redman et al., 2008). Finally, polyandry may also serve to minimise the risk of genetic incompatibility when mating (Redman et al., 2008), as can be the case in free-living organisms (Garcia-Gonzalez and Simmons, 2007).

H. contortus is thought to have originated as a parasite of ungulates initially in South Eastern Africa, and has subsequently spread globally (Salle et al., 2019). Genetically distinct global sub-populations of *H. contortus* have been identified in Southern/Eastern Africa, West Africa, the Mediterranean, Oceania, Asia, and South America. Each lineage has distinct genetic markers identified from whole genome sequencing of isolates, as well as many markers shared between certain lineages based on spread from their original habitat following human migration, and subsequent admixture in the new environment. Spread started from the original population in East Africa to West Africa, the Mediterranean, and South West Asia. Initial spread to Asia appears to have occurred from the original South Eastern Africa lineage (Salle et al., 2019). Worms sampled in Pakistan were found to be genetically positioned between South African and Indonesian isolates (Doyle et al., 2020) indicating a gradual spread outward from the West. Through history, waves of human colonisation and movements of populations, cultural diffusion and the spread of sheep domestication, and the transatlantic slave trade led to the world-wide spread of *H. contortus* from its original habitat. Isolates sampled from Brazil, and West and Central Africa share many genetic markers, indicating a close relationship and likely transmission of West African isolates to the Americas during the transatlantic slave trade (Salle et al., 2019). The admixture seen in the Caribbean is reflected by initial Spanish colonisation bringing *Churra* sheep, followed by subsequent importation of West African

breeds more suited to the climate (Spangler et al., 2017), leading to a mix of West African and Mediterranean *H. contortus* populations, giving rise to the lineages now seen in the colonised areas. Subsequent British colonisation, which brought sheep from Europe into Oceania, is also reflected in the Mediterranean lineage origins of the isolates sampled from Australia (Salle et al., 2019). Finally, worms sampled from the UK and USA were found to be from an ancestral population distributed globally by human movement (Doyle et al., 2020).

1.1.7 Laboratory isolates and genetic crosses of *H. contortus*

In addition to distinct lineages of field populations, a number of laboratory isolates exist. While they all have been isolated from field populations, some have been subsequently maintained and passaged through sheep (Williamson et al., 2011; Doyle et al., 2018; Sargison et al., 2019), while others have been selectively inbred in order to reduce the high intra-isolate sequence variation (Redman et al., 2008; Sargison et al., 2018; Doyle et al., 2020). These laboratory isolates are maintained by different institutions with distinct nomenclature indicating the laboratory group responsible for their maintenance. Isolates maintained at the Moredun Institute, Penicuik, Scotland are denoted by the prefix M (Moredun) followed by Hco (*H. contortus*), an identifying number code, and finally the three-letter code for the isolate in brackets e.g. (ISE) for “Inbred Strain Edinburgh” or (WRS) for “White River Strain” (see Chapter 2.2). The use of one particular inbred isolate, thought to be derived from an anthelmintic susceptible Kenyan field population (Otsen et al., 2001), MHco3(ISE), was instrumental in characterising benzimidazole (BZ) resistance (Kwa et al., 1994), and forms the basis for the current *H. contortus* reference genome (Laing et al., 2013; Doyle et al., 2020). This isolate was inbred via 15 rounds of experimental infection, with broods from a single adult female used to propagate each round (Roos et al., 2004) to generate the ISE line, which was then further inbred by single pair mating to generate the MHco3(ISE.N1) line (Sargison et al., 2018). The White River Strain (WRS)/MHco4(WRS) is resistant to BZ and IVM and was isolated in South Africa (Van Wyk et al., 1987; Van Wyk et al., 1988). The Kokstad Isolate (KOK) is resistant to LEV and also originated in South Africa

(Neveu et al., 2010; Barrere et al., 2014). The Chiswick Avermectin Resistant Strain (CAVR)/MHco10(CAVR), as the name suggests, is Avermectin resistant, arising from an IVM resistant contaminant of laboratory passaged *Trichostrongylus colubriformis* in Australia (Le Jambre, 1995). The UGA/2004 or MHco18(UGA2004) isolate (MHco indicates the line was maintained at the Moredun Institute) is LEV, BZ, and IVM resistant, and was first identified from a resistant field population in the South Eastern USA (Williamson et al., 2011). In addition to these isolates, which are specifically mentioned as they are all used in this study, there are a total of 21 isolates currently maintained in laboratories around the world for research purposes (Sargison et al., 2019; Monash Database). The majority of these have been derived from anthelmintic resistance studies, and are maintained for their resistance and/or susceptibility to specific anthelmintics (Sargison et al., 2019). Finally, recent studies have made use of genetic crosses (Sargison et al., 2018; Doyle et al., 2018) mating two phenotypically and genetically distinct parental isolates together, selecting for the trait of interest, and examining the progeny by Whole Genome Sequencing (WGS). This has proved to be a powerful tool in identifying loci thought to be involved in ivermectin (Sargison et al., 2018; Doyle et al., 2019; Doyle et al., 2021), levamisole (Doyle et al., 2021) and monepantel resistance (Niciura et al., 2019) in *H. contortus*.

1.1.8 Relationship of *H. contortus* to *C. elegans*

H. contortus is a Clade V nematode and closely related to *C. elegans* (Blaxter , 2011; Laing et al., 2013). *C. elegans* serves as a model organism to study many aspects of nematode biology, as well as in wider fields of biology such as neuroscience (Sengupta and Samuel, 2009), evolution (Gray and Cutter, 2014), and ageing (Tissenbaum, 2015) among others. This relatively close relationship to *C. elegans* has been of great value in comparative work (Gilleard , 2013; Burns et al., 2015) and in establishing *H. contortus* as a model organism to study the evolution of parasitism, and the genetic basis of anthelmintic resistance (Kwa et al., 1994; Gilleard, 2013; Laing et al., 2013). *C. elegans* also provides a free-living nematode model which can be cultured *in vitro* throughout its life

cycle, and in which transgenic and functional validation experiments can be carried out (Britton et al., 1999; Redmond et al., 2001; Glendinning et al., 2011; Zhang et al., 2016b). There exists a core of highly conserved nematode genes which are shared between even distant members of the family (Yin et al., 2008). Thus, although there is a great deal of diversity within nematodes, including the independent evolution of parasitism multiple times (Blaxter and Koutsovoulos , 2015; Viney, 2018), there also exists a conserved core of genes and gene families shared between them, which facilitates comparative genomics to a certain degree, particularly when genomic resources for one species lag behind those of another species.

1.1.9 Parasitic nematode genomic resources

The recent completion of the fourth version (V4) of the *H. contortus* genome represents one of the most complete genome assemblies of Clade V nematodes (Doyle et al., 2020). A reduction in size from 370 Mb (Laing et al., 2013) to 283.4 Mb was achieved by a hybrid approach making use of short read and improved long read sequencing technologies, such as PacBio (Rhoads and Au, 2015) followed by manual finishing (Doyle et al., 2020). This approach allowed the elimination of redundant haplotypic sequences (Doyle et al., 2020). In addition to the published genome assemblies discussed above (Laing et al., 2013; Doyle et al., 2020), there exists a well-developed set of genomic and transcriptomic resources available for *H. contortus* research.

WormBase is an integrated *C. elegans* and related nematode genomic resource site (Harris et al., 2020). WormBase Parasite exists due to a decision to expand the mission of the original WormBase project (Howe et al., 2016) from a repository of *C. elegans* genomic resources to include the genomes and associated data of parasitic helminths as well (Howe et al., 2017). Since the publication of the first parasitic nematode genome, *Brugia malayi* (Ghedin et al., 2007), and the publication of the *H. contortus* genome (Laing et al., 2013) several years later, the genomic resources available for parasitic nematode research continue to advance, providing more scope to study parasite biology and anthelmintic resistance. Predicted annotations are also available for the two published genomes for *H. contortus*, from the Wellcome Sanger Institute (Laing

et al., 2013) and the University of Melbourne (Schwarz et al., 2013) in addition to an improved gene annotation file released recently (Doyle et al., 2020). The WormBase team is involved in the curation of gene annotations for *C. elegans*, and a small number of other nematodes (Howe et al., 2012), with the remainder imported from the group responsible for sequencing and publication of the respective genome. WormBase Parasite also provides several free to use web-based tools to explore data, including genome browsers, gene and genome summary pages, BLAST, text and sequence search, and serves as a platform to integrate available functional annotations and gene expression analyses (Howe et al., 2017). In addition to these resources, a full life cycle stage RNA-seq data set is freely available from the European Bioinformatics Institute (EBI) website (Laing et al., 2013).

1.2 Anthelmintic Resistance

1.2.1 Anthelmintic treatment of *H. contortus*

The potential for animal mortalities and the capacity for rapid population growth and contamination of pasture by *H. contortus* underlines the importance and necessity of effective options for the control of haemonchosis (Besier et al., 2016a). However, the efficacy of anthelmintic treatment is hindered by the high prevalence of resistance to the major classes of broad spectrum anthelmintics in *H. contortus*, and further confounded by the growing problem of multidrug resistance (Kotze et al., 2016; Crook et al., 2016; Rose -Vineer et al., 2020).

1.2.1.1 Benzimidazoles (BZs)

Thiabendazole, released in the 1960s, was the first commercially available broad spectrum anthelmintic (Gordon, 1961). Since the 1960s many different BZ drugs have been released and are commercially available for the treatment of various human and veterinary helminth infections. Several BZs have since been discontinued, and the current available range (fenbendazole, oxfendazole,

albendazole, ricobendazole, and mebendazole) were mainly released in the 1970s (McKellar and Jackson, 2004). BZs are effective against many nematodes, both the immature life cycle stages and the mature adult stages of some parasites (Besier et al., 2016a), and some cestodes.

BZs exert their effects by blocking the polymerisation of cellular microtubules by selective binding to nematode β-tubulin (Lacey, 1990; Martin, 1997). Cellular microtubules are formed by the polymerisation of α- and β-tubulin monomers in a dynamic process, driven by polymerisation at the active pole, and depolymerisation at the negative pole. The microtubule complex is stabilised by various microtubule associated proteins. Microtubule formation can be inhibited by binding of BZs to β-tubulin at the positive pole (Martin, 1997). This leads to the inhibition of nematode cellular microtubule formation and thus blocks key cellular functions such as movement of chromosomes during cell division, cytoskeletal structure, intracellular movement of particles such as energy metabolites, and exocytosis leading to cell death. However, the onset of action is relatively slow when compared to other anthelmintics that act on the nervous system (Martin, 1997).

The efficacy of BZs in treating haemonchosis is severely hampered by widespread resistance. Resistance has been documented across Europe (Hoglund et al., 2009; Domke et al., 2012; Pena Espinoza et al., 2014; Mickiewicz et al., 2017), South America (da Cruz et al., 2010; Nunes et al., 2013; Jaeger et al., 2017), Oceania (Baltrusis et al., 2018), and North America (Howell et al., 2008; Williamson et al., 2011; Chaudhry et al., 2014; Mondragón-Ancelmo et al., 2019).

1.2.1.2 Macrocyclic Lactones (ML)

ML were first identified and isolated from the soil bacterium *Streptomyces avermitilis* in the late 1970s (Burg et al., 1979). They are effective against all life cycle stages of nematodes, but are not effective in treating cestodes or trematodes (Campbell et al., 1983). ML are also used to treat ectoparasites of livestock and companion animals (Laing et al., 2017). They are used widely in

the treatment of parasitic nematodes of veterinary importance, and also form the basis of mass drug administration for the treatment and elimination of onchocerciasis and lymphatic filariasis (Geary, 2005; Wilson et al., 2016).

All members of the ML group share disruption of neuronal transmission via potentiation of glutamate gated chloride channels as a common major mode of action (Martin and Pennington, 1988; Martin, 1997). However, there are important pharmacological differences amongst different members of the ML group, which has significant implications in relative potency and mechanism of resistance (Prichard et al., 2012). ML cause flaccid paralysis, inhibition of pharyngeal pumping leading to starvation, and the concurrent expulsion of the parasite from the gastrointestinal (GI) tract (Martin, 1997; Geary and Moreno, 2012).

Resistance to ML presents a problem not only to the livestock industry, but also threatens treatment and eradication efforts for human helminths, with resistance documented in *Onchocerca volvulus* (Osei-Atweneboana et al., 2011; Doyle et al., 2017). Resistance to ML in a population of *H. contortus* is often present in conjunction with resistance to other broad spectrum anthelmintics, such as BZs or LEV, (Howell et al., 2008; da Cruz et al., 2010; Verissimo et al., 2012; Crook et al., 2016; Rose-Vineer et al., 2020). This occurs due to farmers applying the same selective pressure to an existing resistant population with a different compound, ultimately resulting in the development of multidrug resistance. As with BZs, resistance to ML is widespread and documented in GIN in many sheep producing regions globally (Howell et al., 2008; da Cruz et al., 2010; Verissimo et al., 2012). Resistance prevalence does appear to vary by region (Hoglund et al., 2009; Mitchell et al., 2010; Paraud et al., 2010) and multidrug resistance to BZs, ML, and LEV has also been reported in Scotland in *Teladorsagia circumcincta* (Sargison et al., 2007) and is increasing globally (Kotze et al., 2016; Crook et al., 2016; Rose-Vineer et al., 2020).

1.2.1.3 Nicotinic agonists: levamisole (LEV) and pyrantel

Levamisole (LEV) is the most widely used member of this class of anthelmintics in the treatment of small ruminants (Besier et al., 2016a). Pyrantel is widely used in companion, small animal, and equine medicine (Kopp et al., 2007). Pyrantel is also used in the treatment of human helminth infections, particularly hookworms (Kopp et al., 2009). Both LEV and Pyrantel act as a specific selective agonist of the nematode AChR (Aceves et al., 1970; Martin , 1997; Kopp et al., 2009) producing a depolarisation of nematode somatic body wall muscle cells via influx of cations (Harrow and Gration, 1985). The result is spastic paralysis of the worm, detachment from the intestinal wall, and expulsion of the parasite from the GI tract (Martin and Robertson, 2007).

1.2.1.4 Efficacy of LEV

The efficacy of an anthelmintic, such as LEV, is generally determined by the reduction in faecal egg count post-treatment. A drug is regarded as having reduced efficacy when the post-treatment faecal egg count reduction is <95% (with the lower limit of the 95% confidence interval being <90%) (Coles et al., 1992; Coles et al., 2006). Generally, any reduction of <95% in a post-treatment faecal egg count is thus judged to constitute reduced efficacy of the drug within the population. This is generally used in clinical contexts, and reported in surveys of anthelmintic resistance in the field (Kaplan et al., 2020; Geurden et al., 2022).

In addition, in research contexts the efficacy of LEV is often assessed using larval development assays such as the Drenchrite® assay (discussed in detail in 1.5.2.2), with an IC_{50} (i.e. the minimum concentration at which 50% of eggs are inhibited from developing to larvae) of <= 0.78 μM judged to be susceptible, and an IC_{50} of >= 1.56 μM judged to be resistant (Ray Kaplan, personal communication). This method has been used to assess the efficacy of LEV for several of the isolates used within this study, such as MHco18(UGA2004) (Williamson et al., 2011), and field isolates from the USA (Doyle et al., 2021).

1.2.1.5 Mechanism of action of LEV

The nematode AChR is a pentameric ligand gated ion channel, composed of five identical (homomeric) or related (heteromeric) subunits (Duguet et al., 2016). In *H. contortus,* the LEV sensitive acetylcholine receptor is a heteropentamer comprising subunits UNC-38, UNC-29, UNC-63, and ACR-8, wherein one subunit will be present twice to form the pentameric complex (Fauvin et al., 2011; Boulin et al., 2011; Martin et al., 2012; Barrère et al., 2014; Blanchard et al., 2018) (Figure 1.2).

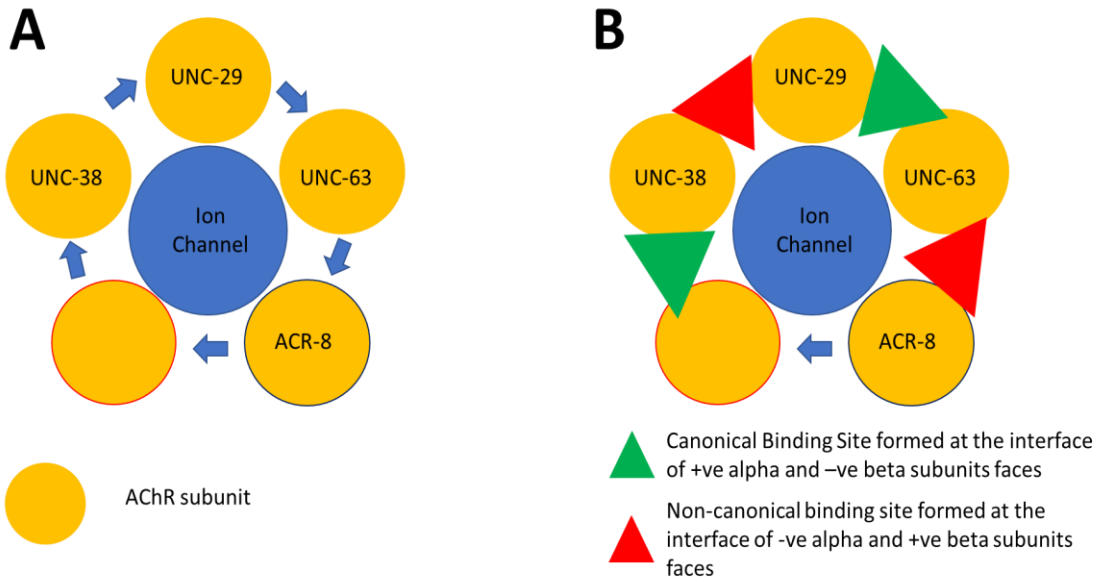

Figure 1.2: Receptor subunit composition and ligand binding in the *Haemonchus contortus* AChR. A: Receptor subunit composition of the *Haemonchus contortus* LEV sensitive AChR. B: Canonical and non canonical binding sites formed at the junctures of alpha and beta subunits in the *Haemonchus contortus* AChR. A canonical binding site is formed at the +ve face of an alpha subunit and the -ve face of a beta subunit, and a non-canonical binding site is formed on the opposite side (i.e. the +ve face of the beta, and -ve face of the alpha subunits). Acetylcholine is known to bind at the canonical binding site, whereas LEV is thought to bind at the non-canonical allosteric binding site (Martin et al., 2012).

Subunit ACR-8 has been shown to play a key role in activation of the AChR by LEV in *H. contortus* in experimental work (Fauvin et al., 2011; Blanchard et al., 2018). Functional reconstitution of AChR in *Xenopus* oocytes showed a 17-fold lower sensitivity of the receptor to LEV when ACR-8 was omitted (Blanchard et al., 2018). LEV is thought to bind at a non-canonical binding site present at the interface of UNC-63 and ACR-8 in the *H. contortus* LEV sensitive AChR (Martin et al., 2012). Pyrantel is believed to exert its effects on the same pharmacological

sub-population of nematode nAChR as LEV (Levandoski et al., 2005; Kopp et al., 2009), although recent *in vivo* RNAi experiments showed that pyrantel and LEV likely act on different receptor subunits (Blanchard et al., 2018).

Generally, resistance to LEV appears to be lower than resistance to BZ and comparable or lower than resistance to ML in many regions (Waghorn et al., 2006; Howell et al., 2008; da Cruz et al., 2010; Verissimo et al., 2012), although in some reports from Australia LEV resistance was higher than ML resistance (Le Jambre et al., 2010). In Europe, resistance to LEV generally remains lower than that of BZ and ML (Rose-Vineer et al., 2020).

1.2.2 Knowledge and understanding of anthelmintic resistance mechanisms and markers in *H. contortus* at the outset of the study

Despite widespread resistance to the three major classes of broad spectrum anthelmintics, the best resolved mechanism of resistance to date is BZ resistance (Kwa et al., 1994). Resistance to BZ is driven by three SNPs in the β-tubulin isotype 1 gene (Kotze et al., 2016). The most common SNP associated with BZ resistance is the F200Y variant (Kwa et al., 1994; Kotze et al., 2012; Kotze et al., 2014). The F167Y variant is less frequent but has, nevertheless, been detected in different species (Hodgkinson et al., 2008). The E198A variant is the least commonly encountered causative SNP for BZ resistance, although this has also been detected in some isolates of *H. contortus* (Ghisi et al., 2007; Kotze et al., 2012). These SNPs can co-occur, although generally one SNP tends to predominate within a population (Avramenko et al., 2019; Quieroz et al., 2020; Melville et al., 2020). There is still considerable heterogeneity amongst populations which complicates any potential molecular diagnosis tool, which would need to be capable of detecting all three SNPs (von Samson-Himmelstjerna et al., 2009b; Avramenko et al., 2019). Different SNPs are thought to be predominant in different regions around the world, and F167Y for example, shows limited expression in certain countries including the USA, Canada, Brazil, Argentina, France and the UK (Barrere et al., 2012; Barrere et

al., 2013; Chaudhry et al., 2014; dos Santos et al., 2014; Redman et al., 2015). E198A has been detected in isolates from South Africa, Australia, China and India (Ghisi et al., 2007; Rufener et al., 2009a; Kotze et al., 2012; Chaudhry et al., 2015; Tuersong et al., 2020).

Significant recent work has focused on genomic approaches to identify potential resistance markers for ML resistance in *H. contortus* (Laing et al., 2016; Doyle et al., 2018; Khan et al., 2020), aided by well-developed genomic resources (Laing et al., 2013; Doyle et al., 2020). Although many potential candidate genes have been identified, no unequivocal markers or mechanisms of resistance have been identified in *H. contortus*, and resistance to ML would appear to be complex and multigenic (Khan et al., 2020; Doyle et al., 2021).

Uncovering resistance mechanisms and genetic markers is of paramount importance in combatting anthelmintic resistance, as it will allow the development of molecular diagnostics for anthelmintic resistance, such as those which have been shown to be effective in published studies for BZ resistance (von Samson-Himmelstjerna et al., 2009b; Zhang et al., 2016b). This allows for improved surveillance of anthelmintic resistance and assessment of the effects of various control programmes, and facilitates investigation of the origin and spread of resistance. In addition, a full understanding of the genetic basis for resistance could allow for the development of new drugs, drug synergists and combinations to bypass or break resistance. However, elucidation of resistance mechanisms is complex. Parasitic nematodes are difficult to study in the laboratory (Gilleard, 2013), as most species cannot be cultured through their full life cycle *in vitro* (Ahmed, 2014). Although *H. contortus* can be effectively cultured from eggs to L_3, maintenance of immature and mature adults usually requires the *in vivo* infection of the primary host (Marks et al., 2019). This requires clearance for animal work and the concomitant ethical concerns involved (Colby et al., 2017). In addition, genomic resources for parasitic nematodes have lagged behind those of other organisms, due to limited investment (Gilleard, 2013), very large genome sizes (Gilleard, 2013; Laing et al., 2013; Doyle et al., 2020) and high levels of polymorphism (Gilleard, 2006; Gilleard, 2013; Laing et al., 2013; Doyle et al., 2020). However, among the parasitic nematodes of medical and veterinary importance, *H. contortus* is one

of the more tractable to study experimentally, and serves as an important model organism in anthelmintic resistance and drug discovery research (Kwa et al., 1994; Gilleard, 2013; Laing et al., 2013; Laing et al., 2016).

Research approaches to determining anthelmintic resistance mechanisms have mainly focused on examination of differences in frequency of SNPs between resistant and sensitive isolates (Prichard, 2001; Eng and Prichard, 2005; Gilleard and Beech, 2007) and identification of polymorphisms in candidate gene sequences after drug selection in experimental infections (Kwa et al., 1993; Prichard, 2001; Beech et al., 2011; Doyle et al., 2021). The latter approach usually only examines a small number of loci and generally candidate gene studies rely heavily on prior assumptions regarding resistance (Gilleard, 2013); these may be informed by comparative work on resistance to the drug in *C. elegans,* as has been the case in many LEV resistance studies (Neveu et al., 2010; Fauvin et al., 2010; Boulin et al., 2011; Sarai et al., 2013; Sarai et al.,2014; Raza et al., 2016). Recent progress in genomic resources has, however, significantly improved prospects for anthelmintic resistance research in *H. contortus*. This, along with improved methods for mating between different isolates (Sargison et al., 2018), has facilitated the use of controlled genetic crosses of resistant and sensitive isolates to identify anthelmintic resistance candidate genes (Niciura et al., 2019; Doyle et al., 2019; Doyle et al., 2021).

1.2.2.1 LEV resistance in *H. contortus*

The mechanisms of LEV resistance in *H. contortus* are not currently completely elucidated, although significant progress has been made in recent years. Initial work focused on comparative studies based on orthologues of genes identified in *C. elegans* LEV resistant mutants (Lewis et al., 1987a; Lewis et al., 1987b; Sangster et al., 1991; Sangster et al., 1998), made necessary by the lack of developed genomic resources for *H. contortus*. This approach, nevertheless, presents some problems and raises the issue of drawing comparisons between *C. elegans* and *H. contortus*. As LEV acts on the nematode nAChR (Aceves et al., 1970) it naturally follows that resistance may well present as mutation or alteration of expression of the receptor subunit(s) to which LEV binds.

1.2.2.2 LEV and ACR-8

This assumption is complicated by the differences in receptor subunit composition between *H. contortus* and *C. elegans*. This is significant, as although both species belong to Clade V, there has been extensive and continued diversification, duplication, and divergence of AChR subunits across *nematoda* (Beech and Neveu, 2015). There are examples of loss of specific subunits and functional receptor types in parasitic nematodes, and receptors which show differing pharmacology and display different subunit composition per species (Williamson et al., 2009; Buxton et al., 2014). Furthermore, the *C. elegans* LEV sensitive AChR has been shown to be pharmacologically distinct from those of parasitic nematodes (Qian et al., 2008; Martin et al., 2012). This point is key when considering ACR-8, the AChR subunit shown to play a key role in LEV mode of action both *in vitro* and *in vivo* in *H. contortus* (Blanchard et al., 2018). This subunit was not identified in LEV resistant *C. elegans* mutants in initial studies (Lewis et al., 1987a; Lewis et al., 1987b; Sangster et al., 1991), where subunits UNC-63, UNC-38, UNC-29, LEV-1 and LEV-8 were identified (Lewis et al., 1987a; Lewis et al., 1987b; Sangster et al., 1991; Sangster et al., 1998). LEV-8, although it plays a key role in LEV sensitivity of the *C. elegans* AChR (Qian et al., 2008), is not present in *H. contortus* (Williamson et al., 2007; Neveu et al., 2010), and its role in the AChR is instead fulfilled by ACR-8 (Neveu et al., 2010; Fauvin et al., 2010; Blanchard et al., 2018). *C. elegans* ACR-8 mutants, lacking a functional ACR-8, were found not to be resistant to LEV (Almedom et al., 2009), despite this subunit being essential for conferring LEV sensitivity to the AChR in *H. contortus* (Blanchard et al., 2018). It has also been demonstrated that the expression of *H. contortus* ACR-8 in *C. elegans* was able to rescue LEV sensitivity in *C. elegans* LEV-8 null mutants. Incorporation of *H. contortus* ACR-8 into the *C. elegans* AChR in functional reconstitution experiments in *Xenopus* oocytes leads to up to a 17-fold increase in sensitivity of the receptor to LEV (Blanchard et al., 2018). In addition, the same study used RNAi in *H. contortus* to silence expression of *acr-8*, leading to a reduced sensitivity to LEV (Blanchard et al., 2018). As such, changes in expression or sequence of ACR-8 may serve as a resistance mechanism and/or marker of resistance to LEV in *H. contortus*. In

addition, the LEV-1 subunit lacks a signal peptide in *H. contortus*, and functional reconstitution experiments indicate it does not incorporate into a functional receptor (Neveu et al., 2010). An extra layer of complexity is added when extrapolating results regarding anthelmintic resistance from *C. elegans* to parasitic nematodes when we consider the multiple gene duplication and expansion events seen in *H. contortus* relative to *C. elegans* (Laing et al., 2013). UNC-29, a component subunit of the LEV sensitive AChR in both *H. contortus* and *C. elegans*, is present as a single copy in *C. elegans*, but expanded to four copies in *H. contortus* (Neveu et al., 2010; Boulin et al., 2011; Duguet et al., 2016), whilst only one (UNC-29.1) has been shown to successfully incorporate into the *H.* contortus AChR (Neveu et al., 2010).

1.2.2.3 Truncated transcripts and altered gene expression patterns

Previous research into LEV resistance in *H. contortus* has focused on several potential markers and/or mechanisms of resistance, built on the foundation of the subunits identified in *C. elegans* LEV resistant mutants. Truncated transcripts have been detected for *unc*-63 (*unc-63b*) (Fauvin et al., 2010), and subsequently for *acr-8* (*acr-8b*) (Neveu et al., 2010; Williamson et al., 2011) in resistant isolates, along with changes in expression of other full length AChR subunits and accessory proteins (Sarai et al., 2013; Sarai et al., 2014; Raza et al., 2016). Changes in expression of P-glycoproteins (Pgps) have also been observed, and have been associated with low level resistance to LEV (Sarai et al., 2014; Raza et al., 2016). Initially, *unc-63b* was thought to present a promising candidate for a resistance mechanism. Functional reconstitution experiments in Xenopus oocytes showed that injection of cRNA (complementary RNA) of both the full length and truncated transcripts led to a dominant negative effect, whereby the product of the truncated transcript preferentially incorporated into the AChR (Boulin et al., 2011). However, there have been some inconsistencies observed in the expression of *unc-63b*, with expression not uniform across resistant isolates examined (Sarai et al., 2013; Sarai et al., 2014). This is also the case for the truncated isoform of *acr-8* (*acr-8b*). Although this isoform has been correlated with a resistant phenotype in some isolates

(Williamson et al., 2011), it is absent in other resistant isolates (Sarai et al., 2013; Sarai et al., 2014), and expressed in a sensitive isolate (Barrere et al., 2014).

1.2.2.4 Intron 2 *acr-8* Deletion

The identification of a variable length deletion in the second intron of the *acr-8* genomic sequence has been correlated with LEV resistance in several isolates (Barrere et al., 2014). This deletion has also been correlated with the expression of the *acr-8b* truncated transcript, and it is thought that the two could be linked (Barrere et al., 2014; dos Santos et al., 2019). The initial study identified the deletion in a number of resistant isolates from various regions around the world, and correlated the presence of the deletion with the expression of *acr-8b* in several isolates. However, one issue encountered in this study was the identification of a LEV sensitive isolate which was homozygous for the deletion, and also found to be expressing *acr-8b*. Another sensitive isolate was also found to be heterozygous for the deletion, but *acr-8b* was not detected in that isolate in the course of the study (Barrere et al., 2014).

1.2.2.5 Prospects for diagnostic markers

When considering the state of understanding of LEV resistance at the outset of the current study, it was clear that significant work remained to fully elucidate a resistance mechanism and an effective genetic marker. The most important discoveries have been the clear *in vivo* confirmation, using RNAi, of the importance of the ACR-8 subunit in conferring LEV sensitivity to the AChR in *H. contortus* (Blanchard et al., 2018). Given the crucial role this subunit has in the mode of action of LEV, it is likely a prudent target for a resistance marker. The expression of truncated *acr-8b* transcripts (Neveu et al., 2010; Williamson et al., 2011), and its correlation with a deletion in intron 2 of the *acr-8* gene (Barrere et al., 2014), are both important steps to our understanding of LEV resistance. However, as these studies showed there are still inconsistencies in the

expression of these potential markers, such as their presence in a definitively LEV sensitive isolate (Barrere et al., 2014), thus signifies these markers require further validation before an effective diagnostic test can be designed.

Finally, recent work employing WGS of pre- and post-LEV treated MHco3/18 genetic cross worms in the course of a live infection identified several non-synonymous SNPs associated with LEV resistance in *H. contortus* (Doyle et al., 2021) which provide fruitful targets for laboratory validation as diagnostic markers of LEV resistance.

1.2.3 Management practices and risk factors in anthelmintic resistance

Anthelmintic resistance is present in most sheep rearing countries, and for countries where multiple cross-sectional studies have been conducted over time, it has been reported that both the incidence and the number of different GIN species displaying anthelmintic resistance is increasing (Rose-Vineer et al., 2020). In addition to understanding molecular and genetic mechanisms underlying anthelmintic resistance, it is also important to understand the risk factors behind the development and spread of anthelmintic resistance, and how these can be managed (Sangster and Gill, 1999; Morgan et al., 2013). This also aligns with the need for improved diagnostics, to allow for effective resistance surveillance and sustainable use of anthelmintics (Kotze et al., 2016; Kaplan , 2020). Thus, using a holistic approach combining effective livestock management, best treatment practices, and a robust and accurate set of diagnostic tools could help limit or control the spread of anthelmintic resistance (Kotze et al., 2020; Kaplan, 2020).

1.2.3.1 Risk factors contributing to the emergence of anthelmintic resistance

A systematic review and meta-analysis of the emergence of anthelmintic resistance in sheep found, of the five risk factors considered, only high frequency of treatment was significant (Falzon et al., 2014). Several further risk

factors were identified as marginal, including mixed species grazing (which has also been reported as protective in some studies), use of long acting drug formulations, and drench-and-shift pasture management. The study also concluded that several current recommendations are not supported by evidence, and that many studies into this area had unclear or high risk of systematic bias (Falzon et al., 2014).

1.2.3.2 Current control measures beyond mass drug treatment

Following the emergence and now widespread incidence of multi-drug resistance to BZs, ML, and LEV (Kotze et al., 2016; Crook et al., 2016; Rose-Vineer et al., 2020), and the rapid development of resistance to newer anthelmintics such as amino acetyl derivatives (AADs) (Van den Brom et al., 2015), effective control of haemonchosis requires a more holistic approach that extends beyond traditional whole flock treatment strategies. Although drug treatment remains the only intervention to eliminate GIN infection in many countries, improper use can have a counterproductive effect. For example, under-dosing often fails to clear the infection, instead placing selective pressure on the population leading to the development of drug resistance (Smith et al, 1999; Shalaby, 2013). Despite this, even correctly dosed drug treatment also exerts selective pressure on the parasite (Falzon et al., 2014), especially when considering the already widespread incidence of anthelmintic resistance. An example of this is the emergence of monepantel resistance on a single farm in the Netherlands, following repeated treatments over an 18-month period, each with declining efficacy (Van den Brom et al., 2015).

Refugia in the context of drug resistance refers to an untreated environment or hosts where selective pressure to the drug is removed, and drug sensitive parasites can be preserved (Van Wyk, 2001; Hodgkinson et al., 2019). In the preceding 20 years, since the first published articles exploring the concept of refugia based approaches into anthelmintic treatment regimens (Van Wyk, 2001), the concept has gained increased interest as a means to slow or halt the spread of anthelmintic resistance (Hodgkinson et al., 2019). Evidence from other biological systems, for example agricultural pests, has shown positive results in

slowing the spread of resistance (Tabashnik, 1994; Tabashnik et al., 2008; Jin et al., 2015). In addition, as discussed by Falzon et al., (2014) the single factor which poses the highest risk to the development of anthelmintic resistance is repeated administration of a drug. Traditionally, control of GIN in livestock has depended mainly on whole-flock/herd administration of broad spectrum anthelmintics. This leads to suppression of susceptible worms, followed by contamination of the pasture exclusively by resistant progeny. However, if drug usage is minimised, then more sensitive worms remain on pasture, for example, and mating between resistant and sensitive individuals would lead to a dilution of resistant alleles within the population as a whole. Conditions such as the environment, the level of mixing between populations, parasite life history traits, and the level of resistance can play a key role in the success of a refugia based approach, and must be better understood in the field. Finally, the presence of a fitness cost for resistance to a drug may also play an important role in refugia based approaches as it will favour retention of the sensitive phenotype (Hodgkinson et al., 2019).

Two methods that aim to apply the concept of refugia in the field are Targeted Selective Treatment (TST) and Targeted Treatment (TT). They both rely on the assessment of clinical signs (indicators) to inform and optimise treatment (Busin et al., 2014; Charlier et al., 2014; Kupcinskas et al., 2017). The TST approach assesses clinical indicators, such as live weight gain (Busin et al., 2014), production efficiency, Faecal Egg Counts (FEC), body condition score, and treats accordingly based on disease severity. The TT approach relies on metaphylactic (i.e. treatment following diagnosis of a condition) treatment of the whole flock or group treatment, based on the assessment of a clinical marker, such as FEC from pooled faecal samples (Kenyon and Jackson, 2012), as opposed to prophylactic treatment where whole flocks are treated without assessing whether the animals are infected or not.

The FAMACHA system uses one specific clinical indicator for control of *H. contortus*. A FAMACHA chart is provided with colour coded scores based on a visual assessment of anaemia which is assessed by the colour of the eye mucous membrane and matched to examples on the FAMACHA chart. This is followed by treatment of only those animals which are anaemic. The FAMACHA system was

shown to be consistent among trainees, even from different background and with different level of knowledge, making it suitable as an effective TST indicator in low resource settings (van Wyk and Bath, 2002). As such FAMACHA is an effective management method to avoid mass drug administration where laboratory diagnosis is not always available or cost prohibitive.

Along with sustainable use of anthelmintics, the use of vaccination against *H. contortus* has several advantages. A vaccine not only offers long term protection against infection, but also eliminates chemical residues left in either meat, or the environment (Matthews et al., 2016). Currently, the Barbervax™ vaccine (Nisbet et al., 2016) is the only commercially vaccine against *H. contortus*. Wirevax™/Barbervax™ is licensed for use in South Africa and Australia (respectively) and can be obtained under veterinary prescription in the UK (Claerebout and Geldhof, 2020). The vaccine is currently produced by the extraction of native antigen from infected sheep, which has some drawbacks as it requires the collection of a large amount of parasite material from an active infection. Efforts have been made to identify and produce recombinant antigens, but this process is still ongoing (Matthews et al., 2016; Nisbet et al., 2016; Claerebout and Geldhof, 2020). Recent identification of a recombinant protein (rHc23) has shown promising early results, but further work is required before this can be deployed (Fawzi et al., 2015; Claerebout and Geldhof, 2020).

Prior to the development of anthelmintics and their widespread availability beginning in the 1960s (Zajac and Garza, 2020), natural or artificial selection against susceptibility to GIN infection was largely the only available practice to improve GIN infection outcomes (Burke and Miller, 2020). There are three phenotypes relating to establishing a GIN infection in animals: resistant, resilient, and susceptible (Woolaston and Baker, 1996). A resistant animal's immune system is capable of reducing or preventing the establishment of infective larvae, and rejecting and expelling those which are already established (Albers et al., 1987). Resilient animals are capable of remaining healthy despite high worm burdens, or in other words are tolerant of the damage or blood loss caused by GIN (Morris et al., 2001). A susceptible animal is characterised as one which is unable to mitigate or prevent the establishment and deleterious effects of GIN infection (Woolaston and Baker, 1996). Selective breeding programmes

have been successfully implemented, and flocks resistant to GIN have been successfully established in Oceania (Vanimisetti et al., 2004a; Vanimisetti et al., 2004b), and South America (Toscano et al., 2019). However, some of these programmes required 10-20 years before achieving a satisfactory outcome (Burke and Miller, 2020), and thus are not applicable in the short term, although in the long term do represent a potential solution to ameliorate production losses due to GIN. However, there is controversy regarding selection of resilient animals, especially in the case of *H. contortus*, as it effectively involves selecting animals capable of living in a state of constant haemorrhage (Le Jambre et al., 1995; Bisset et al., 1996), and under poor nutritional conditions these animals could undergo immune suppression and revert to effective susceptibility (Amarante et al., 2004; Burke and Miller, 2008). These animals are also much more likely to contaminate pasture, due to their tolerance of a high worm burden leading to the shedding of a large quantity of eggs, and they might also be susceptible to other pathogens.

Aside from vaccination and breeding for natural resilience and resistance, there are a number of factors which can improve the tolerance of GIN in small ruminants (Burke and Miller, 2020). Nutrition is a key factor in the tolerance of GIN infection (Coop and Kyriazakis, 1999; Houdijk, 2012) and increased energy and protein in the diet has been shown to improve immune function which in turn impacts the establishment and survival of worms in the host (Houdijk, 2012). The impact of diet is especially important in parasite-susceptible breeds (Steel, 2003). This is especially important when considering the situation in sheep farming regions where subsistence agriculture is practiced, resources are limited, and many people live in a fragile position, occupying marginal land susceptible to drought and climate change, such as the Sahel region in Africa.

Pasture and grazing management can also be an important factor in regulating GIN infections. Approaches such as using different plant species with higher protein content, and multispecies or alternate species grazing of cattle/horses with sheep/goats can provide some benefits by removing the primary host from the pasture for a time (Rocha et al., 2008). However, it must be pointed out that studies have shown conflicting results with regards to multi-species grazing, with one study showing a protective effect and another showing it increased risk

of anthelmintic resistance developing (Lawrence et al., 2006). Similarly, rotational grazing involving, for example, moving lambs to new plots every 3-7 days, and preventing return to the same plot before 28-35 days has been shown to reduce worm infections when compared to control lambs continuously grazed on the same plot (Colvin et al., 2008; Burke et al., 2009).

1.3 Diagnosis of helminth infections

Helminth infections pose a unique set of challenges for diagnosis, in both veterinary and human medicine. Unlike bacterial or protozoan infections, helminths are multicellular organisms and detection is not always carried out directly on the infecting organism itself. When dealing with GIN, the diagnosis is still mainly based on microscopy of eggs passed in stool both in human (Khuruna and Sethi et al., 2017) and veterinary (Coles et al., 1992; Sweeny et al., 2011; Kotze et al., 2016; Kotze et al., 2020) helminthiases. Diagnosis is also complicated by the widespread presence of mixed co-infections of GIN of veterinary importance (Preston et al., 2014; Craig, 2018). Species specific identification based on microscopic morphological analysis is also difficult, with only *Nematodirus* eggs being easily distinguishable from other *Strongyle* spp. eggs. Thus, species specific identification typically requires the culturing eggs to larval stages which can be more easily distinguished via morphology (Preston et al., 2014). Finally, due to the differing pathologies of GINs of veterinary importance species specific identification within mixed co-infection is necessary for appropriate flock management.

1.3.1 Current techniques for the diagnosis of GIN and schistosomiasis

GIN infections in humans are commonly caused by the soil transmitted helminths (STHs), of which the most medically important species are *Ascaris lumbricoides*, *Trichuris trichiura*, *Ancylostoma duodenale, Necator americanus,* and *Strongyloides stercoralis*, with an estimated 50% of the global population

affected or threatened by infection (Pullan et al., 2014). Within endemic areas transmission occurs due to poor sanitation, lack of access to healthcare and overcrowding primarily in lower and middle income countries (LMICs) (Means et al., 2017). STHs infection in children contributes to anaemia, mental, physical, and developmental and growth deficiencies (Pullan et al., 2014; Khurana and Sethi, 2017). Schistosomiasis is caused by the haematophagous parasitic trematode *Schistosoma* spp. Infective larvae penetrate the skin when present in fresh water, with mature adults residing either in the mesenteric (*S. mansoni* and S. *japonicum*) or pelvic (*S. haemotobium*) veins. Females lay eggs, which are then secreted in stool or urine respectively. Eggs which become trapped in surrounding tissue elicit an immune reaction leading to granulomas and consequent intestinal, hepato-splenic, or urogenital disease (Carson et al., 2018). Much like STHs, schistosomiasis causes significant morbidity in infected hosts, particularly children (McManus et al., 2018).

In addition to *H. contortus* discussed above, GIN most commonly associated with ruminant livestock are *Ostertagia, Teladorsagia, Cooperia, Trichostrongylus, Oesophagostomum,* and *Nematodirus* (Preston et al., 2014; Craig, 2018). The eggs of all these genera are similar in appearance and are difficult to distinguish from each other, except for *Nematodirus*, which has distinctive morphology and is significantly larger than the others (Preston et al., 2014; Craig, 2018). Disease in ruminants is rarely due to infection with one species, but often the result of the cumulative effects of a mixed infection, although specific outbreaks due to one species do also occur (Craig, 2018). Identification to species level commonly involves larval culture, taking ~7-10 days for egg hatching, although PCR tests have been developed for species identification of *Strongyle* eggs (Roeber et al., 2012; Roeber et al., 2013a; Bisset et al., 2014). A commercially available (Australia) lectin stain is a recent advance in microscopy-based identification of *H. contortus* eggs (Preston et al., 2014), as commercially available kits were not previously available. Although of benefit for *H. contortus* diagnosis, it is only applicable to this species, and does not resolve other issues, like the labour intensity of coprological analysis for faecal egg counting. It also has the disadvantage of raising the cost of the test.

There are a number of different microscopy-based techniques used in the diagnosis of parasitic GIN infections. The Kato-Katz thick smear is recommended by the World Health Organisation for the diagnosis of STH and Schistosomiasis in endemic regions (Barenbold et al., 2017). Kato-Katz slides are cheap to produce, and the technique yields few false positives, and can be used to detect several co-endemic species (Speich et al., 2015). The technique, however, suffers from poor sensitivity in low intensity infections (Nikolay et al., 2014). A significant drawback of the Kato-Katz technique is the difficulty of standardisation. In addition, when dealing with multiple species co-infecting an individual, false negatives can occur due to different clearing times for eggs from different species (Levecke et al., 2011). This is an issue shared by most microscopy-based techniques for both human and veterinary helminth infections.

The McMaster technique is an alternative method to the Kato-Katz for the diagnosis of STH infections in humans (Levecke et al., 2011), and is used extensively as the method of choice in the diagnosis of veterinary GIN (Coles et al., 1992; Coles et al., 2006; Paras et al., 2018). The McMaster technique begins by suspending a set weight of stool in a set volume of saturated salt solution at room temperature. The faecal suspension is then sieved, removing large particles. Centrifugation, and further sieving steps can be included to increase the clarity of the sample and improve egg collection, if necessary, although this is not usually possible in the field. Aliquots of the filtered solution are then loaded into the counting chambers (X 2) of the pre-produced commercially available McMaster slides. Eggs are then counted under high magnification under a light microscope (Coles et al., 1992). FEC (Faecal Egg Count) as EPG (Eggs per Gram) is calculated by multiplying the egg count by a set multiplication factor of 50. The straining step has shown to be a factor in egg loss, which affects final egg counts for McMaster (Paras et al., 2018).

FLOTAC® is a patented salt flotation-based technique that involves a cylindrical plastic housing in which a set volume of faeces and saturated salt solution are combined and centrifuged. The FLOTAC® device consists of the flotation chamber and an integrated counting chamber with 18 mm X 18 mm ruled grid, which permits the counting of the eggs *in situ* by light microscope (Cringoli, 2006). It has been shown to be more sensitive than Kato-Katz when detecting

STH in the field (Knopp et al., 2009; Albonico et al., 2013) as well as effective in the diagnosis of *H. contortus* (Rinaldi et al., 2015). However, it requires centrifugation, is not 100% sensitive, and often results in reduced egg counts (Glinz et al., 2010; Habtamu et al., 2011). The Mini-FLOTAC® is an extension of the original FLOTAC® protocol. The device comprises the base and reading disk, and accessory key and microscope adaptor. Two 1 ml flotation chambers are present in the reading disk with ruled grids for egg counting. Homogenised faeces and saturated salt solution are added to the reading disk, and flotation occurs therein, allowing direct counting on the reading disk. The Fill-FLOTAC® system is also available which enables the sample collection, weighing, homogenisation, filtration, and filling of the reading chambers integrated into one package (Cringoli et al., 2017). The Mini-FLOTAC® has several advantages over Kato-Katz and McMaster in allowing simultaneous detection of helminth eggs and larvae as well as protozoa and cysts (Barda et al., 2013; Cringoli et al., 2017). Analytical sensitivity is lower than FLOTAC®, however, due to the lower volume of faeces analysed. Fill-FLOTAC® also allows for preservation of faecal samples for later analysis without transfer to another container (Barda et al., 2013; Barda et al., 2014). Like FLOTAC®, mini-FLOTAC® is suitable for the diagnosis of human and veterinary helminths (Rinaldi et al., 2015; Cringoli et al., 2017). The mini-FLOTAC® was found to be the most accurate method of egg counting in a comparative study assessing different methods in ruminant livestock species (Paras et al., 2018).

However, there are a number of disadvantages to the use of the various coprological egg counting techniques. There is a poor correlation between worm burden and egg output for certain species (Preston et al., 2014). In addition, there is significant variation in eggs per sample, which occurs due to aggregation of the eggs and non-random distribution through the faecal sample (Woolaston and Baker, 1996; Hunt and Lello, 2012). In general terms, any microscopy-based diagnosis method is prone to certain limitations which are partly dependent on the competence of the staff, the quality of the microscope used, and whether the infection is endemic to the region and thus likely to be commonly encountered (Solorzano-Garcia et al., 2017; Berzosa et al., 2018). The pre-patent period, prior to the worms reaching sexual maturity and thus not

shedding eggs, is also a significant factor in missed diagnoses (Ghosh et al., 2018). Other issues that can influence the detection of eggs in stool relate to the non-random distribution of the eggs within the sample, and daily fluctuations in egg output by the parasite (Levecke et al., 2011; Levecke et al., 2012; Miller et al., 2012; Lamberton and Jourdan, 2015; Solorzano-Garcia et al., 2017). The fecundity of the parasite itself can also be a factor that complicates detection, for example *H. contortus* is remarkably fecund, with a single female capable of shedding up to 10,000 eggs per day, whereas *Trichostrongulus colubriformis* and *Teladorsagia circumcincta* are significantly less fecund (Steer et al., 2003; Preston et al., 2014).

Further recent improvements in the field have centred on the use of automation to improve the diagnostic accuracy and standardisation of microscopy-based techniques. One such technique is FECPAK^{G2}, which combines the accumulation of GIN eggs into one viewing location (Bosco et al., 2014; Cooke et al., 2015), followed by software-based species identification based on morphology (Jiménez et al., 2016), which is inherently more standardisable.

As such, there is a need to build on recent advances to move toward more accurate and standardisable diagnostic tests, and molecular testing has the capability to transform the field. The increased sensitivity of molecular tests decreases the likelihood of missing low intensity infections, and potentially provides greatly improved monitoring and surveillance of infection for epidemiological and public health purposes. Molecular tests rely on the detection via amplification of genetic material present in the sample. As such, the limit of detection is significantly lower than a human at a microscope is capable of. Molecular tests, such as PCR, are based on primers specifically binding to 18-25 bp long sections of the genome (or transcriptome in the case of reverse transcription-based technology). Although there is the possibility for cross reactivity (i.e. amplification of a related target or species rather than the target or species of interest), this is a problem that should be solved during the optimisation process, and any commercially available kit should have been extensively tested to offset this. Highly conserved regions are generally used for primer design to allay the risk of mutation causing issues in detection. This is

especially important in highly polymorphic organisms such as viruses and certain helminths.

Pre-packaged PCR kits are now available for many infections, and significantly reduce both the training needed to implement the test, and the time to result. However, for PCR, expensive thermocycler equipment is essential, and this restricts its applicability in resource-limited settings. Despite this, molecular testing still has an important role to play in this context, as a confirmatory test in symptomatic patients who are negative under coprological microscopy, and for improved monitoring and surveillance of infections by reference and public health laboratories.

1.4 Molecular diagnosis of helminth Infections

Current molecular diagnosis is dominated by PCR based technologies. The technique is already widely used in many clinical laboratories in the developed world for the diagnosis of bacterial (Tsalik et al., 2018), viral (Peaper et al., 2014), protozoan (Khare et al. 2014), and fungal infections (Khot and Fredricks, 2009).

1.4.1 PCR-based technology

Conventional PCR uses a pair of primers to amplify a specific DNA target sequence, such as a gene or section of a gene (Mullis et al., 1986). The technology has been established for a wide variety of infections across human and veterinary medicine, since its inception in the early 1990s. Conventional PCR can be used to detect helminths in faeces as well as other bodily fluids where certain species, such as microfilariae, are present (Gordon et al., 2011). In addition to conventional PCR, with results confirmed by gel electrophoresis, there exist a variety of modified PCR based techniques used in the diagnosis of helminth infections. Multiplex PCR allows the amplification of more than one target using multiple primer pairs and amplicons of different sizes, although great care must be taken to avoid cross reactivity in the case of related species

(Henegariu et al., 1997). Real time (quantitative) PCR or (rt)-qPCR allows quantification by measuring the accumulation of fluorescent tagged amplicons across the duration of the reaction in “real time”. A standard curve can then be used to determine the original concentration of the target DNA (Wong et al., 2005). Restriction Fragment Length Polymorphism PCR (RFLP-PCR) can be used to discriminate between species and/or alleles based on the digestion of an amplicon by a restriction enzyme. The presence of unique SNPs between alleles of the same species, or conserved sequences between different species allows the selective cutting and distinctive pattern of cut and uncut amplicons which can then be examined on a gel (Rohit et al., 2016).

1.4.1.1 PCR diagnosis of helminths

PCR-based technology has been successfully demonstrated for trematodes such as *Schistosoma* spp. *Fasciola* spp., cestodes such as *Taenia* spp. and *Echinococcus* spp., nematodes such as STHs and the causative agents of lymphatic filiariasis (Gordon et al., 2011) and GINs of veterinary importance such as *H. contortus* (Ljungstrom et al., 2018; Hoglund et al., 2019).

However, despite the establishment of PCR-based tests for most major species of veterinary and human helminths, it has significant disadvantages specifically in the field of helminth diagnosis. The biggest drawback is that most helminth infections in humans are diseases of poverty (Hotez et al., 2008), and by their very nature are present in resource-limited settings. This precludes the use of PCR, requiring expensive thermocycling equipment and further equipment for downstream detection, in the diagnosis of most helminth infections in humans (Deng et al., 2019). The same issues are present in veterinary medicine, especially in the agricultural sector, with many farmers already under significant economic pressure both in the high-income countries and LMICs, therefore highly unlikely to justify the costs necessary for such complex testing. Faeces, and other bodily fluids, are significant sources of PCR inhibitors, and thus their removal and subsequent processing of the sample is necessary (Schrader et al., 2012). This adds a further level of complexity to the performance of the test, necessitating reagents, and trained staff (Deng et al., 2019). This relegates the role of PCR to well supplied reference laboratories, mainly for monitoring and

surveillance purposes carried out by disease control agencies at national and international level, and diagnostic facilities for human medicine in the developed world. Recent improvements and lowering costs of portable PCR machinery have facilitated the use of PCR amplification in more point-of-care (POC)/point-of-need (PON) settings, however, these have largely focused on nanopore amplicon sequencing of PCR products for larger scale surveillance purposes (Radhakrishnan et al., 2019; Acharya et al., 2020) rather than using PCR in isolation.

1.4.2 Isothermal amplification technology

Isothermal amplification technology is a relatively recent introduction. Using different techniques, such as strand displacing polymerases, the thermocycling step in conventional PCR is eliminated, allowing the reaction to proceed at a constant temperature. Reactions proceed faster than conventional PCR, and the elimination of thermocycling equipment removes one of the most significant impediments to developing affordable POC molecular diagnostics (Notomi et al., 2000; Martzy et al., 2019), which meet the WHO ASSURRED guidelines of Affordable, Sensitive, Specific, User-friendly, Robust and Rapid, Equipment-free, Deliverable (Peeling et al., 2006).

1.4.2.1 Loop-mediated isothermal amplification (LAMP)

Loop-mediated isothermal amplification (LAMP) is a nucleic acid amplification test based on the use of a strand displacing polymerase, typically *Bst* (Notomi et al., 2000), although some studies make use of *Bsm* for example (Ding et al., 2021). Use of a strand displacing DNA polymerase allows the elimination of the thermocycling step necessary in conventional PCR for strand separation. *Bst* polymerases typically operate at an optimal temperature of 60-65ºC, and amplification proceeds isothermally (Notomi et al., 2000). It is one of the most widely researched and applied isothermal amplification methods available to date (Martzy et al., 2019).

LAMP makes use of two essential (inner and outer) primer pairs (Notomi et al., 2000), and a third optional (loop) primer pair that can greatly increase the speed of the reaction (Nagamine et al., 2002) (Figure 1.3). Primers are targeted to six distinct sections on the target sequence, F1, F2, and F3 on the forward strand, and B1, B2, and B3 on the reverse strand. Sequences complementary to each of F1-3 and B1-3 are denoted by c, for example B1c is complementary to B1 etc. The reaction initiates upon strand displacement and the concurrent binding of the outer primers, termed F3 and B3, and inner primers, termed FIP and BIP, onto the exposed strand to their respective complementary regions: F3c and B3c for outer primers; F2c and B2c for inner primers. This leads to the amplification of the target region of interest. The initial step of LAMP is analogous to conventional PCR amplification, and yields linear DNA fragments which contain the target sites for further amplification. Both the forward and reverse stand are amplified in this manner by their respective primers. The distinguishing feature of the inner primers (FIP and BIP) is the presence of sequence at the 5' end complementary to sequence lying upstream of the primer binding site at the 3' end of the amplicon. Thus, for example the FIP consists of F1c-F2, as polymerisation proceeds the amplicon contains F1 upstream of F2, which then allows F1c to loop around and self-anneal. The 5' complementary (F1c) sequence then binds to the 3' target sequence (F1) forming a loop structure, with the curved edge of the loop formed by the primer binding sequence itself. This then forms a dumbbell structure, which has exposed primer binding sites for further polymerisation initiation by inner primers. In the following "rounds" of amplification this leads to the formation of increasingly large concatenated loop structure amplicons. At the completion of each "round" of amplification, additional primer binding sites also increase exponentially rather than linearly. If loop primers are included in the reaction, a further binding site is targeted to sequence present on the loop structure between the B2 and B1c or F1 and F2c regions (Figure 1.3). This process leads to the accumulation of 10^9 copies per hour (Notomi et al., 2000; Nagamine et al., 2002).

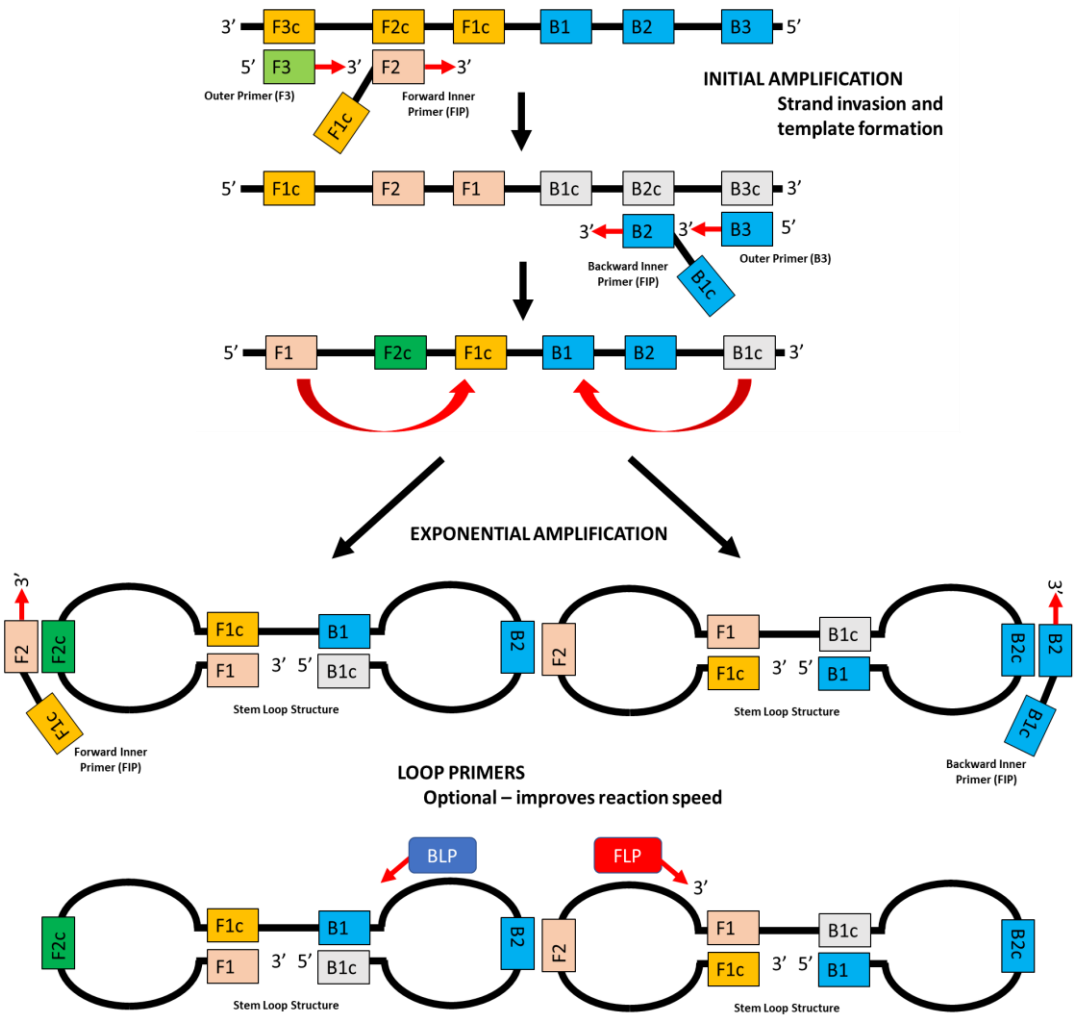

Figure 1.3: Schematic overview of LAMP amplification reaction. LAMP polymerisation begins with the binding of the inner primers to their complementary regions, forming the first stage LAMP amplicon. Self-hybridisation of this amplicon then occurs, yielding the loop amplicon, which contains further sites for polymerisation initiation. Finally, the loop domain itself provides an extra site for the binding of the loop primers, increasing reaction speed.

The complexity of LAMP primers binding to six distinct genomic sequences, and the requirement for the inclusion of self-complementarity, means that manual design of primers would be extremely difficult and time consuming. However, software is available that automates this process, with a varying degree of control over parameters available. The Primer Explorer software, developed by Eiken, is freely available as a web tool (https://primerexplorer.jp/e/). This allows anyone with access to genetic sequences and a computer to design LAMP primers without the need for expensive software licences, and cements its place as an important tool for resource-limited settings. A free to access instruction manual and tutorials are also available. More complex downstream applications of LAMP however, such as allele-specific reactions, will require manual editing

and adjustment of primer designs (Yongkiettrakul et al., 2017; Mohon et al., 2018; Higgins and Smith, 2020).

LAMP is also amenable to multiplexing, and tolerates the simultaneous inclusion of multiple primer sets within the same reaction. This has been applied to viruses with multiple serotypes such as Dengue Virus (Lopez Jimena et al., 2018), HIV (Curtis et al., 2018); SARS-CoV (Kim et al., 2019), Influenza (Chi et al., 2017), detection of different species of *Plasmodium* (Mao et al., 2018), and applied to the detection of bacterial pathogens and antibiotic resistance (Nguyen et al., 2019). A few examples of multiplex LAMP have been demonstrated for helminths, but largely for *Taenia* spp. (Nkouawa et al., 2016; Feng et al., 2017), however, at present this is an area that requires further work and development.

Like most molecular tests, LAMP requires end-point detection to visualise results. Agarose gel electrophoresis has been used in some studies (Elvira-Gonzalez et al., 2017). However, gel electrophoresis is time consuming, and only suitable for use in laboratories. In addition, opening of tubes increases the likelihood of contamination of future reactions with amplicons. Various intercalating fluorescent dyes can be used, which allows the detection of LAMP products either by the naked eye (when UV light is applied) or quantification using qPCR machinery (Oscorbin et al., 2016; Wong et al., 2018). However, care must be taken in choosing the dye, as certain dyes such as SYBR green inhibit the LAMP reaction, and thus must be added to the tube at the end of the reaction (Oscorbin et al., 2016). This has been successfully applied in a number of studies for fluorescent detection and quantification (Wong et al., 2018; Deng et al., 2019; Moehling et al., 2021). Fluorescent dye-based LAMP can also be used in conjunction with smartphone-based applications for field diagnosis, which has successfully been demonstrated for arbovirus diagnosis (Priye et al., 2017; Ganguli et al., 2017) and urinary sepsis (Barnes et al., 2018) to name a few. Various colourimetric dyes are also available, which lead to a colour change in response to the accumulation of $Mg2^{+}$ ions in the reaction solution. The inclusion of dyes such as Calcein, or Hydroxy-Naphthol Blue (HNB) which change colour in response to an increase in metal ions allow this to be detected by the naked eye, or quantified by the use of a colourimeter (Tomita et al., 2008; Goto et al.,

2018). Colourimetric LAMP has been successfully demonstrated for the detection of human and veterinary pathogens (Srisrattakarn et al., 2017; Inoshima et al., 2016; Khangembam et al., 2021). Dyes can be added by the researcher, or commercial kits are available, for example from NEB (NEB #M1800). This approach has significant advantages in that it eliminates the need for any further equipment and is easy to interpret. However, it is not as readily quantifiable as fluorescence-based assays. Colourimetric detection can be used for quantification in a POC setting, as a recently developed smartphone platform termed “smart connected pathogen tracer” has been developed to detect and quantify viral load for HPV and HIV in human clinical samples (Yin et al., 2020). However, this does necessitate the development of specialised software to apply in a POC setting. Current quantification technology based on colourimetric detection lags behind fluorescence detection, as the latter takes advantage of already well-developed resources for qPCR. Smartphone based quantification has also already been demonstrated using the already established method of fluorescence tagged primers on existing qPCR machines, or even read by smartphones (Priye et al., 2017; Huang et al., 2018; Ning et al., 2021). These are emerging technologies that currently require further development before they can be deployed in the field but have the potential to massively improve field-based diagnostics. A further advantage that colourimetric detection has versus fluorescence is that it can be detected by the naked eye, whereas fluorescence detection requires at the very least a source of UV light to excite the fluorophores. The production of magnesium pyrophosphate can also be detected directly as a white precipitate during a LAMP reaction using a turbidimeter (Mori et al., 2001; Mori et al., 2004). However, this method requires some skill and familiarity with the technique, and as such is not as amenable as fluorescence or colourimetry for naked eye detection.

There are, however, some disadvantages to LAMP, although these can be offset by taking appropriate steps. First and foremost, LAMP can be prone to carryover contamination, where amplicons from previous reactions contaminate the environment and lead to false positives. LAMP amplicons are very stable and persist for months, thus it is recommended to open any LAMP tubes far away from set up areas (Tomita et al., 2008). The risk of carryover contamination is

significantly reduced if detection methods that do not require opening of tubes are used, such as colourimetric/turbidity-based methods (Poole et al., 2017; Mauk et al., 2017; Wong et al., 2018). The incorporation of Uracil DNA Glycosylase (UDG), an enzyme that cleaves the Uracil base from the backbone, but leaves natural thymine untouched, and dUTP into the reaction eliminates the issue of contamination, at an increased cost for the extra enzyme. *Bst* preferentially integrates Uracil into amplicons when present in the reaction. If any amplicons then carry over into the next reaction, an initial 25^{o}C incubation, the optimal temperature for UDG, allows the enzyme to cleave any amplicons carried over, leaving the target intact. UDG is then inactivated >50^{o}C (Fallahi et al., 2018). Self-hybridisation of poorly designed/optimised primers can also be an issue, although this can be rectified at the optimisation stage (Meagher et al., 2018). Finally, LAMP is somewhat restricted by the need to locate a minimum of four different primer binding sites within the target region. This can present issues for certain sequences, and increases the risk of primer-primer interactions (Sahoo et al., 2016). The size of the target region is also a factor that must be considered when designing LAMP primers, with products <300 bp ideal, and amplicons >500 bp leading to reduced sensitivity (Li et al., 2016)

To date, LAMP has attracted significant interest for the detection of helminth infections of both human and veterinary importance. Significant progress has led to the development of LAMP assays for parasitic nematodes including STH (Rashwan et al., 2017), filarial worms (Poole et al., 2012; Poole et al., 2017), *Toxocara* spp. (Macuhova et al., 2010; Avila et al., 2021), and *H. contortus* (Melville et al., 2014; Khangembam et al., 2021).

1.4.2.2 Lateral flow (LF) assay LAMP

One of the most significant platforms for use in POC LAMP setting is the lateral flow assay (LFA). The LFA is a paper-based platform which allows the detection of analytes in complex mixtures. LFA is currently widely used in medicine both in hospitals and clinical laboratories, as well as in the field (Koczula et al., 2016). LFA has been used to detect antigen (Boisen et al., 2015; Montesinos et al., 2020) and amplified DNA (Rohrman et al., 2012; Kamphee et al., 2015;

Khangembam et al., 2021), and can be applied to detection of pathogens across a wide range of bodily fluids (Carrio et al., 2015; de Lourdes Moreno et al., 2017), as well as applications in diagnostic tests in veterinary medicine (Nielsen et al., 2009; Burgess et al., 2015; Byzova et al., 2020). LFA has the advantage of being cheap to produce, easy to use with simple readouts, and widely accepted by regulatory authorities as the technology has been tried and tested across many pathogens and fields (Koczula et al., 2016). The LF LAMP assay has also recently been demonstrated for detection of *H. contortus* in field samples (Khangembam et al., 2021).

In an LFA assay, the sample is applied at one end of the paper, often housed in a plastic casing. A buffer is often included with the LFA assay to increase flow along the paper. The supplied buffer also typically contains conjugates such as gold nanoparticles, which aid in visualising the readout on the test strip. DNA probes which recognise a region specific to the LAMP amplicon can be used to immobilise the amplicons onto the test strip, which can then be visualised with biotin for example (Thongkao et al., 2015; Fowler et al., 2016; Huang et al., 2017; Park et al., 2017). Another method involves the labelling of the primers with molecules to which commercial antibodies exist. In this example, one primer is tagged with a generic tag, which will be used to aggregate antibody tagged gold nanoparticles (AuNPs). The other primer is tagged with a specific tag, to which an antibody is conjugated on the test strip. Tagged primer and amplicons are thus immobilised onto the test strip as they pass, however, only amplicons which possess both tags will be visualised, as without the second tag the AuNPs will not aggregate and no colour change will be seen. This method was successfully demonstrated for discriminating between three species of *Entamoeba* (Foo et al., 2017).

1.4.2.3 Other emerging isothermal amplification technologies

Following the publication of the method for LAMP (Notomi et al., 2000), several other methods are rapidly being developed as potential alternatives. Helicase Dependent Amplification (HDA), Recombinase Polymerase Amplification (RPA), Rolling Circle Amplification (RCA), and Cross Priming Amplification (CPA) all

share the common method of target gene amplification at a constant temperature, although their reaction conditions vary significantly (Zhao et al., 2015; Martzy et al., 2019).

Despite the resources for these techniques lagging behind those of LAMP (Martzy et al., 2019), RPA is perhaps the most promising competitor to LAMP to date. First reported in 2006 (Piepenburg et al., 2006), RPA uses bacterial recombinases and single-stranded DNA binding protein to anneal primers to the target sequence, followed by extension and displacement of the native strand by a *Staphylococcus aureus* polymerase. To date RPA is one of the fastest isothermal amplification technologies, and runs at one of the lowest optimal temperatures at 37-42^{o}C (Pipenberg et al., 2006; Lobato and O'Sullivan, 2018). The inclusion of fluorescent probes allows detection and monitoring of the reaction in diagnostic settings (Dahler et al., 2016) and it has been demonstrated successfully to detect many different pathogens (Lobato and O'Sullivan, 2018). In addition, RPA can be used with existing PCR primers. However, reports have shown the reaction can be inhibited by the presence of background DNA found, for example, in blood samples (Rohrman and Richards-Kortum, 2015). In addition, it has been reported that it is difficult for inexperienced users to distinguish between the small difference in fluorescence signal between negative and positive results, and therefore requires a portable optical device to read results with certainty (Cha et al., 2020).

1.5 Diagnosis of resistance

1.5.1 Diagnosis of anthelmintic resistance

Resistance of a pathogen to chemotherapy in its simplest form can be defined as a reduction in efficacy of a drug as compared to the reported efficacy at the time of release (Kotze et al., 2016). Current clinical diagnosis of anthelmintic resistance and measurement of the efficacy of anthelmintic treatment relies primarily on the microscopy based Faecal Egg Count Reduction Test (FECRT) in both human and veterinary medicine (Coles et al., 1992; Levecke et al., 2011;

Kotze et al., 2014). In addition, controlled comparisons of post-mortem worm counts are also used in veterinary medicine and research, however this method requires elective euthanasia of the animals (Coles et al., 2006; Kotze et al., 2014) and as such it is preferrable to avoid where possible due to animal welfare concerns.

1.5.1.1 Faecal egg count reduction test (FECRT)

The FECRT is currently the only on-farm diagnostic test available for assessing anthelmintic resistance (Kotze et al., 2016; Kaplan, 2020). FECRT is also used extensively when monitoring anthelmintic efficacy in mass administration programmes for the treatment of human helminthiases (Levecke et al., 2011; Vercruysse et al., 2011; Levecke et al., 2015) and represents the only widely available diagnostic test for assessing the efficacy of anthelmintic treatment in humans in human helminth endemic regions.

FECRT involves the collection of pre- and post-treatment samples, followed by direct microscopic analysis to compare egg counts (per gram) between the two samples (Coles et al., 1992; Coles et al., 2006; Kotze et al., 2016). Time to result is dependent on the time taken for the drug to exert its effects, as assessment of the post treatment sample must take place after sufficient time has elapsed for this to occur. In some cases, such as ML, the longer assessment period is to allow resistant worms to resume egg laying, which is impeded by the presence of ML, though the worm is not fully paralysed or expelled from the host. In the case of LEV, it is recommended to wait 7 days before examining the post treatment sample (Coles et al., 2006). Faecal samples are collected per rectum on day 0 (of treatment), followed by a second collection from the same group of animals post-treatment (Coles et al., 1992; Coles et al., 2006). An untreated control group should also be included for evaluation of natural changes in egg output during treatment (Coles et al., 1992). Egg reduction is calculated by first determining the arithmetic mean (X), 95% confidence interval, and percentage reduction. Percentage reduction is given by the following formula:

$$Percentage\ Reduction = 100(1 - (X_{\text{treated}}/X_{\text{control}}))$$ (Coles et al., 1992)

Resistance is defined as present if the percentage reduction is <95%, and the confidence interval for a 95% reduction in egg output is <90% (Coles et al., 1992; Coles et al., 2006).

Although its use is widespread and the practice is well established, the FECRT suffers from the same issues as the FEC used for diagnosis of helminth infections, such as subjectivity and imprecision of egg counting techniques (Levecke et al., 2011; Levecke et al., 2012). There is also a lack of an agreed standard egg count method, whether using the geometric or arithmetic mean, as well as the use of single vs pooled samples which further complicates the interpretation of results (Dobson et al., 2009; Falzon et al., 2015). There have been recent attempts to introduce software and other technological solutions to improve the accuracy and standardisation of the FECRT (Torgerson et al., 2014). However, this test still lacks the specificity and sensitivity of a well-designed and optimised molecular test. Finally, in the case of BZs at least, resistance is not detectable when present in <25% of the population (Martin et al., 1989; McIntyre et al., 2018). This is a serious concern, as the FECRT is likely to miss the early emergence of resistance, increasing selection pressure of the compound on the parasite population. Thus, FECRT remains useful in the present day only in the absence of effectively resolved genetic markers and concurrent developed molecular tests.

1.5.2 Other *in-vitro* bio-assay methods

There is also a variety of *in vitro* methods for diagnosis of resistance. However, these are largely based on comparing the hatching of eggs and/or the development and/or motility of free-living larvae in the presence and absence of the drug of interest. These methods have been used in studies elucidating

resistance mechanisms (Sarai et al., 2013; Sarai et al., 2014; Raza et al., 2016). However, they are time consuming and must be carried out in a laboratory setting, which makes them unsuitable as POC tests.

1.5.2.1 Egg hatch assay (EHA)

EHA was first described in 1976 for the detection of resistance to BZs in *H. contortus* and *T. circumcincta* (Le Jambre, 1976), followed by Dobson et al. (1986) who developed the assay for LEV. In brief, the assay involves hatching eggs under a concentration of the drug of interest, such that 99% of eggs should not hatch at this concentration in a fully susceptible isolate. The percentage of eggs hatching under these conditions is then used as an estimate for the proportion of resistant individuals within a population (Kotze et al., 2016). The procedure has been standardised for the diagnosis of BZ resistance in *H. contortus* by European laboratories, with a published standard operating procedure available (von Samson-Himmelstjerna et al., 2009a).

1.5.2.2 Larval development assay (LDA)

LDA was initially developed in the 1990s to evaluate the resistance status of nematodes isolated in Australia. The LDA works on the same principle as the EHA, but extends to the development from eggs to infective L_3 larvae (Kotze et al., 2016). There are several different variants on the basic assay. These are largely divided along the lines of using liquid media (Taylor, 1990), or moist agar (Gill et al., 1995). The assay has been demonstrated as capable of discriminating between resistant and susceptible isolates of *H. contortus* for the major drug classes BZs, ML, and LEV (Gill et al., 1995), and recently has been shown to be effective in identifying monepantel resistance (Raza et al., 2016). LDA however, is no longer appropriate for use with *T. circumcincta*. In general the LDA is now only used to assess resistance in *H. contortus*, and is rarely used for other GINs of veterinary importance (Kaplan et al., 2007; Dolinska et al., 2012; Dolinska et al., 2013).

1.5.2.3 Larval motility and larval migration inhibition assays (LMA and LMIA)

The larval motility and migration assays are often employed as drug development screening tools for drug discovery programmes. They are also used frequently in anthelmintic resistance research beyond drug development settings, and played a role in a number of publications elucidating determinants of LEV resistance (Blanchard et al., 2018) and ML resistance (Kotze et al., 2006; Demeler et al., 2013; George et al., 2018).

At their base, each variation of the assay fundamentally assesses the same action: the active movement of motile L_3 larvae across or through a medium that would otherwise impede their passive movement. The medium can vary, for example the assay can involve the migration out of an agar block (d'Assonville et al., 1996), or migration through soft agar followed by through a fine mesh (Kotze et al., 2006). The assay can also take the form of migration from one side of a mesh to the other suspended in liquid (Demeler et al., 2010). Measurement of motility/migration can be conducted by a simple visual end point assessment by light microscopy. This involves counting the number of larvae that have migrated vs those that have not, which can then be communicated as a percentage and an estimation of the prevalence of resistance within the population. In addition, motility can also be assessed through software, which either tracks the movement of individuals via high-definition video in the case of the WormAssay (computer programme) and Worminator (integrated platform with WormAssay software) (Marcellino et al., 2012; Storey et al., 2014) or in the case of the Automated Larval Motility Assay (ALMA) that tracks motility based on autofluorescence (Blanchard et al., 2018). The latter method has the advantage of being more objective in its assessment versus direct eye microscopy as it is connected to video which can then also be quantified. It is, however, more expensive, and requires complex machinery and software unavailable in many laboratories.

1.5.3 Molecular diagnosis of anthelmintic resistance

Currently, commercial kits for molecular testing of anthelmintic resistance are not available. This is largely due to the varying levels of understanding of resistance determinants for each major class of drug. The uncertain nature of resistance mechanisms for LEV and ML precludes the development of effective molecular tests until such a time as mechanisms or markers are validated (Kotze et al., 2016; Kotze et al., 2020).

The elucidation of three SNPs (F167Y, E198A, and F200Y) determined to be causative for BZ resistance has allowed the development and publication of a number of molecular methods for the detection of BZ resistance in *H. contortus* and other GIN (Kotze et al., 2016; Ramunke et al., 2016). However, these SNPs show varying levels of prevalence worldwide, with some SNPs predominant in different regions (Barrere et al., 2012; Barrere et al., 2013; Chaudhry et al., 2014; dos Santos et al., 2014; Redman et al., 2015; Ramunke et al., 2016). Thus, for any commercial test to be successful, it would need a multiplex format to effectively detect all three SNPs.

A variety of methods have been successfully demonstrated for the detection of BZ resistance in *H. contortus*. Real Time-PCR (RT-PCR) was initially demonstrated by Alvarez-Sanchez et al. (2005) and Allele Specific PCR (AS-PCR) for the F200Y SNP by Coles et al. (2006). AS-PCR works by the inclusion of mismatches relative to the *wt* or resistant sequence which leads to amplification only in the case of the desired allele (Figure 1.4A). Various AS-PCR methods have since been demonstrated for example for E198A (Chen et al., 2009; Zongze et al., 2018) and F200Y (Chagas et al., 2016). Restriction Fragment Length Polymorphism-PCR (RFLP-PCR) (Tiwari et al., 2006; Ghisi et al., 2007) has also been demonstrated as effective for diagnosing BZ resistance. RFLP-PCR involves the amplification of the sequence of interest, followed by restriction digest of PCR products (Figure 1.4B). It, therefore, requires the presence of a restriction site unique to one allele to be effective. Although RFLP-PCR is an invaluable tool for confirming genotype when designing and optimising AS-PCR or when no other test is available, it is time consuming and expensive when compared to AS-PCR, requiring the purchase of restriction enzymes and an extra digestion step.

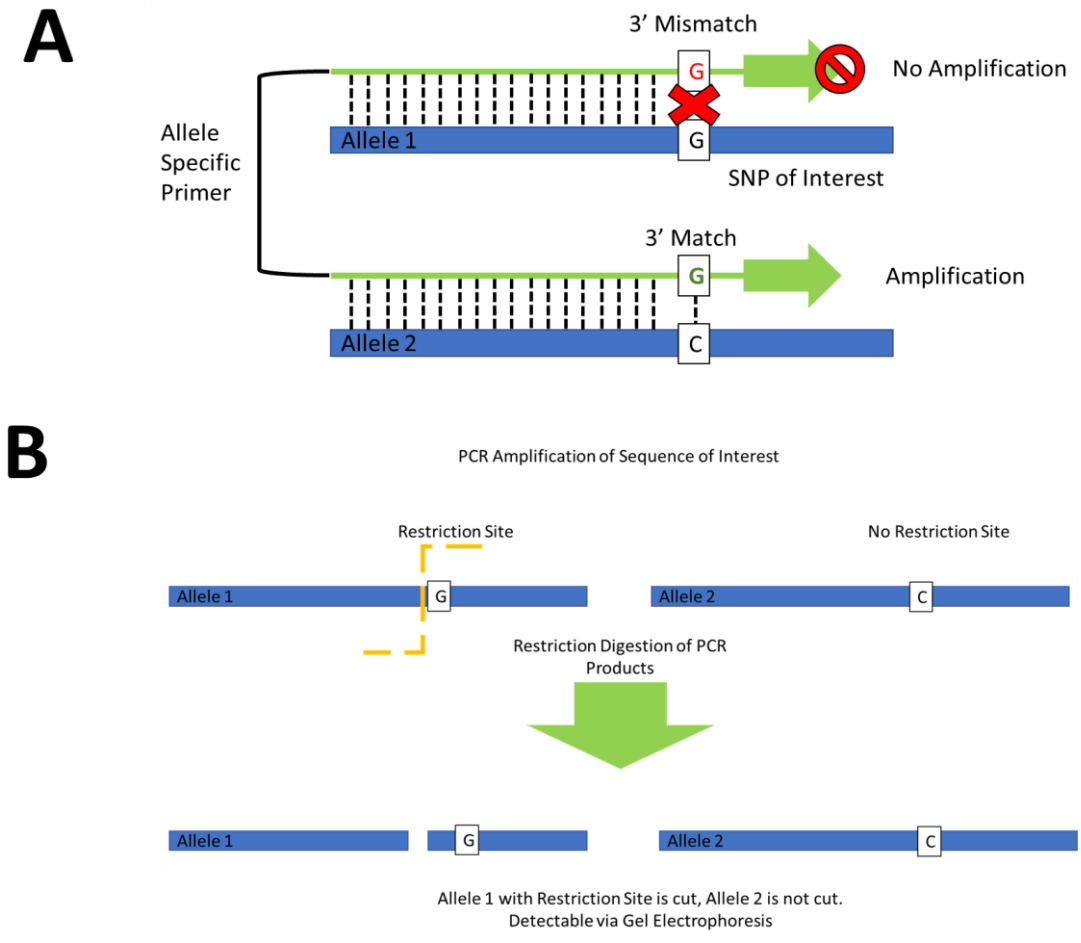

Figure 1.4: Summary schematic representation of two allele specific PCR techniques.

A: Schematic representation of allele specific PCR illustrating the principle of an allele specific primer. 3' Mismatch leads to amplification in only the allele of interest.

B: Schematic representation of Restriction Fragment Length Polymorphism PCR. Initially sequence of interest is amplified by PCR, then followed by restriction digest of PCR products. Only one allele will be cut which can then be identified on an agarose gel.

In addition to PCR based technologies, a pyrosequencing-based approach was demonstrated by von Samson-Himmelstjerna et al. (2009b). Pyrosequencing works by the principle of "sequencing by synthesis" wherein the addition of the correct nucleotide to the pre-prepared DNA template of the sequence of interest leads to the release of pyrophosphate. This, in turn, is converted to ATP by ATP-sulfurylase and thus provides energy to a luciferase enzyme which then generates detectable light (Ronaghi, 2001). Using pyrosequencing, it has been possible to integrate and multiplex all three SNPs for both isoforms of β-tubulin. Pyrosequencing has been validated for use under field conditions (i.e. the

collection of samples from the field rather than laboratory-maintained isolates) (McIntyre et al., 2018; Hinney et al., 2020). Since its inception as a diagnostic tool for BZ resistance, pyrosequencing has been demonstrated as an effective tool to monitor the frequency of resistance alleles within a population. However, despite its advantages as a method, pyrosequencing suffers from the same drawbacks as any other laboratory-based test. It requires expensive equipment and reagents, trained staff, and is not suitable for POC or point-of-need deployment.

Finally, a recently published study demonstrated the first example of a LAMP assay for the detection of the E198A SNP in *H. contortus* (Tuersong et al., 2020). Allele Specific LAMP (AS-LAMP) operates on the same principle as AS-PCR, wherein artificially introduced resistant allele specific mismatches lead to amplification of only the allele of interest. The assay described by Tuersong et al. (2020) was combined with colourimetric detection by the inclusion of HNB dye. Thus, this test represents an important step towards a POC test for BZ resistance. Field samples from sheep and goats in eight regions within the People's Republic of China were analysed, with 100% concordance seen versus E198A AS-PCR (Zhang et al., 2018). However, one serious limiting step to the development of a truly on-farm molecular test for resistance diagnosis in *H. contortus* is DNA extraction. Although Tuersong et al. (2020) describe an assay amenable to POC detection, their method relies on proteinase K digestion of single worms followed by spin column DNA purification using Wizard DNA clean-up (Promega) (Gasser et al., 2006; Avramenko et al., 2015; Tuersong et al., 2020). Finally, initial groundwork has been laid for the multiplex allele-specific LAMP detection of three BZ resistance SNPs (F200Y, E918A, and F167Y), however, this was not fully optimised and served as a proof-of-concept design employing synthetic DNA (Costa-Junior et al., 2022).

1.5.4 Nemabiome

Nemabiome is a recently developed next-generation sequencing approach which combines the principles developed for microbiome sequencing and applies it to species specific identification of parasitic nematodes. Barcoded PCR amplicons target the highly conserved ITS2 rDNA locus (Avramenko et al., 2015) which can

then be used for species specific identification during downstream analysis, allowing for an accurate “nematode biome” to be constructed for livestock (both sheep and cattle) or wild animals (Avramenko et al., 2015; Avramenko et al., 2018; Santa et al., 2021; de Seram et al., 2022). Nemabiome has also been adapted to the detection of BZ resistance SNPs in parasitic nematodes (Avramenko et al., 2019; Melville et al., 2020; Evans et al., 2021). Another significant advantage of the Nemabiome approach is that, compared to nucleic acid amplification test (including isothermal) based diagnostics for BZ resistance (Alvarez-Sanchez et al., 2005; Tiwari et al., 2006; Walsh et al., 2007; Tuersong et al., 2020; Costa-Junior et al., 2022), there is no requirement to individually develop and optimise each, which is labour intensive and may not be economically viable for minor markers of resistance (Roeber et al., 2013b). Thus, Nemabiome presents a significant advantage when assessing the presence of minor, or multiple markers, on a large scale by allowing cost effective and fast multiplexable amplicon sequencing (Avramenko et al., 2019). However, despite recent drops in cost of next-generation sequencing technology, this technique still remains cost-prohibitive for anything other than large-scale surveillance or research studies. It is also not currently applicable to single-worm genotyping applications, due to the high cost and labour requirements necessary to barcode individual primers.

1.5.5 Molecular diagnosis of resistance at the point-of-care

Although a molecular test can greatly improve the sensitivity and specificity of detection of a pathogen, ultimately this benefit will only be relevant if the test can be delivered in such a way that is amenable to fast and low-cost diagnosis. These parameters naturally vary depending on the environment and industry in which the test is deployed. Eliminating the use of expensive equipment is one of the most important aspects to developing POC tests. The advent of isothermal amplification has been one of the most significant steps to making molecular diagnosis truly POC by eliminating the need for expensive thermocycling equipment necessary for conventional PCR. The development of colourimetric visualisation of isothermal amplification and lateral flow-based technology combined with isothermal amplification has been another invaluable step, which

eliminates the need for fluorescence or gel electrophoresis-based methods to detect nucleic acid amplification. In addition to these well characterised methods of POC detection there are a number of emerging technologies and methodologies that have the potential to further transform the field of POC diagnosis. The proliferation of relatively inexpensive smartphones in the last decade has also opened up new avenues for POC diagnosis, placing high definition camera technology, mobile internet, and high computing power into the hands of technicians and medical professionals working in the field.

1.5.5.1 Detection of genetic polymorphisms as resistance markers

One of the most common genetic markers for resistance across most species of pathogen are SNPs. SNPs have been identified for BZ resistance in *H. contortus* (Kwa et al., 1994), and major human pathogens such as *Plasmodium* spp. resistance against multiple anti-malarial drugs (Damena et al., 2019); *Mycobacterium tuberculosis* (Fakhruzzaman et al., 2019), and hospital acquired pathogens such as methicillin resistant *Staphylococcus aureus* (MRSA) (Prosperi et al., 2019), and *Acetinobacter baumanii* (Lee et al., 2018). Thus, the detection of SNPs predictive of resistance is of great value for POC diagnosis of resistance.

SNP markers can be detected by Allele Specific-LAMP (AS-LAMP) (reviewed by Gill and Amree, 2020) and are the most technically demanding types of polymorphism to detect, as primers must be designed with artificial mismatches in a manner similar to AS-PCR. Optimisation is a time-consuming process, that can require the testing of multiple different primer sets in order to determine the ideal combination and number of mismatches (Chahar et al., 2017; Mohon et al., 2018; Chahar et al., 2019).

Insertions and deletions (indels) are a common genetic polymorphism used as resistance markers. Indels are not technically demanding to detect using molecular testing. Indels can be detected by placing primers within the deleted sequence, or spanning the inserted sequence, which allows for effective disruption of amplification in the presence or absence of the indel, respectively. An example of this protocol has been successfully demonstrated for β-lactamase carbapenemase resistance gene in the nosocomial pathogen *Acetinobacter baumanii* (Mu et al., 2016). Resistance in many bacterial species can also be

conferred by horizontally or vertically transferred resistance plasmids, which can accumulate multiple resistances on a single plasmid, or multiple resistance plasmids (Qi et al., 2016). These resistance mechanisms are detected in the same way as any LAMP assay, by designing LAMP primers specific to the gene or sequence of interest and present no practical challenge beyond conventional LAMP assay optimisation.

1.5.6 End-point point-of-care detection methods for genetic polymorphism resistance markers

A LAMP assay in and of itself is not necessarily a POC test, unless the end-point detection method yields a result that can be analysed at the POC or PON. The simplest method for end-point detection for POC/PON LAMP is the aforementioned colourimetric detection (Tanner et al., 2015), which allows naked eye visualisation of the result. This has been successfully applied, for example, for the detection of multi-drug resistance in *Mycobacterium tuberculosis* (Lee et al., 2010) and K76T SNP in the *Plasmodium falciparum* chloroquine resistance (*Pfcrt)* gene (Chahar et al., 2017).

1.5.6.1 Lateral flow assays for point-of-care resistance diagnosis and SNP genotyping

In addition to colourimetric detection, lateral flow assays have successfully been demonstrated for POC LAMP resistance diagnosis. The dihydrofolate reductase thymine synthase (*dhfr-ts*) gene, confers pyrimethamine resistance in *P. falciparum* in various field samples collected in different regions. Different mutations in *dhfr-ts* give rise to varying levels of resistance, from low-level resistance to a single drug up to high level resistance, when multiple mutations are present in a single allele (Basco et al., 1996; Rastelli et al., 2000; Chusacultanachai et al., 2002; Sridaran et al., 2010). Current detection relies on either *in vitro* culture with pyrimethamine and measurement of a phenotype, WGS, or RT-PCR (Rantala et al., 2010; van Lenthe et al., 2019). A multiplex LAMP assay was designed to detect each of four causative SNPs conferring

differing levels of pyrimethamine resistance using the genome of a quadruple mutant *dhfr-ts* strain of *P. falciparum*. Primers were then conjugated with an FITC probe, which allowed detection by lateral flow strip (Yongkiettrakul et al., 2017).

1.5.6.2 Modified LAMP primer enzymatic cleavage-based technologies

Several studies have demonstrated an improvement to LAMP technology which improves SNP genotyping by using a modified loop primer with a mismatch at the 3' end, coupled with the addition of an extra proof-reading polymerase, which leads to the cleavage of the 3' end in the case of a mismatch (Kuzuhara et al., 2005; Chen et al., 2020; Higgins and Smith, 2020; Ding et al., 2021). This probe cleavage is then used to genotype SNPs with emission of fluorescence. This also has the advantage of eliminating the need for time consuming optimisation of an allele specific primer set with artificial mismatches. At present, detection has only been demonstrated using a quencher and fluorophore. The liberation of the fluorophore from the quencher upon cleavage at the mismatch site leads to fluorescence which can be detected by qPCR machinery (Higgins and Smith, 2020; Chen et al., 2020; Ding et al., 2021), although the technology could also be amenable to lateral flow detection or smartphone based fluorescence detection (Ganguli et al., 2021).

Other studies have shown in principle how this type of cleavage can also be adapted for lateral flow end point detection. A technique known as Specific High Sensitivity Enzymatic Reporter unLOCKing (SHERLOCK), which utilises the collateral cleavage of a reporter molecule by a CRISPR-Cas system (Gootenberg et al., 2017). A CRISPR sequence is designed to recognise the target sequence by the inclusion of a PAM Spacer motif (a guide sequence that targets the Cas protein to the sequence of interest) which is then recognised by Cas proteins for cleavage. The target sequence is amplified by RPA in order to generate sufficient template. As the target sequence is cleaved, the reporter molecule is conjugated with fluorophore and the quencher cleaved by the Cas protein. This is then detected by a fluorescence reader, and allows for relative quantification of the sample. In addition, it was shown that cleavage of the reporter was

detectable by lateral flow. This was achieved by two antibody test lines on the strip, one for the quencher, and one for the fluorophore. Thus, if the reporter molecule has not been cleaved, it would be captured by the first antibody, but if it has been cleaved, then one half will be captured there, and the other half by the second antibody (Gootenberg et al., 2017). Thus this principle is amenable to adaptation for cleavage of a 3' mismatched loop primer, as well as having its own intrinsic value as a POC test in itself. The lateral flow SHERLOCK technique was then successfully adapted by Sullivan et al., (2019) for the detection of White Spot Virus in Shrimp using a colourimetric paper based lateral flow assay. The application of SHERLOCK and LFA to RPA solves many of the issues which hamper RPA development into a fully POC amenable assay, as typically RPA require fluorescence detection and expensive machinery, whereas this technique offsets this. However, one issue that remains is the necessity of designing and including PAM spacer motifs into the product, which adds an extra level of complexity to the technique. It also raises the issue of indirect detection, which can present some problems for the accuracy of the test, as the target sequence itself is not directly detected. Nevertheless, as this technology improves and more examples are demonstrated, it also has the potential to transform resistance diagnosis at the POC/PON.

1.5.6.3 Quantification at the point-of-care/need

The above methods of end-point detection are sufficient for simple yes/no tests, which simply provide an answer as to whether resistance is present in a given sample population. However, in certain circumstances it is important to understand the proportion of a population that is resistant, say in the case of emerging resistance. Alternatively, it may be necessary to quantify the resistance to different drugs in a multi-drug resistant population. A significant hindrance on developing POC quantitative molecular tests has been end-point detection method for quantification (Nanayakkara and White, 2019). Many studies to date have demonstrated fluorescence-based quantification methods using redesigned loop primers as molecular beacons (Liu et al., 2017) or a variety of fluorogenic probes (Kubota et al., 2011; Jenkins et al., 2011;

Nanayakkara and White, 2019). Although these studies have all demonstrated effective detection in the laboratory, this has usually required the use of qPCR machinery for fluorescence detection, which requires further engineering of POC devices to work in the field.

In the past five years numerous studies have been published demonstrating how quantitation can be carried out with handheld devices, in many cases by modifying smartphone cameras to do the work of qPCR machinery. These are emerging technologies, and currently most studies have focused on end point detection for presence of an infection, rather than resistance.

Nevertheless, the technology demonstrated in the following studies is fully amenable to adaption for use on any LAMP assay optimised for resistance detection.

Urinary sepsis is a complex and potentially lethal syndrome which can be caused by several different bacterial species, wherein diagnosis is usually based on culture of urine samples (Simerville et al., 2005). A smartphone-based LAMP (SmaRT-LAMP) assay was developed using previously published primer sets for multiple pathogens causative for urinary sepsis infections. A qLAMP device was then built using an aluminium sample block placed on a commercial hotplate and encased within a carboard housing painted black to prevent ingress of light. An LED light source was then fixed inside the box, and a Samsung Galaxy smartphone with light filter was used to detect emitted green light from the reactions. They demonstrated quantitative real time analysis using an app designed as part of the study (Bacticount – made available on the Google Play app store). Intra-species differentiation and quantitation was shown to be accurate compared to conventional qPCR, and the entire qLAMP apparatus could be manufactured for $100 and run on a simple DC power source (Barnes et al., 2018). The same fluorescence detection principle was also previously applied to the multiplex detection of Zika Virus (ZIKV), Dengue Virus (DENV), and Chikungunya Virus (CHIKV) in a single sample. These three viruses all have similar symptoms in most cases, however, in 1-2% of cases DENV can cause severe haemorrhagic fever, whereas ZIKV can cause birth defects. All three co-circulate in many parts of the world, thus differentiation is important for triage and treatment. The study demonstrated the use of a portable “LAMP box”

powered by a USB power bank and made use of a custom smartphone app and a novel algorithm to increase the detection of fluorescence signals. The technique was able to multiplex and accurately quantify the three viruses in a single sample (Priye et al., 2017). Another study demonstrated a Bioluminescence Assay in Real Time LAMP assay (BART-LAMP) in an attempt to eliminate the need for a light source and optical filters for fluorescent-based detection (Priye et al., 2017); heating of the LAMP reaction was carried out without electricity using an exothermic chemical reaction. This study also used a custom designed app, and showed accurate quantitative detection of ZIKV and HIV in urine and saliva within 45 minutes (Song et al., 2018). Finally, a recent study demonstrated a quantitative molecular detection platform named “Smart Connected Pathogen Tracer” (SCPT) which uses smartphone camera-based colour analysis to quantify HPV DNA, and HIV RNA from clinical samples using a colourimetric LAMP assay. Sensitivity was comparable to conventional qPCR machinery (Yin et al., 2020). Finally, POC qLAMP for SARS-CoV-2 has also been demonstrated (Ganguli et al., 2020).

The global proliferation of the SARS-CoV-2 pandemic has been a significant driver of interest in the application of a variety of nucleic acid amplification tests to detection of the SARS-CoV-2 virus. There have been extensive examples of both LAMP and PCR based assays (Augustine et al., 2020; Rai et al., 2021; Pu et al., 2022; Kang et al., 2022), with significant translational potential beyond SARS-CoV-2 diagnosis.

A wealth of emerging technological solutions have been demonstrated for LAMP to date, however, there is a lack of studies demonstrating the application of these technologies to resistance diagnosis. This is the next logical step in which research needs to move. Given the amenability of these techniques to both quantification and multiplexing, the ideal standard to move towards would be a test which provides both detection of the pathogen, and quantification of any known resistance markers in a single sample population. At the time of writing, all the necessary tools are available to make this possible, for specific pathogens. All that is required is to combine and optimise the existing technology, and although this still poses a significant challenge, also highlights

how new technology can open previously inaccessible areas to molecular diagnosis.

1.5.6.4 DNA extraction at the point-of-care/need

DNA (or RNA) extraction at the POC/PON is another significant hurdle in producing a truly POC/PON test, which can give a result at the site of sample collection with minimal extra processing or expensive machinery. The extent of the challenge depends on the organism. For example, a paper in which a SARS-CoV-2 LAMP assay with integrated sample processing and smartphone-based readout in a plastic cartridge-based system demonstrated a result within 30 mins of sample collection (Ganguli et al. 2020). Thus, this study shows a significant improvement as, prior to the COVID-19 pandemic, in most diagnostic virology laboratories viral RNA was extracted with dedicated extraction kits that use silica-based membrane extraction requiring centrifugation steps (Klenner et al., 2017). However, the lysis of virions and DNA/RNA extraction presents significantly less technical challenges than DNA extraction from other organisms. Many virions can be effectively lysed by heat, often at temperatures below that of isothermal amplification technologies such as LAMP. As such there has been a proliferation of direct detection POC assays demonstrated for various viral infections in recent years such as those for Arboviruses (Mehta et al., 2019; Seok et al., 2020) and the recently emerged SARS-CoV-2 (Wee et al., 2020; Ganguli et al., 2020; Wei et al., 2021) based on simple thermal lysis.

There is also a variety of protocols developed which make use of paper-based technology for DNA/RNA extraction at the POC. These studies have mostly concentrated on viral infections wherein clinical samples (blood or saliva for example) are pipetted onto a specialised paper membrane such as Whatman filter paper or polyether sulfone (PES) (Rodriguez et al., 2015), which is then used as the site for *in situ* lysis of the virions within the sample. This can be carried out using specialised viral lysis buffers, such as those containing guanidine isothyocynate (Cao et al., 2012). The DNA/RNA can then be either eluted from the paper medium, or it can be combined with LAMP (Rodriguez et al., 2015) or RPA (Naik et al., 2019) for *in situ* amplification. *In situ* RNA

extraction and LAMP amplification was successfully demonstrated for Influenza A virus (H1N1) and combined with a lateral flow assay for a sample collection to result in a POC test (Rodriguez et al., 2015). Integrated *In situ* nucleic acid extraction and amplification using paper-based technology has also been successfully demonstrated for HPV 16 (Rodriguez et al., 2016), and rotavirus A (Ye et al., 2018).

Bacterial samples present a greater challenge than viral samples from which to extract DNA due to the presence of cell membrane and cell walls in many bacteria, with certain bacteria such as those able to sporulate proving refractory to both chemical and enzymatic lysis (de Bruin et al., 2019). Thus, simple paper based *in situ* lysis is not practical for many bacterial samples. This has been worked around by the development of more specialised systems based on mechanical or chemical lysis within integrated microfluidic devices. A system has been developed for the multiplexed DNA extraction and LAMP detection of *Escherichia coli, Klebsiella pneumoniae,* and *Staphylococcus aureus* (Hui et al., 2018). Bacterial culture samples were passed three times through a chitosan-modified glass filter paper pad to lyse cells, followed by water washes, with the resulting eluate aspirated into adjacent capillaries with LAMP reagents for *in situ* amplification in the same device. End point detection was carried out using an integrated smartphone detection (Hui et al., 2018). A similar protocol has been developed for respiratory samples for *Steptococcus pneumoniae* and *Mycoplasma pneuomoniae,* wherein a paper and polymer based microfluidic chip used on-chip magnetic particle-based nucleic acid extraction, followed by *in situ* LAMP amplification. Detection of amplification was accomplished by a combination of colourimetric dye and integrated smartphone readout (Wang et al., 2019a). However, although these solutions are functional and well designed, they add complexity to the extraction process. Although it is advantageous to carry out the whole process at the POC, if this requires complex bespoke designed instruments then it may be more cost effective to sacrifice patient-side detection for a better equipped diagnostic laboratory. This issue is particularly important in resource limited settings.

Finally, the detection of helminth infections, both human and veterinary, presents a unique set of difficulties compared to viral and bacterial infections.

Non-invasively collected (i.e. faecal/urine instead of post mortem or surgically collected) samples typically yield low quantities of target DNA, as well as the high potential for contamination with bacterial nucleic acids (Doyle et al., 2019) in addition to copious PCR inhibitor molecules (Monteiro et al., 1997). Contamination with non-target DNA from faecal bacteria is a potential issue when dealing with sequencing-based technologies (Doyle et al., 2019), although prior target amplification by LAMP can offset this issue if using a portable sequencer for diagnostics (Imai et al., 2017; Runutwene et al., 2018; Imai et al., 2020. Helminth eggs are typically small, and not present in a comparable quantity within a sample to viruses or bacteria, which limits the amount of DNA and genome copies available. Helminth eggs are also generally durable and resistant. A recent study by Doyle et al. (2019) compared the efficacy of various helminth DNA extraction protocols, namely *Nexttec*, *Bloodspot, CGP* (Moore, 2018), *ForensicGem (FGM),* and *PicoPure*. However, this was done for WGS rather than POC diagnosis, thus the end point requirements were somewhat different. Nevertheless, as a survey of available helminth egg DNA extraction methods it is valuable. However, although there are relatively fast extraction protocols available such as the *Bloodspot* extraction, these still require centrifugation steps, or protease digestion followed by heat inactivation, in the case of the *CGP* protocol (Moore, 2018; Doyle et al., 2019). This presents a significant barrier to truly POC diagnosis of helminth infections. Current successfully developed LAMP assays for *Necator americanus* (Mugambi et al., 2015) or *H. contortus (*Melville et al., 2014) rely on proteinase K digestion and/or centrifugation. The same issue is true for an RPA assay developed for *Schistosoma haemotobium*, which relies on DNA extraction with centrifugation (Rostron et al., 2019). This is an unfortunate consequence of both the complexity of the organisms that cause human and veterinary helminth infections, and a lack of funding and research being dedicated to helminth infections.

1.5.6.5 Portable NanoPore MinION sequencing and LAMP

Finally, one of the latest developments with the potential to completely transform the future of diagnostics and resistance surveillance has been the introduction of small portable sequencers like the Oxford Nanopore MinION (Jain et al., 2016). The MinION weighs 90g, and works on the basis of nanopore flow cell which sequences bases by changes in ionic current of each nucleotide (Branton et al., 2008; Manrao et al., 2012; Deamer et al., 2016). Single strand sequencing occurs as the DNA stand passes through the nanopore, and extremely long read lengths are possible via this method (Jain et al., 2016). MinION has recently been shown to be a valuable tool in genomic surveillance during outbreaks and for rapid identification in areas with limited resources such as during the Ebola outbreak in 2014 (Hoenen et al., 2016; Quick et al., 2016). However, one drawback of MinION sequencing is it shows much higher error rates than other next generation sequencing technology (Jain et al., 2016; Imai et al., 2020). The accuracy of the R9.4 flow cell is reported to be ~90% (Imai et al., 2020) and thus due to this high error rate regions must be sequenced with at least 50-fold read coverage to accurately determine genotypes. This is where the advantage of combining the assay with LAMP becomes clear, as LAMP generates large quantities of concatenated amplicons, with multiple repeats of the target sequence (Notomi et al., 2000). Thus, when LAMP is used to enrich the target DNA it can then be combined with MinION sequencing allowing for incredible depth sequencing carried out in the field. LAMP-MinION has previously been used for serotyping of DENV (Yamagishi et al., 2017) and genotyping of *Plasmodium* (Sugano et al., 2015). A recent study combined LAMP with MinION for surveillance and monitoring of potential molecular markers of artemisin (ART) resistance in *P. falciparum* (Imai et al., 2020). Mutations in the *kelch13* propeller gene have previously been associated with ART resistance *in vitro* and *in vivo* (Ndwiga et al., 2021). The study combined LAMP which was used to amplify a target sequence for the *kelch13* gene, coupled with sequencing with MinION, eliminating the need for multiple allele specific primers, and enabling a large volume of sequencing data to be collected from clinical samples. The entire pathway from DNA extraction to variant calling was completed in 3 hours (Imai et al., 2020). Although at present the MinION costs between £1000-5000

and £90 for a single flow cell, this is still significantly less than even a moderately priced qPCR or conventional PCR thermocycler. In addition, the ability to carry out long read sequencing in the field is an incredibly valuable tool, which has the potential to eliminate the need for conventional sequencing and PCR in resource limited settings in the future.

1.5.6.6 Reagents and sample processing

The technologies discussed thus far have focused on solving the problem of the detection of the genetic polymorphism at POC/PON, and how that detection is then read by the technician or healthcare professional. However, all these technologies are only useful if genetic material can be obtained that is appropriate for use with a molecular test. Furthermore, if the primary site of testing is located in difficult to access settings, or settings with extreme resource limitations such as lack of electricity, then most molecular tests at that point become unfeasible, even with isothermal amplification.

1.5.6.7 Elimination of cold chain storage

Elimination of the cold chain is one of the most challenging problems when dealing with POC/PON testing in many rural and resource limited settings (Carter et al., 2017). In many regions where the need for POC/PON testing is greatest there is also poor transportation infrastructure, lack of electricity and, to complicate the problem further, extreme temperatures, which can be in excess of 40^{o}C in many tropical and sub-tropical areas.

Lyophilisation is a process by which LAMP reagents are freeze dried and can then be stored at 4^{o}C, ambient temperature, or 37^{o}C for up to one month without loss of sensitivity (Chen et al., 2016; Chen and Ching, 2017). When stored at 4^{o}C reagents were stable for up to 3 months (Chen et al., 2016). A further study reported that lyophilised LAMP reagents can be stored at 25-45^{o}C for up to 72h without loss of sensitivity (Hayashida et al., 2015). The addition of a dialysis step to remove glycerol and the inclusion of the non-reducing sugar trehalose

has been shown to improve the stability of lyophilised reagents, as was successfully demonstrated for a LAMP assay designed to detect the Zaire Ebola Virus. LAMP reagents lyophilised under this protocol were found to be stable for up to 55 days at 10^{o}C and up to 20 days at 40^{o}C (Carter et al., 2017). Lyophilised LAMP was subsequently successfully demonstrated for a variety of major pathogens and neglected tropical diseases common in resource limited settings such as malaria (Lucchi et al., 2016), *Coxiella burnetti* (Chen et al., 2016; Chen and Ching, 2017), *Vibrio cholerae* (Syafirah et al., 2018), ZIKV (Escalante-Maldonado et al., 2019), and a multiplexed assay for the arboviruses ZIKV, DENV and CHIKV (Yaren et al., 2017), and Buruli Ulcer Disease (Beissner et al., 2015).

1.6 Concluding remarks

Given the widespread loss of economic activity, extensive morbidity, and poor quality of life caused by chronic infections in humans, and damage to livelihoods and food security by veterinary helminths, there is an urgent need to expand on recent successes in improving helminth diagnosis, and translate emerging technologies to develop truly POC/PON diagnostics. Finally, this also highlights the importance of a one health approach to medicine, particularly in LMICs where large numbers of people are at risk of zoonotic infections and are highly dependent for their day to day survival on the health of their livestock animals.

1.7 Aims of the study

The principal aims of the current study are as follows:

1. Examine putative markers of resistance to determine if any of these can serve as a diagnostic marker of LEV resistance, and if not then identify an alternative marker of resistance

2. Validate the chosen marker of resistance using single worm analysis

3. Develop a laboratory-based gold-standard diagnostic assay to genotype populations

4. Develop a proof-of-concept POC amenable diagnostic test for eventual use in the field

5. Explore further putative resistance markers identified in WGS data

2 Materials and Methods

2.1 Animal handling and ethics statement

All animal experimental procedures were examined and approved by the Animal Welfare Ethical Review Board of the Moredun Research Institute, Penicuik, Scotland. All experiments were conducted under approved UK Home Office licenses following the Animals (Scientific Procedures) Act of 1986 (Home Office licence number: PPL 60/03899).

2.2 Parasitic nematode isolates

2.2.1 *Haemonchus contortus* isolates

A summary of all *H. contortus* isolates used within the current study is listed in table 2.1, including the origin of the isolates and LEV efficacy of the isolates where available.

Table 2.1: Summary of *Haemonchus Contortus* isolates used in the study

Isolate	Name in Text	Resistance Status	Comments	LEV Efficacy (where available)
MHco18(UGA2004)	MHco18(UGA2004)	LEV, BZ, IVM Resistant	Resistance developed in the field (USA) (Williamson et al., 2011), isolated and passaged in laboratory since 2004. Maintained at the Moredun Research Institute.	Drenchrite® ED_{50} – 6.98 μM (Williamson et al., 2011)
MHco3(ISE)	MHco3(ISE)	Sensitive to all major classes of anthelmintics	Originally isolated from Eastern Africa (Hoekstra et al., 1997; Roos et al., 2004), inbred and passaged in laboratory. Further inbred to generate MHco3(ISE).N1 used to create draft *H. contortus* genome (Laing et al., 2013). Maintained at the Moredun Research Institute.	Reported as LEV susceptible (Hoekstra et al., 1997; Roos et al., 2004; Laing et al., 2013; Doyle et al., 2021), however, detailed LEV efficacy data is not available
MHco3/18 (F3 Generation)	MHco3/18	LEV, BZ, IVM Moderately Resistant	Genetic cross of MHco3(ISE) and MHco18(UGA2004) (Doyle et al., 2018). Maintained at the Moredun Research Institute.	LEV administration during passage of the F2 generation (July 2016) led to a 94.92% reduction in EPG 3 days post-treatment; 92.65% reduction in EPG 7 days post-treatment; 89.71% 13 days post-treatment; 81.15% reduction in EPG 21 days post-treatment

White River Strain MHco4(WRS)	MHco4(WRS)	BZ, IVM Resistant LEV Sensitive	Isolated from a field population in Transvaal, South Africa (Van Wyk et al., 1989; Jeannin et al., 1990). Laboratory passaged following isolation. Maintained at the Moredun Research Institute.	LDT (Lacey et al., 1990) LD_{50} 1.4 μM reported by Le Jambre et al. (1995)
Chiswick Avermectin Resistant strain MHco10(CAVR)	MHco10(CAVR)	IVM Resistant LEV and BZ Sensitive	Isolated as IVM resistant extraneous contaminant of a laboratory passaged *Trichostringylus colubriformis* strain in Australia (Le JAMBRE, 1993). Laboratory passaged following isolation. Maintained at the Moredun Research Institute.	LDT (Lacey et al., 1990) LD_{50} 0.58 μM reported by Le Jambre et al. (1995)
Kokstad	KOK	LEV, IVM, BZ Resistant	Isolated from South Africa and Laboratory passaged following isolation. Maintained at INRA, Tours, France (Fauvin et al., 2010)	Reported as LEV resistant (Neveu et al., 2007), however, detailed LEV efficacy data is not available
Farm 001	Farm 001	LEV Resistant	Field isolate derived from a farm in the Southern United States with treatment history of LEV, phenotypically LEV resistant	DrenchRite® ED_{50}: 9.36 μM (Doyle et al., 2021; Farm 4 in that study)
Farm 002	Farm 002	LEV Susceptible	Field isolate derived from a farm in the Southern United States without treatment history of LEV, phenotypically LEV susceptible	DrenchRite® ED_{50}: 0.57 μM) (Doyle et al., 2021; Farm 7 in that study)

2.2.2 *Teladorsagia circumcincta* Isolates

A summary of all *T. circumcincta* isolates used within this study is listed in table 2.2, including the origin of the isolates and LEV efficacy data of these isolates where available.

Table 2.2: Summary of *Teladorsagia circumcincta* isolates used in the study

Isolate	Name in Text	Resistance Status	Comments	LEV Efficacy
MTci2	MTci2	BZ, IVM, LEV Susceptible	Isolated from a field population in England before 1970, and originally maintained at the Central Veterinary Laboratories, Weybridge, UK. until 2000, (Turnbull et al., 2019) Phenotypically susceptible to all major anthelmintics (Skuce et al., 2010; Bartley et al., 2015; Turnbull et al., 2019). Laboratory passaged following isolation. Maintained at the Moredun Research Institute since 2000 (Skuce et al., 2010; Turnbull et al., 2019).	Reported as LEV susceptible (Skuce et al., 2010; Bartley et al., 2015; Turnbull et al., 2019), however, detailed LEV efficacy data is not available
MTci5	MTci5	BZ, IVM, LEV Resistant	Isolated from a Scottish lowland sheep farm. Phenotypically resistant to BZ, IVM, LEV (Sargison et al., 2001; Bartley et al., 2005; Bartley et al., 2015). Laboratory passaged following isolation. Maintained at the Moredun Research Institute.	Reported as LEV resistant (Sargison et al., 2001; Bartley et al., 2005; Bartley et al., 2015), however, detailed LEV efficacy data is not available

2.3 Bioinformatics

2.3.1 Whole genome sequencing data analysis

Visualisation of *H. contortus* WGS data was carried out in Geneious 11.1.5 (Biomatters Ltd, Auckland, New Zealand) using BAM files kindly provided by Dr Stephen Doyle, Wellcome Sanger Institute. The BAMs were generated from WGS of pools of 200 L_3 from MHco18(UGA2004), MHco3(ISE), and pre-/post-LEV MHco3/18 F3 as described in Doyle et al. (2020) and Doyle et al. (2022), and MHco4(WRS) and MHco10(CAVR) as described by Redman et al. (2012). Reads were then mapped to the WBPS16 assembly (https://ftp.ebi.ac.uk/pub/databases/wormbase/parasite/releases/WBPS16/species/haemonchus_contortus/PRJEB506/haemonchus_contortus.PRJEB506.WBPS16.genomic.fa.gz) and annotations (https://ftp.ebi.ac.uk/pub/databases/wormbase/parasite/releases/WBPS16/species/haemonchus_contortus/PRJEB506/haemonchus_contortus.PRJEB506.WBPS16.annotations.gff3.gz) the *H. contortus* genome and uploaded to Geneious 11.1.5. Subsequent further custom annotations of the BAM files were carried out to mark areas of significant polymorphism (SNPs or deletions of interest), and primer bind loci. A full summary of all WGS data used in the current study is listed in table 2.3.

Table 2.3: ENA accession identification and further information for WGS data used in the study

Sample	Sample Identification	ENA Accession	Resistance Status	Reference
MHco3(ISE)	MHCO3_P0_L3_n200_01	ERS2532042	BZ, IVM, LEV Susceptible	Doyle et al. (2022)
MHco18(UGA2004)	MHCO18_P0_L3_n200_IVM_01	ERS2532043	BZ, IVM, LEV Resistant	Doyle et al. (2022)
MHco3/18 F3 XQTL Pre	XQTL_F3_L3_n200_control_pre_01 XQTL_F3_L3_n200_LEV_pre_01	ERS1420441 ERS1420447	BZ, IVM, LEV Moderately Resistant	Doyle et al. (2022)
MHco3/18 F3 XQTL Post	XQTL_F3_L3_n200_control_post_01 XQTL_F3_L3_n200_LEV_post_01	ERS1420442 ERS1420448	BZ, IVM, LEV Moderately Resistant	Doyle et al. (2022)
US Farm Samples	Hc_RKUSA_L3_n200_FARM_004 Hc_RKUSA_L3_n200_FARM_007	ERS3655682 ERS3655687	LEV resistant LEV susceptible	Doyle et al. (2022)
MHco4(WRS)				Redman et al. (2012)
MHco10(CAVR)				Redman et al. (2012)

2.3.2 Generation of isolate specific and inter-isolate consensus sequences

Generation of consensus sequences for each isolate from pooled WGS of 200 L_3 was carried out in Geneious 11.1.5 utilising the in-built Geneious Prime automatic consensus sequence generation feature. Subsequent inter-isolate consensus sequences of genomic loci of specific interest (e.g. for primer design (2.7), alignment of amplicon sequencing) were generated by extraction of isolate specific consensus sequences of the genomic locus of interest, followed by multiple alignment of isolate specific consensus sequences using MUSCLE 3.8.425 alignment to generate inter-isolate consensus sequences (maximum number of iterations: 8).

2.3.3 Amplicon sequencing analysis

Amplicon sequencing data generated to confirm target amplification for primer validation was analysed in Geneious 11.1.5. Files in ".fasta" format (Eurofins, Ebersberg, Germany, were uploaded directly to Geneious. Consensus sequences generated for the target region (as described in 2.3.1) were used as a reference sequence to which amplicons were mapped using the in-built Geneious Mapper: highest sensitivity/slow; iterate up to 5 times. Generated alignment files were then visualised in Geneious Prime 11.1.5 or Geneious Prime 2022.1.1.

In the case of *acr-8b* amplicon analysis, the published *acr-8b* transcript (GU168770; Kokstad) was aligned to the genomic locus in Chromosome V (Exon 1: 32,223,583-32,530,834 bp; Exon 2: 32,529,053-32,530,110 bp; retained intron locus: 31,228,631-31,229,046 bp) corresponding to exons 1 and 2 and the retained intron 2 locus of *acr-8*. Sequencing results were then multiple aligned to this using MUSCLE alignment tool in Geneious.

2.3.4 TRACY analysis of Sanger sequencing chromatograms

Amplicon sequencing generated for SNP genotyping and variant calling in adult *H. contortus* surviving LEV treatment for S168T in *acr-8* (HCON_00151270), and putative minor LEV resistance associated SNPs identified in *lev-1.1* (HCON_00107700) and *kdin-1* (HCON_00107560) was carried out via chromatogram trace analysis using TRACY (Rausch et al., 2020) analysis tool Pearl (https://www.gear-genomics.com/pearl/). Target of interest consensus reference sequences corresponding to the target of interest, i.e the genomic locus containing the SNP which is amplified by the respective amplicon sequencing primers (2.7.2.7), were extracted from consensus sequences generated by alignment of WGS from MHco18(UGA2004) and MHco3/18 to the WBPS16 MHco3(ISE) genome assembly in Geneious 11.1.5. Extracted sequences were saved in the ".fa" file format. Target of interest consensus reference sequences and ".ab1" trace files from Sanger sequencing (Eurofins, Ebersberg, Germany) were then uploaded to TRACY analysis tool Pearl to generate variant tables and associated chromatograms for visual analysis.

2.3.5 Bioinformatic analysis of RNA sequencing data

Haemonchus contortus RNA-seq data from a previous study (Laing et al., 2013) was used in this study. RNA-seq data from the MHco3(ISE) isolate was available for eggs, L_1, L_3, L_4, adult males and adult females in triplicate per life cycle stage, with each replicate a different single pool of worms from an individual host. Fastq files were uploaded from EMBI website directly to the University of Glasgow Galaxy instance (http://heighliner.cvr.gla.ac.uk/) using accession numbers detailed in Table 2.4. BAM files were generated by aligning reads to the *H. contortus* reference genome (PRJEB506; WormBase parasite GCA_000469685.2; WBSP16) using HISAT2 (Kim et al., 2015) with default parameters. ".GFF3" annotation file for WBSP16 PREJB506 *H. contortus* genome annotations were converted to ".GTF" file format using GFFread to facilitate downstream analysis using featureCounts (Liao et al., 2014). Normalised fragments per kilobase of exon per million mapped (FPKM) counts were generated for all gene models (annotation: WBPS16; 2021-04-WormBase parasite) using Cuffnorm (Trapnell et al., 2010). The featureCounts tool was

then run on BAM files to generate a count of differentially expressed genes (output format: Gene-ID "\t" read-count (DESeq2 IUC wrapper compatible); create gene length file: False), for differential gene expression using DESeq2 with gene ID of genes of interest specified as listed in Table 2.5. DESeq2 (Love et al., 2014) was carried out to create pairwise comparisons (Factor 1/2: featureCounts input file life cycle stage; Choice of input data: Count data; Files have header: True). Data for genes of interest were extracted manually and visualised in R (ggplot2) for windows (https://cran.r-project.org/bin/windows/base/) using code written during the course of this study (Appendix 1). The Galaxy workflow is summarised online; full workflow and parameters available at: http://heighliner.cvr.gla.ac.uk/u/aantonopoulos1/w/rnaseqanalysis.

Table 2.4: RNA-seq data from different *Haemonchus contortus* life cycle stages with accession numbers for EBI

Life cycle stage	Isolate	Reads	Accession number (EBI)
Eggs	MHco3(ISE).N1	11,176,696	ERS209876
Eggs	MHco3(ISE).N1	13,251,399	ERS209877
Eggs	MHco3(ISE).N1	16,670,770	ERS209878
L1	MHco3(ISE).N1	13,117,727	ERS209879
L1	MHco3(ISE).N1	11,670,618	ERS209880
L1	MHco3(ISE).N1	15,327,087	ERS209881
L3	MHco3(ISE).N1	14,929,822	ERS092633
L3	MHco3(ISE).N1	14,369,393	ERS092634
L3	MHco3(ISE).N1	16,187,935	ERS092635
L4	MHco3(ISE).N1	12,074,945	ERS209882
L4	MHco3(ISE).N1	15,890,211	ERS209883
L4	MHco3(ISE).N1	14,195,344	ERS209884
Adult Female	MHco3(ISE).N1	11,626,701	ERS209885
Adult Female	MHco3(ISE).N1	14,034,068	ERS209886
Adult Female	MHco3(ISE).N1	11,989,468	ERS209875
Adult Male	MHco3(ISE).N1	16,292,131	ERS092624
Adult Male	MHco3(ISE).N1	15,372,590	ERS092625
Adult Male	MHco3(ISE).N1	16,391,491	ERS092626
Adult Female (gut dissected from adult female)	MHco3(ISE)	15,266,731	ERS092636
Adult Female (adult)	MHco3(ISE)	14,533,415	ERS092637
Adult Female (gut dissected from adult female)	MHco3(ISE)	14,469,949	ERS092638

Table 2.5: Summary of acetylcholine receptor subunits and associated genes chosen for bioinformatic analysis of life cycle stage expression.

Gene	Gene Name	Function	Comments
unc-63	HCON_00024380	AChR alpha type subunit	Implicated in LEV resistance via truncated transcript expression (Fauvin et al., 2010) and required for functional receptor reconstitution (Boulin et al., 2011).
acr-8	HCON_00151270	AChR alpha type subunit	Implicated in LEV resistance, essential for LEV sensitivity in AChR and functional receptor reconstitution (Boulin et al., 2011; Blanchard et al., 2018), identified in LEV selection locus in MHco3/18 (Doyle et al., 2021).
unc-29.1-4	HCON_00003530 HCON_00003560 HCON_00003520 HCON_00003570	AChR non-alpha type subunit	Multiple functional duplications of unknown function and role (Laing et al., 2013).
lev-1.1	HCON_00107700	AChR non-alpha type subunit	Identified in *C. elegans* LEV resistant mutagenesis screen (Lewis et al., 1980; Lewis et al., 1987a; Fleming et al., 1997), but lacks signal peptide (Laing et al., 2013), and not required for receptor assembly in functional reconstitution (Boulin et al., 2011), identified in LEV selection locus in MHco3/18 (Doyle et al., 2021).
lev-1.2	HCON_00107690	AChR non-alpha type subunit	Functional duplication of *lev-1* with unknown function (Laing et al., 2013), identified in LEV selection locus in MHco3/18 (Doyle et al., 2021).
unc-38	HCON_00017680	AChR alpha type subunit	Identified in *C. elegans* LEV resistant mutagenesis screen (Lewis et al., 1980; Lewis et al., 1987a; Fleming et al., 1997), required for functional receptor reconstitution (Boulin et al., 2011).
ric-3	HCON_00102850	AChR associated accessory protein	Chaperone protein promoting correct AchR folding in endoplasmic reticulum in *C. elegans* (Millar, 2008), required for functional receptor reconstitution (Boulin et al., 2011).
Unc-50	HCON_00080500	AChR associated accessory protein	Golgi localised protein thought to play a role in AChR protein regulation (Eimer et al., 2007), required for functional receptor reconstitution (Boulin et al., 2011).
Unc-74	HCON_00162770	AChR associated accessory protein	Thioredoxin protein related to human TMX3 (Halevi et al., 2002; Halevi et al., 2003) required for functional receptor reconstitution (Boulin et al., 2011).

2.4 Nucleic acid extraction

2.4.1 DNA and RNA extraction

2.4.1.1 RNA extraction

RNA was extracted from a pool of 20 *H. contortus* adult males or a pool of ~10,000 L_3 (previously stored in liquid nitrogen) by a modified Trizol extraction. Briefly, a sterile pestle and mortar were chilled with liquid nitrogen and a frozen pellet of *H. contortus* worms was added and ground thoroughly to a powder, after which 800 μl of Trizol (Invitrogen, 15596026) was added and mixed. The Trizol/ground worm suspension was removed to a DNAse/RNAse-free Eppendorf tube and left at room temperature (R/T) for 5 min to complete tissue dissociation. Chloroform (200 μl) was then added and the tube shaken vigorously for 15 s to mix, incubated at R/T for 3 min, and centrifuged at 12000 x g for 15 min at 4ºC. The aqueous upper layer was removed to a fresh tube and isopropanol (500 μl) added, incubated for 10 min at R/T and then centrifuged at 12000 x g for 15 min at 4ºC. The supernatant was aspirated, followed by the addition of 70% ethanol (1 ml) which was then centrifuged at 7500 x g for 5 min at 4ºC. Finally, the supernatant was aspirated on a light box, after which the RNA pellet was left to air dry for 2 min until all ethanol had evaporated. The RNA pellet was resuspended in 10-30 μl of DNAse/RNAse-free water and incubated on a heat block for 5 min to dissolve fully. The RNA concentration was calculated using a Qubit Spectrophotometer (Qubit RNA HS kit Invitrogen, Q32852).

2.4.1.2 gDNA extraction

Extraction of gDNA was carried out on frozen aliquots of ~10,000 L_3 by a modified phenol chloroform extraction protocol. Initially, 20 μl (20 mg/μl) Proteinase K (Invitrogen, 25530015) suspended in 300 μl of lysis buffer (200 mM NaCl, 100 mM Tris-HCl, 30 mM EDTA, 0.5% SDS) was added to a pellet of L_3 and incubated at 55ºC for 2 hr, after which 10 μl of RNase A was added and

incubated at 37ºC for 10 min in order to eliminate any residual RNA and ensure only pure gDNA was present in the final product. Phenol/chloroform/isoamyl alcohol (25:24:1; 550 μl) was then added, the resultant mixture was then shaken and incubated at R/T for 5 min. Following incubation, the mixture was centrifuged at 14,000 x g for 15 min at R/T. The top layer was then removed to a fresh tube, into which chloroform (200 μl) was then added. The resultant mixture was then centrifuged at 14,000 x g for 15 min at R/T. The top layer was again removed to a fresh tube to which was added 0.1 volume of 3 M sodium acetate, three volumes of 100% molecular grade ethanol, and 2 μl glycogen. This was then incubated O/N at -80ºC. Following O/N incubation the mixture was centrifuged at 14,000 x g at 4ºC for 45 min, after which the supernatant was aspirated and 70% ethanol (500 μl) (70% molecular grade ethanol, 30% DNase/RNase free water) was added, followed by a final centrifugation at 14,000 x g at 4ºC for 5 min. The pellet of gDNA was then air dried and resuspended in 10 μl of elution buffer (10 mM Tris-Cl, pH 8.5). The DNA concentration was calculated using a Qubit Spectrophotometer (Qubit dsDNA HS kit Invitrogen, Q33231) and diluted to 100 ng/μl.

2.4.1.3 Single worm crude proteinase K lysis

Crude lysates of single worms were produced using a modified proteinase K lysis reaction. 10 μl of lysis buffer (100X stock solution: 1000 μl Direct PCR Lysis Reagent (Cell) (Viagen, 301-C), 50 μl 1 M DTT (Invitrogen, P2325), and 10 μl Proteinase K (100 mg/ml) (Invitrogen, 25530049) was aliquoted per well of a 96 well PCR plate. One L_3 was picked into each well in a volume of <1 μl of water. Lysates were incubated at 60ºC for 2 hr, followed by 85ºC for 45 min to denature the Proteinase K. Crude lysates were stored at -20ºC. Dilution plates of crude lysates were produced by diluting 1 μl of crude lysate in 9 μl of DNase/RNase free water and stored at -20ºC.

2.4.2 cDNA synthesis

Total RNA was initially treated with DNAse I (NEB, M0303S) as follows: 1 μl of DNAse I 10X buffer, 1 μl of DNAse I, and 1 μg of RNA were combined and made up to 10 μl with DNAse/RNAse-free water then incubated at 37°C for 30 min. EDTA was then added to a final concentration of 5 mM and incubated at 75°C for 10 min to heat inactivate the DNAse I.

Synthesis of cDNA from DNAse-treated RNA was carried out as follows: 0.5 μg of DNAse-treated RNA was combined with 1 μl 50 μM oligo dT (Promega, C1101), 1 μl 10 mM dNTP mix (Promega, U1511) and made up to 10 μl with DNAse/RNAse-free water, incubated at 65°C for 5 min, then placed on ice for >1 min. The resultant mix (10 μl) was then combined with 1 μl 10X Superscript buffer, 1 μl Superscript III reverse transcriptase (RT) (Invitrogen, 12574-026) (or 1 μl DNAse/RNAse-free water in the no RT sample), 4 μl 25 mM $MgCl_2$, 2 μl 0.1 M DTT, and 1 μl RNAse OUT (Invitrogen, 10777-019), then incubated at 50°C for 50 min, then 85°C for 5 min to inactivate the RT. RNAse H (1 μl; Invitrogen, EN0201) was then added to remove any residual RNA and incubated at 37°C for 20 min. Synthesised cDNA was stored at -20°C.

2.5 Cloning

Target sequences were amplified using either Phusion Green HiFi PCR (2.8.2.2) or Flexi Taq Go PCR (2.8.2.1). Target sequences were then purified using a QIAquick PCR Purification Kit (Qiagen 28104) according to manufacturer's instructions if only a single amplicon of expected size was present. In the case of multiple amplicons of different sizes, the band of expected size for the target sequence was excised from 2% agarose gel and purified using a QIAquick PCR and Gel Cleanup Kit (Qiagen 28506) according to manufacturer's instructions. Purified target sequences were cloned using TOPO™ TA Cloning™ with One Shot™ TOP10 chemically competent *E. coli* cells (Invitrogen, k450001), or with XL10 Gold Ultracompetent Cells (Agilent, 200317) according to manufacturer's instructions. Colonies were then cultured on 100 μg/ml ampicillin (Sigma Aldrich, A9518) LB-Agar. In some cases, initial colony screening was carried out

by blue/white screening using X-gal (Promega, V3941). Colony screening was most often carried out by direct colony PCR: one colony was picked into a volume of 10 μl DNase/RNase free water, incubated at 95°C for 5 min, followed by Phusion Green HiFi PCR (1.7.1.2) with the target sequence primer set, or by Restriction Fragment Length Polymorphism (RFLP) (2.8.3) in some cases. Colonies found to contain the correct insert were then subcultured on 100 μg/ml ampicillin LB agar.

2.6 Plasmid DNA purification

Putification of plasmid DNA was carried out using a QIAprep Spin Miniprep Kit (Qiagen, 27106X4) according to manufacturer's instructions. Starting material was 5 ml of 100 μg/ml ampicillin LB broth inoculated with a single colony and incubated at 37°C O/N.

2.7 Primers

2.7.1 PCR primer design and validation

All primers were designed initially *in silico* using WGS data from MHco3(ISE); MHco18(UGA2004), and MHco3/18 (refer to WGS section above). Consensus sequences generated from WGS data from MHco3(ISE), MHco18(UGA2004), and MHco3/18 were used to ensure no significant polymorphism affected primer binding and to create a primer sets which were functional on all three isolates. Primer candidates were BLASTed against *H. contortus* PRJEB506 genome (WormBase Parasite: https://parasite.wormbase.org/Haemonchus_contortus_prjeb506/Tools/Blast?db=core) to validate binding to correct loci and minimise non-specific binding at secondary loci. In specific cases, primer sequences were modified based on WGS data from MHco4(WRS) and MHco10(CAVR) (2.3.1) (Redman et al., 2012). PCR products were capillary sequenced (Eurofins, Ebersberg, Germany) to ensure the correct products were amplified in each isolate.

2.7.2 General primer design parameters

The following general parameters were followed (where possible) to ensure optimal primer design: terminal 3' bases GC, G, or C; GC content of 40-60% (with a minimum of 35%); primers predicted in Geneious Prime 11.1.5 (Biomatters Ltd, Auckland, New Zealand) to form hairpin loops or self-dimers were excluded; long stretches (>4 bp) of repeating nucleotides were avoided; genomic loci showing high sequence polymorphism relative to the MHco3(ISE) PRJEB506 reference sequence were avoided, as were genomic loci showing moderate to frequent instances of deletions; primers were optimally sited within exons; SNPs of interest were kept >20 bp from the most 3' nucleotide of the nearest primer to ensure detection of the SNP was possible in sequencing reads; T_ms of primers were kept between 50-60°C; differences in T_m between forward and reverse primers were kept within ~2°C of each other. However, this list of criteria represents an optimal guideline which it was not possible to follow in all instances due to demands of the specific assay and the genomic locus amplified.

2.7.3 Specific PCR primer design parameters

In addition to the general parameters for primer design outlined in 2.7.2, reaction and assay specific design parameters and considerations were necessary to take into account the optimisation requirements of the diverse and differing PCR reactions and target sequences undertaken in this study.

2.7.3.1 Truncated transcript *acr-8b* primers

The first published transcript sequence for *acr-8b* (GU168769), detected in the Kokstad isolate, was initially used for primer design. This transcript was then aligned to the *acr-8* (HCON_00151270) genomic locus comprising exons 1 (PRJEB506: 31,530,758-31,530,834), 2 (PRJEB506: 31,529,953-31,530,110) and cryptic exon 3b/retained intronic locus (PRJEB506 31,526,848-31,527,252). Primers were designed to amplify *acr-8b* by siting the reverse primer within the retained intronic sequence. This ensured that *acr-8* would not also be detected.

2.7.3.2 Transcript *acr-8* and *lev-1.1* primers

Published transcript sequences for *acr-8* (EU006785) and *lev-1.1* (GU060987) were initially used to guide primer design. Candidate primers were manually designed based on the published transcripts following general primer design guidelines outline in 1.7.2. Consensus sequences were then generated for the CDS of *acr-8* (HCON_00151270) and *lev-1.1* (HCON_00107700) from MHco18(UGA2004) and MHco3(ISE). Primer candidates were then annotated to consensus CDS sequences in Geneious Prime 11.1.5 to account for potential polymorphism at primer bind loci. Location of exons were annotated to extracted CDS and primers designed to overlie exon junctions where possible, to prevent amplification of residual genomic DNA during PCR. Where it was not possible to overlie an exon junction, primers were sited in exons separated by long intronic sequences (>500 bp) to prevent amplification of residual genomic DNA during PCR.

2.7.3.3 Intron 2 deletion in *acr-8* primers

Primers Hco-Indel-F and Hco-Indel-R were designed 50-100 bp up and downstream of the site of the deletion in the second intron of *acr-8* (HCON_00151270) (Figure 2.1A). The primer set was designed to allow for size discrimination by gel electrophoresis between the full length and deletion amplicons, which should differ in size by 63-98 bp depending on the size of the

deletion present (Barrere et al., 2014; Doyle et al., 2021). A deletion internal primer, Hco-Indel-Ins-R (Figure 2.1B) was then designed as a confirmatory primer which would discriminate between the deletion and full-length alleles. Hco-Indel-Ins-R was designed in the reverse orientation to pair with Hco-Indel-F and was sited within the 51 bp sequence shared between all reported deletions. This ensured that natural variation in the size of the deletion would not confound results.

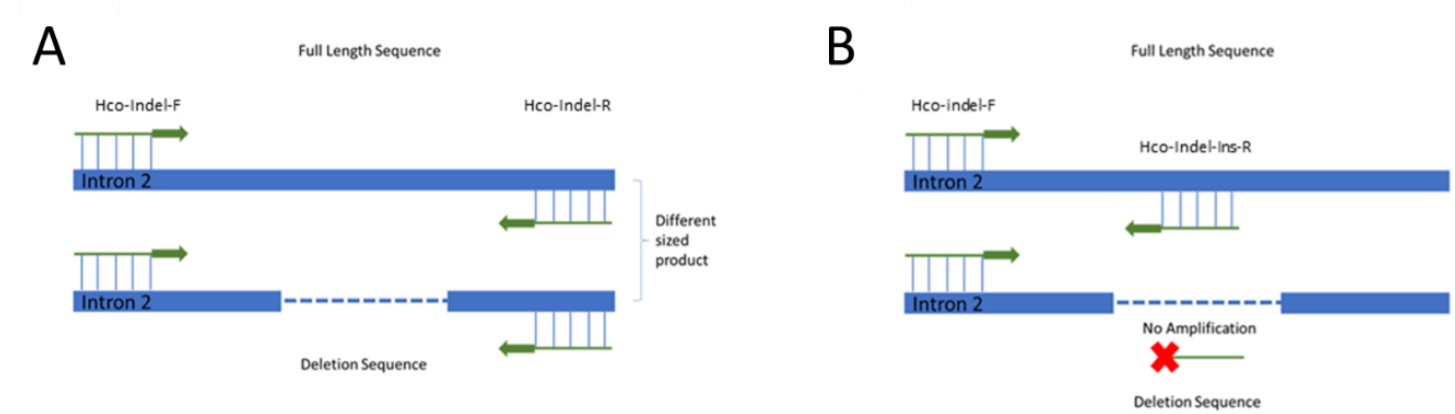

Figure 2.1: Summary of *acr-8* intron 2 deletion PCR function. A: Indel spanning primers (Table 6: Hco-Indel-R and Hco-Indel-F). If the deletion allele is present, then the product size will differ from the full-length allele, allowing for a size discrimination PCR to determine the genotype of the individual. B: Deletion specific primer (Table 1: Hco-Indel-Ins-R). The deletion specific primer lies wholly within the deleted sequence, thus blocking amplification if the deletion is present.

2.7.3.4 Exon 4 *acr-8* primers

Primers were designed to amplify exon 4 of *acr-8* due to the presence of SNP S168T within this exon. Initial design focused on the generation of an amplicon conducive to RFLP. Therefore, primers Hco-Intron4-F and Hco-Exon4-R were designed 100-150 bp up and downstream of S168T, in order to yield an amplicon of sufficient size to allow for restriction fragment size discrimination on an agarose gel. Primer Hco-Exon4-F was designed to be sited slightly upstream of Hco-Intron4-F so at to allow a semi-nested PCR to increase the quantity of template available for RFLP. To further facilitate RFLP visualisation, primers were not spaced evenly, so as to give one larger, and one smaller restriction fragment when the SNP was present. Forward primers (Hco-Intron4-F, Hco-

Exon4-F) were sited within the intron upstream of exon 4. The reverse primer was sited in exon 4 within 10 bp of the end of the exon.

2.7.3.5 S168T allele specific PCR primers

Allele specific primers were designed so the most 3' nucleotide of the primer would anneal to the S168T SNP site (PRJEB506: 31,521,884; S168T G/S168 C). This mismatch/match constituted the allele specific nucleotide, which differentiated the resistant from the susceptible primer set. For the resistant primer Hco-S168T-R, the terminal 3' nucleotide will match with S168T (C-G), and vice versa for the susceptible (G-C), Hco-S168-R. Allele specific primers were designed to be paired with existing exon 4 primers described in 2.7.3.4. Initially, only a single mismatch at the most 3' nucleotide was trialled, but this did not yield allele specific discrimination, following this, mismatches were successively added at the 2nd, 2nd and 3rd, and finally the 2nd and 5th positions relative to the 3' end. Mismatches were designed to ensure maximum inhibition of polymerisation, thus T-T and A-A mismatches were used at the relevant positions, as this offered the best chance of allele specific discrimination due to stronger inhibition between T-T/A-A mismatches than to T-C/A-G (Liu et al., 2012). The final design with 2nd and 5th position mismatches (Figure 2.2) was found to offer the best results as assessed by allele discrimination of cloned sequences.

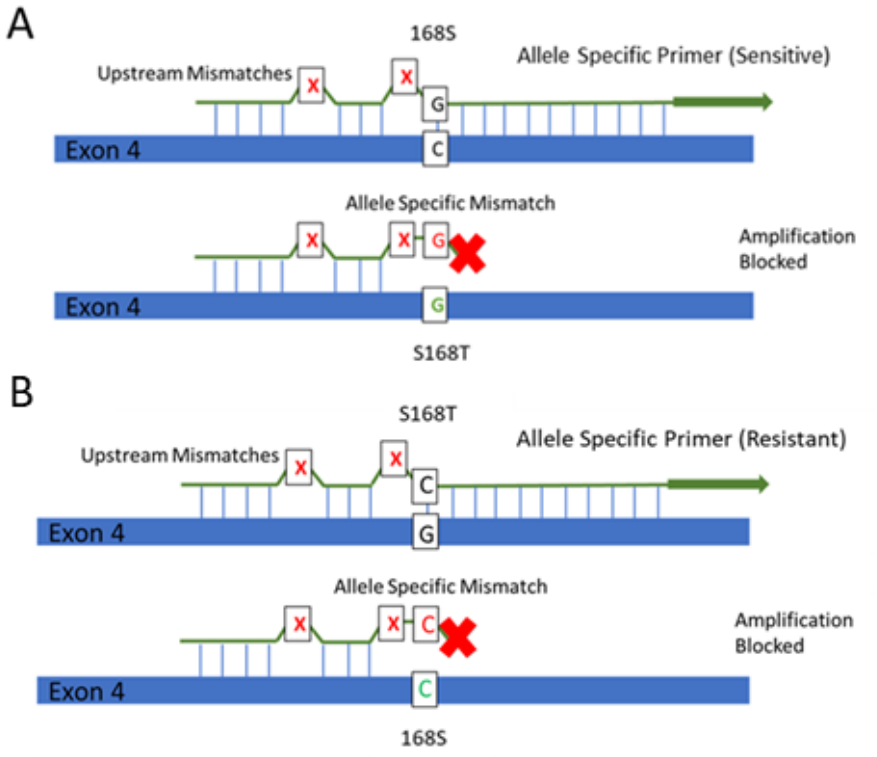

Figure 2.2: Schematic representation of allele specific primer function. Upstream mismatches at the 2nd and 5th position from the 3' end of the primer are identical in each set (A: susceptible or B: resistant; GCT or GGT respectively) and lead to maximum destabilisation of the polymerisation complex in the case of an allele specific mismatch at the 3' position. The 3' mismatch is allele specific, and as shown by the example above will prevent polymerisation and amplification if the S168T variant is present in the susceptible primer (A). In the resistant primer the opposite occurs (B).

2.7.3.6 Deoxyinosine modified primers

Following the observation of poor specificity and sensitivity of Hco-168T-R and Hco-168S-R when assaying MHco4(WRS) and MHco10(CAVR) individuals, primers were modified with a deoxyinosine [I] base. Modification was based on WGS from MHco4(WRS) and MHco10(CAVR) showing synonymous SNPs present at the 3rd and 11th bases (5'-3') at the primer bind site. [I] bases were then substituted at these positions producing primer set Hco-168T[I]-R and Hco-168S[I]-R with the [I] base present at the 3rd base. Hco-168T[II]-R and Hco-168S[II]-R possess [I] bases at the 3rd and 11th position. No primer was tested with [I] at the 11th base only as sensitivity and specificity was restored by inclusion of a single [I] at the 3rd position.

2.7.3.7 Minor LEV resistance associated SNP panel amplicon sequencing primers

Primers designed for amplicon sequencing of genes *kdin-1* (HCON_00107560), *lev-1.1* (HCON_00107700, *lev-1.2* (HCON_00107690) were targeted to amplify template containing specific SNPs of interest identified in Doyle et al. (2021) (Appendix 2). Primers were designed to lie within the exons of the gene of interest, as the position of the SNP relative to the primer was not as restricted as in 2.7.3.4 as downstream allele specific PCR is not required. However, the primer bind site was sited a minimum of 20 bp from the SNPs of interest to ensure that the SNPs are detectable within sequencing reads. No SNPs of interest were present in *unc-63* and *unc-29.1*, thus the regions of sequence most amenable to standard primer design parameters (avoiding long runs of identical nucleotides, GC content ~40-60%, terminal G/C nucleotide etc) were chosen and primers designed within these loci. Primers were tested on MHco3(ISE) and MHco18(UGA2004) individuals and products capillary sequenced (Eurofins, Ebersberg, Germany) to confirm the correct template amplification. Nested primers for *kdin-1* and *lev-1.1* were designed to partially overlay the original primers, ending 5-10 bp downstream or upstream for the forward and reverse primers respectively.

2.7.4 PCR primer sequences

Table 2.6: Summary of PCR primers used in the study

Primer Name	Target	Sequence (5′-3′)[a,b]	Annealing Temperature (T_A) [qPCR T_A – if applicable]	Reference[c]
Hco-acr8b-F[c]	*acr-8b* transcript	GATTTCACCGAAAGCTTATGCG	53ºC [60ºC]	Sarai et al., 2013
Hco-acr8b-R[c]	*acr-8b* transcript	TTCTTGCCGTCATTACACCC	53ºC [60ºC]	Sarai et al., 2013
Hco-acr8b-UoG-F	*acr-8b* transcript	ATGCTGAACAACTCTATGAGG	50ºC	
Hco-acr8b-UoG-R	*acr-8b* transcript	GCTCAAGAAGAACCTGGAGAG	50ºC	
Hco-lev1tr-F	*lev-1.1* transcript	CGACCGAGACGTAAGAACATCC	58ºC [60ºC]	
Hco-lev1tr-R	*lev-1.1* transcript	ATGTGCAAACCCACCCCATACC	58ºC [60ºC]	
Hco-Indel-F	*acr-8* intron 2 deletion	ATTCTTGCCGTTATTACACC	56ºC	
Hco-Indel-R	*acr-8* intron 2 deletion	GGAGGGAGCTTCAGCTTTT	56ºC	
Hco-Indel-Ins-R	*acr-8* intron 2 deletion	GCGATATAACAGCAGTTAAC	56ºC	
Hco-Exon4-F	*acr-8* exon 4	AGATAGGAGAAGTGGCGC	55-58ºC	
Hco-Intron4-F	*acr-8* exon 4	GTGATTTCGTGCAGAGATAGG	55-58ºC	
Hco-Exon4-R	*acr-8* exon 4	AATTATGAGCGATGCCCTC	55-58ºC	
Hco-168T-R	S168T GGT	TTAAAATTTGGAGGAaGGtc	60ºC	
Hco-168S-R	168S GCT	TTAAAATTTGGAGGAaGGtg	60ºC	
Hco-168T[I]-R	S168T GGT	TT[I]AAATTTGGAGGAaGGtc	60ºC	
Hco-168S[I]-R	168S GCT	TT[I]AAATTTGGAGGAaGGtG	60ºC	
Hco-ITS2-F	*H. contortus* ITS2	GTTACAATTTCATAACATCACGT	50ºC	Redman et al., 2008
Hco-ITS2-R	*H. contortus* ITS2	TTTACAGTTTGCAGAACTTA	50ºC	Redman et al., 2008
Hco-β-Tubulin-F	*H. contortus* β-Tubulin	CGTTGTTCCATCACCCAAGGTATC	55ºC	Marks , 2016

Hco-β-Tubulin-R	*H. contortus* β-Tubulin	CAAGGTGGTTGAGATCTCC	55ºC	Marks, 2016
Hco-kdin1-F	*H. contortus kdin-1* exon 25	GCACAGGATTTAATTTCAAAGGC	56ºC	
Hco-kdin1-R	*H. contortus kdin-1* exon 25	AATGACCAAGAGCGAGCC	56ºC	
Hco-kdin1Nest-F	*H. contortus kdin-1* exon 25	GGATTTAATTTCAAAGGCTACGG	56ºC	
Hco-kdin1Net-R	*H. contortus kdin-1* exon 25	TCGATCTCGAATGCTCGC	56ºC	
Hco-lev1.1-F	*H. contortus lev-1* exon 4	TGCTGACGGCAACTACG	59ºC[d]	
Hco-lev1.1-R	*H. contortus lev-1* exon 5	CTGCATCGCTGATCATCG	59ºC[d]	
Hco-lev1.1Nest-F	*H. contortus lev-1* exon 4	AATCTTTCCTTGTTGAGCTTGG	56ºC	
Hco-lev1.1Nest-R	*H. contortus lev-1* exon 5	ACGTACCAATAATGCAACTGC	56ºC	
Hco-lev1.2-F	*H. contortus lev-1* functional duplication exon 4	ATAGGCGATACGATTTGTGC	59ºC[d]	
Hco-lev1.2-R	*H. contortus lev-1* functional duplication exon 4	TGGTTATGATGTTGAGCACG	59ºC[d]	
Hco-unc63-F	*H. contortus unc-63*	CAAATAGAACACAAGAGCCG	59ºC[d]	
Hco-unc63-R	*H. contortus unc-63*	CCATTTGAGAAGAAAAACGCTC	59ºC[d]	
Hco-unc29.1-F	*H. contortus unc-29*	CGTTGGTGTTTGGATCATGG	59ºC[d]	
Hco-unc29.1-R	*H. contortus unc-29*	CTACCCATTCTGCTTGTTCG	59ºC[d]	
Hco-unc29.1-Rii	*H. contortus unc-29*	ACTCCGTTTATTAACTAGTGATGC	59ºC[d]	
Tci-Intron3-F	*T. circumcincta acr-8* exon 3	CTCTGTGATTCTTTACACGG	56ºC	
Tci-Exon3-F	*T. circumcincta acr-8* exon 3	AGAGAGCTGGGACAAGGC	56ºC	
Tci-Exon3-R	*T. circumcincta acr-8* exon 3	CGTTAGTGTATCACAATGGC	56ºC	

Tci-S168T-R	*T. circumcincta* S140T	TGAAATTTGGAGGAaGGtc	56°C-60°C
Tci-S168-R	*T. circumcincta* S140	TGAAATTTGGAGGAaGGtG	56°C-60°C

a: [I] denotes deoxyinosine base

b: mismatches relative to the reference sequence (PRJEB506; MHco3(ISE)) are denoted by lower case

c: current study unless stated otherwise

d: requires GC buffer

2.8 PCR

2.8.1 qPCR

qPCR was carried out using Brilliant III Ultra Fast qPCR Master Mix (Agilent 600880) according to manufacturer's instructions. Primers Hco-acr8b-F and Hco-acr8b-R (Sarai et al., 2013), or Hco-lev1tr-F and Hco-lev1tr-R were used to amplify *acr-8b* or *lev-1.1* respectively. A total of 1 μl of cDNA per reaction was used as amplification template from either pooled mixed adult, adult male, or L_3 generated as described in 1.4.2. RNA was treated with DNAse I during extraction to eliminate potential gDNA carry over as described in 1.4.1. qPCR was run on an Agilent Mx3005P qPCR system, and results were visualised in MxPro 4.10.

2.8.2 Conventional PCR

2.8.2.1 Taq Go Flexi PCR

PCR with GoTaq G2 Flexi DNA polymerase (Promega, M7801) was carried out in a final volume of 20 μl as follows: 1 μl of DNA template was added to 5 μl 5X Green GoTaq Flexi Buffer, 8 μl 25mM $MgCl_2$, 1 μl 10 mM dNTP mix (Promega, U1511), 1 μl F and R primer (100 pmol), 0.25 μl GoTaq G2 Flexi DNA polymerase, 3.75 μl DNAse/RNAse-free water and amplified as follows: initial denaturation 95°C for 2 min, followed by 40 cycles (denaturation 95°C for 30 s, primer annealing temperature (T_A) °C for 30 s, and extension 72°C for 15 s with a final

extension at 72°C for 5 min). PCR products were visualised on a 2% agarose gel stained with SYBR Safe DNA Gel Stain (Thermo Fisher, S33102).

2.8.2.2 Phusion HiFi PCR

Phusion High-Fidelity PCR was carried out using either Phusion Green (Sigma, F534S) or Phusion HiFi (NEB, M0530S) polymerase, using the protocol described below.

Phusion High-Fidelity Polymerase PCR was carried out in a final volume of 20 μl as follows: 4 μl 5X HF/GC or Green HF/GC reaction buffer, 0.4 μl 10 mM dNTP mix, 0.2 μl F and R primer (100 pmol), 0.2 μl Phusion DNA Polymerase, 15 μl DNAse/RNAse-free water. The PCR programme was as follows: initial denaturation for 30 s at 98°C, followed by 35 cycles (denaturation 98°C for 10 s, annealing T_A °C for 10 s, and extension 72°C for 10 s) with the final extension 72°C for 5 min. PCR products were visualised on a 2% agarose gel stained with SYBR Safe DNA Gel Stain.

2.8.2.3 Nested Phusion HiFi PCR

Nested HiFi PCR for Sanger sequencing amplification for template enrichment of *kdin-1* and *lev1.1* was carried out using primers Hco-kdin1Nest-F/R or Hco-lev1Nest-F/R, with the reaction as described in 2.8.2.2 except using 1 μl of 1:20 diluted first round PCR (Hco-kdin-1-F/R or Hco-lev1.1-F/R) product as DNA template. PCR products were visualised on a 2% agarose gel stained with SYBR Safe DNA Gel Stain.

2.8.3 RFLP

RFLP for the detection of S168T in *H. contortus* was carried out using a two-step nested GoTaq Flexi PCR (2.8.2.1). The first round PCR used primers Hco-Intron4-F/Hco-Exon4-R and the second round used Hco-Exon4-F/Hco-Exon4-R. The final product was digested for 7-12 hr at 37°C with *Ava*II (NEB, R0153L). All digested RFLP products were visualised on a 4% NuSieve gel (Lonza, 50090) stained with SYBR Safe DNA Gel Stain.

2.8.4 AS-PCR

2.8.4.1 H. contortus

AS-PCR for the detection of S168T in *H. contortus* was carried out using a two-step nested PCR. First round Phusion High Fidelity PCR was carried out to amplify the *H. contortus acr-8* exon 4 template. Semi-nested second round AS-PCR using allele specific primers was then carried out to discriminate between alleles. Primer sets were initially tested using the second round semi-nested AS-PCR on cloned exon 4 fragments containing the S168T or S168 alleles derived from MHco18(UGA2004) and MHco3(ISE) respectively. Template enrichment was not necessary at this stage due to abundant S168T and S168 containing plasmid (2.5) purified via Mini-prep (2.6). Extensive optimisation of reaction conditions was then carried out to determine the optimal primer set combination and reaction temperature. A touchdown PCR step was included to improve specificity. Primer and dNTP concentrations were also reduced from standard manufacturer recommendations for DreamTaq PCR protocols to further improve reaction specificity.

First round amplification of the exon 4 template from crude lysates of individual L_3 or adults (2.4.1.3) using Phusion Green High-Fidelity Polymerase (Sigma, F534S) was carried out in a final volume of 20 μl as follows: 4 μl 5X HF reaction buffer, 0.2 μl 10 mM dNTP mix, 0.1 μl Hco-Intron4-F/Hco-Exon4-R primers (100 pmol), 0.2 μl Phusion DNA Polymerase, 14.5 μl DNAse/RNAse-free water. The PCR programme was as follows: initial denaturation for 30 s at 98°C, followed by 35 cycles (denaturation 98°C for 10 s, annealing (T_A)°C for 10 s, and extension 72°C for 10 s) with the final extension 72°C for 5 min.

The second round PCR using allele specific primers Hco-168T-R or Hco-168S-R, or Hco-168T[I]-R or Hco-168S[I]-R was carried out using DreamTaq DNA polymerase (Thermo Scientific, EP0702) PCR in a final volume of 20 μl as follows: 2 μl 10X reaction buffer, 0.05 μl 10 mM dNTP mix, 0.025 μl Hco-Exon4-F/Hco-168T-R or Hco-Intron4-F/Hco-168S-R (100 pmol), 0.2 μl DreamTaq polymerase, 16.9 μl DNAse/RNAse-free water, and 1 μl of the first-round PCR product diluted 1:10-1:20. The PCR programme using a touchdown PCR was as follows: initial

denaturation for 2 min at 95°C, followed by 12 touchdown PCR cycles (denaturation 95°C for 30 s, annealing first temperature 68°C for 30 s, annealing last temperature 60°C (decreasing in equal increments per cycle) for 30 s, and annealing (T_A)°C for 10 s, extension 72°C for 15 s), with the final extension of 72°C for 5 min. PCR products were visualised on a 2% agarose gel stained with SYBR Safe DNA Gel Stain (Thermo Fisher, S33102).

2.8.4.2 T. circumcincta

AS-PCR was developed for the detection of a serine to threonine substitution at the equivalent position to S168T in *T. circumcincta*. The AS-PCR used a two-step nested PCR protocol adapted from the assay in *H. contortus* (2.8.2.2). Initially, Phusion Green High-Fidelity polymerase PCR was carried out to amplify the exon 3 fragment as described for *H. contortus*. Following this, the second-round allele specific PCR was initially tested as described in 1.8.4.1. Second-round allele specific PCR was also tested as follows: 25-35 PCR cycles (denaturation 95°C for 30 s, annealing temperature 56°C-60°C for 30 s, and extension 72°C for 15 s) with the final extension of 72°C for 5 min. PCR products were visualised on a 2% agarose gel stained with SYBR Safe DNA Gel Stain (Thermo Fisher, S33102).

2.9 Sequencing

2.9.1 Capillary (Sanger) sequencing

Capillary Sanger sequencing was carried out by MWG Eurofins, Ebersberg, Germany, according to manufacturer's instructions for individual PCR products or plasmid DNA samples (tube-seq service) or 96 well plates of PCR products (plate-seq service). All PCR products sequenced were purified using QIAquick PCR purification kit according to manufacturer's instructions or were purified by Eurofins prior to sequencing.

2.10 LAMP

2.10.1 LAMP primer design

2.10.1.1 ProofMan LAMP primer design

ProofMan LAMP primers were designed initially in primer explorer V5 (Eiken Chemicals, Japan; http://primerexplorer.jp/lampv5e/index.html) to amplify the genomic locus of *acr-8* exon 4 (2.8.2.2) containing S168T. This region was chosen as it allowed for the use of cloned sequences generated with the validated exon 4 primer set Hco-exon4-F/R. Initial primer design was carried out under default parameters set by the program. The site of the SNP S168T was marked as a polymorphism, and primers designed accordingly.

Three candidate primer sets were generated, however, only one generated primer set had the S168T SNP site on the loop domain, therefore, this primer set was taken forward (Figure 2.3), as this was a necessary prerequisite for probe design. Further factors were also considered, namely: design was limited to exon 4 of *H. contortus acr-8*, as this is the location of the SNP of interest, in addition, polymorphism within the intron also restricted design, preventing the siting of primers within this region. Overall, this restricted the available the target region for LAMP primer design to that of the existing exon 4 template amplified by PCR primers Hco-Intron4-F and Hco-Exon4-R.

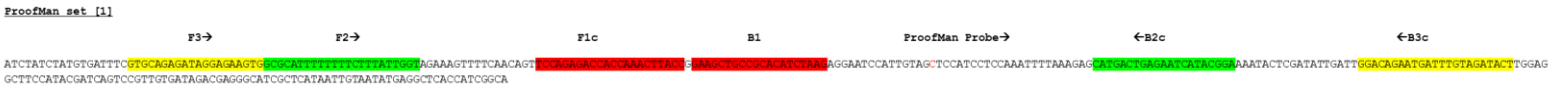

Figure 2.3: Schematic representation of primer bind sites for ProofMan primer set PM1.

2.10.1.2 ProofMan LAMP probe design

ProofMan probes were designed to overlie the S168T locus with the SNP present at the 3’ end of the probe. Probe design was restricted by a number of factors, including the limited space available between the B1c and B2 regions, which through their self-complementarity will anneal during amplicon formation

forming the loop domain of the LAMP amplicon. Genomic region available for probe design: 31,521,758–31,522,025 bp; PRJEB506. The region between B1c/B1 and B2/B2c (Figure 2.4) was chosen as the optimum site as this allowed for probes to be designed within a sufficiently long LAMP amplicon loop domain to allow for a probe of sufficient length to ensure efficient and specific binding to template target. Following design parameters established by comparison with the ProofMan probe reported by Ding et al. (2021) (probe length, overall T_m), several probes were designed and tested. Details of all LAMP primers are summarised in Table 7.

All probes were provided by Eurofins MWG, Ebersberg, Germany.

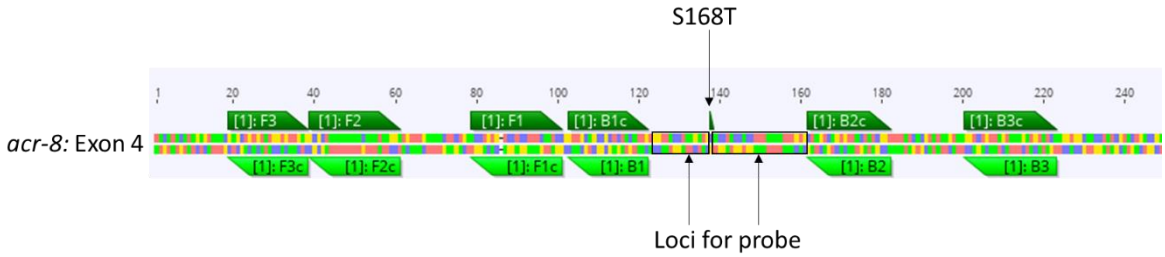

Figure 2.4: Schematic of acr-8 exon 4 region and ProofMan primer set PM1. Primer bind sites visualised in Geneious Prime 11.1.5.

2.10.1.3 LEC-LAMP primer design

LEC-LAMP primer sets were generated in primer explorer V5 as described in 2.10.1.1. LEC-LAMP primers were designed in collaboration with Dr Owen Higgins, NUI Galway, Ireland. Following the generation of basic primer sets these were manually edited to best suit the parameters of the assay design. Manual editing was carried out by either increasing or decreasing the length of individual primers within the primer set. This was done in order to synchronise T_ms of the various primers. Higher T_ms allow for a more specific reaction. Primers were designed with both the LEC-LAMP and ProofMan assays in mind, in order to evaluate each assay. Although the loop section (Figure 2.5) is smaller than during initial ProofMan primer design (Figure 2.4), the area has been designed for a probe of maximum T_m within the parameters of the primer set.

Figure 2.5: Schematic representation of primer bind sites for LEC-LAMP primer set.

2.10.1.4 LEC-LAMP probe design

LEC-LAMP probes were designed to overlie the S168T locus with the site of the SNP situated 3-4 bp upstream of the 5' end of the LEC-LAMP probe. The 6-8 bp region lying immediately upstream of and including the most 5' end of the LEC-LAMP probe, termed the cleavage arm, contains the cleavage recognition site for *endonuclease* IV, an abasic site which is flanked by the S168T locus. The most 5' and 3' nucleotides of the cleavage arm are labelled with the quencher and fluorophore moieties respectively. Fluorophore and quencher moieties are preferentially conjugated to a T nucleotide for optimal probe performance (Higgins, personal communication). Care was also taken to avoid an overlap of greater than 4-5 bp between the 3' end of the LEC-LAMP probe and the 5' end of the B2c region (Figure 2.6), as this can negatively affect probe performance (Owen Higgins, personal communication). Alteration of the orientation of the abasic site in relation to the SNP (i.e the SNP either 5' or 3' to the abasic site), and the overall length of cleavage arm were considered when undertaking resistant probe redesigns for probes R-R5 (Table 7). Probes were designed with T_ms that synchronised with LEC-LAMP primers, and that were >= 65°C to ensure maximal specificity at the higher temperatures recommended for LEC-LAMP reactions (Higgins and Smith, 2020; Owen Higgins, personal communication).

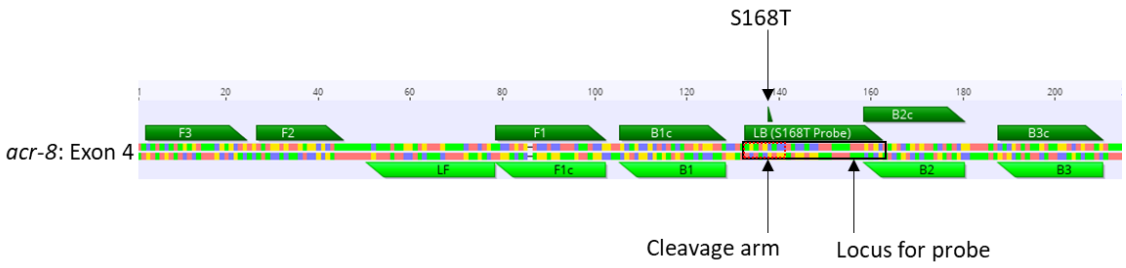

Figure 2.6: Schematic of acr-8 exon 4 region and LEC-LAMP primer set and probe. Primer bind sites visualised in Geneious Prime 11.1.5

2.10.2 LAMP primer sequences

Table 2.7: Summary of LAMP primers used in the study

Primer Name	Target	Sequence (5′-3′)[a,b]	Optimal Amplification Temperature
ProofMan F3	*acr-8* exon 4	GTGCAGAGATAGGAGAAGTG	60-65ºC
ProofMan B3	*acr-8* exon 4	AGTATCTACAAATCATTCTGTCC	60-65ºC
ProofMan FIP	*acr-8* exon 4	GGTAAGTTTGGTGGTCTCTGGAGCGCATTTTTTTTCTTTATTGGT	60-65ºC
ProofMan BIP	*acr-8* exon 4	GAAGCTGCCGCACATCTAAGTCCGTATGATTCTCAGTCATG	60-65ºC
ProofMan Probe 1 (PM1)	*acr-8* S168T allele	BHQ1-TT[I]AAATTTGGAGGATGGAG-FAM	60-65ºC
ProofMan Probe 2 (PM2)	*acr-8* S168T allele	BHQ1-TTAAAATTTGGAGGATGGAG-FAM	60-65ºC
ProofMan Probe 3 (PM3)	*acr-8* S168T allele	FAM-AAATTTGGAGGATGGAG-BHQ1	60-65ºC
ProofMan Probe 4 (PM4)	*acr-8* S168T allele	FAM-CTCTTTAAAATTTGGAGGATGGAG-BHQ1	60-65ºC
ProofMan Probe 5 (PM5)	*acr-8* S168T allele	FAM-CTCCATCCTCCAAATTTTAAAGAGCA-BHQ1	60-65ºC
LEC LAMP F3	*acr-8* exon 4	CTATCTATGTGATTTCGTGCAG	65ºC
LEC LAMP B3	*acr-8* exon 4	CATTCTGTCCAATCAATATCGAG	65ºC

LEC LAMP FIP	*acr-8* exon 4	CGGTAAGTTTGGTGGTCTCTGGAATAGGAGAAGTGGCGCATT	65°C
LEC LAMP BIP	*acr-8* exon 4	GCTGCCGCACATCTAAGAGGAATCGTATGATTCTCAGTCATGCTC	65°C
LEC LAMP FLP	*acr-8* exon 4	ACTGTTGAAAACTTTCTACCAATAAAGA	65°C
LEC LAMP S168 (S)	*acr-8* S168 allele	BHQ1-TGTA(dSpacer)C(FAM-dT)CCATCCTCCAAATTTTAAAGAGC	63-67°C
LEC LAMP S168T (R)	*acr-8* S168T allele	BHQ1-TGTA(dSpacer)G(HEX-dT)CCATCCTCCAAATTTTAAAGAGC	66-67°C
LEC LAMP S168T (R2)	*acr-8* S168T allele	BHQ1-TTGTA(dSpacer)C(HEX-dT)CCATCCTCCAAATTTTAAAGAGC	66-67°C
LEC LAMP S168T (R3)	*acr-8* S168T allele	BHQ1-TTGTA(dSpacer)C(HEX-dT)CCATCCTCCAAATTTTAAAGAGCA	66-67°C
LEC LAMP S168T (R4)	*acr-8* S168T allele	(BHQ1)TAGG(dSpacer)CCA(HEX-dT)CCTCCAAATTTTAAAGAGCA	63°C
LEC LAMP S168T (R5)	*acr-8* S168T allele	(BHQ1)ATTGTA(dSpacer)G(HEX-dT)CCATCCTCCAAATTTTAAAGAGC	65-67°C
LEC LAMP FIP Lateral flow (LF-FIP)	*acr-8* exon 4	FITC-CGGTAAGTTTGGTGGTCTCTGGAATAGGAGAAGTGGCGCATT	63-7°C
LEC LAMP S168T Lateral Flow (LF Probe)	*acr-8* S168T allele	Biotin-TGTA(dSpacer)CTCCATCCTCCAAATTTTAAAGAGC	67°C

a: [I] denotes deoxyinosine base

b: dSpacer denotes abasic site, dT denotes moiety attachment to T nucleotide, BHQ1 denotes black hole quencher 1, FAM denotes 6-Carboxyflurescein, HEX denotes hexachloroflourescein, FITC denotes Flourescein isothiocyanate, Biot denotes Biotin

c: current study unless stated otherwise

2.10.3 LAMP primer validation

2.10.3.1 ProofMan LAMP primer validation

Colourimetric LAMP reactions were carried out as follows using WarmStart Colorimetric LAMP kit (NEB M1800S) in a final volume of 25 μl: WarmStart Colorimetric LAMP 2X Master Mix 12.5 μl, 10X LAMP primer mix 2.5 μl, DNA template 1 μl, made up to 25 μl with DNase/RNase free water.

Initial primer validation for the ProofMan LAMP primer set was carried out using WarmStart Colorimetric LAMP kit. Gradient LAMP was used to establish the optimal temperature for the reaction, which was functional from 60-65°C. To determine primer set efficiency, primers were tested on exon 4 PCR products from individual L3 from MHco18(UGA2004) and MHco3(ISE), pre- and post-LEV treated MHco3/18, MHco10(CAVR) and MHco4(WRS). Optimal reaction temperature was established at 64°C.

Following initial optimisation on PCR products, primers were tested on crude lysates using 1ul and 2ul of undiluted crude lysate and the WarmStart Colourimetric LAMP kit (NEB, M1800S).

2.10.3.2 LEC-LAMP primer validation

As LEC-LAMP was not initially designed for end point naked eye fluorescence detection, this primer set was directly validated using DNA intercalating LAMP Fluorescent Dye (NEB B1700S) to measure DNA amplification on an MxPro Mx3005P qPCR machine. Cloned sequences (2.6) of exon 4 fragments amplified by Phusion High Fidelity PCR (2.8.2.2) encoding S168T and S168, from MHco18(UGA2004) and MHco3(ISE) respectively, were used. A no template (negative) control sample was also included to ensure no non-specific amplification occurred.

2.10.4 LAMP reaction conditions and optimisation

2.10.4.1 ProofMan LAMP reaction

ProofMan LAMP reactions were carried out in a final volume of 25 μl as follows: 2.5 μl 10X reaction buffer (ProofMan Reaction buffer: 200 mM Tris-HCl (pH 8.8), 100 mM $(NH4)_2SO_4$, 50 mM $MgSO_4$ and 600 mM KCl) or *Bsm* Reaction Buffer (ThermoFisher, EP0691), 0.8 mM dNTP, 4U *Bsm* polymerase (ThermoFisher EP0691), 2.5 μl 10X LAMP primer mix (final concentrations: FIP/BIP: 0.8 μM, 0.2 μM F3/B3), 1M Betaine (Sigma-Aldrich B0300-1VL), 0.1-1U *Pfu* polymerase (Promega M7741), 0.4-1.2 μM ProofMan probe (PM1-5; Table 7), 1 μl DNA template, made up to 25 μl with DNase/RNase free water. ProofMan LAMP reactions were run at a range of temperatures from 50°C-67°C.

2.10.4.2 ProofMan LAMP reaction buffer optimisation

The composition of the buffer used in ProofMan LAMP reactions was extensively optimised. Ding et al. (2021) describe the use of a custom buffer with the following components: 200 mM Tris-HCl (pH 8.8), 100 mM $(NH4)_2SO4$, 50 mM $MgSO_4$ and 600 mM KCl. This buffer is termed ProofMan buffer from hereafter. This differed from both the *Bsm* polymerase buffer (Thermofisher) and the *pfu* polymerase buffer (Promega). Each buffer was tested during initial optimisation.

2.10.4.3 LEC-LAMP reaction

LEC-LAMP reactions were carried out as follows in a final volume of 25 μl: 2.5 μl 10X Isothermal Reaction Buffer (NEB M0538S), 0.8 mM dNTP, 1 μl *Bst*2.0 DNA polymerase (NEB M0538S), 1.5 μl $MgSO_4$ (NEB M0538S), 2.5 μl 10X LAMP primer mix (final concentrations: 0.8 μM FIP/BIP, 0.2 μM F3/B3, 0.4 μM LF), 1 μl *Endonuclease* IV (NEB M0304S), 0.4 - 0.8 μM LEC probe, 1 μl DNA template, made up to 25 μl with DNase/RNase free water. LEC LAMP reactions were run at a range of temperatures from 63°C-68°C to determine the optimal temperature for each of the LEC-LAMP probe (S, R1-5; Table 7).

2.10.5 Lateral flow LEC-LAMP

2.10.5.1 Assay design

The initial LEC-LAMP assay design focused on detection of the S168T allele. To convert to a lateral flow assay, the LEC-LAMP probe was modified to remove the fluorophore and quencher moieties, retaining the abasic *endonuclease IV* recognition site. Biotin is present on the 5' end of the lateral flow probe (Figure 2.7A), allowing for immobilisation on the lateral flow strip (Disposable Nucleic Acid Detection Strip, D003-03, Ustar Biotechnologies, Hangzhou, China). The forward inner primer (FIP) is then conjugated with a fluorescein isothiocyanate (FITC) moiety which allows aggregation with gold conjugated nano-particles (AuNPs) leading to colour change (Ferreira Carlos et al., 2017; Rasmi et al., 2021). Only amplicons with both the FITC and Biotin moieties will lead to visualisation of colour change on the lateral flow strip (Figure 2.7B/C). Cleavage only occurs when S168 allele is present, leading to SNP specific detection.

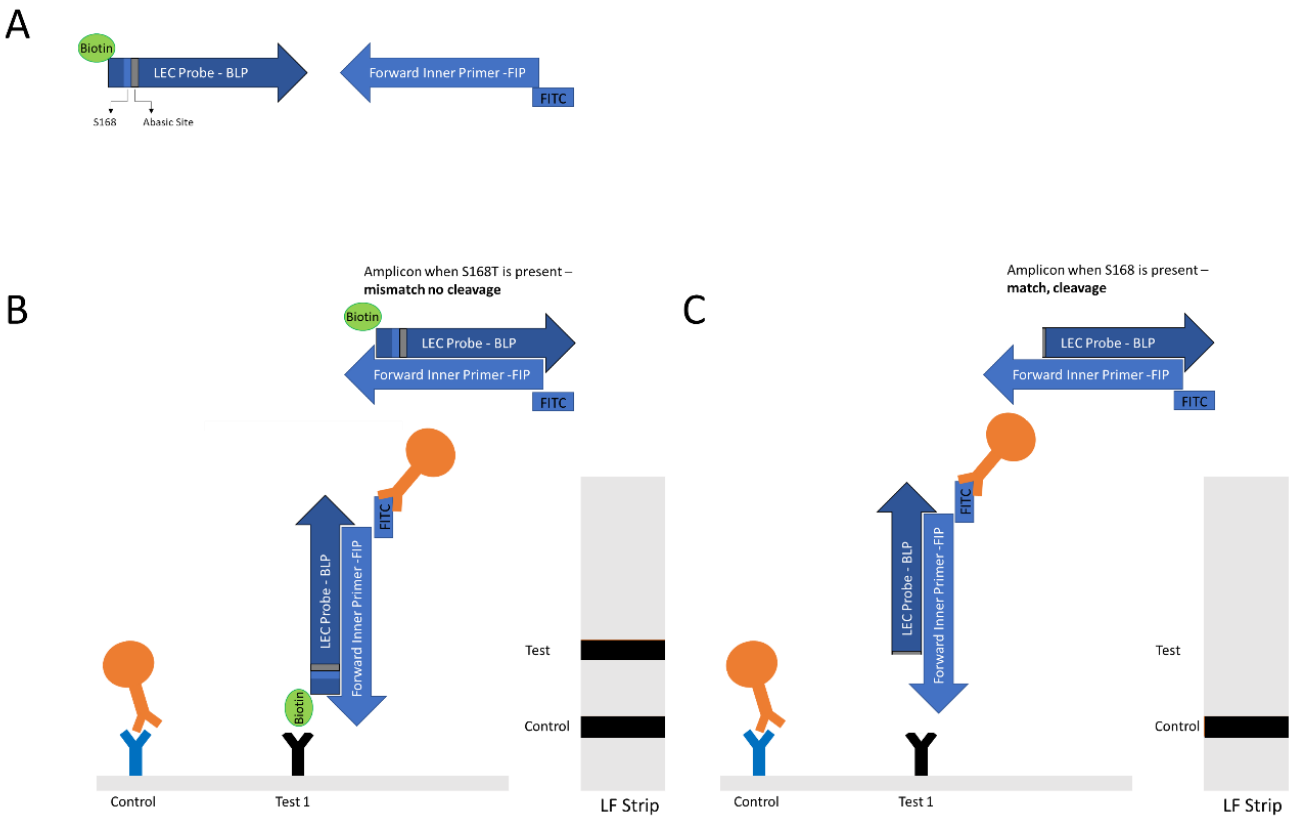

Figure 2.7: Schematic representation of the initial assay design for S168T specific lateral flow LEC LAMP.

A: Schematic representation of the FIP and probe used for LEC-LAMP lateral flow. LEC probe is tagged with Biotin, FIP with FITC.

B: Schematic representation of amplicon formed when S168T is present, with no cleavage occurring. The amplicon is then immobilised by streptavidin on the lateral flow strip, where anti-FITC conjugated antibodies localise, leading to colour change.

C: Schematic representation of amplicon formed when S168T is absent. The amplicon is not immobilised by streptavidin as biotin moiety has been cleaved off. No colour change is seen.

2.10.5.2 Lateral flow reaction

Lateral flow LEC-LAMP was carried out initially as described in 1.11.3.3. Following this, different primer and probe concentrations were also trialled in order to increase specificity. Initially the concentration of the LEC probe was lowered to 0.04 μM. The concentration of the FITC labelled FIP was lowered to 0.08 μM and 0.72 μM unlabelled FIP was included in the reaction to maintain the

overall concentration of both labelled and unlabelled FIP at 0.8 μM. Finally, the concentration of the LEC-LAMP LF probe was lowered to 0.004 μM and FITC labelled FIP to 0.008 μM and included within a standard LEC-LAMP reaction as described in 2.11.3.3.

3 Investigation of Putative Levamisole Resistance Markers in *Haemonchus contortus*

3.1 Introduction

3.1.1 LEV and the nematode AChR

LEV acts as a selective agonist of nematode acetylcholine receptors (AChR) (Aceves et al., 1970) which produces depolarisation of nematode somatic body wall muscle cells due to an influx of cations (Harrow and Gration, 1985). This then leads to spastic paralysis, allowing expulsion of the parasite from the gastrointestinal tract (Martin and Robertson, 2007). The nematode neuromuscular system responds to and senses the environment via fast synaptic transmission. The neurotransmitter acetylcholine (ACh) plays a key role in this. Control of body muscle contraction is mediated by pentameric ligand-gated ion channel receptors in *C. elegans*, and other nematodes. Five related or identical subunits combine to form a pentamer. The extracellular domain of the receptor exposes the binding site for the activating neurotransmitter at the junction of two subunits. The transmembrane domain is embedded in the post-synaptic junction and forms the ion channel (Duguet et al., 2016).

Analysis of ribosomal DNA evolution places nematodes into five clades, with clade I the most basal and clade V the most recent (Blaxter, 1998). Both *H. contortus* and *C. elegans* belong to clade V. Diversification, duplication, and divergence of AChR subunits has continued throughout the evolution of nematodes (Beech and Neveu, 2015) (Figure 3.1). Both free living and parasitic nematodes possess multiple AChR subtypes, which are composed of different combinations of subunits. The LEV sensitive AChR subunit composition in *C. elegans* is a homomeric receptor of fixed composition, whilst parasitic nematodes show loss of certain subunits, and multiple duplication of others. In *C. elegans* for example the LEV sensitive AChR contains subunit LEV-8, which is absent in *H. contortus*, the role instead filled by the paralogue ACR-8. In addition, LEV sensitive AChR *unc-29* shows multiple instances of duplication (Laing et al., 2013; Duguet et al., 2016) (Figure 3.2). In addition, the AChR associated subunit LEV-1, which in *C. elegans* is an integral part of the LEV

sensitive AChR (Boulin et al., 2008; Li et al., 2015) lacks a signal peptide in *H. contortus* (Laing et al., 2013) and its role in this species remains to be fully elucidated. The *lev-1* gene also shows evidence of functional duplication in *H. contortus* (Laing et al., 2013; Doyle et al., 2021) (Figure 3.2). These are referred to hereafter as *lev-1.1,* and *lev-1.2* for the functional duplication.

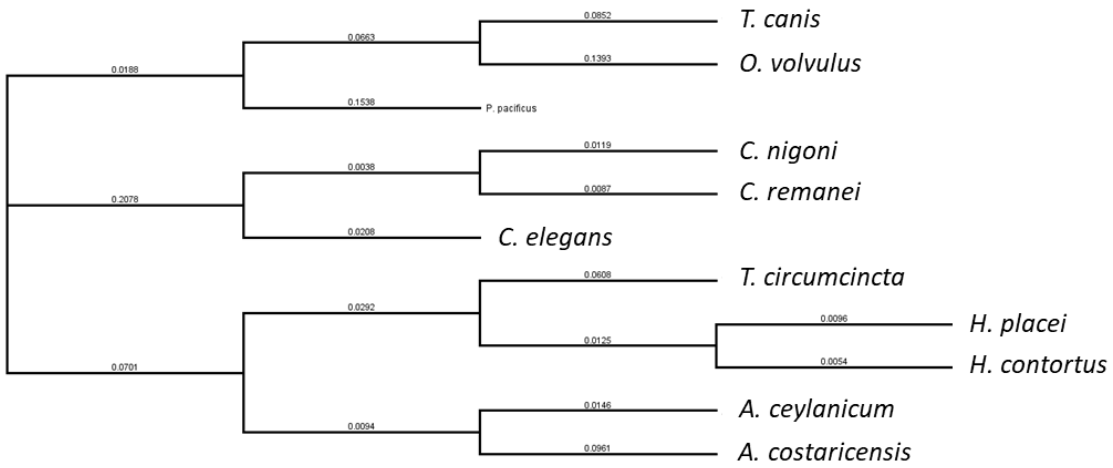

Figure 3.1: Phylogenetic tree of ACR-8 and ortholog protein sequence in selection of clade V nematodes. Relative distance is shown by numbers on branches. Blosum62 neighbour joining global alignment tree with free end gaps, using Jukes-Cantor genetic distance model. Created in Generous 11.1.5. *Haemonchus contortus* shows the highest level of sequence similarity with *Haemonchus placei* and *Teladorsagia circumcincta*. Significant divergance is seen relative to free living nematodes *Caennorhabditis* spp.

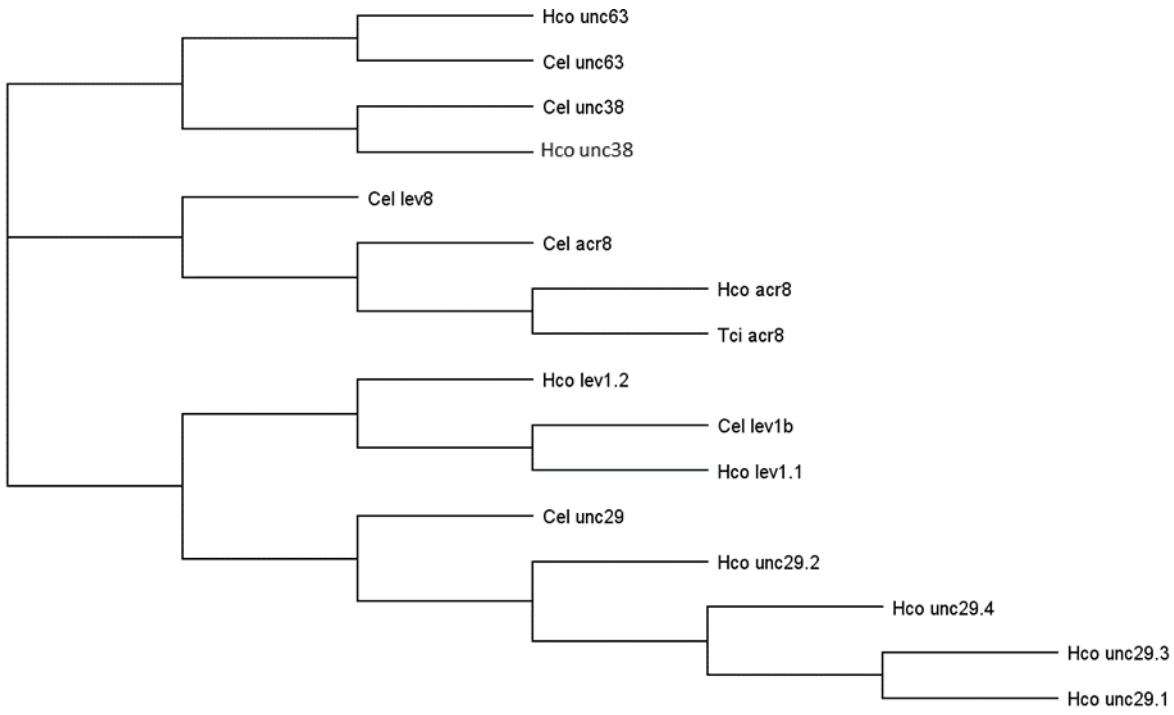

Figure 3.2: Phylogenetic tree of sequence similarity of AChR subunits between *Haemonchus contortus* and *Caenorhabditis elegans*. *Teladorsagia circumcincta* is also included for illustrative purposes. Blosum62 neighbour joining global alignment tree with free end gaps, using Jukes-Cantor genetic distance model. Created in Generous 11.1.5.

Further elucidation of receptor composition and subunit function has been driven by functional reconstitution experiments in *Xenopus* oocytes (Boulin et al., 2008; Neveu et al., 2010; Boulin et al., 2011). By utilising this system, it was determined that a combination of the five subunits (LEV-8, UNC-38, UNC-29, UNC-63, and LEV-1) identified in *C. elegans* in a mutant screen for LEV resistance, and three ancillary proteins (UNC-50, UNC-74, and RIC-3) are necessary for a functional *C. elegans* LEV sensitive AChR to assemble in *Xenopus* oocytes (Boulin et al., 2008). Orthologues of all these genes, except *lev-8,* were detected in *H. contortus*, *T. circumcincta* and *T. colubriformis* (Neveu et al., 2010).

The ACR-8 subunit in *H. contortus* was later identified as a *C. elegans* LEV-8 orthologous AChR subunit by comparative transcriptomic analysis (Fauvin et al., 2010). *H. contortus* thus lacks LEV-8, with ACR-8 substituting for the role played by LEV-8 *in vivo*. The ACR-8 subunit was subsequently shown to associate with UNC-63, UNC-29, and UNC-38 to form a functional AChR (Boulin et al., 2011). Further functional reconstitution experiments showed a key role for ACR-8 in

conferring LEV sensitivity to the AChR. It was shown first that parasitic nematode ACR-8 is able to replace the *C. elegans* LEV-8 subunit when expressed in *Xenopus* oocytes, and strikingly when *H. contortus* ACR-8 was present it led to a significant increase (approaching that of ACh) in sensitivity of the receptor to LEV. The same level of sensitivity to LEV was not seen when the *C. elegans* ACR-8 subunit was present in the reconstituted receptor (Boulin et al., 2011). RNAi silencing of *Hco-acr-8* also conferred LEV-R in *H. contortus* larvae, and the expression of *H. contortus acr-8* in a *lev-8* null *C. elegans* mutant led to the rescue of LEV susceptibility (Blanchard et al., 2018), indicating a crucial role for ACR-8 in conferring LEV sensitivity to the parasitic nematode AChR.

Studies to date have found a number of potential mechanisms of LEV resistance involving these key AChR subunits in *H. contortus*. Truncated transcripts of *unc-63* and *acr-8*, termed *unc-63b* and *acr-8b* respectively, have been identified in resistant isolates (Neveu et al., 2010; Fauvin et al., 2011; Boulin et al., 2011). The truncated *unc-63b* was initially detected in resistant *T. circumcincta*, *T. colubriformis*, and *H. contortus* (Neveu et al., 2010). Functional reconstitution experiments also described a dominant negative effect when *unc-63* and *unc-63b* were co-expressed inhibiting the formation of functional receptor (Boulin et al., 2011). Quantitative analysis of expression patterns found a consistent down-regulation of *unc-63* in the two most resistant isolates examined (Sarai et al., 2013). However, in MHco18(UGA2004) *unc-63b* was not detected whereas both *acr-8* and the truncated *acr-8b* transcript were significantly up-regulated in resistant L_3 larvae, but undetectable in susceptible isolates (Williamson et al., 2011). qPCR analysis of *H. contortus* L_1, L_3, and adults from three LEV-R (two highly resistant and one moderately resistant) and a sensitive strain revealed a great variation in expression pattern of *acr-8, acr-8b, unc-63,* and *unc-63b* across life cycle stage and isolate relative to the sensitive isolate (Sarai et al., 2013).

Although the altered expression of subunits, and the expression of truncated AChR subunit transcripts, has been correlated with resistance in multiple studies, the results are variable. Nevertheless, the role of *acr-8b* in LEV resistance requires further investigation. Despite inconsistent detection of *acr-8b*, multiple studies have nonetheless correlated its expression with a LEV

resistant phenotype (Neveu et al., 2010; Williamson et al., 2011; Sarai et al., 2013; Sarai et al., 2014). Furthermore, AChR subunits are known to be expressed at significantly lower levels than ubiquitously expressed genes, as the AChR is localised to neuromuscular junctions (NMJs) in the body wall muscle (Martin and Robertson, 2007; Duguet et al., 2016). This may complicate detection of *acr-8b* and does not necessarily preclude its expression in those isolates. The expression of *acr-8b* may also act in a dominant negative manner (i.e. the truncated/mutant protein preferentially integrates into the receptor compared to the full length/wild-type protein), as has been observed for *unc-63b* (Boulin et al., 2011). This may indeed come with a fitness cost, although this again is not conclusively demonstrated in studies to date.

The *acr-8b* transcript is composed of 256 bp corresponding to exons 1 and 2 of the *acr-8* gene, and a 356 bp sequence (Figure 3.3) which did not align to any known published transcripts of *acr-8* at the time of publication. Subsequent analysis revealed this sequence to be the result of alternative splicing leading to an intron retention event (Fauvin et al., 2011). The presence of this deletion was also correlated with *acr-8b* expression in qPCR experiments (Barrere et al., 2014). A subsequent study examined the effect of LEV selective pressure on free-living larvae *in vitro* over nine generations (Sarai et al., 2015). This led to the induction of high-level resistance in the L_3 larvae, and while the expression of AChR subunit and ancillary proteins increased by up to five-fold by the ninth generation, adult worms did not display any resistance, and indeed showed an inverse expression pattern of the same genes (particularly *unc-63* and *acr-8*). Previous comparisons of drug sensitivity in different life cycle stages had shown that adults and larvae of the same isolate were both resistant to LEV *in vivo*. However, in the *in vitro* experiment, resistance was only present in the pressured (larval) stage. Of particular interest was the downregulation of *acr-8* in later generations of L_1, but upregulation in adult males. Overall, *acr-8b* expression showed an increasing trend from generation one to nine in larvae, but was not detectable in adults (Sarai et al., 2014). That study revealed the ability of worms to regulate receptor subunit expression in a stage-specific manner in response to drug pressure.

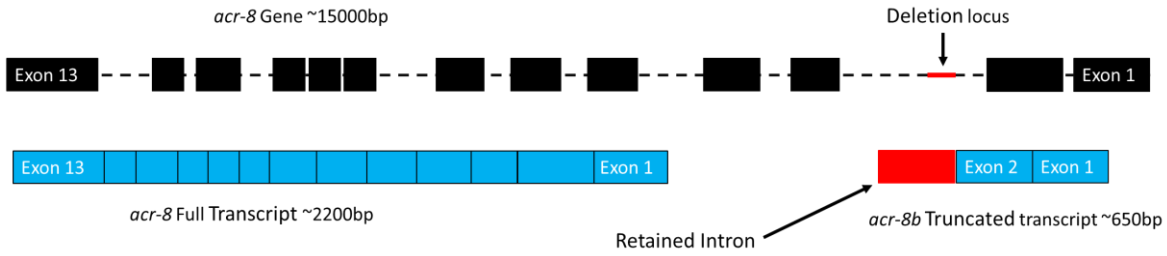

Figure 3.3: Schematic representation of the *acr-8* gene, *acr-8* transcript, and relationship to *acr-8b* transcripts including the retained intronic sequence. The full length *acr-8* transcript encompasses 13 exons (blue), whereas the truncated *acr-8b* transcript comprises exons 1 and 2 (blue) in addition to a retained intronic sequence (red). This retained intron corresponds to the locus surrounding the intron 2 deletion in *acr-8*.

3.1.2 Summary

The groundwork laid down over the previous 20 years and summarised above, together with more recent experiments, have pointed to a key role of the subunit ACR-8 in conferring sensitivity to LEV (Blanchard et al., 2018). There are a number of ways in which parasites can modulate drug resistance such as changes in target gene expression, alterations at binding site, and various efflux mechanisms (Kotze et al., 2016). Evidence thus far has indicated two specific mechanisms potentially involved in LEV resistance, namely the expression of a truncated *acr-8b* transcript correlated with a LEV-R phenotype, and the presence of a 63 bp deletion at the retained intron locus correlated with *acr-8b* expression (Barrere et al., 2014).

3.1.3 Aims

In this part of the study, the aim was to examine putative resistance markers *acr-8b* and the intron 2 deletion in *acr-8* to elucidate their role in LEV resistance. Initially the aim was to quantify expression of *acr-8* and *lev-1.1* by qPCR in LEV resistant and susceptible MHco18(UGA2004) and LEV susceptible MHco3(ISE) L_3 larvae and adult males. Expression of *acr-8b* in LEV resistant MHco18(UGA2004) and LEV susceptible MHco3(ISE) L_3 larvae and adult males was then assessed by PCR. Finally, the aim was to investigate individual genotype of *H. contortus* L_3 in relation to the *acr-8* intron 2 deletion using single worm PCR.

By integrating these data, the aim was to further elucidate the role of *acr-8b* in LEV resistance and determine whether the *acr-8* intron 2 deletion would be suitable as molecular diagnostic marker of LEV resistance.

3.2 Results

3.2.1 Bioinformatic analysis of AChR subunits implicated in LEV resistance

The foundation for this part of the study was based on the results of whole genome sequencing of an *H. contortus* genetic cross (MHco3(ISE) females x MHco18(UGA) males) hereafter referred to as MHco3/18. Sequencing analysis of pools of 200 L_3 pre and post LEV treatment was carried out by Doyle et al. (2021) with the aim of identifying regions under selection following LEV treatment. Two regions of significant genetic differentiation (i.e. quantitative trait loci (QTL)) were identified in the post-treatment sample relative to the pre-treatment sample (Figure 3.4). The major QTL, identified on Chromosome V, corresponds to the region containing *H. contortus acr-8* (HCON_00151270). A second QTL on chromosome IV (Figure 4) overlies the locus of the *lev-1.1* (HCON_00107700) AChR subunit which has been implicated in LEV-R in *C. elegans* mutants (Neveu et al., 2010), and found to be differentially regulated in LEV-resistant *H. contortus* larvae (Sarai et al., 2013; Sarai et al., 2014). Following the identification of regions containing these two AChR subunits as under selection, the expression of AChR subunits in LEV resistant and LEV susceptible worms was investigated.

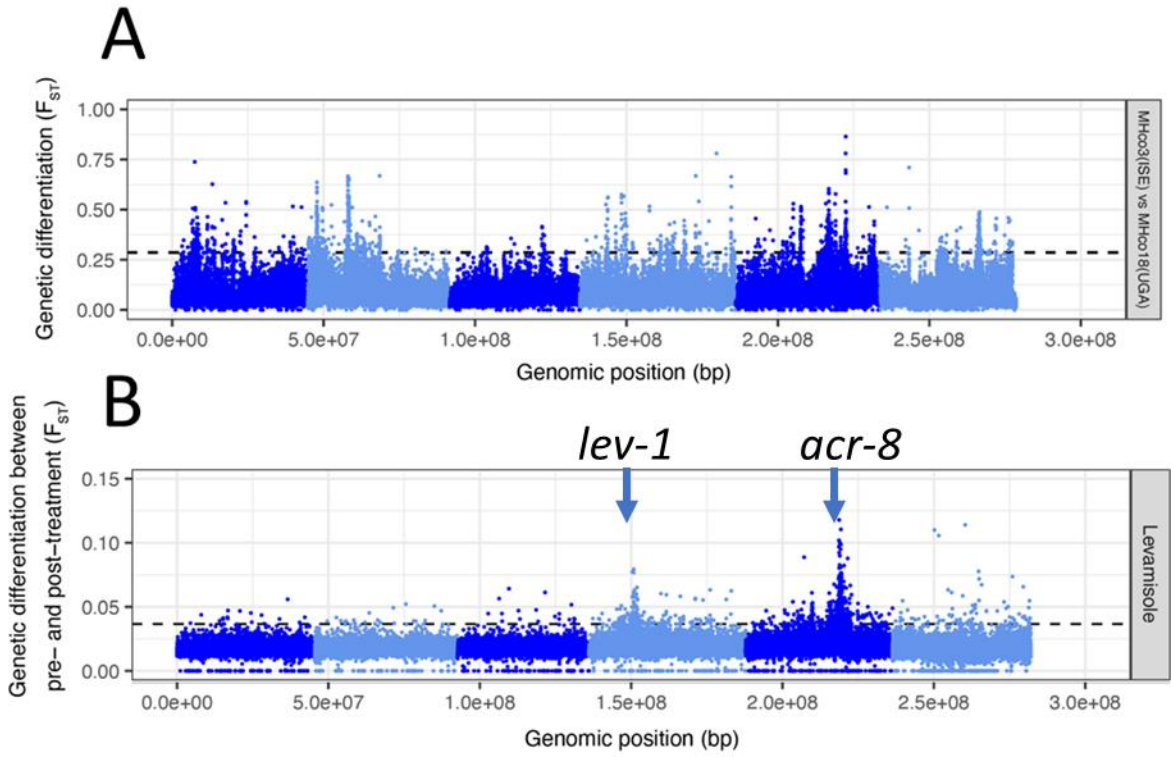

Figure 3.4: F_{ST} Plot showing genetic differentiation in *Haemonchus contortus* MHco3/18 following whole genome sequencing of L_3 F3 progeny from one animal pre- and post-levamisole treatment. A: Genome wide comparison of untreated parental strains MHco3(ISE) and MHco18(UGA2004). Significant variation is seen throughout the genome. B: Comparison of genome wide differences pre and post single LEV treatment in F3 progeny. Chromosome IV and V show peaks corresponding to regions under genetic differentiation following LEV treatment relative to pre-treatment sample. Loci for *lev-1* and *acr-8* are found within these peaks. (Adapted from Doyle et al. (2022) with author permission).

3.2.2 RNA-Seq Analysis

Expression of specific subunits involved in AChR assembly and function was analysed using previously published RNA-seq data (Laing et al. 2013) from eggs, L_1, L_3, L_4, adult males (whole body tissue), and adult females (with data from both whole body tissue, and with the gut/eggs excised) of MHco3(ISE) (for EBI accession numbers see Chapter 2; Table 4). All pairwise comparisons were normalised to eggs in order to visualise changes across life cycle stages. Subunits *acr-8*, *lev-1.1*, and *lev-1.2* were chosen due to their presence in the two QTL identified following LEV selection of MHco3/18 (Figure 34). The remaining AChR subunits (UNC-29.1-4, UNC-63, UNC-38) and accessory proteins (RIC-3, UNC-50,

UNC-74) were chosen as they have been shown to be necessary for receptor assembly and composition in *C. elegans* and *H. contortus* (Neveu et al., 2010; Fauvin et al., 2010; Boulin et al., 2011).

Overall expression of all AChR subunits and associated accessory proteins was low when compared initially to β-tubulin, a ubiquitously expressed cellular protein (see Appendix 3 for β-tubulin expression graph). This was expected, as neuronal tissue is highly specialised. The AChR receptor subunits and accessory protein expression was then analysed separately from β-tubulin.

Initially raw FPKM data were analysed to give a general overview of life cycle stage specific expression of the AChR, and associated genes of interest. Overall trends showed a decline in expression as the life cycle progresses, with particularly pronounced declines seen in accessory proteins *ric-3*, *unc-50*, and *unc-74* from eggs to adult stages. However, *unc-50* was a notable exception, with expression being relatively high in the adult male. Expression of *ric-3* was also relatively high in the L_3. LEV resistance associated subunits *unc-63, lev-1.1,* and *acr-8* showed low expression throughout the life cycle overall, with particularly low expression observed in the adult female. Expression of *lev-1.1,* however, was most pronounced in the L_3, showing a strong skew to expression in this life cycle stage. The *lev-1.2* transcript showed an expression pattern that largely mirrored that of *lev-1.1*, except for conspicuously low expression in the L_3, and high expression seen in the L_4. This isoform could, therefore, be involved in an L_4 specific process, however, further investigation is necessary. Subunit *unc-38* also showed an expression profile skewed to the L_3, with relatively low expression overall in the remaining life cycle stages. The expression profile of *unc-29.1* and *unc-29.2* showed highest expression in the adult male, whereas *unc-29.3* and *unc-29.4* both showed relatively low expression in all life cycles stages, with little observable detection in larval stages and adult females. Finally, the adult female showed the lowest expression overall of all AChR subunits and accessory proteins (Figure 3.5).

Relative expression patterns of the AChR subunits and accessory proteins was then examined to determine if any of the genes of interest were significantly downregulated in a life cycle stage specific manner (Figure 3.6). These results were consistent with the raw counts, with significant ($p < 0.05$) downregulation seen for LEV resistance associated AChR subunits *unc-63, lev-1.1,* and *acr-8* in the adult female compared to eggs. Significant downregulation of all genes of interest was seen in the adult female, except *unc-50* and *unc-29.2*. Adult males and L_4 showed a significant upregulation of *unc-29.1* and *unc29.2*, whereas L_3 showed a significant upregulation of *lev-1.1*. Finally, L_1 did not show a markedly different expression profile overall to eggs, with the exception of a significant downregulation of *unc-74*, which is downregulated across all life cycle stages in relation to expression levels in eggs.

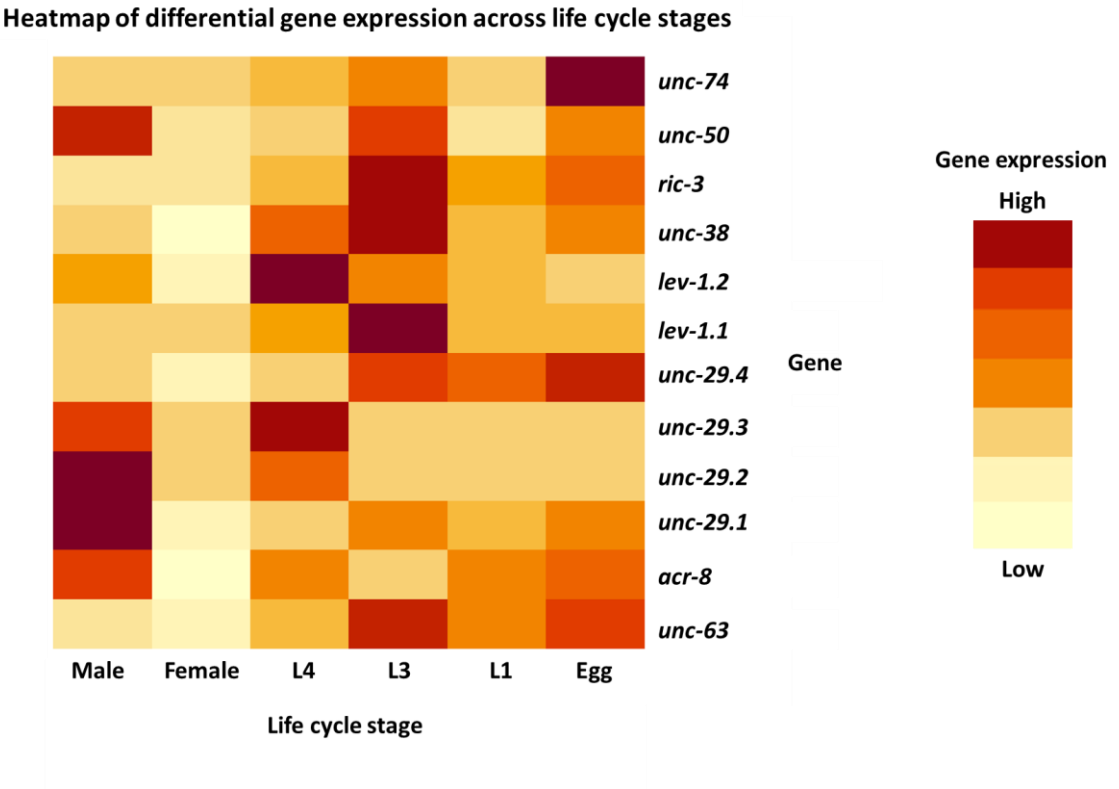

Figure 3.5: Heatmap showing differential life cycle stage specific expression of AChR and associated genes in different life stages of *H. contortus*. Darker indicates higher expression, whereas lighter indicates lower expression.

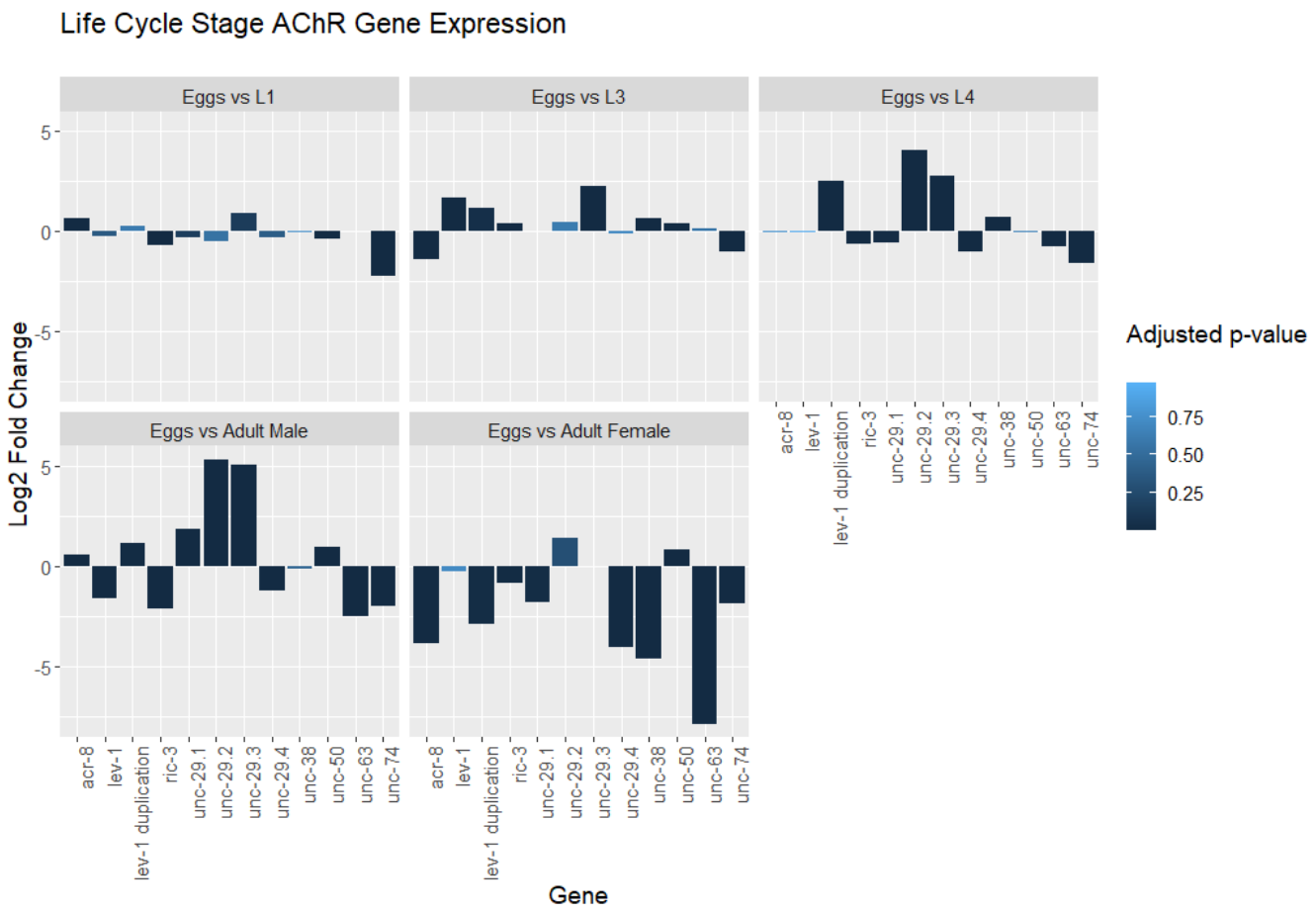

Figure 3.6: Bar chart showing log_2 fold change (y axis) expression changes across life cycle stage (x axis) of AChR and AChR associated subunits. Bars are shaded according to the adjusted p-value, with darker bars indicating a lower adjusted p-value. Each panel represents a pairwise comparison of life cycle stage expression vs eggs.

3.2.3 Comparative RT-qPCR analysis of *acr-8*, and *lev-1.1* expression in L_3 and adult LEV resistant MHco18(UGA2004) and LEV susceptible MHco3(ISE)

The expression of *acr-8* and *lev-1* was then compared between MHco3(ISE) and MHco18(UGA2004) (Figure 3.7). RT-qPCR was carried out using cDNA from mixed adults, as no significant sex differential expression was seen for *lev-1*, and as *acr-8* expression in females was undetectable using the current assay (see Appendix 4). MHco18(UGA2004) L_3 showed a 1.8-fold upregulation of *lev-1* and a 3.2-fold downregulation of *acr-8* (Figure 3.7B) relative to MHco3(ISE). This represented a significant differential expression of both *acr-8* and *lev-1* in resistant L_3. MHco18(UGA2004) adults showed a 1.4-fold downregulation of *lev-1* and a 0.2-fold downregulation of *acr-8* relative to MHco3(ISE).

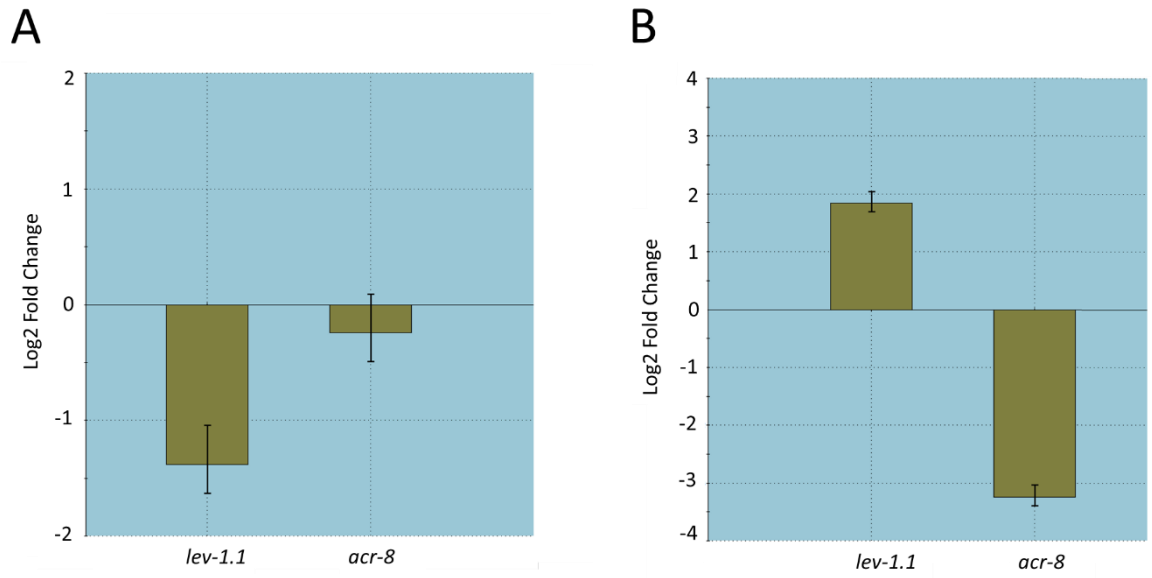

Figure 3.7: Differential expression of *lev-1.1* and *acr-8* in MHco3(ISE) and MHco18(UGA2004).

qPCR Log_2 fold change of relative expression of *lev-1.1* and *acr-8* in MHco3(ISE) vs MHco18(UGA2004):

A: Log_2 fold change expression in adult MHco3(ISE) vs MHco18(UGA2004) *lev-1.1* and *acr-8* (tubulin, GPD normalisers).

B: Log_2 fold change expression in L_3 MHco3(ISE) vs MHco18(UGA2004) *lev-1.1* and *acr-8* (tubulin, GPD normalisers).

3.2.4 Detection of *acr-8b* in MHco18(UGA2004) and MHco3(ISE) L_3

Expression of the truncated *acr-8b* was then compared by conventional PCR in MHco18(UGA2004) and MHco3(ISE). Expression was examined in mixed adults or males only. However, *acr-8b* was not detectable with primer set Hco-acr8b-UoG. The *acr-8b* transcript was detected in both MHco18(UGA2004) and MHco3(ISE) pooled L_3 (~10,000) using the previously published Hco-acr8b (Sarai et al., 2013) primer set (Figure 3.8). In addition, the presence of bands smaller than expected for Hco-acr8b were of interest. Bands from both amplicons were purified and sequenced to confirm target identity. All amplicons were found to align to exons 1 and 2 of *acr-8*. The expected size amplicon from MHco18(UGA2004) aligned to the retained intron locus of *acr-8*. Both the expected size and the larger than expected amplicons of MHco3(ISE) also aligned to the retained intron locus of *acr-8b* in the reverse primer amplicon (Hco-acr8b-R). Sequence corresponding to the retained intron locus was detected in both (expected size and larger than expected size) MHco3(ISE) amplicons. The retained intronic sequence differed between the expected size and the smaller size amplicon of MHco3(ISE), indicating variation within this isolate. Notably, both MHco3(ISE) and MHco18(UGA2004) showed a deletion relative to the published transcript between the retained intron locus and exon 2. Finally, there were several SNPs relative to the reference genomic sequence present across all isolates, and several SNPs unique to each isolate, indicating that a level of variability at the single nucleotide level also exists between isolates.

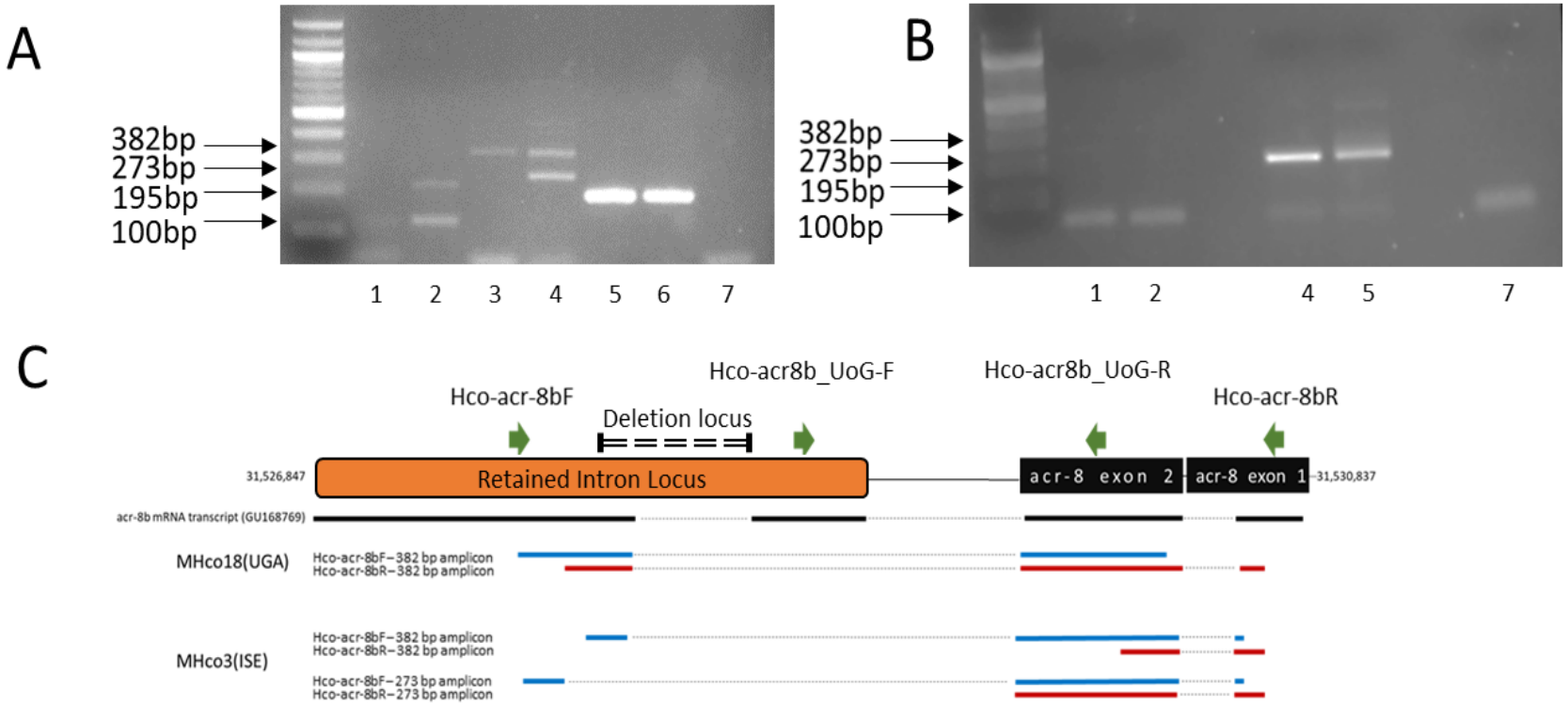

Figure 3.8: Summary of *acr-8b* detection in MHco18(UGA2004) and MHco3(ISE).

A: PCR detection of *acr-8*b in L_3 *H. contortus* cDNA from MHco3(ISE) and MHco18(UGA2004) isolates on a 2% agarose gel. 1: MHco18(UGA2004) (Hco-acr8b-UoG), 2: MHco3(ISE) (Hco-acr8b-UoG), 3: MHco18(UGA2004) (Hco-acr8b), 4: MHco3(ISE) (Hco-acr8b), 5: MHco18(UGA2004) (β -tubulin), 6: MHco3(ISE) (β -Tubulin), 7: No template control (β -Tubulin primers).

B: PCR detection of *acr-8*b in adult male *H. contortus* cDNA from MHco3(ISE) and MHco18(UGA2004) isolates on a 2% agarose gel. 1: MHco18(UGA2004) (Hco-acr8b), 2: MHco3(ISE) (Hco-acr8b), 3: MHco18(UGA2004) (β -tubulin), 4: MHco3(ISE) (β -Tubulin), 5: No template control (β -Tubulin primers).

C: Illustration of results from sequenced PCR products (Figure 2A). Retained intron (orange box) and genomic loci of exon 1 and 2 (black boxes) and partial flanking sequences of intron 2 are shown (black line). A published *acr-8b* transcript (GU168769) is shown below the genomic loci schematic. Sequencing reads: blue represents forward reads; red represents reverse reads. Gaps relative to the reference sequence are represented by dashed black lines. Deletion locus corresponds to the entire region where the variable length (63 – 97 bp) deletion is located.

3.2.5 Deletion in *acr-8* intron 2 correlated with LEV resistant phenotype.

Analysis of WGS from the pre- and post-LEV treated MHco3/18 isolate (Figure 3.9) revealed the presence of a 97 bp deletion present in the second intron of *H. contortus acr-8*. The presence of the deletion appeared to be under LEV selection in post-treatment MHco3/18. This deletion overlies the deletion previously identified in resistant isolates (Barrere et al., 2014). Although there is naturally some difference in sequence, as the deletion identified post-LEV treatment of the cross is 31bp larger, they both share a significant (~60%; see Appendix 5 for alignments) portion of sequence identity and are present in the same locus.

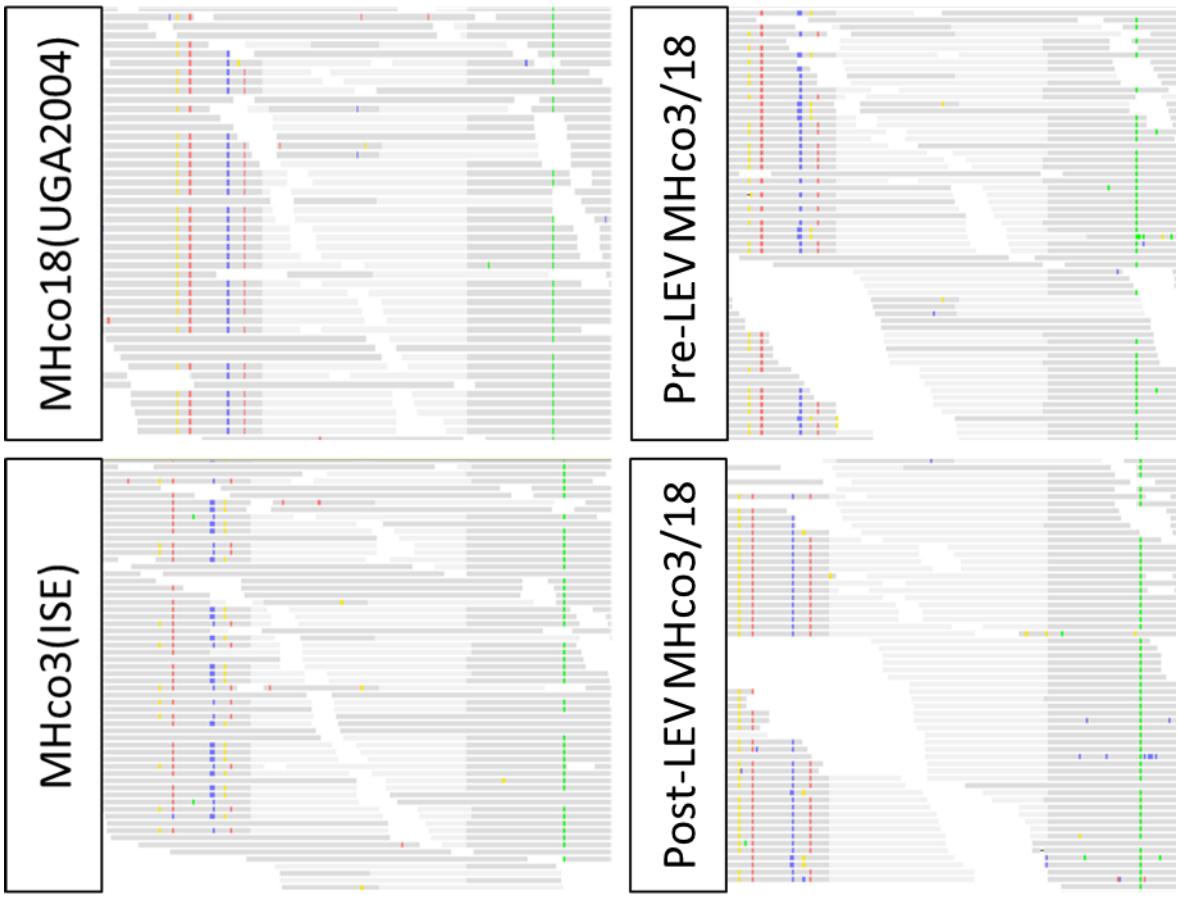

Figure 3.9: Schematic representation of reads spanning the intron 2 deletion locus. A clear deletion is visible in the centre of the image, which increases in prevalence following LEV treatment. The deletion is also present in both MHco3(ISE) and MHco18(UGA2004). Increase in the prevalence of the deletion is most apparent post-LEV treatment, indicating this locus may be under LEV selection.

The presence of the *acr-8* intron 2 deletion was then investigated in individual L_3 by size discrimination PCR to determine the proportion of homozygotes and heterozygotes in test populations of MHco18(UGA2004) and MHco3(ISE). Initial experiments revealed 90% of MHco18(UGA) sampled (n=32) were homozygous for the deletion (DD). The same experiment however, found that 56% of MHco3(ISE) were homozygous for the deletion (n=32). The remainder were almost exclusively heterozygous (DF), with few individuals homozygous for the full-length allele (FF) detected within either population.

To determine if this high percentage of DD individuals in MHco3(ISE) was due to sampling, a larger number (n=92) of MHco3(ISE) L_3 were assayed and a further 69% of individuals were found to be DD (Figure 3.10A). Amongst the total population (n=124) of MHco3(ISE) assayed 64% were found to be DD (Figure 3.10B).

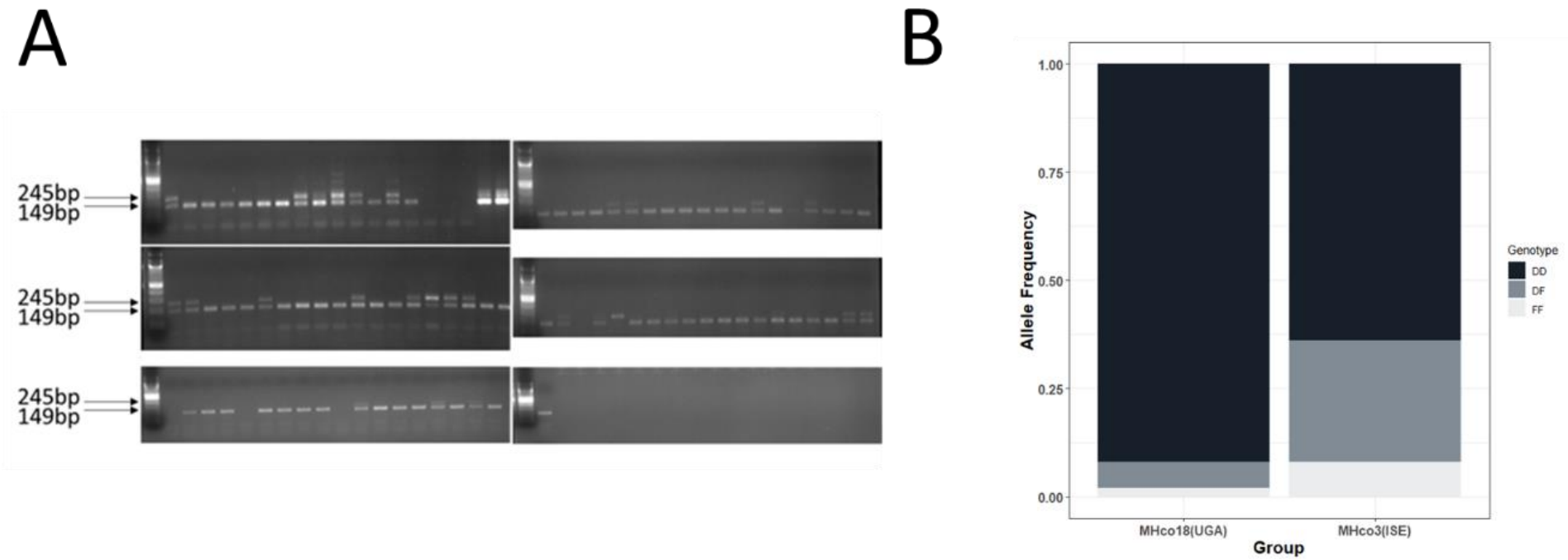

Figure 3.10: Summary of genotyping results by indel size discrimination PCR.

A: Size discrimination PCR for *acr-8* intron 2 deletion in *H. contortus*. Large sample (n=94) of MHco3(ISE) using size discrimination Indel PCR using primers Hco-Indel-F and Hco-Indel-R. From Antonopoulos et al. (2022)

B: Bar chart showing proportion of deletion (D) and full-length (F) allele detected by indel PCR in LEV resistant MHco18(UGA2004) and LEV susceptible MHco3(ISE). DD represents homozygous deletion, DF heterozygous, and FF homozygous full-length.

Finally, as validation, a primer was designed within the deleted sequence in order to confirm the size discrimination PCR genotype. This primer was sited within the 51bp sequence shared between all three deletions identified thus far (see Chapter 2.3.1; 2.7.3.3). This was to ensure that any potential variation in the size of the deletion would not affect detection. The primer was then tested side by side with the deletion spanning primers (Figure 3.11). The same individual MHco3(ISE) and MHco18(UGA2004) L_3 were assayed with each of the two primer sets. The results were found to be 100% concordant (n=6) confirming that the alleles being detected throughout were correctly identified.

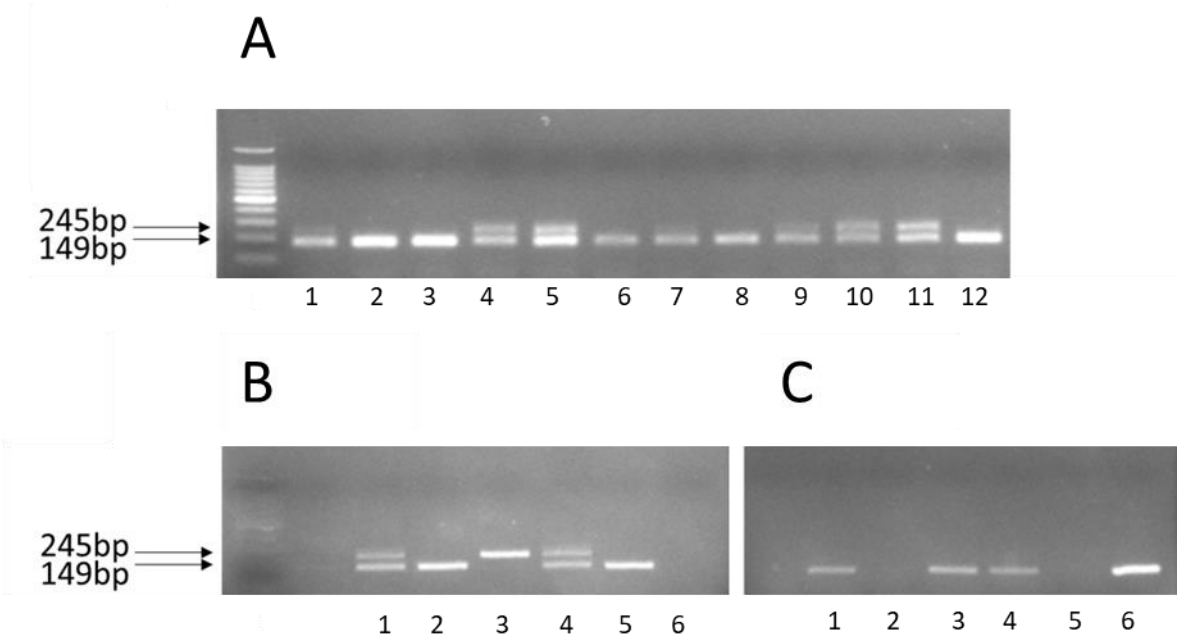

Figure 3.11: Indel PCR genotype confirmation by semi nested deletion PCR.

A: Example gel of single worm PCR of MHco18(UGA2004) and MHco3(ISE) with indel primer set. Odd numbers: MHco18(UGA2004). Even numbers: MHco3(ISE). 245 bp band corresponds to full length allele, 149 bp band corresponds to deletion allele.

B & C: Single worm indel (Hco-Indel-F/Hco-Indel-R) and indel internal confirmation (Hco-Indel-F/Hco-Indel-Ins-R) PCR. B: indel primer set. C: indel internal primer set. Gel 1: 1,3,5 MHco18(UGA2004), 2,4; MHco3(ISE) single L3, 6 NTC. Gel 2: 1,3,5 MHco18(UGA2004); 2,4 MHco3(ISE) single L3 worms; 6 Positive Control (100 ng pooled L3 MHco3(ISE) gDNA. (Reproduced from Antonopoulos et al., 2022 with author permission)

3.3 Discussion

In this section of the study, differential expression of AChR subunits and LEV resistance associated genes in *H. contortus* RNA-seq data was initially investigated. Data showed a strong life-cycle stage and sex-specific expression of several AChR subunits. Differential expression *in vivo* was then examined by qPCR for *acr-8* and *lev-1.1* in adult and L_3 MHco18(UGA2004) and MHco3(ISE). A significant down-regulation of *acr-8* in MHco18(UGA2004) relative to MHco3(ISE) was observed. The expression of the truncated transcript *acr-8b* was detected in L_3 of both MHco18(UGA2004) and MHco3(ISE) but not in adults of either isolate. In addition, unique splice variants of *acr-8b* were detected in MHco3(ISE) L_3. Finally, the presence of the *acr-8* intron 2 deletion was examined by size discrimination single worm PCR, and validated with a deletion specific PCR. Extensive single worm genotyping revealed an overwhelming majority of MHco18(UGA2004), and a majority of MHco3(ISE) L_3 showed a DD genotype.

While the initial groundwork carried out in elucidating LEV resistance has been extremely valuable in identifying a key role for ACR-8 in conferring LEV sensitivity to the AChR in *H. contortus* (Fauvin et al., 2010; Blanchard et al., 2018), the use of genetic crossing provides an important additional tool in identifying genomic loci involved in anthelminthic resistance (Sargison et al., 2018; Doyle et al., 2019; Doyle et al., 2021). By using this approach to complement the data generated to date, is has been possible to identify *acr-8* as an important target for intensive investigation into potential LEV resistance markers.

Initially the expression of AChR subunits involved in receptor assembly, function, and in resistance, were examined by reanalysis of RNA-seq data from various life cycle stages (Laing et al., 2013). Expression profiles of *unc-38*, *ric-3,* and *lev-1*, showed high expression in larval stages, peaking in the L_1 and L_3 (or L_4 in the case of *lev-1.2*), and a sharp decline in the adult stages. In *C. elegans* UNC-38, and LEV-1 are two of the five subunits necessary for the formation of a LEV sensitive receptor (Green et al., 1981; Fleming et al., 1997; Qian et al., 2008).

RIC-3 is a transmembrane protein, which acts as a molecular chaperone during the maturation and processing (folding and assembly) of AChRs in the endoplasmic reticulum (ER) (Millar, 2008). AChR subunits are characterised by a complex membrane topology and folding of each subunit is a slow and inefficient process (Green and Millar, 1995). Correct processing requires the formation of appropriate subunit-subunit interactions in order to form a functional pentameric AChR (Green and Millar, 1995). Mutations in *C. elegans ric-3* lead to reduced cell surface expression of LEV-1 (Gottschalk and Schafer, 2006), and LEV-1 has been shown to co-assemble with UNC-38, while mutation of *unc-38* also leads to altered expression of LEV-1 (Fleming et al., 1997; Lansdell et al., 2005). The similar expression profiles of *unc-38, ric-3,* and *lev-1* in *H. contortus* identified here raises an interesting question regarding the potential role of *lev-1* in resistance. As *lev-1.1* is thought to lack a signal peptide (Neveu et al., 2010; Laing et al., 2013), it is unclear whether it is trafficked to the cell surface and forms part of a functional receptor in *H. contortus*, or whether the signal peptide is redundant due to its necessary association with UNC-38 during assembly in the ER. Furthermore, the role of the *lev-1.2* in this process remains to be elucidated, with differing expression profiles observed for each isoform. There appears to be an L_4 specific expression pattern for *lev-1.2*, and this requires further investigation. The expression profile of *lev-1.1* is also markedly different from *acr-8*. Further questions remain regarding the plasticity of receptor composition, and the relative stability of neuronal receptor quantity, but these questions lie beyond the scope of this study.

A pilot study was then carried out to validate RNA-seq data *in vitro*, and examine expression profiles of QTL genes *lev-1.1* and *acr-8*. Initial qPCR data showed higher expression of *lev-1.1,* and lower expression of *acr-8,* in LEV resistant MHco18(UGA2004) L_3 relative to the LEV susceptible MHco3(ISE) L_3. This is noteworthy especially in light of the significantly higher expression of *lev-1.1* seen in L_3 in RNA-seq data, as there appears to be a strong skew towards expression in this life cycle stage. Early studies showed that L_3 from a LEV resistant isolate did not show strong phenotypic resistance during larval migration assays, judged by motility inhibition (Sangster et al., 1988). It is of note here that larval migration assays (LMA) can be used on L_3 (Demeler et al.,

2010; Raza et al., 2016), but LEV slows movement rather than leading to total paralysis. Thus, this comes with the caveat that it can be somewhat harder to accurately assess degrees of LEV resistance using the LMA on L_3. The induction of L_3-specific resistance when selection pressure is applied to the larvae *in vitro* (Sarai et al., 2014), taken alongside the differential expression of these subunits may imply that mechanisms of resistance and/or inducible tolerance of LEV differ between free-living larvae and adult worms. Thus, increased expression of *lev-1.1* in MHco18(UGA2004) L_3, and a concomitant downregulation of *acr-8* may represent an L_3 specific resistance mechanism driven by the alteration of receptor composition. This was not seen in qPCR data from MHco18(UGA2004) adults, where no significant difference was observed in the expression of *acr-8* or *lev-1.1* relative to the LEV susceptible MHco3(ISE) adults. This may also indicate that adults do not display the same level of neuronal plasticity as larvae, and thus inducible tolerance mechanisms may not be possible in the later life-cycle stages. This has significant ramifications regarding emergence of resistance and the significance of altered expression patterns for treatment and management decisions.

A pronounced sex-dependent expression profile for *acr-8* was also observed in RNA-seq data, with significantly higher expression in adult males compared to adult females. Adult males showed the overall highest expression of *acr-8*, followed by the larval stages and eggs, while expression of *acr-8* was not readily observable in adult females. This is in line with a potential sex-specific expression of genes, although such a strong differential expression is most often related to sex-specific and developmentally expressed proteins in *C. elegans* (Boeck et al., 2016), and *H. contortus* (Laing et al., 2016). However, a sex-specific expression pattern for a receptor subunit shown to play a key role in conferring LEV sensitivity to the AChR (Boulin et al., 2011; Blanchard et al., 2018) is of particular interest both to understanding the mechanism of LEV resistance and the efficacy of LEV *in vivo*. However, there has been no evidence published to date which suggests a sex-specific role for ACR-8, and this differential expression may be life cycle stage specific rather than sex-specific across all life cycle stages, with a sex-specific expression pattern only emerging in the adult stages. Some *trichostrongylidae* have been shown to display a sex-

dependent sensitivity to LEV (Dash, 1985), which could relate to the lifestyles of the males and females. *H. contortus* is known to display polyandrous mating behaviour (Redman et al., 2008; Doyle et al., 2018; Sargison et al., 2018), with multiple males mating with a single female. This necessitates a more motile male, leading to increased movement in the abomasum, and concurrent disruption of feeding and mucosal attachment (Gilleard and Redman, 2016). The increased motility of male worms may explain the sex-specific expression pattern of *acr-8*. This would be in line with the observation that expression of *acr-8* is also relatively high in the larval stages, excluding the L_3 stage which shows relatively low expression. This drop in expression may be explained by the lack of feeding behaviour in the L_3 (Veglia, 1915; Yang et al., 2017).

The initial identification of *acr-8b* in L_3 of the LEV resistant MHco18(UGA2004) (Williamson et al., 2011) and the expression of *acr-8b* in several additional LEV resistant strains (Sarai et al., 2013; Sarai et al., 2014; Barrere et al., 2014) presented the possibility that *acr-8b* may constitute a marker and mechanism of LEV resistance. However, some inconsistencies were observed, such that in one highly resistant isolate (LV – "LevR"), *acr-8b* was not detectable by qPCR (Sarai et al., 2013). The *acr-8b* transcript was also detected in one LEV susceptible (CRA) isolate (Barrere et al., 2014). Nevertheless, the bulk of evidence suggested a role in LEV resistance for *acr-8b*.

However, as part of this study, expression was also detected in L_3 of the LEV susceptible MHco3(ISE) isolate, which has not previously been reported. Barrere et al. (2014) assayed MHco3(ISE) but did not detect the transcript, however, this may have been due to template quantity, rather than lack of expression (β-*tubulin* positive control shows markedly weak banding compared to in other samples) (Barrere et al., 2014) in the MHco3(ISE) sample. Based on PCR carried out as part of this study, and re-analysis of RNA-seq data, AChR subunits are expressed at very low levels, and a sample with weak detection of a ubiquitously expressed gene (Hco-β-*tubulin)* would be unlikely to have sufficient template material to detect expression of *acr-8b*. Following detection of this transcript with primers designed as part of this study (Hco-acr8bUoG-F/Hco-acr8bUoG-R), a previously published primer set (Hco-acr8b-F/Hco-acr8b-R) (Sarai et al., 2013) was employed as validation. Following confirmatory Sanger sequencing

additional variation in the sequence composition and length of the retained intronic sequence was observed in the L_3 of the MHco3(ISE). In addition, amplicons of differing sizes were detected in MHco3(ISE). This is in line with Barrere et al. (2014), who also examined *acr-8b* expression in several resistant and susceptible isolates, with variation in amplicon sizes also observed in the LEV susceptible CRA isolate (Barrere et al., 2014). It was not possible to detect *acr-8b* expression in adults of either MHco3(ISE) or MHco18(UGA2004), which is consistent with previous work (Sarai et al., 2014). This presents *acr-8b* as having a potential life-cycle stage specific expression pattern, which may be related to larval stage inducible tolerance, as has been observed in some studies (Sarai et al., 2014). The mechanism may also be splice variant specific, however, this is not confirmed at the time of writing, and would need to be investigated further to drawn any concrete conclusions.

The detection of *acr-8b* in a further susceptible isolate is significant, as it precludes its use as a reliable marker of resistance. Expression could be attributed to a small population of resistant individuals, as has been observed in a moderately resistant (WAL) population (Sarai et al., 2013; Sarai et al., 2014) which contains a distinct subset of highly resistant individuals. However, the population overall shows an estimated ~80% susceptibility to LEV (Sarai et al., 2013). The field isolate from which ISE was derived originated in the 1950s from a field population which predates the introduction of modern anthelmintics, and there are no documented instances of resistant sub-populations. Variable expression patterns of *acr-8b* in resistant isolates also constitutes an area of interest. In one highly resistant (LV) isolate, which is estimated to be ~90% LEV resistant, *acr-8b* was not detectable in the adult stages. The same study detected *acr-8b* in the WAL isolate (Sarai et al., 2013), but a follow up study by the same authors failed to detect *acr-8b* again in the same isolate (Sarai et al., 2014), indicating variable expression even within the same isolate.

Finally, it has been suggested that worms expressing the truncated *acr-8b* transcript will express receptors lacking a functional ACR-8, and thus be less sensitive to LEV (Barrere et al., 2014). However, both in the present study, and in several others (Sarai et al., 2013; Sarai et al., 2014; Barrere et al., 2014), populations of resistant worms have been found to express both the truncated

acr-8b and the full-length *acr-8*. Theoretically, the truncated ACR-8b protein could substitute for ACR-8 in a dominant negative manner, as has been shown to occur with UNC-63b in *Xenopus* oocytes (Boulin et al., 2011). However, if this were to occur *in vivo*, then we would expect to see some level of resistance in the CRA and MHco3(ISE) isolates, both of which have been shown to express *acr-8b* (Barrere et al., 2014; present study), unless there are other regulatory mechanisms at play which are yet to be elucidated, potentially involving post-translational processing. Expression was reported as relatively low in CRA (Barrere et al., 2014), and this may help explain the lack of resistance in the CRA isolate, although if a highly resistant subpopulation were present, as is the case in the WAL isolate (Sarai et al., 2013; Sarai et al., 2014), then *acr-8b* should nevertheless be detectable if its expression were driving resistance. CRA was isolated in 1984 and has been subsequently maintained and passaged in sheep 75 times, and it seems highly unlikely that a resistant subpopulation would have evaded detection for three decades and across multiple anthelmintic resistance studies.

Finally, potential alternate splice variants of *acr-8b* in MHco3(ISE) L_3 were detected. This raises additional questions regarding the role of *acr-8b*, and truncated transcripts in general, on the physiological function of AChR receptors in *H. contortus*. It may be that these transcripts play a regulatory role in receptor assembly and expression independent of LEV resistance, but which under the right circumstances can become up-regulated to offer some level of LEV resistance. However, significant further work, beyond the scope of this study, would be necessary to elucidate this mechanism. As a final point, this could be integrated into future controlled infection studies, combined with RNA-seq and qPCR, which could yield significant data regarding the role of truncated transcripts and altered transcript expression within resistant isolates of *H. contortus*.

These findings do not preclude *acr-8b* playing a role in a mechanism of LEV resistance, but cumulatively, the detection of *acr-8b* in two fully LEV susceptible isolates, transient or inconsistent detection in a moderately LEV resistant isolate, and undetectable in a highly resistant isolate all indicate it would not serve as an effective marker of resistance. In addition, identification of multiple

potential splice variants would further complicate the picture. This, coupled with the inherent impracticality of working with RNA transcripts for a practical (for veterinary medicine) diagnostic test, demonstrate alternative markers should be sought.

The variable length deletion in intron 2 of *acr-8* was then examined. Barrere et al. (2014) originally described the presence of the deletion in five LEV resistant and six LEV susceptible isolates from diverse geographical origins, including the MHco18(UGA2004) and MHco3(ISE) isolates used in this study. Three of these isolates, Cedara, RHS6, and MHco18(UGA2004), have previously been shown to express *acr-8b* (Fauvin et al., 2010; Neveu et al., 2010; Williamson et al., 2011). Analysis of WGS from MHco3/18 identified a 97bp deletion present in the second intron of *acr-8* (Doyle et al., 2021). The deletion in intron 2 of *acr-8* has previously been detected in both resistant and susceptible isolates (Barrere et al., 2014; dos Santos et al., 2019). However, it was thought that the presence of the deletion in susceptible isolates likely represented heterozygous individuals which would still be phenotypically LEV susceptible (Barrere et al., 2014; dos Santos et al., 2019). This was based on expectations of LEV resistance being recessive (Dobson et al., 1996; Sangster et al., 1998). Single worm PCR was carried out in the initial study by Barrere et al. (2014), however, sample sizes were small (two individuals from six isolates; four LEV resistant and two LEV susceptible). The MHco3(ISE) examined showed one DF individual, and one FF individual, whereas for CRA both individuals were both FF. Pooled samples of ten adults were also assessed, and all but Kokstad (LEV resistant, and encoding only the deletion allele) were found to be encode both alleles (Barrere et al., 2014). A subsequent study in Brazil used this deletion as a genetic marker for LEV resistance and designed a qPCR assay to quantitate the prevalence of the deletion in field samples. dos Santos et al. (2019) reported a relatively high incidence (~30%) of the deletion allele in MHco3(ISE) control population. However, both studies found a statistically significant correlation between the deletion and LEV resistance (Barrere et al., 2014; dos Santos et al., 2019).

Primers were therefore designed spanning the *acr-8* intron 2 deletion to investigate this, and amplicons sequenced from MHco18(UGA2004) and MHco3(ISE). A 67 bp deletion was detected in these isolates. This differed from

the 63 bp deletion reported by Barrere et al. (2014), and the 97 bp deletion found in MHco3/18 (Doyle et al., 2021). A core sequence of 51 bp is shared by all three deletions. Some variation in length of the deletion has already been documented (Barrere et al., 2014), thus, this finding was in line with expectations. Single worm PCR was then carried out to determine the genotype of individual L_3 within MHco18(UGA2004) and MHco3(ISE) test populations. It was necessary to determine if previous reports of the presence of the deletion were indeed due to heterozygosity in the susceptible populations. Data generated as part of this study showed that the deletion would not serve as a diagnostic marker, due to the presence of >60% individuals being DD in the MHco3(ISE) population tested. The single worm PCR data herein is in line with a more recent study by Baltrusis et al. (2021) examining the presence of the deletion in Swedish field isolates of *H. contortus*. Contrary to previous studies (Barrere et al., 2014; dos Santos et al., 2019), the authors found no correlation between a LEV resistant phenotype and the presence of the deletion in a population. They observed that sample populations found to have a high proportion of the deletion allele did not survive LEV treatment (Baltrusis et al., 2021). The previous association of the deletion with a LEV resistant phenotype can in a large part be explained by natural variations of the proportion of the deletion allele within populations. This led to a correlation appearing to be present, but which does not stand on further examination. This also highlights the critical importance of carrying out larger scale single worm PCR, as was done here, and the use of pre- and post-LEV treatment samples (Baltrusis et al., 2021).

3.3.1 Concluding remarks

The expression patterns of AChR receptor subunits in *H. contortus* was initially examined, with a particular focus on the truncated *acr-8b* transcript as a potential diagnostic marker of LEV resistance. Although *acr-8b* was indeed expressed in the resistant MHco18(UGA2004) isolate, it was also detected in LEV susceptible MHco3(ISE) L_3. This finding was further complicated by what appears to be multiple splice variants of differing sizes expressed in this isolate, in addition to a high degree of sequence variation. As such these data do not

suggest that *acr-8b* would serve as an effective marker of LEV resistance at the present time. However, its mechanistic role in LEV resistance, and the AChR in general remain to be elucidated. The deletion in intron 2 of *acr-8* was then examined. Although a statistical correlation with LEV resistance was initially reported by some studies (Barrere et al., 2014; dos Santos et al., 2019) data here, and that of Baltrusis et al. (2021), did not support these findings. Furthermore, data generated in this study also demonstrates the value of single worm genotyping in order to fully elucidate potential genetic markers of resistance. In conclusion, alternative markers must be sought for LEV resistance in *H. contortus*.

4 Restriction Fragment Length Polymorphism and Allele Specific PCR for the Detection of the S168T Variant in *Haemonchus contortus*.

4.1 Introduction

4.1.1 S168T variant

The S168T variant is a recently reported non-synonymous SNP present in exon 4 of the *acr-8* (HCON_00151270) gene (Doyle et al., 2021). It is strongly associated with a LEV resistant phenotype in *H. contortus*, and its frequency was found to increase following LEV selection of a multi-drug resistant genetic cross (Doyle et al., 2021; Antonopoulos et al., 2022). This variant encodes a serine-threonine change at codon 168 of the *H. contortus acr-8* gene. *In silico* analysis has shown that this serine residue is highly conserved amongst parasitic nematodes (Figure 4.1; Doyle et al., 2021). In addition, the threonine residue has been identified at this position in a resistant isolate of *T. circumcincta* (Choi et al., 2017), indicating that this may be associated with a resistant phenotype in other closely related parasitic nematodes.

Figure 4.1: Conservation of serine 168 residue at analogous positions in homologous proteins across selected *Nematoda* spp. The serine residue is highly conserved in closely related Clade V nematodes, except for *Caenorhabditis* spp. where a threonine residue is present at the analogous position.

4.1.2 SNP genotyping

Haemonchus contortus is a diploid organism, and the heritability of LEV resistance has long been thought to be recessive (Dobson et al., 1996; Sangster et al., 1998). To further investigate the inheritance of LEV resistance, and develop a sensitive molecular assay, it was therefore important to use single worm genotyping to validate the S168T variant.

Since its inception in the 1980s (Mullis et al., 1986), PCR has been adapted to a wide variety of uses beyond simple amplification of target sequences. One of these applications, now widespread, is SNP genotyping. As SNPs can be involved in a variety of clinically relevant mechanisms in pathogen biology, including both drug resistance and pathogenesis, their detection has become a mainstay of diagnostic medicine (Matsuda, 2017).

A wide variety of different PCR based methodologies are now available for the detection of SNPs in a given sample organism, each with its own advantages and disadvantages.

4.1.3 Restriction fragment length polymorphism

Restriction fragment length polymorphism (RFLP) is one of the quickest and simplest PCR based SNP genotyping assays to develop (Pourzand and Cerutti, 1993; Gasser and Chilton, 1995), requiring only template enrichment followed by restriction digest and gel electrophoresis. RFLP can be used to discriminate different alleles in a single PCR reaction. However, it requires that a restriction site be introduced by the SNP of interest, which may not occur in every case. Furthermore, restriction sites may be present, but require restriction enzymes that are not widely available commercially, are particularly expensive, or that require reaction conditions that are impractical for routine use, which can complicate and limit use of the assay.

Initially, once a restriction site has been identified in the target sequence, PCR primers are developed which amplify the region of interest. The amplicon should be of sufficient length to allow identification of variants by gel electrophoresis.

Generation of insufficient product or very short amplicons may cause issues with visualisation, as will a restriction site placed too far at either end of the amplicon in question, making identification of cut products difficult. In addition, although easy to develop (if commercially available restriction enzyme sites are present), the assay can be time consuming, as it requires digestion steps in addition to PCR. As such, its role in SNP genotyping is largely restricted to research settings, as it is not a viable protocol for large scale or high throughput diagnostic applications.

There has been considerable prior application of RFLP in a wide variety of organisms and conditions, particularly, for *H. contortus*, and BZ resistance. Early studies made use of RFLP to discriminate between β-tubulin alleles in resistant and susceptible isolates and were instrumental in early work investigating BZ resistance (Lubega et al., 1994; Kwa et al., 1994). Since then, RFLP has been widely used to assess the presence of different resistance SNPs related to BZs in *H. contortus* (Tiwari et al., 2006; Nabavi et al., 2011; Shokrani et al., 2012; Chandra et al., 2015; Nagy et al., 2016; Kowal et al., 2016). The technique has also been demonstrated for screening for BZ resistance in human soil transmitted helminths such as *Necator americanus* and *Ascaris lumbricoides* (Zuccherato et al., 2018) and other veterinary pathogens such as *Ancylostoma braziliense* (Furtado et al., 2019).

4.1.4 Allele-specific PCR

Allele specific PCR is a rather broad umbrella term covering a variety of PCR based techniques for allelic discrimination. In the most general terms this is accomplished by the preferential amplification of the allele of interest and/or the suppression of amplification of the allele which is not of interest. The most salient of these for the purposes of the current study is amplification refractory mutation system (ARMS) PCR, which will hereafter be referred to simply as allele specific (AS)-PCR. This technique makes use of a primer set which amplifies only in the presence of the desired allele. This is accomplished by the inclusion of a SNP specific mismatch which only allows for annealing and polymerisation of the allele specific primer when the SNP is present. There are a variety of possible

mismatch combinations, which will vary based on the specific demands of the assay design (Bui et al., 2009; Liu et al., 2012). It is also necessary to consider the destabilising potential of nucleotide mismatches. For example, a C-C, or A-A and a C-T or A-G mismatch do not behave identically, and thus the choice of nucleotide-nucleotide mismatch can influence the overall dynamics of a primer (Liu et al., 2012). Assay conditions can also be influenced by a number of factors including: the polymerase, the temperature requirements of the reactions, the polymorphism of the sequence, and quality and quantity of template (Bottema and Sommer, 1993; Sullenberger and Maine, 2018).

Tetra-ARMS PCR, as the name suggests, is a variant of AS-PCR which makes uses of four primers (Sullenberger and Maine, 2018). It is a well-established technique which has recently been demonstrated for the benzimidazole resistance SNP E198A in *H. contortus* (Zongze et al., 2018) and F200Y in *A. lumbricoides* (Furtado et al., 2019). In addition to the allele specific primers (one each for resistant and/or sensitive alleles) tetra-ARMS also includes a set of outer primers which amplify the genomic region surrounding the SNP of interest. The allele specific primer then pairs with either a forward or reverse outer primer, which drives the allele specific reaction. Tetra-ARMS PCR has several key advantages over standard AS-PCR, which is of particular interest when conducting single worm genotyping. Assays for detecting both alleles can be run in a single tube if resistant and susceptible primers are orientated in opposite directions (i.e. one forward the other reverse), and the SNP of interest is not equidistant between the outer forward and reverse primers (which can be avoided by taking account of this during the design process). This allows for multiplexing, as two different amplicon sizes will be produced by each of the allele specific-outer primer pairs (i.e forward or reverse outer primer). However, if both primers are orientated in the same direction, no size differentiation will be possible, as pairing will be with the same outer primer in the case of each allele specific primer. The inclusion of the outer primers allows for the enrichment of template in the case of low abundance samples, such as encountered when dealing with single nematode L_3, and it also adds a level of multifunctionality to the assay, as the template produced by the outer primers can be used for sequencing (Sullenberger and Maine, 2018).

4.1.5 Summary

The S168T variant is a non-synonymous SNP identified in exon 4 of the *H. contortus acr-8* gene, which was found to be under LEV selection in the MHco3/18 genetic cross (Doyle et al., 2021). Previous studies have also identified a serine-threonine coding change at the corresponding locus in LEV resistant *T. circumcincta* (Choi et al., 2017). The serine residue at this position is highly conserved across Clade V parasitic nematodes. The S168T variant, therefore, presents as an ideal putative marker of LEV resistance in *H. contortus*, but requires validation in individual worms prior to use as a diagnostic marker.

4.1.6 Aims

This section of the study aimed to validate the S168T variant as a genetic marker of LEV resistance in *H. contortus*. This was to be accomplished by developing a laboratory based RFLP assay to allow genotyping of resistant and susceptible individual worms from laboratory isolates. Individual worm genotypes would confirm population-level (pooled worm) WGS data which indicated a strong correlation between a LEV resistant phenotype and the presence of the S168T variant. Following this, the aim was to develop a faster and more accurate AS-PCR assay which could be used as a robust proof-of-concept molecular diagnostic assay to genotype laboratory and field populations. Finally, the adaption of the AS-PCR to genetically divergent LEV susceptible populations of *H. contortus*, and preliminary adaptation to the closely related and economically important *T. circumcincta* were explored.

4.2 Results

4.2.1 S168T

A single non-synonymous SNP in exon 4 of *acr-8* (PRJEB506: HCON_00151270; 31,521,884 bp; GCT-GGT) that is under LEV selection in the MHco3/18 genetic cross was recently reported (Doyle et al., 2021); re-analysis of pooled WGS showed that, in the F3 generation, S168T was present in ~35% of reads from the population pre-LEV selection, increasing to ~70% of reads in the population post-LEV selection. Re-analysis of WGS sequencing data from LEV resistant (MHco18(UGA2004)) and LEV susceptible (MHco3(ISE), MHco4(WRS), MHco10(CAVR)) isolates found S168T is present in ~70% of MHco18(UGA2004) reads, and absent in MHco3(ISE), MHco4(WRS), and MHco10(CAVR) reads.

4.2.2 Restriction fragment length polymorphism

The GCT-GGT (S168T) mutation at codon 168 introduces an *Ava*II restriction site, which allowed development of an RFLP assay to genotype individual MHco3(ISE), MHco18(UGA2004), and MHco3/18 L_3. A 237 bp product encompassing exon 4 and a small section of intronic sequence upstream of exon 4 of *acr-8* was amplified, followed by digestion with *Ava*II (Figure 4.2A). Single worm RFLP was then carried out to predict the genotype of a sample of LEV resistant or susceptible L_3. The S168T variant was not detected in any of the MHco3(ISE) worms, while 75% (n=58) of MHco18(UGA2004) worms (n=77) showed the presence of at least one GGT allele, of which 38% of worms were identified as homozygous for GGT (n= 30) and 36% were of ambiguous genotype with at least one allele GGT (n= 28) (Figure 4.2B). For ambiguous genotypes, it was difficult to ascertain with certainty whether an individual was homozygous or heterozygous due to incomplete and variable digestion of PCR product (Figure 4.2A, gel 3).

A total of 40 F3 generation MHco3/18 L_3 without LEV selection were also analysed by RFLP; ~50% of individuals were of ambiguous genotype, and ~2% homozygous for the GGT allele, with the remainder (~48%) homozygous GCT (Figure 4.2A).

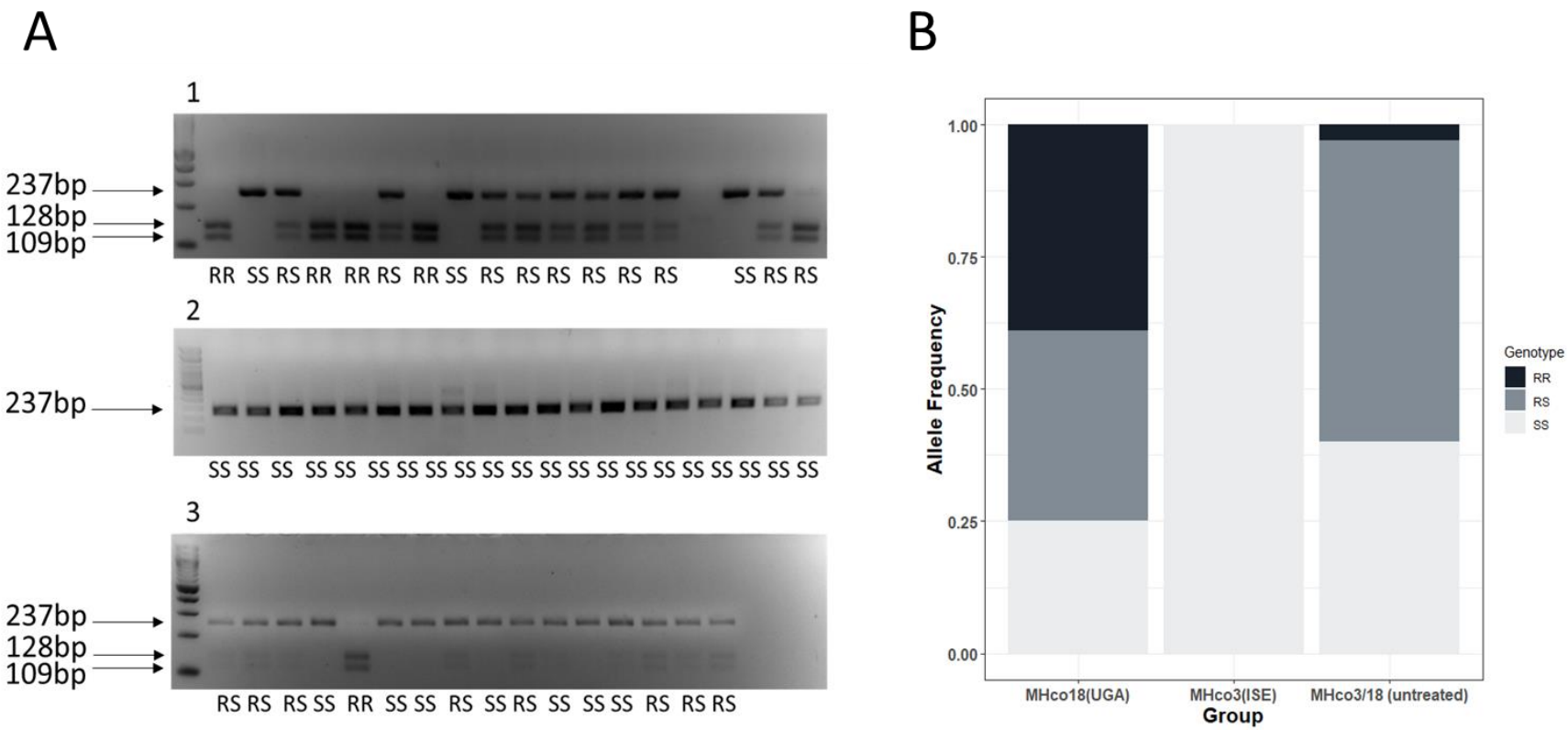

Figure 4.2: Summary of RFLP analysis for the detection of S168 (S/susceptible – GCT) and S168T (R/resistant – GGT) allele in MHco18(UGA2004), MHco3(ISE) and MHco3/18 L_3.

A: Detection of GGT by single L_3 *Ava*II RFLP in LEV resistant MHco18(UGA2004), LEV susceptible MHco3(ISE), and MHco3/18 genetic cross. 1: MHco18(UGA2004); 2: MHco3(ISE); 3: MHco3/18. 237 bp bands represents 168S GCT sequence. 128 bp and 109 bp bands represent GGT sequence. Predicted genotypes RR: homozygous GGT; RS: heterozygous; SS: homozygous GCT.

B: Bar chart showing proportion of GGT allele detected by RFLP in LEV resistant MHco18(UGA2004), LEV susceptible MHco3(ISE), and MHco3/18 genetic cross. Y axis: genotype frequency. Fill: Genotype frequency

4.2.3 AS-PCR

4.2.3.1 Optimisation of reaction conditions using cloned sequences S168T and S168

Cloned *acr-8* exon 4 amplicons were initially used for PCR optimisation to provide abundant template and remove the complexity of sequence polymorphism in individual worms. Gradient PCR was used to identify the optimal annealing temperature of 60^{o}C. Different combinations of allele specific and forward primers were also extensively tested before arriving at the pairing of Hco-Intron4-F + Hco-168S-R for the detection of S168 allele, and Hco-exon4-F + Hco-168T-R for the detection of the S168T allele. There is, thus, a size discrepancy between the resistant (132 bp) and susceptible (148 bp) PCR products. As shown in Figure 3, following this optimisation process allele specific discrimination was established using cloned exon 4 fragments containing the exon 4 S168 (GCT) and S168T (GGT) alleles. However, some faint non-specific amplification was seen in the resistant primer (Figure 3A).

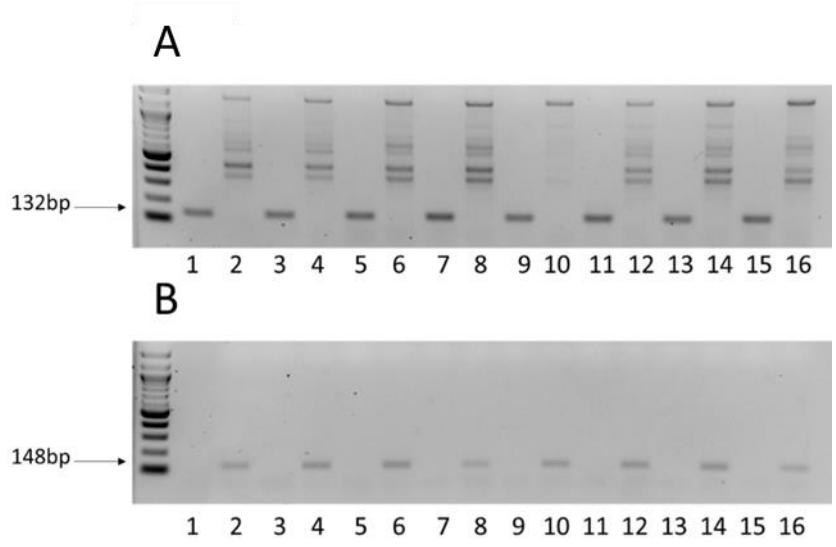

Figure 4.3: Example gel showing allele specific discrimination between S168T and S168 alleles using cloned *acr-8* exon 4 template.

A: Cloned S168T (GGT) sequence assayed with Hco-Exon4-F + Hco-168T-R (odd numbers) and Hco-Intron4-F + Hco-168S-R (even numbers). Lane 16 shows non-specific amplification.

B: Cloned S168 (GCT) sequence assayed with Hco-Exon4-F + Hco-168T-R (odd numbers) and Hco-Intron4-F + Hco-168S-R (even numbers).

4.2.3.2 Single worm AS-PCR

Following optimisation, a single worm two-step nested AS-PCR protocol, comprising initial high fidelity polymerase amplification of *acr-8* exon 4 template, followed by AS-PCR for each allele to determine genotype, was validated (Figure 4.4) to detect the presence of S168T from single L_3 lysates. MHco18(UGA2004) showed a high proportion of heterozygotes (RS), as well as subsets of both homozygous S168T (RR) and homozygous S168 (SS) individuals. The MHco3(ISE) population showed 100% SS individuals. This, along with good correlation with RFLP results, indicated that the AS-PCR was capable of accurately discriminating between the S168 and S168T alleles and was ready to be used to measure the frequency of S168T in different populations.

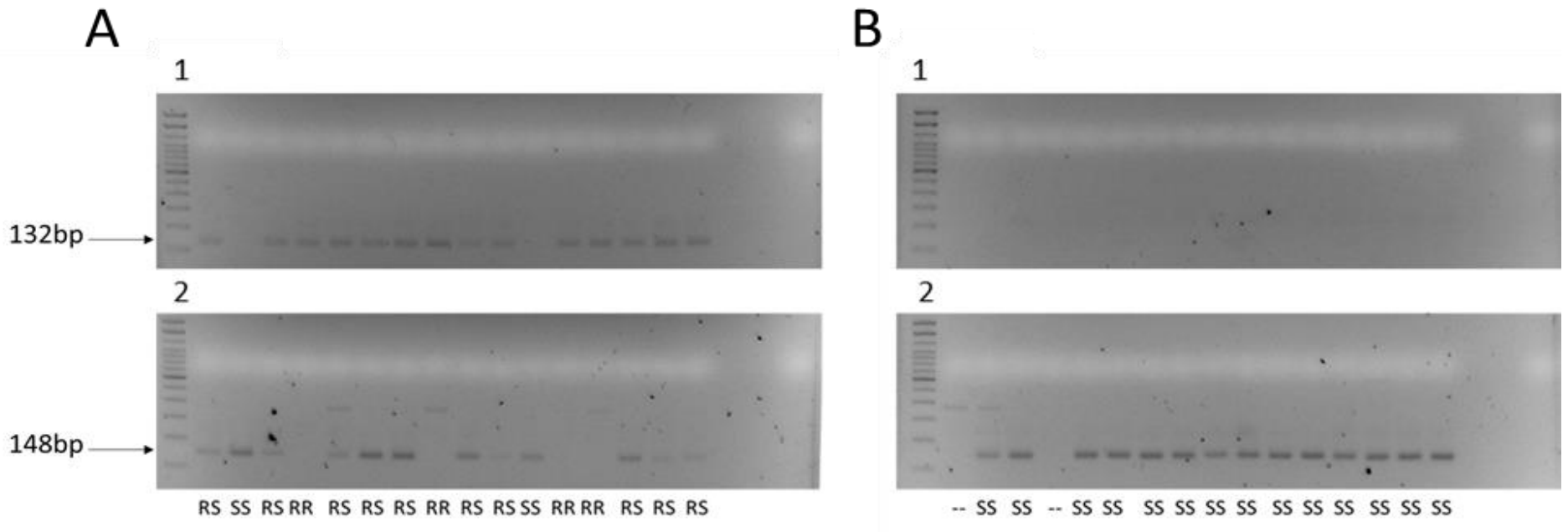

Figure 4.4: Single worm AS-PCR to discriminate between LEV resistant and LEV susceptible L_3 of *H. contortus.*

16 LEV resistant MHco18(UGA2004) and 16 LEV susceptible MHco3(ISE) L_3 were analysed on 2% agarose gel using the two-step nested AS-PCR for the detection of S168T. Each gel corresponds to a primer set amplifying either the S168T or the S168 allele for either MHco18(UGA2004) or MHco3(ISE).

A: MHco18(UGA2004): 1: Exon 4Fw + Hco-168T-R. 2: Hco-Intron4-F + Hco-168S-R.

B: MHco3(ISE): 1: Exon 4Fw + Hco-168T-R. 2: Hco-Intron4-F + Hco-168S-R.

Predicted genotypes RR: homozygous GGT; RS: heterozygous; SS: homozygous GCT. "- -" indicates this individual did not amplify by PCR.

4.2.4 Genotyping L_3 using AS-PCR

Following optimisation of the AS-PCR, it was used to genotype a range of populations with known LEV phenotypes to further validate the efficacy of the S168T allele as a robust marker of LEV resistance (Figure 4.5). Initially, the laboratory populations used in the optimisation process were genotyped (see Appendix 6 for full tables). All MHco3(ISE) individuals were SS (n=38). MHco18(UGA2004) was found to be a mixed population, with a high proportion of individuals (94%, n=71) with the S168T variant. Around one third of MHco18(UGA) individuals assayed were found to be RR, with only 6% found to be SS. In F3 progeny of the MHco3/18 genetic cross, the proportion of individuals encoding at least one S168T (R) allele increased from 58% (n=85) to 75% (n = 79) following LEV treatment of the F2 adults during an *in vivo* infection. RR individuals increased from 9% to 16% following LEV administration. The pre-treatment population was found to be within Hardy-Weinberg equilibrium (HWE) (χ^2=0.81, p=0.29) whereas the post-treatment population was not in HWE (χ^2=3.26, p=0.043), with an excess of heterozygotes found in the post-treatment population.

Finally, field populations of *H. contortus* from two farms located in the Southern United States were analysed for the presence of S168T. DrenchRite® larval development assays showed Farm 001 had an EC_{50} of 9.36 μM for LEV, and was predicated to be resistant, whereas Farm 002 had an EC_{50} of 0.57 μM and was predicted to be LEV susceptible. 80% of individual L_3 assayed from Farm 001 (n=59) were found to encode at least one S168T allele, of which 32% were RR, 48% RS, and the remaining 20% SS. Farm 002 was found to be 100% SS (n=38).

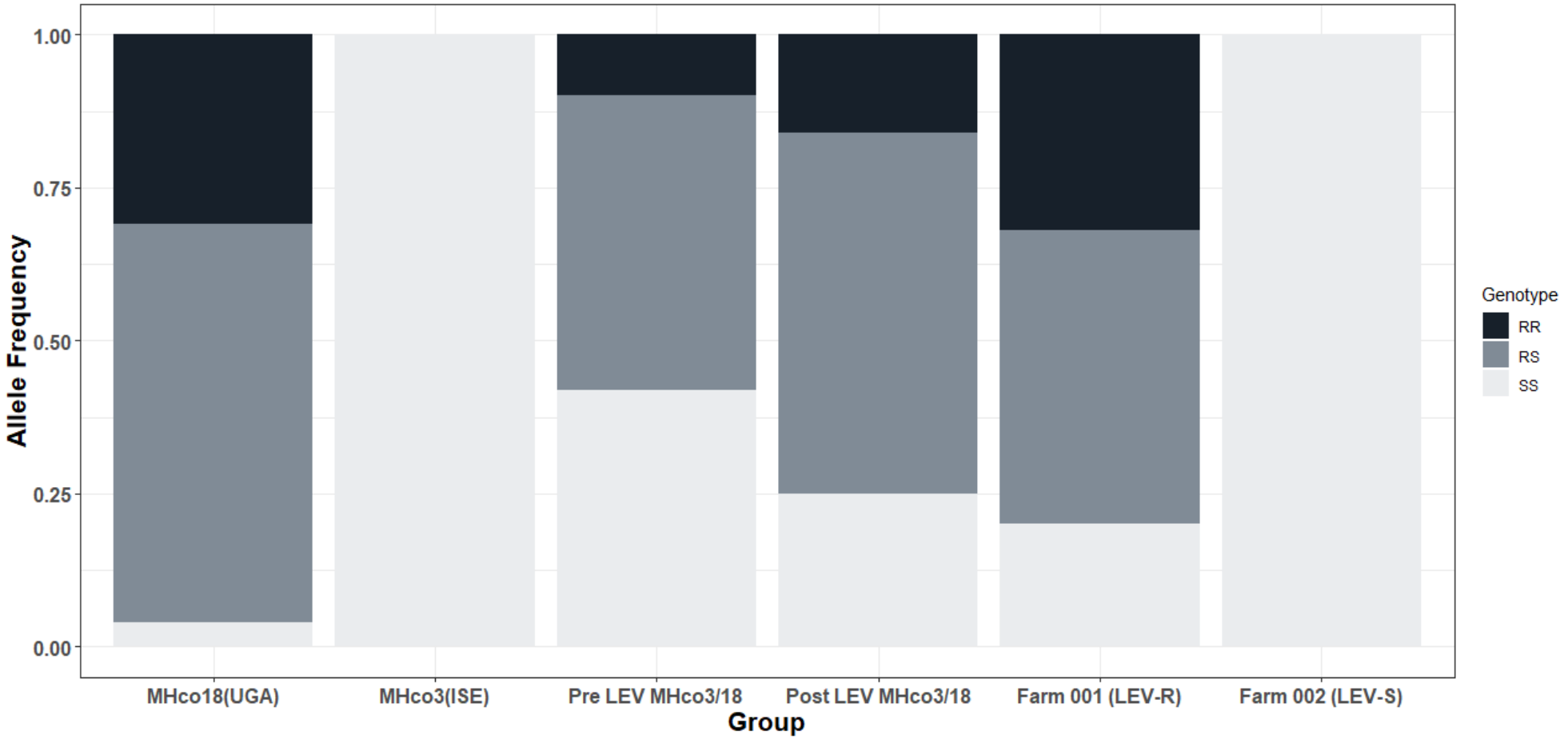

Figure 4.5: Bar chart showing the proportion of S168T and S168 alleles detected by AS-PCR within LEV resistant and LEV susceptible laboratory and field isolates. X axis: *H. contortus* isolate. Y axis: allele frequency. Fill: genotype frequency.

However, in Farm 002 a high degree of amplification failure was observed, with only 59% of wells amplifying (n=64). A control PCR using species identification primers targeting the highly conserved ITS2 locus (Redman et al., 2008) was then used on the 64 L_3 assayed from Farm 002 to ascertain whether amplification failure was due to polymorphism at the *acr-8* locus or due to sample quality. ITS2 PCR showed a strong correlation between the band intensity observed in the AS-PCR and the ITS2 PCR. As these PCRs each target distinct regions of the genome, this indicated that poor amplification was likely the result of sample quality, not polymorphism at the *acr-8* locus in this case (Figure 4.6).

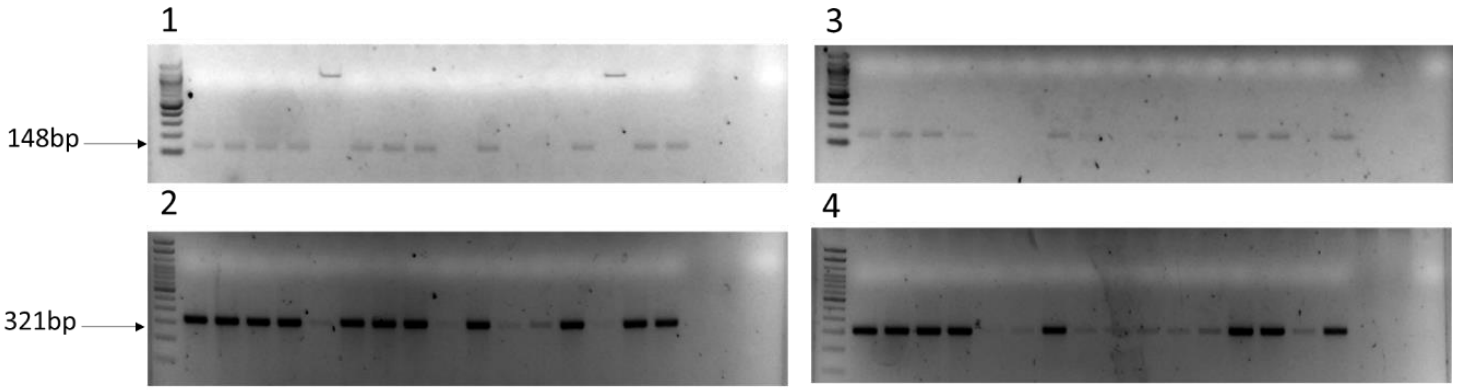

Figure 4.6: Comparison of first round AS-PCR amplification of *acr-8* exon 4 template and ITS2 species identification PCR in Farm 002 L_3.

Farm 002 was amplified by AS-PCR (1 and 3; Hco-168S-R (sensitive) primer; 148 bp band) and ITS2 species identification PCR (2 and 4; 321 bp band) and 100% concordance between poor amplification by AS-PCR and ITS2 PCR indicating a lack of genetic material is responsible for failure of those wells to amplify.

4.2.5 Genotyping adults surviving LEV treatment using AS-PCR

If LEV resistance is recessive and conferred by a single locus (S168T), all progeny of the MHco3/18 cross post LEV treatment would be expected to be RR, which was not the case. In order to explore this finding, S168T AS-PCR was used to genotype five adult female heads (to avoid contamination with fertilised eggs) and five adult male MHco3/18 surviving LEV administration *in vivo* (Figure 4.7). It was found that 2/4 of females and 3/5 of males assayed were RR. It was found that 1/4 of females were RS, and 1/4 SS. In total 5/9 of surviving adults were

RR, with 2/9 either RS or SS. Amplification failed for one female, which was likely due to poor template quality.

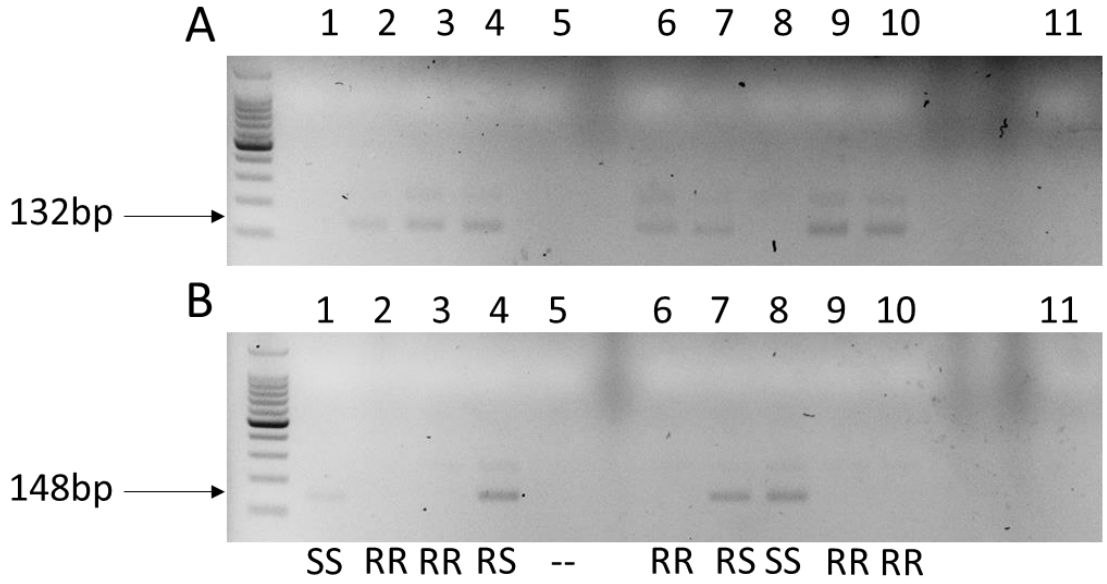

Figure 4.7: AS-PCR of five individual adult male and five individual adult female MHco3/18 worms surviving LEV administration *in vivo*.

Primers used in panel A: Hco-Exon4-F + Hco-168T-R; primers used in panel B: Hco-Intron4-F + Hco-168S-R. Lanes 1-5: adult female MHco3/18; lanes 6-10: adult male MHco3/18. 11: no template control. Predicted genotypes RR: homozygous GGT; RS: heterozygous; SS: homozygous GCT. "- -" indicates this individual did not amplify by PCR. Only wells that amplified by PCR are counted in the final percentage counts.

4.2.6 Deoxyinosine modification of primers for optimisation of S168T allele detection in divergent LEV susceptible isolates

Following demonstration of good performance of the S168T AS-PCR on laboratory isolates and field isolates from the Southern United States, the assay was tested on two distinct and geographically separated LEV susceptible isolates. Pooled WGS data (Redman et al., 2012; Gilleard, 2013) showed that the S168T variant is absent in both the MHco10(CAVR) and MHco4(WRS) populations.

The exon 4 template was successfully amplified in the first round using primers Hco-Intron4-F + Hco-Exon4-R, however, the second-round allele specific reaction showed no amplification in MHco4(WRS) and MHco10(CAVR). The dilution factor was then lowered from 1:20 to 1:8 to determine if amplification would occur in

the presence of more abundant first round exon 4 template. This led to second-round amplification, however, the reaction was no longer specific, with non-specific amplification seen using the resistant primer, and general inconsistent amplification across both primers and isolates (Figure 4.8A, B). Analysis of published WGS data at the *acr-8* exon 4 locus in MHco4(WRS) and MHco10(CAVR) showed synonymous SNPs present at the 11th and 17th bases from the 5' end of the primer bind site for the allele specific primers. Deoxyinosine bases [I] were then substituted at the 11th and the 11th and 17th positions (Hco-168T/S[I]-R and Hco-168T/S[II]-R respectively) and tested under the standardised AS-PCR conditions established previously. This yielded a substantial improvement in both specificity and consistency of the assay (Figure 4.8C, D). However, some non-specific amplification was still detectable in both isolates (Figure 4.8C MHco4(WRS) lane 1, MHco10(CAVR) lane 6).

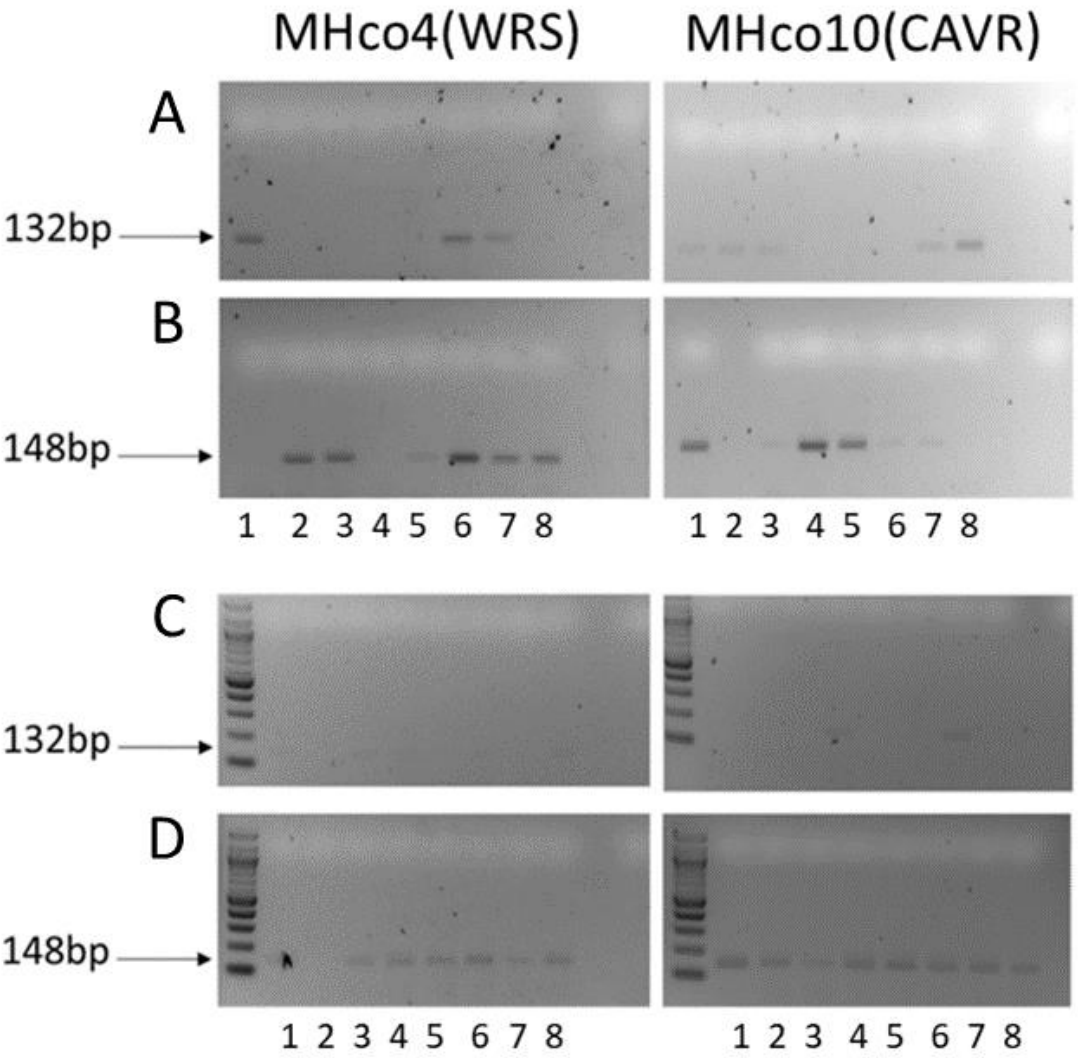

Figure 4.8: Comparison gel showing difference between unmodified (Hco-168T-R/Hco-168S-R) and deoxyinosine modified (Hco-168T[I]-R/Hco-168S[I]-R) primers on geographically divergent LEV susceptible isolates MHco4(WRS) and MHco10(CAVR):

Each gel corresponds to a primer set amplifying either the S168T or the S168 allele for either MHco4(WRS) or MHco10(CAVR. The top panel represents the unmodified AS-PCR primers (A: Hco-168T-R, B: Hco-168S-R) trialled on MHco4(WRS) and MHco10(CAVR) isolates (first round product diluted 1:8). The lower panel represents the deoxyinosine modified AS-PCR (C: Hco-168T[I]-R, D:Hco-168S[I]-R) primers trialled on MHco4(WRS) and MHco10(CAVR) (first round product diluted 1:20).

X axis 1-8: individual L_3 worms. The same eight individual L_3 from each isolate were used to compare primer sets.

4.2.7 *T. circumcincta* AS-PCR

Following the establishment of the S168T AS-PCR in *H. contortus,* a modified version of the assay was trialled on *T. circumcincta.* The SNP responsible for the equivalent serine-threonine substitution in *T. circumcincta* lies with exon 3 of *acr-8.* Initial experiments focused on amplification of the exon 3 fragment using a variety of primer pairs (Figure 4.9), showing moderate to good amplification using primer pair Tci-Exon3-F + Tci-Exon3-R. However, non-specific amplicons larger than the expected size in most individual L_3 assayed were present (Figure 9A 1-8, B 1-8). Primer pair Tci-Intron4-F + Tci-Exon3-R showed less consistent amplification, but with an absence of non-specific amplification (Figure 4.9A 9-16, B 9-16).

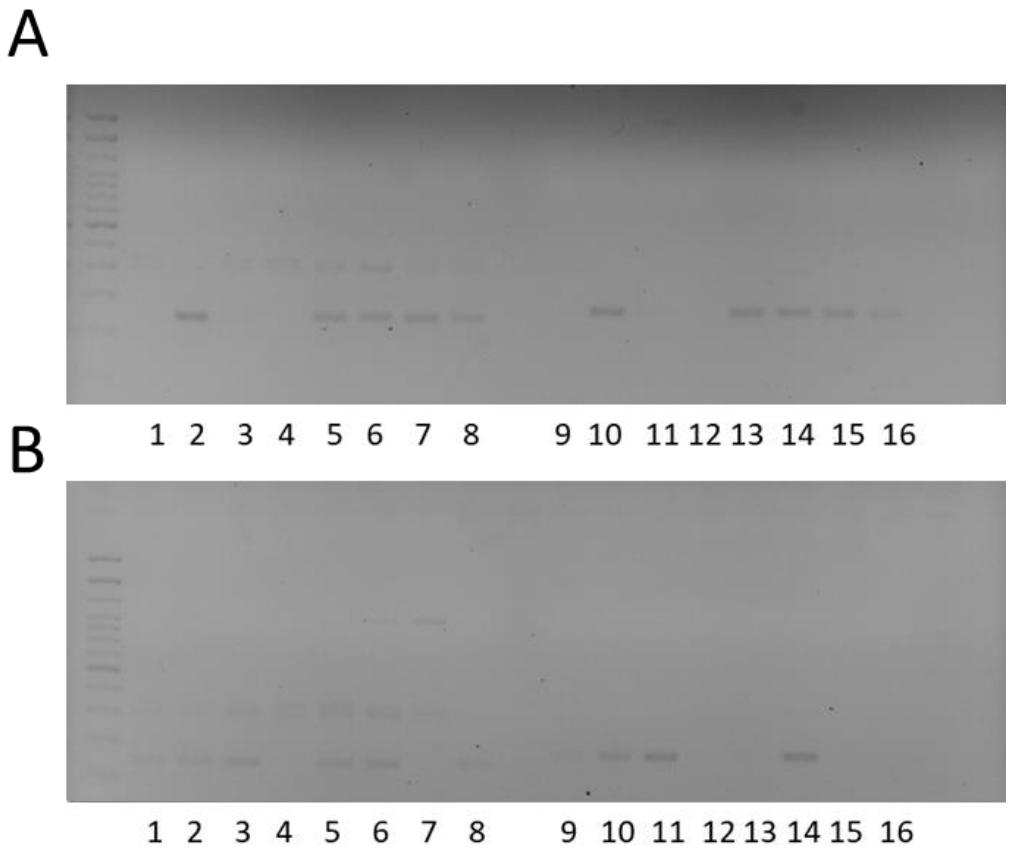

Figure 4.9: *T. circumcincta* exon 3 primer pairing comparison PCR.

A: MTci2. 1-8: Tci-Exon3-F + Tci-Exon3-R. 9-16: Tci-Intron4-F + Tci-Exon3-R. B: MTci5. 1-8: Tci-Exon3-F + Tci-Exon3-R. 9-16: Tci-Intron4-F + Tci-Exon3-R.

Second round allele-specific PCR was then carried out with *T. circumcincta* AS-PCR primers Tci-S168T-R or Tci-S168-R using diluted first round PCR products (Figure 9). Primer pairing of allele-specific primers with either Tci-Intron3-F or Tci-Exon3-F was evaluated. However, following repeated testing at various temperatures, it was not possible to achieve allele specific amplification with either of these primer pairs in MTci2 and MTci5.

4.3 Discussion

In this section of the study, the presence of the S168T variant in LEV resistant MHco18(UGA2004) L_3, and the absence of the S168T variant in LEV susceptible MHco3(ISE) was validated, confirming WGS data. A two-step nested PCR and RFLP was designed for this purpose, which were then used to genotype MHco3(ISE), MHco18(UGA2004), and MHco3/18 populations. This indicated that S168T would likely serve as an effective marker of LEV resistance in *H. contortus*. A two-step nested AS-PCR for the detection of S168T in laboratory and field populations was then developed, which was used to measure allele frequencies in phenotyped populations. A high proportion of L_3 within resistant populations encoded the variant, whereas it was notably absent in all susceptible populations assayed. However, there were still a significant number of homozygous S168 individuals in F3 progeny of the MHco3/18 genetic cross following LEV treatment. AS-PCR was then used to assay MHco3/18 adults surviving LEV treatment, which identified the presence of homozygous S168 individuals. Finally, preliminary experiments adapting the *H. contortus* S168T AS-PCR to *T. circumcincta* were also carried out, although further work is necessary to adapt the assay to *T. circumcincta*.

This section of the study sought to characterise the S168T variant in laboratory and field isolates of *H. contortus* to explore its potential as a diagnostic marker of LEV resistance. S168T is the only non-synonymous SNP present in *H. contortus acr-8* in the available WGS data, and its presence is strongly correlated with a LEV resistance phenotype. S168T is entirely absent from L_3 of all LEV susceptible isolates and identified in a moderate to high proportion of individuals in all LEV resistant isolates examined in this study. This raises the possibility of S168T constituting a robust marker for LEV resistance. The practicality of developing a proof-of-concept diagnostic assay for the detection of S168T in *H. contortus* was explored, making extensive use of single worm genotyping and building upon previous work based on pooled WGS (Doyle et al., 2021).

Early-stage genotyping was initially facilitated by the identification of an *Ava*II restriction site at the S168T locus, allowing for the development of an RFLP

assay. Using the RFLP, it was possible to genotype large samples of L_3 of MHco3(ISE), MHco18(UGA2004), and the MHco3/18 genetic cross. Sequencing data showed the S168T variant was present in a large majority of reads (~70%), with our RFLP genotyping data showing a similar proportion of S168T overall within the population; approximately 75% of L_3 analysed showed at least one allele S168T. A high proportion of both homozygous (RR) and heterozygous (RS) S168T individuals was also seen. This was in line with sequencing data, where S168 was only present in ~25% of reads examined in MHco18(UGA2004). Conversely, it was not expected to detect the S168T variant in any of the MHco3(ISE) individuals assayed by RFLP, as the S168T variant was also completely absent from sequencing data. Finally, the genotype data showed that a high proportion (60%) of individuals in the MHco3/18 population encoded the S168T variant, although the majority of those encoding S168T were estimated to be heterozygous (56%). Only a very small proportion of homozygous S168T individuals (4%) were observed, which presented a markedly different genotype profile to the parental resistant population, MHco18(UGA2004). However, despite the utility of the RFLP, the technique showed some drawbacks, the most significant of which related to the inability to clearly distinguish heterozygotes from homozygotes due to incomplete digestion of first round template. Attempts at optimising the RFLP with increased concentrations of restriction enzyme and longer incubations failed to resolve the issue. Despite this limitation, the RFLP was capable of correctly identifying the presence of the S168T allele in an individual L_3. However, it was necessary to develop an assay that showed greater specificity and accuracy for genotyping. A further drawback of the RFLP was its time-consuming protocol, an intrinsic limitation of this assay. Following two rounds of PCR it was necessary to run the *Ava*II digestion overnight (7-12h) for optimal digestion efficiency. Longer digestion times were found to lead to non-specific digestion, shorter digestion times were found to lead to incomplete digestion, and a single step PCR did not yield sufficient template for accurate visualisation following restriction digest. When compared to sequencing, which has a comparable time-to-result RFLP offers less information than sequencing, with a comparable, albeit somewhat faster, turnaround time: 12-24h for RFLP vs 24-36h for Sanger sequencing, and a week or potentially slightly longer for Illumina MiSeq. Set up for sequencing is equally labour intensive, however, the

increase in quantity of information acquired from sequencing offsets this. Nevertheless, the development of the RFLP assay allowed detection of the presence at least one S168T allele in individual worms, which was essential for validating this marker, and formed the rationale for proceeding to develop a higher throughput diagnostic test.

Thus, in order to develop an assay that could offer efficient and accurate large-scale genotyping of individual L_3 for the S168T variant, which could serve as a proof-of-concept assay for use in a research laboratory setting, the development of an allele specific PCR was undertaken. Initially a number of different primer designs were trialled (Chapter 2.7.3), wherein the number of mismatches relative to the reference or consensus sequence of MHco3(ISE) and MHco18(UGA2004) was altered, the orientation of the primers (i.e. forward or reverse), and their respective pairing with generic exon 4 primers. Following extensive testing, the final design of the AS-PCR primers (Hco-S168-R and Hco-S168T-R) was chosen, with mismatches at the 2nd and 5th position from the 3' nucleotide, with a SNP specific mismatch present at the most 3' nucleotide. Successful demonstration of allele specific discrimination with this primer set using cloned exon 4 fragments from MHco18(UGA2004) was then possible, and, following further optimisation, successful allelic discrimination using single worm exon 4 PCR products for both laboratory and field isolates. The final protocol established for the AS-PCR has a turn-around time of 5-6h, including lysis of L_3, with a per reaction cost (<£3), lower than that of Sanger sequencing. This allows for fast, cheap, and accurate genotyping of populations of *H. contortus* for the S168T variant.

Optimisation of the AS-PCR for single worm exon 4 PCR products allowed the genotyping of single worms from a range of LEV susceptible and resistant *H. contortus* populations in a 96 well plate format. Initial genotyping of the MHco3(ISE) isolate was in line with expectations, as no evidence was found of the S168T variant in any individuals assayed, confirming both published WGS data and the RFLP data. The MHco18(UGA2004) isolate, however, showed a higher proportion of individuals encoding S168T (94%) compared to RFLP (75%). This discrepancy may be due to an underestimation of the allele frequency by

the RFLP caused by incomplete digestion. However, it could also potentially be due to intra-batch variation, as in both the RFLP and the AS-PCR, some variation in allele frequency was seen within each sub sample of ~30-48 L_3 assayed (See Appendix 6). Nevertheless, across both assays, a very high proportion of MHco18(UGA2004) individuals encoding the S168T variant were detected.

Sampling was then expanded to examine how LEV treatment affected the proportion of individuals encoding S168T by assaying MHco3/18 L_3 collected pre- and post-LEV treatment during an *in vivo* infection. WGS data previously generated for pooled populations showed that the proportion of S168T within the population increased following LEV administration (Doyle et al., 2021). Prior to LEV administration, 58% of individuals encoded S168T, of which 9% were homozygous. Post-LEV administration this increased to 75% of individuals, of which 16% were homozygous. This confirmed the sequencing data, which also showed an increase in the proportion of S168T within the post-treatment population of MHco3/18. However, the proportion of heterozygous and homozygous individuals was not known and, following a single LEV treatment, an overall increase in both genotypes was demonstrated. The proportion of S168T in two field populations from farms in the Southern United States was then examined making use of field isolates that had previously been phenotypically characterised as resistant or susceptible using a commercial (DrenchRite®) assay. No evidence of the S168T variant encoded by any individuals on the susceptible farm was found, while ~80% of individuals from the resistant farm encoded the S168T variant, with ~30% being RR. This population had been subjected to multiple rounds of LEV selection over the course of several years (Ray Kaplan, personal communication). The S168T frequency in the population was similar to that of MHco18(UGA2004), which is also derived from a phenotypically LEV resistant field population in the Southern United States (Williamson et al., 2011).

One observation of particular interest seen in both the post-treatment MHco3/18 and the LEV resistant farm population was the high proportion of homozygous S168 (SS) individuals, 25% and 20% respectively. A small proportion of MHco18(UGA2004) (6%) were also found to be homozygous S168. As LEV

resistance is thought to be recessive (Dobson et al., 1996; Sangster et al., 1998), this merited further investigation, as it suggests that heterozygous and/or homozygous S168 adults are surviving LEV treatment. As all the L_3 populations examined were the progeny of the adult population subjected to treatment, the adult worms recovered post-mortem from the *in vivo* infection were then genotyped. Five males, and five females were examined, with one female worm failing to amplify. Overall, the majority of adults surviving LEV treatment were indeed homozygous for S168T (56% (5/9) total; 50% (2/4) females; 60% (3/5) males). However, the remaining adults were found to be either heterozygous or homozygous S168 (22% each). Although based on a small number of samples, this provides an explanation for the relatively high proportion of homozygous S168 and heterozygous individuals within LEV selected resistant populations. As mating is polyandrous in *H. contortus*, females show a high degree of fecundity (Redman et al., 2008; Gilleard and Redman, 2016). It also points toward LEV resistance being multigenic, with the possibility of additional resistance mutations yet to be elucidated. In line with this hypothesis, a second QTL was identified in the MHco3/18 isolate following LEV treatment which contains *lev-1*, a gene previously implicated in LEV resistance in *C. elegans* (Fleming et al., 1997; Qian et al., 2008). However, a mechanistic role for *lev-1.1* in the AChR is as yet unclear, with reconstitution experiments in *Xenopus* oocytes showing *H. contortus* LEV-1 is not essential for functional receptor assembly (Boulin et al., 2011). Further evidence in support of S168T as the major marker of LEV resistance is the presence of a serine-threonine change at the analogous position of *acr-8* in LEV resistant *T. circumcincta* (Choi et al., 2017; Doyle et al., 2021). In *H. contortus*, S168T is also at fixation in the LEV resistant Kokstad population (Cedric Neveu, personal communication), although unfortunately L_3 from this population were not available for genotyping with the AS-PCR. This observation suggests S168T likely could constitute the major marker of LEV resistance, however, evidence from the controlled genetic cross infection suggests that it may take multiple generations of selection for this to occur, during which time minor markers of resistance may also play a role. In summary, S168T appears to be the major determinant of resistance (in the isolates examined herein), with mutations in *lev-1* potentially encoding for minor determinants of resistance,

which may or may not be cumulative with S168T, or other mutations as yet undiscovered. This possibility will be considered further in Chapter 6.

Global populations of *H. contortus* are known to exhibit a high level of genetic diversity (Yin et al., 2013; Salle et al., 2019). Thus, given the need for genetic markers of LEV resistance to be used globally it was necessary to examine genetically divergent LEV susceptible populations to determine the robustness of the AS-PCR. To this end, MHco4(WRS) and MHco10(CAVR) from South Africa, and Australia respectively were used, as these represent genetically and geographically separated populations in relation to the isolates examined thus far. AS-PCR initially showed poor specificity and sensitivity on these isolates, the cause of which was determined to be SNPs within the allele specific primer binding sites in these isolates. Substitution of deoxyinosine bases at these positions was then trialled, following a methodology previously demonstrated for a multi-serotype DENV PCR (Wang et al., 2000). Deoxyinosine bases allow for universal base pairing with a preferential order (I-C>I-A>I-G/I-T) (Case-Green and Southern, 1994). The introduction of a deoxyinosine base at the site of the most frequent SNP (Hco-168T[I]-R/ Hco-168S[I]-R) resulted in a marked improvement in both the specificity and sensitivity of the assay. Non-specific binding was largely eliminated, although faint traces were still present in 3/16 individuals. Overall consistency of the reaction was also markedly improved, with similar band intensity seen in all eight individuals from each of the two isolates, in contrast to the much more variable result seen previously with unmodified primers. Inclusion of a second deoxyinosine base however, severely compromised the reaction, with no amplification seen. Thus, further optimisation will be necessary for a truly universal *H. contortus* primer set, but this nevertheless provides a first proof-of-concept demonstration of the use of deoxyinosine modification to improve specificity in a highly polymorphic parasitic nematode. Moving forward it may be necessary to develop region specific primers to improve diagnostic efficacy, and this can be accomplished by making use of previously sequenced isolates in the *H. contortus* global diversity database (Salle et al., 2019). However, this was beyond the scope of the current study and should be explored in future work.

Finally, it was decided to explore adaptation of the AS-PCR to *T. circumcincta,* a closely related clade V parasitic nematode, and an important pathogen of small ruminants in Northern Europe (Dobson et al., 1996; Steer, 2005; Roeber et al., 2013a). *T. circumcincta* also shows significant polymorphism (Choi et al., 2017), similar or greater than *H. contortus* (Redman et al., 2015). The genome resources are less advanced than that of *H. contortus*, as *T. circumcincta* is not used as a model organism, and is restricted geographically to temperate climates, rather than the widespread global distribution of *H. contortus* (Roeber et al., 2013a; Bartley et al., 2015). Following a similar design strategy to that used with *H. contortus*, a two-step nested AS-PCR was designed, with primers of similar T_m and length to those used with *H. contortus*. Allele specific primers followed the same pattern of two upstream mismatches and a terminal SNP specific mismatch. Care was taken to match nucleotide-nucleotide mismatches as closely as possible to those used in *H. contortus*, as this also affects primer dynamics (Liu et al., 2012). It was found that the first-round primers amplified *T. circumcincta acr-8* exon 3 fragment, albeit with some non-specific amplification in one primer pair. However, it was not possible to achieve allele specific amplification without significant further optimisation, which was beyond the scope of this part of the study. Further work should initially centre on sequencing the *T. circumcincta* exon 3 locus amplified by the first round primers designed herein, to build a clearer picture of the nucleotide composition at the primer bind site. Sequencing would also allow confirmation of the presence of the S168T analogous variant in MTci5, as previous work centred on a multi-drug resistant field isolate from New Zealand (Choi et al., 2017) which likely shows significant genetic differentiation compared to MTci5, which was isolated from a multidrug resistant field population in Scotland (Sargison et al., 2001; Bartley et al., 2005; Bartley et al., 2015). Nevertheless, the first-round primers will be of use in future work either optimising an AS-PCR or for use in Nemabiome based amplicon sequencing approach (Avramenko et al., 2015; Avramenko et al., 2019; Melville et al., 2020).

4.3.1 Concluding Remarks

To conclude, this section of the study sought to validate the novel LEV resistance marker S168T, and to design a robust proof-of-concept diagnostic test capable of accurately genotyping large samples of *H. contortus* L_3. The development of an RFLP assay allowed for initial confirmation of WGS, followed by the optimisation of an AS-PCR for larger scale accurate and fast genotyping of *H. contortus* populations. The S168T variant was detected in all resistant populations examined and was found to be absent in all LEV susceptible populations examined. This presents a significant advantage over previous markers, which showed inconsistent and variable detection. It was also discovered that there were individual worms that lacked the S168T variant that survived LEV treatment, which demonstrates that other resistance mechanisms are present in the MHco18(UGA2004) population. Finally, deoxyinosine modification of primers presents a solution to genetic polymorphism within primer bind loci in divergent isolates of *H. contortus*.

5 Evaluation of two novel loop-mediated isothermal amplification technologies for the detection of S168T in *Haemonchus contortus*

5.1 Introduction

5.1.1 Loop-mediated isothermal amplification (LAMP) and resistance diagnosis

The isothermal nucleic acid amplification method, loop-mediated isothermal amplification (LAMP), has been widely applied to the diagnosis of resistance, especially within the field of bacteriology, where antibiotic resistance is rampant and mechanisms are well understood and characterised (Vasala et al., 2020). However, adaptation to the diagnosis of antiparasitic resistance has been somewhat slower. In part, this can be explained by the fact that most parasitic diseases affecting humans, such as malaria, leishmaniasis, trypanosomiasis, and the numerous human helminth infections, tend to disproportionately affect people in lower- and middle-income countries (LMICs), with many classed as neglected tropical diseases (NTD) (Taylor and Smith, 2020). Veterinary medicine has many challenges comparable to NTDs in resource limited settings, not to mention the overlap that exists between the two fields due to zoonoses, often due to close proximity between humans and animals in rural and/or LMIC settings.

The first application of LAMP for the detection of anthelmintic resistance in *H. contortus* was demonstrated for the E198A BZ resistance SNP (Tuersong et al., 2020). LAMP primers were designed to distinguish between the E198A and *wild-type* β-tubulin alleles, combined with the use of the hydroxy naphthal blue (HNB) colourimetric dye, which allows for naked eye differentiation of negative and positive results. More recently, a study by Costa-Junior et al. (2022) designed multiple LAMP primer sets to target all three SNPs involved in BZ resistance: E198A, F200Y, and F167, with a proposed application towards colourimetric detection and multiplexing of these primers. These examples highlight the principal challenge when translating SNP detection from conventional PCR to

LAMP: the design of allele-specific primers. This is a well-established process when designing PCR primers (Liu et al., 2012; Sullenberger and Maine, 2012), as these can be manually designed or using software and only require a forward and reverse primer (Sullenberger and Maine, 2018; Antonopoulos et al., 2028; Chapter 4). However, the requirement for two essential (outer and inner) and one optional (loop) primer pair in LAMP (Notomi et al., 2000; Nagamine et al., 2002) significantly complicates the design process. The location of the SNP mismatches is also a confounding factor, with studies reporting differing results depending on the location of mismatches on the primer (Badolo et al., 2012; Yongkiettrakul et al., 2017; Chahar et al., 2017; Mohon et al., 2018; Costa-Junior et al., 2022), and the position of mismatches on the inner (FIP, BIP) and/or outer (F3, B3) LAMP primer pairs (Yongkiettrakul et al., 2017; Chahar et al., 2017; Mohon et al., 2018). This highlights the difficulty in producing a standardised pipeline for primer development (Malpartida-Cardenas et al., 2018; Malpartida-Cardenas et al., 2019).

A recently emerging novel group of LAMP techniques centres on the use of an enzymatic cleavage-based loop primer labelled with fluorophore and quencher moieties to perform the work of SNP genotyping. These techniques function in a manner analogous to the well-established TaqMan PCR methodology with enzymatic cleavage of a reporter oligo, usually a primer/probe, used to directly measure sequence specific fluorescence. SNP genotyping with TaqMan PCR utilises a wild-type, and/or a SNP specific probe to differentiate between alleles. Each probe, when fully bound to the target sequence, leads to the activation of the 5' nuclease activity of the Taq DNA polymerase, cleaving the reporter dye from the hybridised probe. Binding of the probe in the presence of a SNP mismatch relative to the probe sequence alters binding, which then blocks cleavage of the probe (Rojas Garcia et al., 2005; Shen et al., 2009). As the polymerases typically used in LAMP assays lack exonuclease activity (Notomi et al., 2000), this necessitates the inclusion of an additional protein to carry out the work of enzymatic cleavage (Higgins and Smith, 2020; Chen et al., 2020; Ding et al., 2021).

ProofMan LAMP is an example of an enzymatic cleavage LAMP technique which uses a dual labelled loop primer with quencher and fluorophore moieties at the 5'

and 3’ ends, and *pfu* polymerase which recognises simple double stranded DNA mismatches. A single mismatch at the 3’ end corresponding to the target SNP forms the recognition site for *pfu* mediated enzymatic cleavage. If no mismatch is present, then the dual labelled probe simply remains bound *in situ* to the loop domain of the LAMP amplicon. However, in the case of cleavage, the dissociation of the 3’ moiety from the 5’ moiety (fluorophore/quencher) leads to detectable fluorescence. The probe can then simultaneously serve as a loop primer, accelerating the reaction if the SNP is present (Chen et al., 2020; Ding et al., 2021) (Figure 5.1). This technique has recently been applied to the detection of SARS-CoV-19 synthetic RNA with both qPCR machinery-based fluorescence and naked eye fluorescence detection under UV light (Ding et al., 2021).

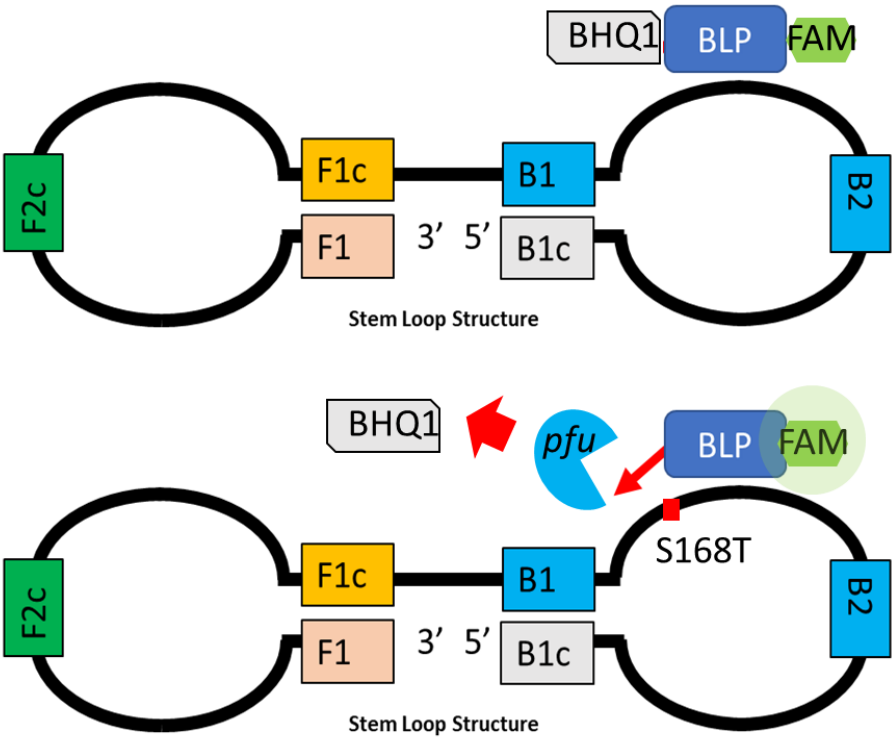

Figure 5.1: Simplified schematic representation of ProofMan LAMP. When the S168T SNP is not present the tagged loop primer binds to the loop domain, but no fluorescence is detected, as the tag is not cleaved. When the S168T SNP is present however, the dsDNA SNP mismatch is recognised by *pfu* polymerase which cleaves the BHQ1 molecule from the 3’ end leading to detectable fluorescence. This also allows the tagged loop primer to drive further DNA polymerisation previously blocked by the presence of a 3’ tag.

Loop-primer endonuclease cleavage (LEC)-LAMP functions in an analogous manner to ProofMan LAMP, except that the loop primer probe employs a more complex and sequence specific design. Enzymatic cleavage is carried out by *endonuclease* IV, which recognises and cleaves abasic sites in dsDNA form (Higgins and Smith, 2020). An abasic site can form spontaneously in cells as a consequence of destabilisation of the N-glycosyl bond by certain types of DNA damage, or by glycosylases. This leads to loss of the purine or pyrimidine moiety, leaving only the sugar phosphate backbone. The *endonuclease* enzyme recognises the presence of an abasic site within dsDNA and excises it as part of the normal cellular repair process *in vivo* (Thompson and Cortez, 2020). However, this will not occur in single stranded DNA. The LEC-LAMP probe is therefore designed to accommodate this mechanism of cleavage. Thus, a mismatch relative to the target SNP or *wild-type* sequence is flanked by an abasic site, with 5' quencher, and the fluorophore immediately adjacent to the abasic site. When the probe binds to the sequence, if a match is present at the SNP locus on the amplified target sequence, the probe will fully hybridise, forming a dsDNA abasic site adjacent to the SNP locus. The *endonuclease IV* enzyme will then cleave at this site, dissociating fluorophore from quencher, and leading to a detectable signal (Figure 5.2). However, if a mismatch is present at the SNP locus on the target sequence, this prevents the formation of a double stranded abasic site at the adjacent position, preventing cleavage (Higgins and Smith, 2020). Quencher and fluorophore are designed to sit optimally within 5-6 bp of the 5' end of the probe (Higgins, personal communication). This section of the probe containing the quencher and fluorophore labels, abasic site, and SNP site is termed the cleavage arm. LEC-LAMP has been successfully demonstrated for both singleplex and multiplex detection of clinically relevant human pathogens *Neisseria meningitidis*, *Haemophilus influenzae,* and *Streptococcus pneumonia*, and SNP genotyping capabilities of this technology has been exemplified using synthetic DNA templates (Higgins and Smith, 2020).

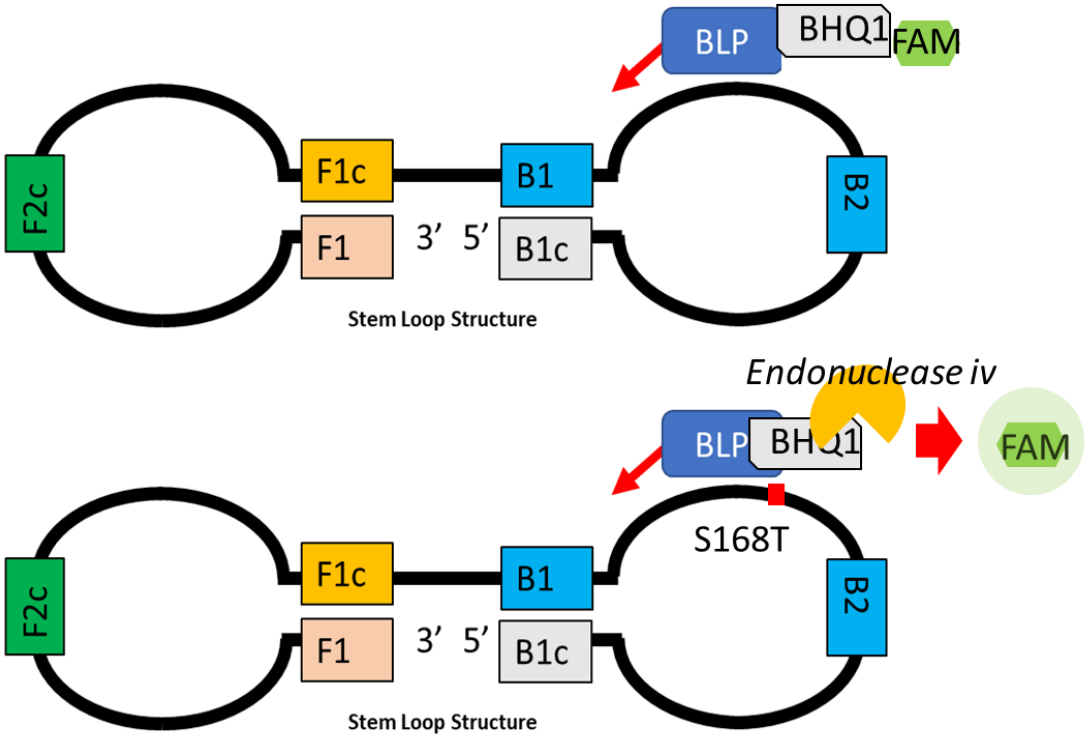

Figure 5.2: Simplified schematic representation of LEC-LAMP. When S168T SNP is not present, the tagged loop primer binds to the loop domain, but no fluorescence is detected, as the tag is not cleaved. When S168T is present however, the abasic site and flanking SNP mismatch is recognised by *endonuclease IV* which cleaves the FAM molecule from the 5' end leading to detectable fluorescence.

The ProofMan LAMP and LEC-LAMP technologies also have the advantage of being applicable for downstream amplicon sequencing. As enzymatic cleavage LAMP amplifies the target gene fragment, and with allele-specific detection then measured based on the cleavage of the dual labelled primer/probe, this facilitates the use of LAMP amplicons generated by the LEC-LAMP reaction to be sequenced in a downstream amplicon sequencing protocol. LAMP amplicon sequencing for resistance detection has previously been demonstrated for antiparasitic resistance diagnosis for *Plasmodium* and *Leishmania* (Imai et al., 2017; Imai et al., 2018; Runtuwene et al., 2018).

LAMP has also significant potential for downstream development as a point-of-care (POC) technology (Augustine et al., 2020; Moehling et al., 2021) for improving the diagnosis of anthelmintic resistance (Kaplan, 2020).To date, few LAMP assays have been validated for the detection of anthelmintic resistance. LAMP has been

investigated for the detection of *H. contortus* DNA in ruminant faeces by targeting the ITS2 region (Melville et al., 2014), which is highly conserved and widely used for species identification PCR (Redman et al., 2008). Subsequent work showed the adaptability of the ITS2-targeting LAMP primer set to a more POC setting by inclusion of colourimetric and lateral-flow endpoint detection for identification of *H. contortus* in farm samples (Khangembam et al., 2021).

Enzymatic cleavage LAMP assays also have the potential to be adapted for POC applications. Gootenberg et al. (2018) describe a modification of an alternate isothermal amplification technique, recombinase polymerase amplification (RPA), where a dual labelled reporter molecule, is enzymatically cleaved via a modified CRISPR-Cas system, where the cleaved reporter products are then detected using a lateral flow platform. Thus by replacing the fluorophore/quencher labels with moieties to allow detection with widely available streptavidin/biotin kits, this could potentially allow for rapid laboratory-based quantitative detection, as well as field based, POC naked eye detection.

5.1.2 Aims

In this part of the study, the aim was to apply the enzymatic cleavage-based LAMP technologies, ProofMan LAMP and LEC-LAMP, to the detection of the S168T variant in *H. contortus,* based on the previously validated AS-PCR (Antonopoulos et al., 2022). Evaluation of the adaptation of these assays to POC amenable end-point detection was also explored.

5.2 Results

5.2.1 ProofMan LAMP

ProofMan LAMP primers (Chapter 2.10.1.1) were initially tested in the absence of the allele-specific probe to confirm specific amplification of the target allele using *Bst* 2.0 polymerase and colourimetric end-point detection. ProofMan LAMP primers were tested against *acr-8* exon 4 template PCR products from MHco3/18, MHco4(WRS) and MHco10(CAVR) to determine primer specificity. Target amplification was seen across all isolates except MHco4(WRS), in which only 2/3 individuals amplified (Figure 5.3).

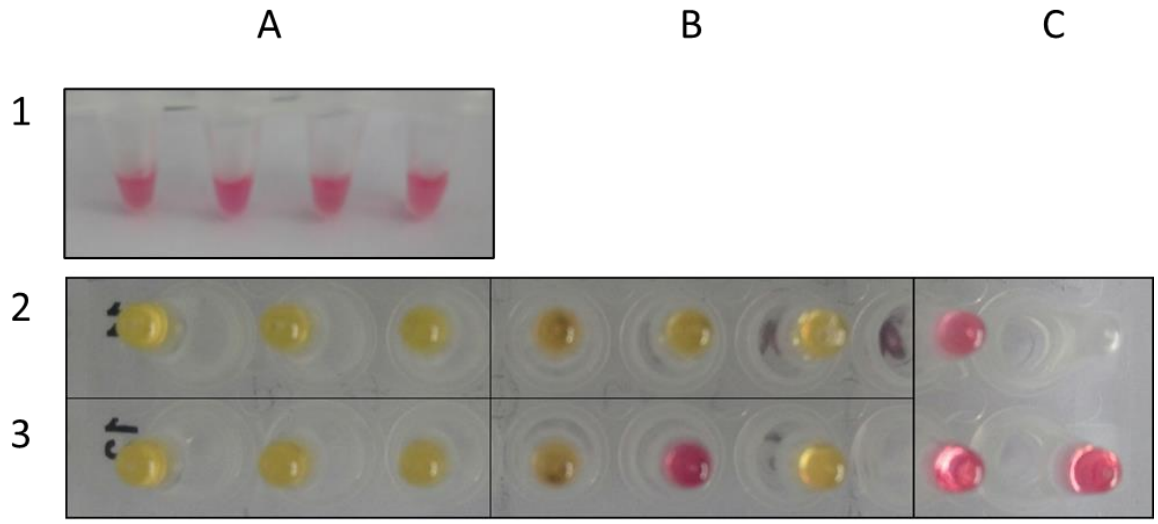

Figure 5.3: Colourimetric validation of ProofMan LAMP primer set PM1.

1A: Initial no template control reaction.

2A: Pre-LEV treated MHco3/18. 2B: MHco10(CAVR)

3A: Post-LEV treated MHco3/18. 3B: MHco4(WRS).

2-3C: No template control.

The assay was then tested on cloned sequences of the S168T and S168 alleles to determine optimal reaction conditions and probe design. Initial experiments were carried out with *Bsm* polymerase, as described in Chen et al. (2020) and Ding et al. (2021) using either the kit provided *Bsm* reaction buffer, or a custom buffer as described in Ding et al. (2021) (see Chapter 2.10.4.2). Each buffer was tested to

determine which gave the best amplification and overall fluorescence detection (Figure 5.4). No fluorescence was detected when using the *Bsm* buffer (ThermoFisher, EP0691) (Figure 5.4 1A).

ProofMan buffer optimisation was then carried out on cloned target sequences to determine the optimal buffer for the reaction and amplification of target template and detection of fluorescence. Fluorescence was detectable by naked eye when using both the *pfu* (Figure 5.4 2A) and the ProofMan (Figure 5.4 3A) buffers. Agarose gel electrophoresis was then carried out to determine if amplification was taking place. Amplification only occurred at higher temperatures using the *pfu* buffer (Figure 5.4 2B). The ProofMan buffer showed the best overall performance. Amplification was detectable in both the S168T and S168 sequences (Figure 5.4 3B). Non-specific fluorescence was also strongly visible under UV light in all samples (Figure 5.4).

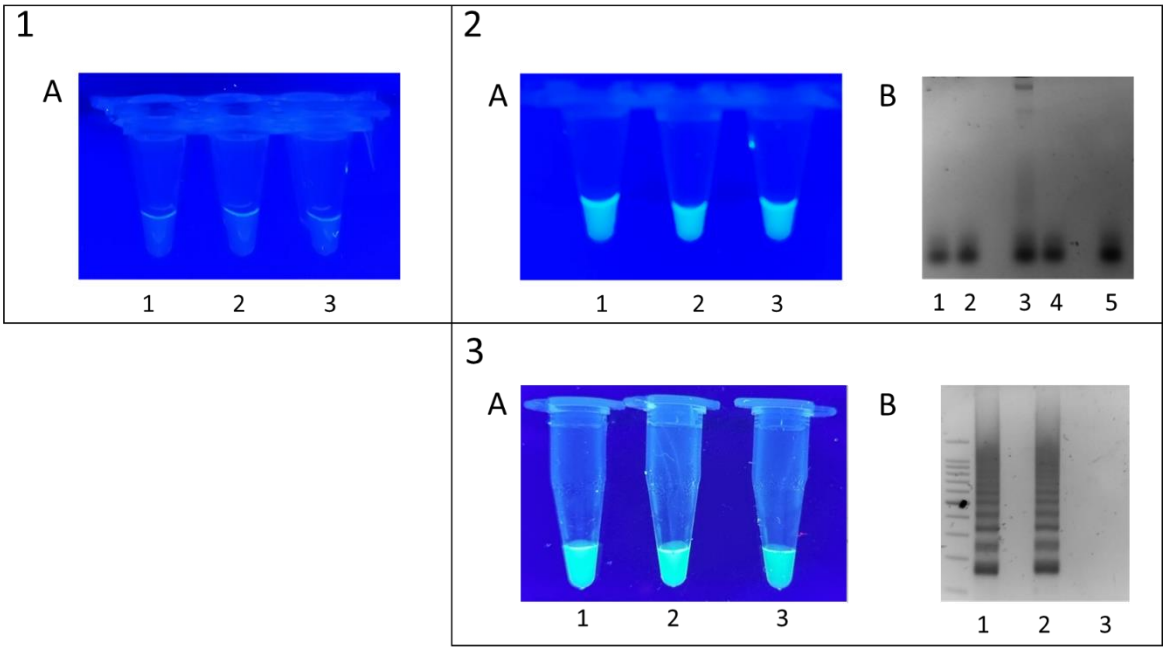

Figure 5.4: Comparison of different buffers on naked eye UV light and gel detection of ProofMan LAMP reaction using probe 1 at 1.2μM.

1A: *Bsm* kit buffer (Thermofisher) UV detection. 1: S168T sequence. 2: S168 sequence. 3: no template control.

2A: *Pfu* kit buffer (Promega) UV detection. 1: S168T sequence. 2: S168 sequence. 3: no template control. 2B: Gel comparison of different reaction temperatures with *pfu* buffer amplification; 1: S168T sequence 61°C. 2: S168 sequence 61°C. 3: S168T sequence 64°C. 4: S168 sequence 64°C. 5: No template control.

3A: ProofMan buffer (Ding et al., 2021) UV detection. 1: S168T sequence. 2: S168 sequence. 3: no template control. 3B: Gel electrophoresis of 3A.

5.2.1.1 ProofMan probe cleavage validation

A variety of different probe designs were used during probe validation. The general rationale for probe design centred on eliminating self-dimer formation, as this could lead to non-specific cleavage of probe dimers, and ensuring an appropriate T_m for the reaction. Probe 1 (Chapter 2.10.1 for a full description of probe design and sequences) showed a linear increase in fluorescence as the reaction progressed. This was equal in both the S168T and S168 template samples. The same linear increase in fluorescence was also seen in the no template (negative) control, and this was initially attributed to the cleavage of primer dimers formed due to the deoxyinosine base present in probe 1. This did not occur in either probe 2 (identical to probe 1 less the deoxyinosine base) or probe 3, which showed no linear increase in fluorescence as the reaction progressed. Fluorescence remained constant throughout the reaction for probes 2 and 3, which indicated that no sequence specific cleavage occurred (for full data for probes R1, R2 and R3 see Appendix 7). A range of temperatures were then trialled during optimisation in an attempt to improve specificity of the reaction. Probe 1 continued to show primer dimer cleavage across the range of temperatures tested. Probe 2 did not show any appreciable sequence specific fluorescence despite confirming amplification in the presence of S168T and S168 template at 55^{o}C. Although there was a difference in fluorescence levels between the S168T, S168 and the no template control, this was constant from the beginning of the reaction and thus did not represent sequence specific detection.

Following this it was decided to increase the T_m of future probes in an effort to improve target hybridisation and cleavage. Probe 4 was designed and tested at a higher temperature. The reaction trialling probe 4 showed a small sigmoidal curve in the S168 sequence sample at around cycles 40-50, representing a degree of sequence specific cleavage, although not to a diagnostic level (Figure 5.5A, B and C).

The potential for naked eye fluorescence detection as reported by Ding et al. (2021) was then evaluated. However, background fluorescence (Figure 5.5D, a1

b1) from the FAM fluorophore moiety was encountered which would make any sequence specific naked eye detection difficult at the concentrations of probe described therein (0.4 μM–1.2 μM). Thus, it was not possible to apply the assay described by Ding et al. (2021) to detection of the *H. contortus* S168T allele.

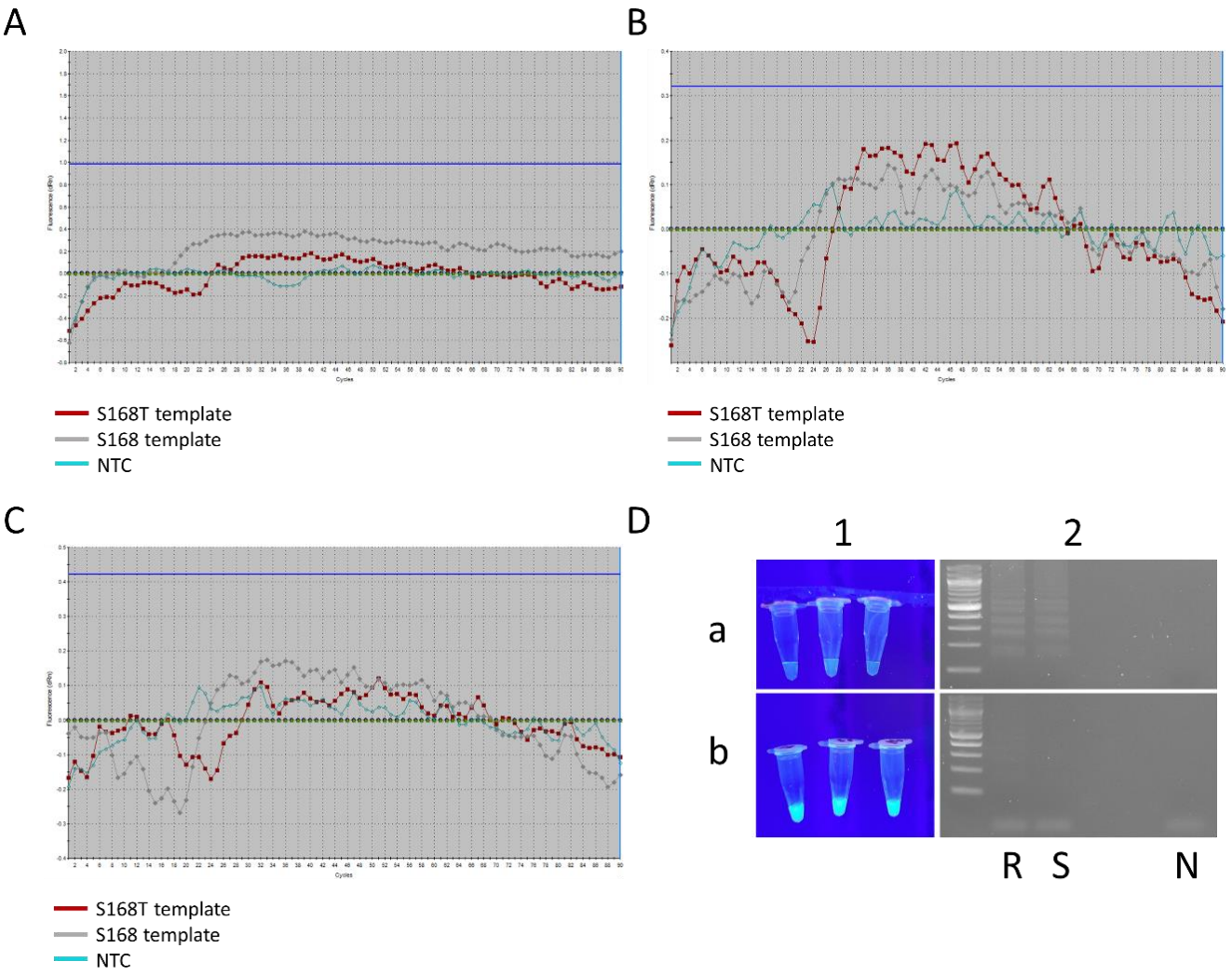

Figure 5.5: Summary of results of the ProofMan LAMP assay for detection of S168T.

A: Initial ProofMan assay using probe 4 at 65ºC showing partial allele-specific discrimination between the S168 template (grey line) and the S168T template (red line).

B and C: ProofMan assay repeated replicates using probe 4 at 65ºC to validate partial allele-specific discrimination seen in A.

D: Comparison of 1: naked eye fluorescence using different probe concentrations with strong background fluorescence seen in both. 2: gel electrophoresis to confirm amplification of target sequence using probe 4 at 65ºC. a: 0.4 μM probe 4. B: 1.2 μM probe 4.

New LAMP primers were then designed to allow a larger space on the loop domain for a longer probe with higher T_m. A new probe with a higher T_m was then designed and tested. In addition, the assay was also tested using *Bst* 2.0 polymerase (NEB) to determine if this would improve reaction specificity. However, allele-specific discrimination was not possible under the conditions described here (Appendix 7).

5.2.2 LEC-LAMP

LEC-LAMP primers (Chapter 2.10) were initially tested in the absence of the allele-specific probe to confirm specific amplification of the target allele. The LEC-LAMP reaction was run using S168T and S168 allele plasmids, with fluorescence data showing good amplification in the case of both cloned sequences, and no amplification in the no template (negative) control. The optimal reaction conditions for the LEC-LAMP primers were established at 65°C (Figure 5.6).

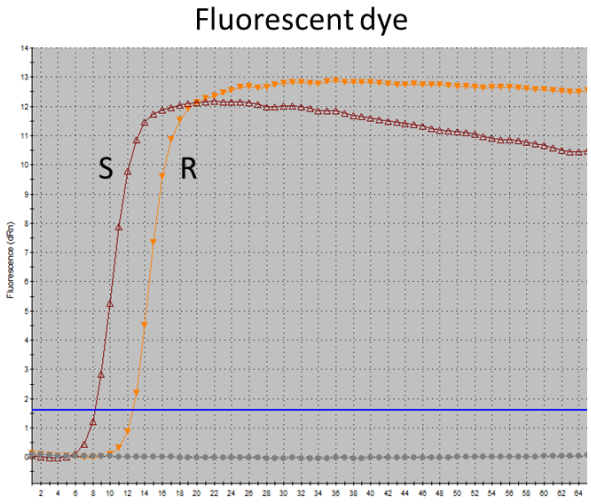

Figure 5.6: Validation of LEC-LAMP primer set using DNA intercalating dye. R corresponds to S168T plasmid DNA, S corresponds to S168 plasmid DNA. Sigmoidal amplification is seen in both reactions indicating DNA amplification is taking place as expected.

Amplification of the *acr-8* exon 4 template using single L_3 crude lysates was then trialled, however, amplification was not possible from single worm lysates under the current assay conditions (Appendix 7).

5.2.2.1 LEC-LAMP Validation and Optimisation

Strong allele-specific discrimination was seen initially in the susceptible probe (S), (Figure 5.7A and C), and amplification of the target template by the LEC-LAMP primer set was confirmed using DNA intercalating dye (Figure 5.7B and D). The optimal temperature for the S probe was established at 66-67ºC. No difference in the specificity and sensitivity of the probe was seen within this temperature range (Figure 5.7A and C) however, below this range (<66ºC) the probe did not perform as well (although allele-specific discrimination was not compromised). Above this range (>=68ºC), amplification was compromised (Appendix 7).

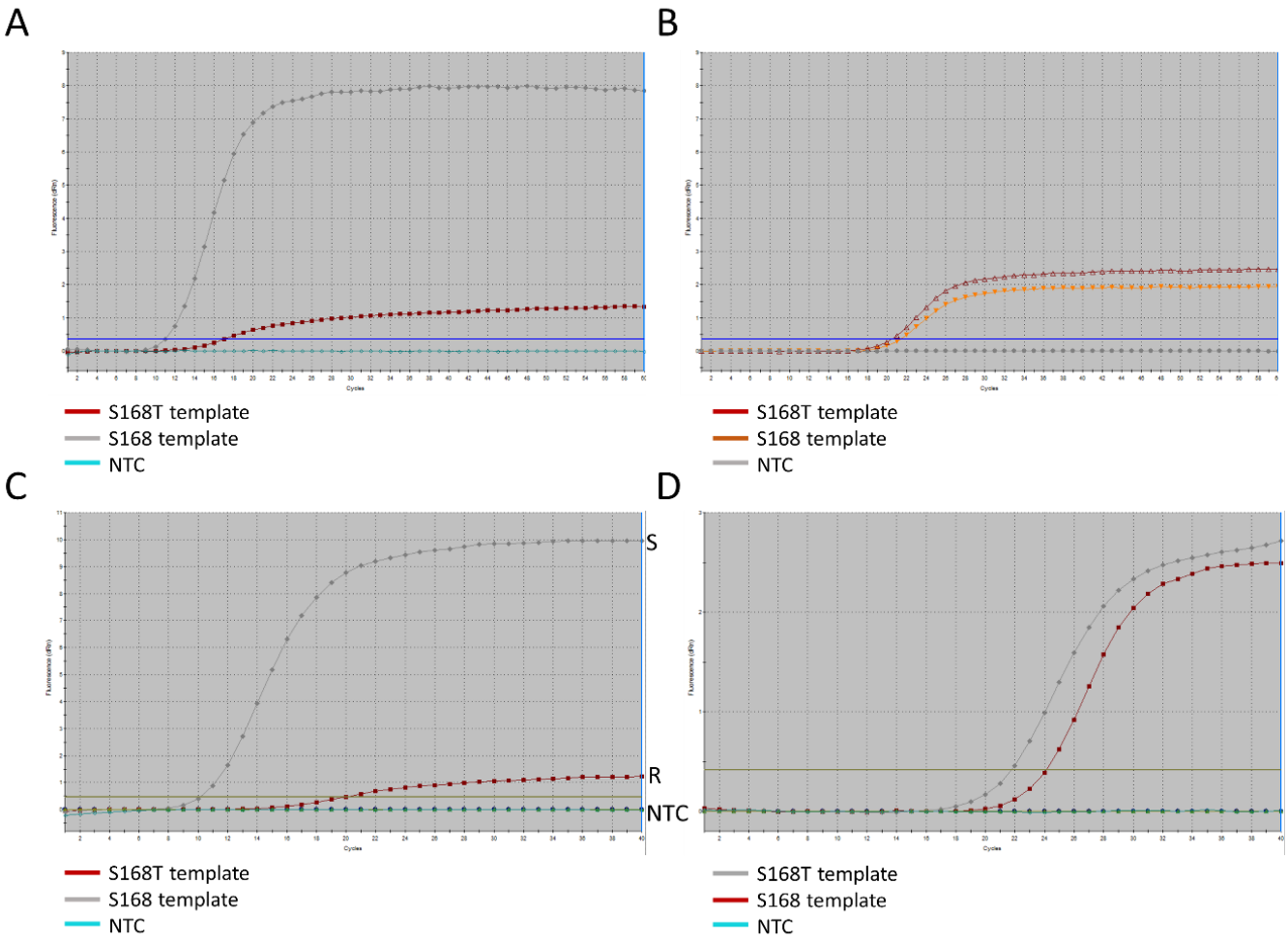

Figure 5.7: Comparison of temperature of LEC-LAMP assay for the discrimination between the S168T and S168 allele using susceptible LEC-LAMP probe S (A and B) and amplification of the *acr-8* exon 4 template (C and D).

A: LEC-LAMP susceptible probe, 67ºC. B: LEC-LAMP primer set with DNA intercalating LAMP dye, 67ºC.

C: LEC-LAMP susceptible probe, 66ºC. D: LEC-LAMP primer set with DNA intercalating LAMP dye, 66ºC.

The first resistant probe (R) was trialled under the same conditions as the S probe. The initial probe design (see Chapter 2.10) of the R probe was identical to that of the S probe, excluding the single base change made at the site of the S168T variant. Initially, poor specificity was seen in the R probe (Figure 5.8A) compared to that seen in the S probe (Figure 5.7A and C). Following this, a single base was added to the 5' end of the R probe, to produce a modified resistant probe (R2). This led to a significant improvement in probe specificity and performance (Figure 5.6C), however, allele-specific discrimination was still below requirements for the purposes of a diagnostic assay.

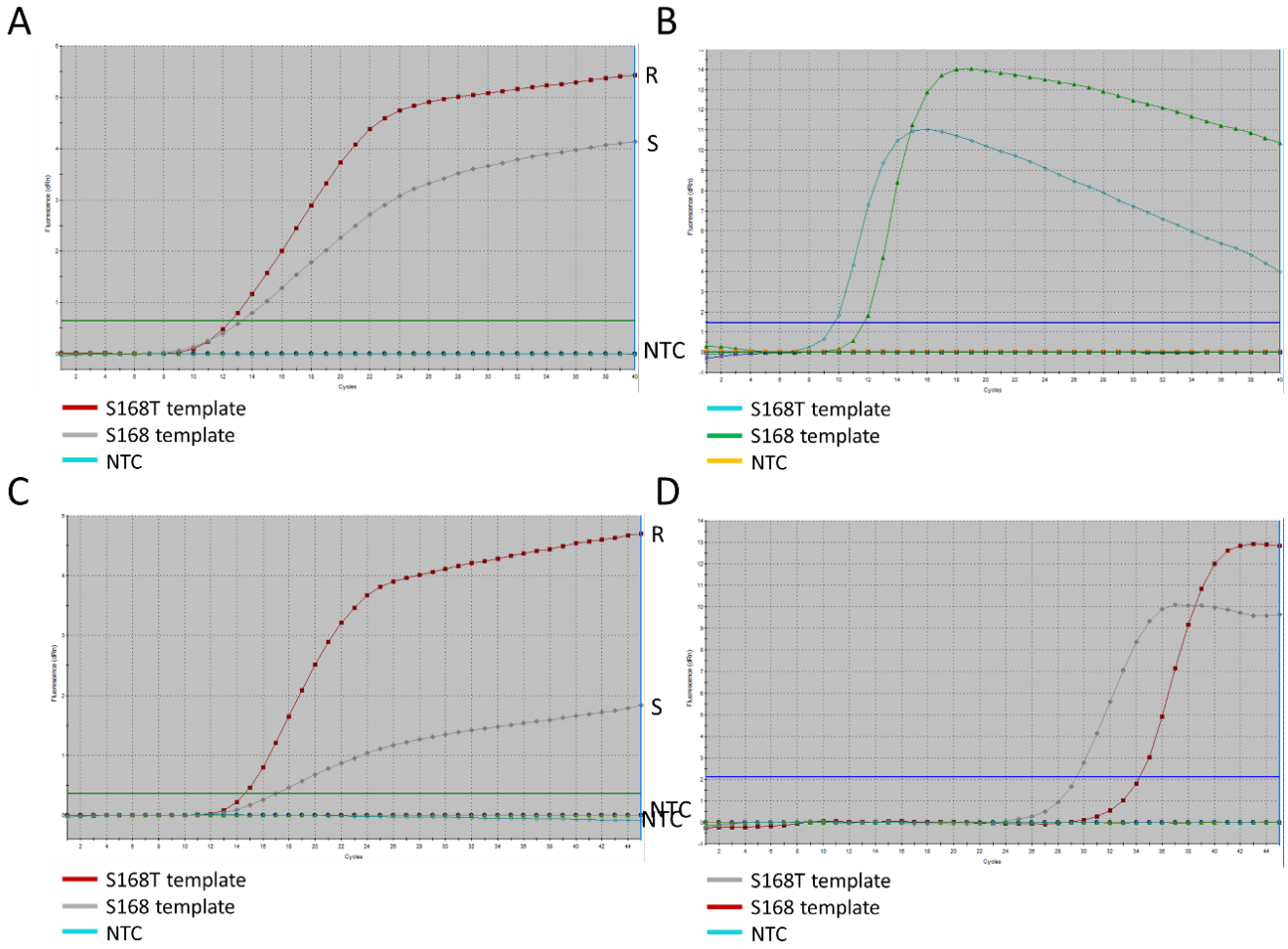

Figure 5.8: Comparison of specificity of the resistant LEC-LAMP probe R (A) and modified resistant LEC-LAMP probe R2 (B) for the discrimination between the S168T and S168 allele following modification of probe design.

A: LEC-LAMP resistant probe R, 66ºC. B: LEC-LAMP primer set with DNA intercalating LAMP dye, 66ºC.

C: LEC-LAMP resistant probe R2, 66ºC. D: LEC-LAMP primer set with DNA intercalating LAMP dye, 66ºC.

Three further modifications (see Chapter 2.10.1.4) were therefore made to the R probe: R3; R4; R5. Probes R3 and R5 showed no appreciable improvement from that seen in R2 (Figure 5.9A and C). The fluorescence pattern seen with R4 at 66ºC indicated that 66ºC was not the optimal temperature for this probe (Figure 5.9B).

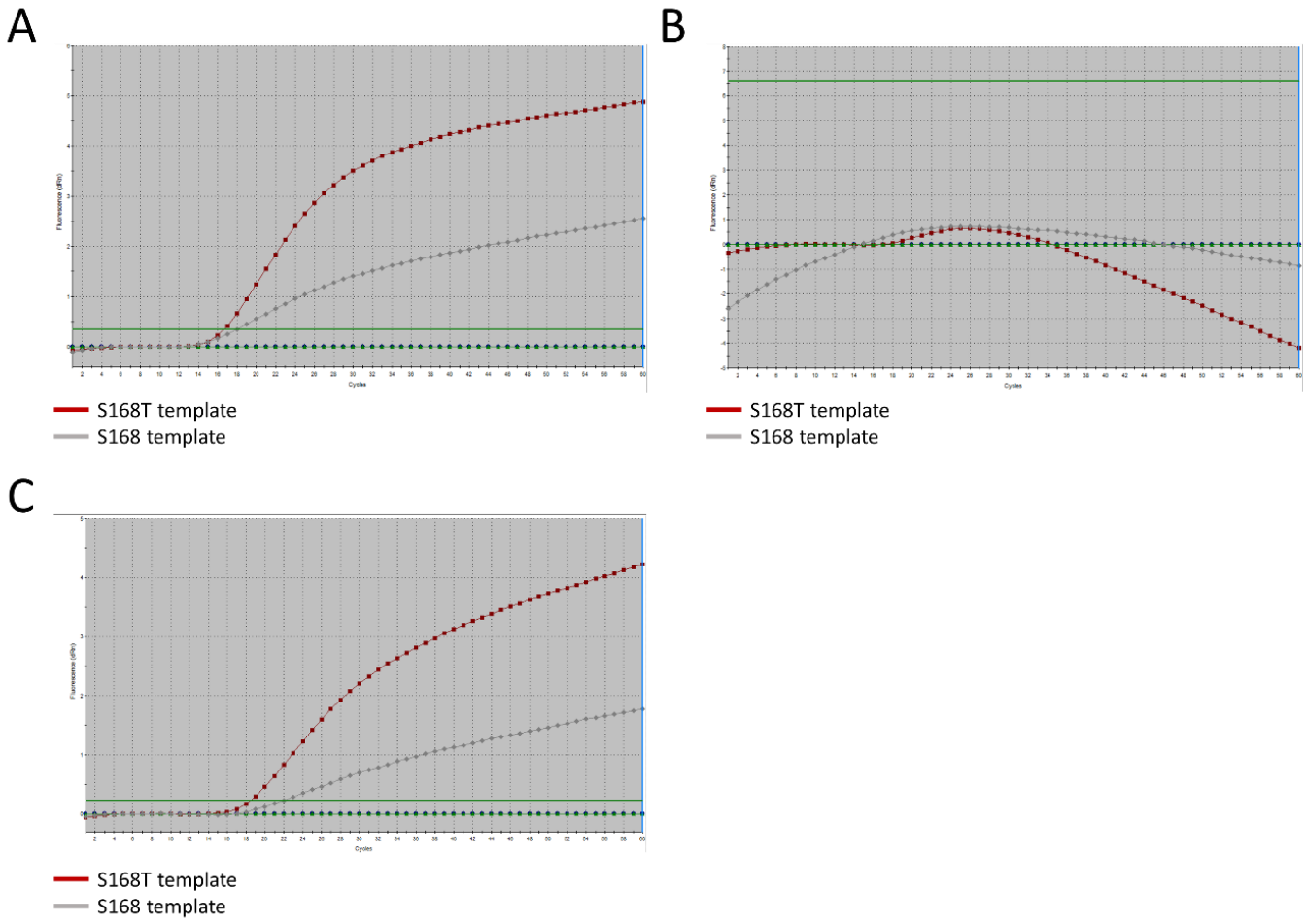

Figure 5.9: Comparison of modified resistant LEC-LAMP probe R3 (A) modified resistant LEC-LAMP probe R4 (B) and modified resistant LEC-LAMP probe R5 (C) for discrimination between the S168T and S168 allele at 66ºC.

The T_m of R4 was predicted to be lower than that of R3 and R5. Therefore, R4 was subsequently tested at 65ºC. At this temperature R4 showed clear allele-specific discrimination (Figure 5.10A), albeit with weak overall fluorescence. This indicated that for R4 improved allele-specific discrimination is possible at a lower reaction temperature. At 63ºC improved allele-specific discrimination was observed (Figure 5.10B). Although overall fluorescence was lower than that seen other probes, clear discrimination was possible between the two alleles using cloned sequences. The optimal temperature for R4 was established at 63ºC.

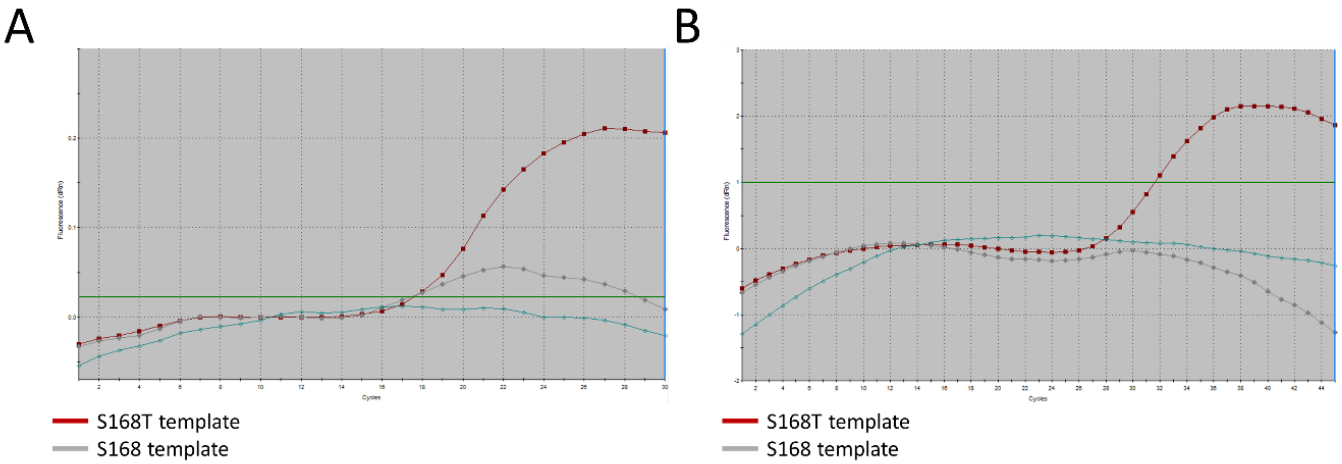

Figure 5.10: Comparison of the effect of lowering reaction temperature to 65ºC (A) and 63ºC (B) on modified resistant LEC-LAMP probe R4 for discrimination between the S168T and S168 allele at 66ºC.

5.2.3 Multiplex LEC-LAMP

The S168T LEC-LAMP was then trialled as proof-of-concept multiplex assay towards resistance allele quantification within mixed populations using probes S and R4. Initially the reaction temperature was set at 63ºC as this was determined to be the optimal temperature for probe R4. Results showed that the reaction was amenable to duplexing under the current assay design at 63ºC (Figure 5.11), with discrimination possible between the resistant and susceptible alleles, even at different ranges of temperatures for the S probe.

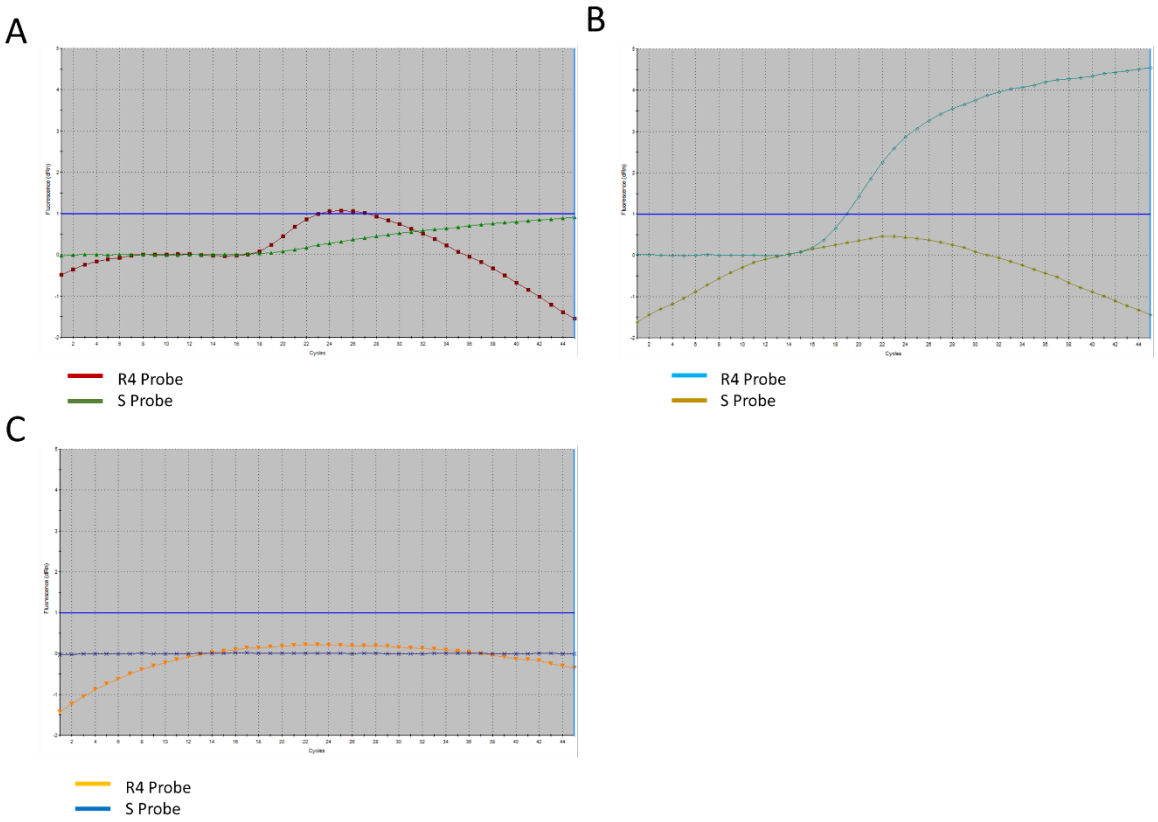

Figure 5.11: Multiplex (duplex) LEC-LAMP using susceptible and modified resistant probe R4 to simultaneously detect the S168T and S168 alleles within a single tube assay at 63ºC. Susceptible and modified resistant probe R4 were combined in single tubes, with one tube receiving the cloned S168T (A) allele, one tube receiving the S168 cloned allele (B) and one tube as the no template control (C).

A: Detection of the S168T allele in a single tube assay by susceptible probe and modified resistant probe R4

B: Detection of the S168 allele in a single tube assay by susceptible probe

C: No template control

5.2.4 Point-of-care (POC) end-point detection for LEC-LAMP

The singleplex LEC-LAMP assay was then evaluated for POC amenable end-point detection. Initially this focused on adaptation to a lateral-flow platform using the S probe. The correct amplification of template and the detection of the S168 allele by the S probe was confirmed via fluorescence detection using qPCR (Figure 5.12A). Following this, LEC-LAMP products were transferred to the lateral-flow strip (Figure 5.12B). It was then possible to confirm detection of the *acr-8* exon 4 fragment corresponding to uncleaved dual labelled (FITC and Biotin) LAMP amplicons (Figure 5.12B: 1 and 2), with no detection in the no template control (Figure 5.10B: 3). Although serving as confirmation of template amplification, the lateral-flow was not allele-specific due to the detection of uncleaved dual labelled LAMP amplicons in both the S168T and the S168 templates. Finally, naked eye visualisation of LEC-LAMP S probe products under UV light was evaluated (Figure 12C). It was possible to observe a slight difference in overall fluorescence between the S168 (Figure 5.12C, 1), the S168T (Figure 5.12C, 2) and the no template control (Figure 5.12C, 3) samples. However, this difference in fluorescence was insufficient for clear discrimination between the two alleles.

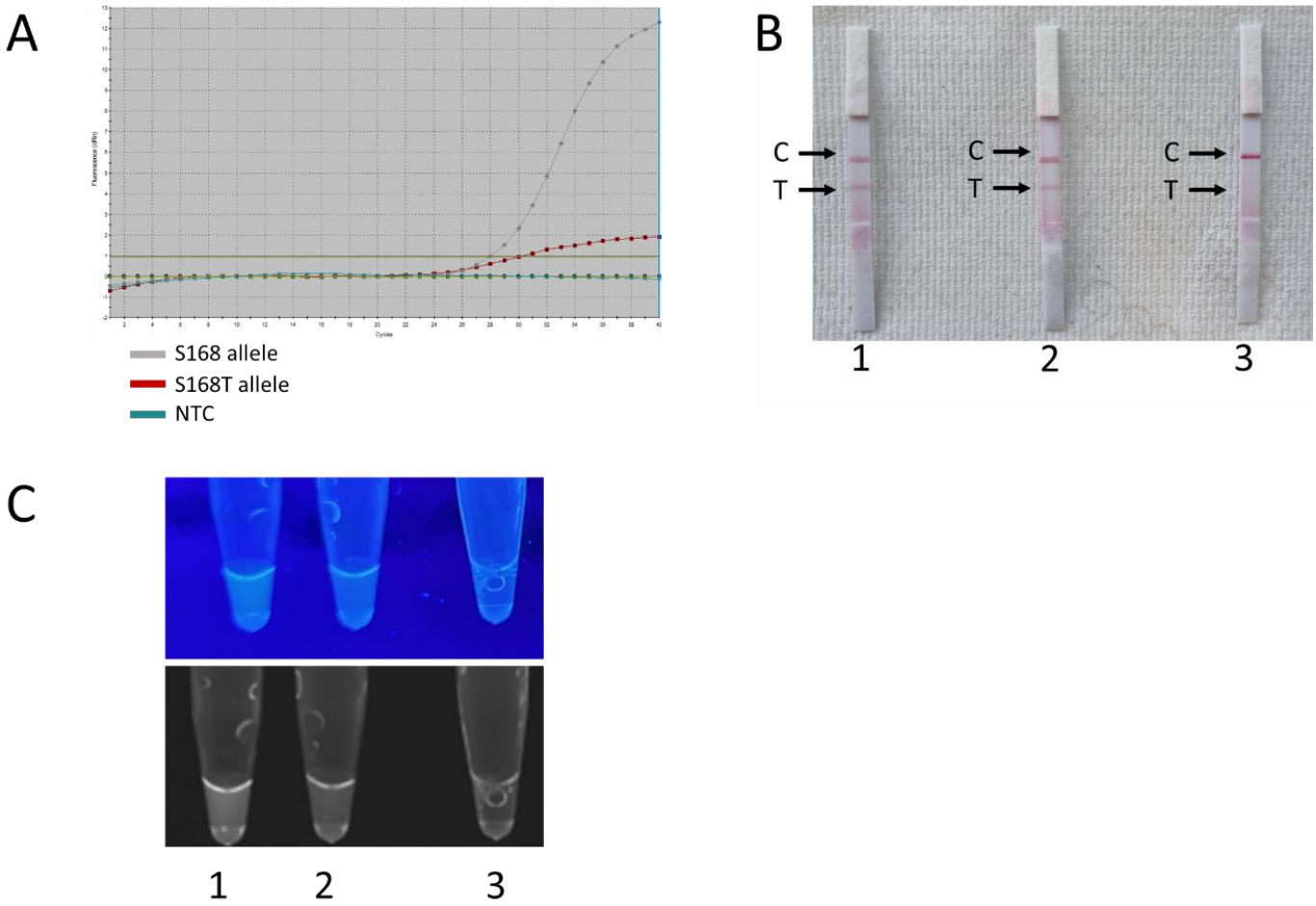

Figure 5.12: Evaluation of POC amenable end-point detection for LEC-LAMP detecting the S168 allele using cloned *H. contortus acr-8* exon 4 fragment plasmid.

A: Discrimination via qPCR machine fluorescence between S168T and S168 allele by S probe

B: Lateral-flow LEC-LAMP by lateral-flow modified biotin labelled S probe and FITC labelled BIP. 1: S168 allele plasmid. 2: S168T allele plasmid. 3: no template control. C – control line: indicates that the lateral-flow assay is functional. T – test line: indicates that the amplicon has been detected.

C: Naked eye fluorescence discrimination between the S168T and S168 alleles using LEC-LAMP S probe. Top image: UV light box. Bottom image: UV gel doc (UVITEC Firereader).

The same samples were used for all images in this panel.

5.3 Discussion

The evaluation of two recently developed SNP specific enzymatic cleavage-based LAMP technologies, ProofMan LAMP and LEC-LAMP, for the detection of the S168T allele in *H. contortus* was carried out. Following several design and optimisation attempts, it was not possible to develop a successful ProofMan LAMP assay for the detection of the S168T allele in *H. contortus*. A similar approach was followed for LEC-LAMP optimisation, which showed clear allele-specific discrimination between cloned sequences of the S168T and S168 allele, while providing the first example of a proof-of-concept LEC-LAMP assay for simultaneous *H. contortus* (via amplification of the *acr-8* exon 4 fragment) and levamisole resistance (via SNP specific fluorescence) detection. The LEC-LAMP assay was then incorporated into a single tube multiplex assay, with each probe successfully discriminating between S168 and S168T within the same reaction, although significant further optimisation is necessary for the S168T probe. Finally, lateral-flow and naked eye visualisation of fluorescence under UV light were evaluated for POC amenable end-point detection. Using a commercially available lateral-flow platform it was possible to detect the *acr-8* exon 4 fragment, however, this was not allele-specific. Using naked eye fluorescence visualisation, a subtle difference was visible between the S168 and S168T alleles, however, this was not sufficient for clear allelic discrimination.

The initial choice to focus on the ProofMan assay (Chen et al., 2020; Ding et al., 2021) was based on its simple design, easy to source reagents, dual labelled oligo probe, and *pfu* polymerase, which are recognised criteria for fast, cheap, and instrument free molecular diagnostics (Kotze et al., 2016; Kotze et al., 2020; Kaplan, 2020). Initial experiments focused on probe design, however, despite extensive evaluation of multiple different probe variations, none offered consistent allele-specific discrimination. Similarly, when a redesign of the LAMP primer set, and the trialling of a different polymerase was undertaken allele-specific discrimination still was not possible. Although a difference was detected in fluorescence between the two alleles, this was not of sufficient magnitude to be of value as a diagnostic tool. Naked eye fluorescence detection described by Ding et al. (2021) was also examined. This would present a significant advantage

for use in POC settings in veterinary medicine, particularly as veterinarians routinely use a UV light device known as Wood's lamp for the diagnosis of ringworm (Kristensen and Krogh, 1981; Cafarchia et al., 2004; Newbury et al., 2015). However, due to inefficient probe cleavage it was not possible to detect any difference between the two alleles. Although considerable efforts were made to replicate the conditions described by Ding et al. (2021), the original publication focused on the use of ProofMan LAMP for the detection of SARS-CoV-2, not *H. contortus*. Furthermore, Ding et al. (2021) made use of commercially produced, locally available synthetic DNA, rather than a cloned PCR product from a single individual worm. It is, therefore, not possible to discount some degree of polymorphism leading to non-specific cleavage at the probe binding site due to the *pfu* cleavage recognition site of simple dsDNA nucleotide-nucleotide mismatch. The restricted nature of the area of sequence available for primer design (due to the location of the S168T SNP locus so near to the intron), further complicates ProofMan optimisation. In addition, the different reagents and suppliers used herein may have also influenced the reaction optimisation. This is one of the inherent difficulties to be overcome when adapting emerging and novel technology to significantly different organisms. Due to the constraints of this study, it was not feasible to initially attempt to replicate the results of Ding et al. (2021) using synthetic SARS-CoV-2 DNA exactly as described, although future attempts to adapt the ProofMan assay may benefit from adopting this approach first. This would allow for any potential unforeseen variables relating to the use of different reagents from different suppliers potentially affecting the reaction performance to be accounted for prior to adaptation to a different organism.

The adaptation of the LEC-LAMP assay for the detection of the S168T variant in *H. contortus* was also explored. Due to the more sequence specific probe design and with the inclusion of an abasic site, and the 5-7 bp cleavage arm which forms the sequence specific cleavage recognition locus, LEC-LAMP offers the potential for increased SNP specificity for enzymatic cleavage of the labelled loop primer/probe. There is still potential for false positives if a secondary SNP lies immediately adjacent to the abasic site, on the opposite side to the SNP of interest within the cleavage arm of the probe. This is significant due to the highly polymorphic nature of *H. contortus* (Laing et al., 2013; Doyle et al., 2020),

particularly as there are known to be several moderate frequency synonymous SNPs within the probe binding locus in MHco4(WRS) and MHco10(CAVR) (Antonopoulos et al., 2022). These SNPs, however, do not lie within the LEC-LAMP cleavage arm and thus are unlikely to affect probe cleavage. Nevertheless, probe binding may still be affected by polymorphism outside the cleavage arm, and this could also lead to false negatives in highly divergent isolates. Future work adapting any LEC-LAMP assay to field populations would need to take this into account, and could make use of the well-developed genomic resources available for *H. contortus* including the global genetic database (Salle et al., 2019) to ensure that probes were unlikely to lead to excessive false positives. It may also be necessary to develop region specific probes, as per AS-PCR (Antonopoulos et al., 2022).

The LEC-LAMP S probe, designed to detect the S168 allele, showed immediate and clear allele-specific discrimination between S168 and S168T alleles (Figure 5.7). Optimisation of the LEC-LAMP R probe, however, proved more difficult, and necessitated several probe redesigns. This could be partly explained by the sensitivity to single base changes of the probe. The initial R probe trialled was identical to the S probe except for the alteration of the base at the S168T SNP site (C to G). While this did not lead to any significant change in the overall T_m of the probe, the specificity was, nevertheless, compromised, which necessitated a redesign of the probe entirely. Allele-specific discrimination became possible following alteration of the architecture of the cleavage arm in probe R4, which significantly altered the overall binding dynamics of the probe. This highlights the key role of the composition and design of the cleavage arm in the design of LEC-LAMP probes. Although it was possible to reach allele-specific discrimination with R4, this remains sub optimal for applications beyond a proof-of-concept demonstration of the potential of the assay. Following the success obtained in adding a single base to the original R probe at the 5’ end (R2) this could also be applied to R4, and it may be of value to explore combining aspects of assay design from all probes thus far trialled. There are therefore significant avenues for further research to be pursued. Despite the redesigns necessary for the R probe however, this assay design still offers a faster and more efficient path to SNP specific differentiation than that of conventional AS-PCR, with specific

discrimination between two alleles achieved with fewer design steps required to arrive at a proof-of-concept assay (Yongkiettrakul et al., 2017; Chahar et al., 2017; Mohon et al., 2018; Malparteda-Cardenas et al., 2018; Malparteda-Cardenas et al., 2019; Costa-Junior et al., 2022).

One of the principal advantages LEC-LAMP offers is also the potential for quantification of resistance alleles within a sample via fluorescence emitted during probe cleavage, thus, any future optimisation efforts should focus on producing a highly specific probe for the S168T allele. This would allow for the development of a test which could offer a viable alternative to the current diagnostic tests, due to their heavy reliance on thermocycler equipment or lack of quantification capability (Kotze et al., 2016; Kotze et al., 2020).

Demonstration of the amenability of the assay to multiplexing is also significant and an important further step towards the improvement of diagnosis of multiple anthelmintic resistance within the field of veterinary medicine. It raises the possibility of combining several different drug resistance SNPs within a single assay. This would require the introduction of multiple primer sets, which has already been demonstrated as applicable in LEC-LAMP (Higgins and Smith, 2020) and could initially be combined with the fully resolved markers of BZ resistance (E198A; F200Y; and F167Y) with the addition of the S168T SNP.

Finally, LEC-LAMP offers the possibility of POC detection using lateral-flow platforms. Lateral-flow also has the significant advantage of having been extensively used and familiar to large numbers of the general population due to the SARS-CoV-2 pandemic and subsequent adoption of widespread RDT testing and self-testing (Kierkegaard et al., 2021; Denford et al., 2021).This possibility was explored from the adaptation of the previously published *H. contortus* ITS2 species identification primer set (Melville et al., 2014) to lateral-flow for the detection of *H. contortus* in Hungarian field isolates (Khangembam et al., 2021). Initial experiments showed accurate and specific detection of exon 4 *acr-8* cloned fragment, however, this was not yet allele specific. This is likely due to the design of the assay, as detection is based on uncleaved dual FITC and biotin labelled amplicon using a modified version of the S probe labelled with a single biotin

moiety. As cleavage occurs in the presence of the S168 allele in the S probe, dual labelled amplicons correspond to uncleaved probe, and therefore, the presence of the S168T allele. However, this assumes full cleavage of all dual labelled amplicons, which represents an unforeseen flaw in the initial assay design. As the NEB *endonuclease* IV enzyme has an optimal temperature of 37ºC the efficiency of the reaction is likely to be reduced at 63ºC, whilst the quantity of LAMP amplicons produced in a standard LAMP reaction likely exceeds the capacity of the enzyme to cleave within the time frame of the reaction (40 mins). A future improvement to the assay design is the inclusion of a second digoxigenin moiety upstream of the 5' biotin moiety and the cleavage site, combined with a multiplex detection lateral-flow platform with test lines for both biotin and digoxigenin. This would ensure that both cleaved and uncleaved probe would be detectable. Nevertheless, the current assay offers the first example of a clear demonstration of the applicability of LEC-LAMP to lateral-flow end-point detection. The current detection of the *acr-8* exon 4 fragment is also significant as it serves as confirmation of the detection of *H. contortus* DNA by lateral flow. This lays the groundwork for further improvement and the possibility of simultaneous POC detection of *H. contortus* and the S168T variant in the near future.

5.3.1 Concluding remarks

Herein, two enzymatic cleavage-based LAMP technologies were evaluated for the detection of the S168T allele in *H. contortus*. ProofMan LAMP was not applicable for the detection of S168T in *H. contortus*, while LEC-LAMP was able to discriminate between the two alleles using cloned sequences, and the efficacy of this assay for the detection of S168T was demonstrated as a proof-of-concept. Thus, it was possible to demonstrate its amenability to multiplexing and the potential for application of LEC-LAMP to end-point POC detection.

6 Preliminary Investigation of LEV Resistance Associated SNPs in a Second QTL in *H. contortus*

6.1 Introduction

6.1.1 Background

Recent work has demonstrated the high likelihood of LEV resistance being multigenic (Doyle et al., 2021; Antonopoulos et al., 2022); although S168T is likely the major determinant of resistance in MHco18(UGA2004), some SS adults can still survive LEV treatment (2/10 adults examined by AS-PCR (Chapter 4)). Therefore, to accurately diagnose LEV resistance with a molecular assay, a panel of markers may be required, particularly for diverse field populations.

Analyses of WGS data from pre- and post-LEV treated MHco3/18 populations identified a second QTL on chromosome IV (Doyle et al., 2021). This peak is known to contain two paralogues of *lev-1* (*lev-1.1* (HCON_0010770) and *lev.1.2* (HCON_00107690)), a gene previously associated with LEV resistance in *C. elegans* (Fleming et al., 1997; Qian et al., 2008). The role of *lev-1.1* and *lev-1.2* in LEV resistance in *H. contortus* is not currently well understood. For both paralogues, the conceptual translation (LEV-1) is thought to lack a signal peptide so will not necessarily form part of the functional receptor *in vivo* (Laing et al., 2013). Functional reconstitution experiments have also shown that *H. contortus* LEV-1 is not necessary for the formation of a functional receptor in a *Xenopus* oocyte heterologous expression system (Boulin et al., 2011). However, this does not rule out a role for *lev-1.1* or *lev-1.2* in resistance, as they could fulfil an ancillary role in receptor assembly, therefore, still contributing to a resistant phenotype (Kotze et al., 2014). The *lev-1.2* (HCON_00107690) gene is a tandem duplication which lies immediately upstream of the *lev-1.1* locus (Laing et al., 2013; Doyle et al., 2021). The mechanistic role of this duplication is unknown at present, however, non-synonymous SNPs that increase in frequency under LEV selection of the genetic cross have been identified within both genes (Doyle et al., 2021). Another gene present within the LEV resistance associated QTL is a

homologue of *C. elegans kdin-1* (Doyle et al., 2021). Although *kdin-1* has not been well characterised to date in *H. contortus*, in *C. elegans* it has been shown to be associated with neuronal signalling, particularly in oxygen sensitive neurons, and is thought to be involved in neuroplasticity (Li et al., 2020). *kdin-1* may also play a role in transcriptional activation in response to stress and in adaptation (Serobyan et al., 2020). This may be significant within the context of LEV resistance in *H. contortus*, given the main target of LEV is the cholinergic neuronal system within body wall muscle (Aceves et al., 1970, Boulin et al., 2011; Martin et al., 2012). Non-synonymous SNPs that increase in frequency with LEV selection in the genetic cross have also been identified in *kdin-1*.

6.1.2 Incorporation of resistance markers to Nemabiome sequencing

The use of next-generation sequencing technology to study the microbiome of humans, its role in health, and species composition is well established at the time of writing (Davidson and Epperson, 2018). The Nemabiome approach uses the principles developed for microbiome sequencing and applies them to identification of parasitic nematode species by making use of barcoded PCR amplicons targeting the highly conserved ITS2 rDNA locus (Avramenko et al., 2015). This allows for the construction of an accurate and detailed picture of the species composition in samples collected from livestock (sheep and cattle) or wild animals (Avramenko et al., 2015; Avramenko et al., 2018; Santa et al., 2021; de Seram et al., 2022).

Although initially developed for species identification in mixed infections, the technique is also applicable to resistance surveillance. This has facilitated the rapid adoption of the Nemabiome approach to target BZ resistance SNPs. The initial proof-of-concept demonstration of this approach centred on examining the prevalence of BZ resistance SNPs F167Y, E198L, and F200Y in β-tubilin isotype 1 (Avramenko et al., 2019), which are among the most common and well-established markers of BZ resistance. Nemabiome presents a significant advantage when assessing the presence of multiple markers, in multiple species, on a large scale by allowing cost effective and fast multiplexable amplicon sequencing. This is especially significant for BZ resistance, as one of the

confounding issues in BZ resistance diagnosis has been multiplexing the three most important SNPs (F167Y, F200Y, E198A) in an assay. One study took a longitudinal approach, measuring FEC, species composition, and BZ resistance genotypes in *T. circumcincta* over a four-year period in a single Scottish sheep farm (Evans et al., 2021). The study found that F200Y was present at near fixation within the *T. circumcincta* population studied. It was also found that there was little evidence of BZ susceptibility despite the use of targeted treatment. This last finding is of particular interest as it highlights the importance of integrating these data into future management practices, which currently are often informed only be FEC. Finally, the Nemabiome approach has also been applied to monitoring BZ resistance in *Nematodirus battus* on UK sheep farms, with the study documenting early emergence of resistance (Melville et al., 2020). This is especially significant as resistance was not reported until 2010 for *N. battus* in the UK (Mitchell et al., 2011). The study showed evidence of the presence of known BZ resistance SNPs appearing in the populations of *N. battus* examined (Melville et al., 2020), further highlighting that the Nemabiome approach is amenable to use across a range of species.

In addition to identification of resistance SNPs, species diversity following anthelmintic treatment has also been assessed using the Nemabiome approach. Initially this was trialled on GIN diversity following anthelmintic treatment in beef calves, with the authors reporting clinically significant changes in species composition in mixed co-infection (Avramenko et al., 2017). Another study used a similar approach of integrating FEC data along with clinical history and Nemabiome sequencing in sheep farms in western Canada (Queiroz et al., 2020). Using this approach, the authors were able to detect the early emergence of LEV resistance in *T. circumcincta,* by speciating post treatment samples. A subsequent study applied the same methodology to Swedish sheep and goat herds. The authors reported detection of nematode DNA in samples despite FEC scores being below the McMaster threshold. This study further highlights the inaccuracy of the FECRT, and the value of carrying out sequencing-based surveillance even without known resistance markers, as this points to the potential emergence of resistance in these samples.

Finally, another significant advantage of the Nemabiome approach is that, compared to PCR (including isothermal) based diagnostics for BZ resistance (Alvarez-Sanchez et al., 2005; Tiwari et al., 2006; Walsh et al., 2007; Tuersong et al., 2019; Costa-Junior et al., 2022), there is no requirement to develop and optimise each assay individually, which is labour intensive and may not be economically viable for minor markers of resistance.

All studies to date using Nemabiome have used previously validated markers of resistance for the BZs. Therefore, to facilitate the expansion of the Nemabiome approach to LEV resistance it is necessary to develop a panel of validated markers of LEV resistance. Single worm sequencing is essential for investigating multigenic mechanisms of resistance and for the development of robust markers that could later be incorporated into a diagnostic assay. Next generation sequencing-based technologies are not practical or cost effective for genotyping individual worms, largely due to the extra cost associated with barcoding amplicons during library preparation, so marker development requires a different approach.

6.1.3 Aims

In this section of the study, the aims were to investigate the multigenic nature of LEV resistance and to design a panel of LEV resistance markers for future integration into the Nemabiome platform. This was to be accomplished by initially developing a panel of validated primer sets to amplify the genes of interest. Following this, a pilot study using Sanger sequencing was used to determine if an estimate of genotype of test populations in relation to each of the SNPs of interest could be generated.

6.2 Results

6.2.1 Marker panel selection

Markers were selected from the most highly differentiated SNPs following LEV selection in the MHco3/18 cross, as described in Doyle et al. (2021), with analysis of WGS data to assess amenability of surrounding sequence for primer design.

In *lev-1.1* (HCON_00107700), two SNPs were identified. A synonymous variant, D100 (C-T; 14,994,914, CMH p value: 5.48×10^{-17}), was initially selected as it showed a strong association with LEV resistance in the MHco3/18 population and was present in all resistant isolates except Farm 001 (LEV-R). A non-synonymous D118E (C-A; 14,994860, CMH p value: N/A) variant was also chosen as it lies upstream of the D100 (C-T) variant, and although it is does not increase in frequency following LEV treatment of MHco3/18, it is present in all resistant isolates examined, and absent in MHco3(ISE).

In *lev-1.2* (HCON_00107690), a N243I variant (A-T; 14,984,937 bp, CMH p value: 5.75×10^{-7}) and an I239V (A-G; 14,984,876, CMH p value: 0.0023932) variant were taken forward for further analysis.

In *kdin-1* (HCON_00107560), a R934H variant (G-A; 14,781,344 bp, CMH p value: 1.02×10^{-21}), a T933 variant (G-A; 14,781,343 bp, CMH p value: 0.00172831), and an E938E variant (G-A; 14,781,357, CMH p value: 3×10^{-5}) were taken forward for further analysis.

6.2.2 Allele frequencies of LEV resistance associated SNPs in *kdin-1, lev-1.1, lev-1.2,* and *acr-8*

LEV resistance associated SNPs identified in *kdin-1* (E933 (G-A); R934H; T938 (G-A)), *lev-1.1* (D100 (C-T); D118E), and *lev-1.2* (N243I, I239V) were examined in WGS to assess allele frequencies in pooled samples. Initially, WGS data from

MHco18(UGA2004), MHco3/18 pre- and post-LEV treatment, and MHco3(ISE) populations were examined, and compared to one phenotypically LEV resistant, and two LEV susceptible farm isolates (Figure 6.1). Overall, when considering both laboratory and US farm isolates, the S168T, D100 (C-T), R934H, and N243I variants showed the strongest associations with LEV resistance. These variants were present at moderate-high frequency in all resistant isolates, and either absent or present at very low frequency in susceptible isolates. The D100 (C-T) variant showed the strongest association with LEV resistance of the synonymous variants examined, showing a pronounced increase following LEV treatment.

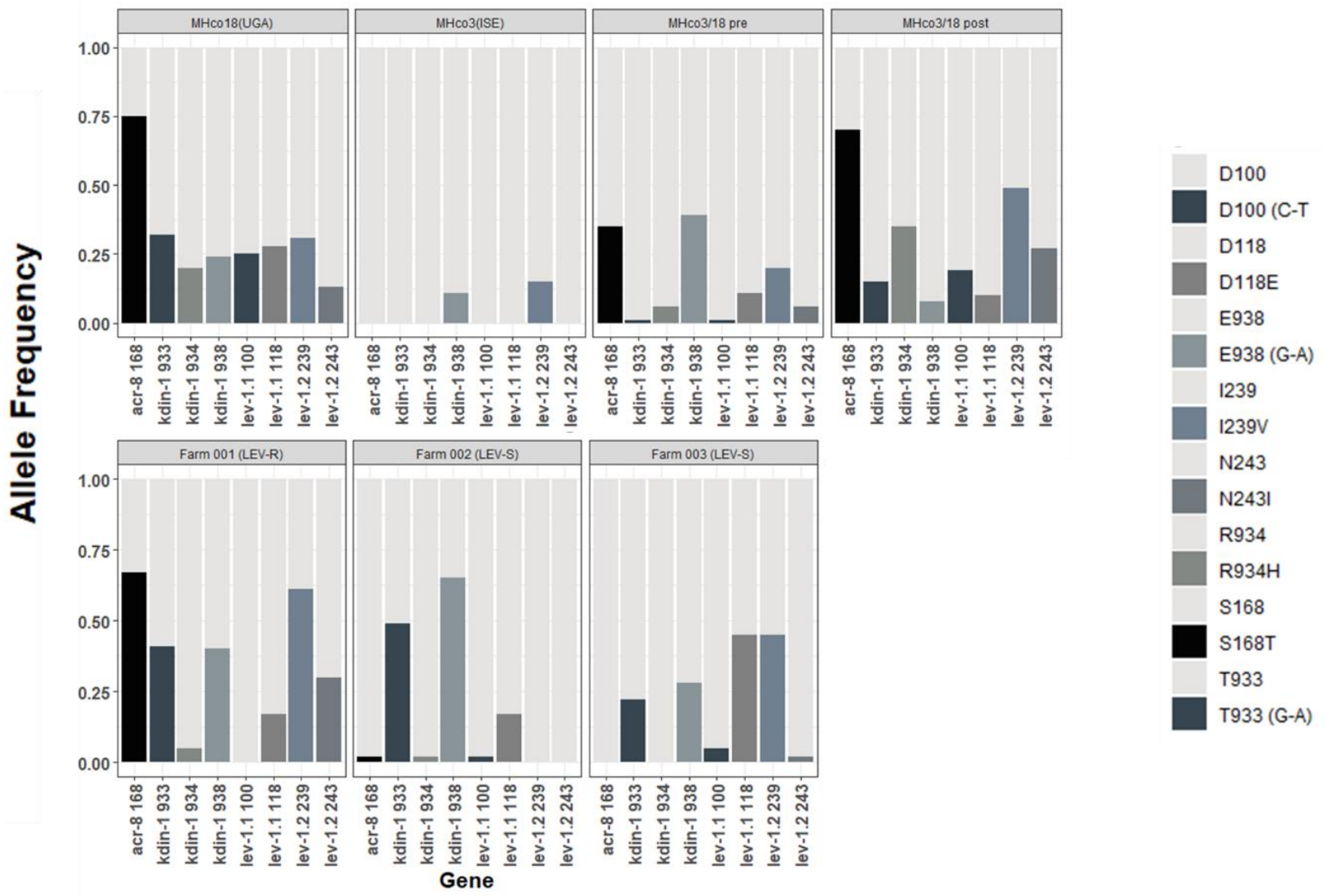

Figure 6.1: Summary of synonymous and non-synonymous SNP frequency identified in *acr-8*, *lev-1.1, lev-1.2,* and *kdin-1* in WGS data from laboratory and US farm isolates

6.2.3 Between-species diversity of LEV resistance associated SNPs in *kdin-1*, *lev-1.1*, and *lev-1.2*

Amino acid sequence diversity was then examined between nematode species for non-synonymous LEV resistance associated SNPs R934H (*kdin-1*), D118E (*lev-1.1)*, and N243I (*lev-1.2)*.

The R934H variant occurs at moderate frequency following LEV selection of the MHco3/18 population (Doyle et al., 2021), and is absent in MHco3(ISE) and Farm 002. The D118E variant, although not shown to increase in frequency following LEV selection of MHco3/18 (Doyle et al., 2021), is nonetheless present in WGS data from all resistant isolates examined, and absent from all laboratory susceptible isolates examined, but was however, found in LEV susceptible farm populations from the USA. The N243I variant is present at moderate frequency in all LEV resistant isolates examined, and absent or present at very low (<5%) frequency in all susceptible isolates examined.

The arginine residue at position 934 in KDIN-1 is highly conserved across Clade V nematodes (Figure 6.1). *H. placei* and *H. contortus* show a near identical amino acid sequence surrounding R934H, indicating a high degree of conservation between these species. Due to the highly fragmented nature of the *T. circumcincta* draft genome, equivalent sequence could not be identified, although a ~250 amino acid fragment of *T. circumcincta kdin-1* was identified. R934 is predicted to lie within the KAP P-loop domain, which is predicted to face the cytoplasm and is thought to be involved in the NTP-dependent regulation of assembly of membrane associated signalling complexes involved in neuronal growth and development (Aravind et al., 2004). This could suggest a potential role in AChR membrane insertion and receptor complex assembly.

The aspartic acid residue at analogous position 118 in LEV-1, however, is less well conserved across the same group of species, with the glutamic acid variant present in the predicted LEV-1 sequences for both *T. circumcincta* and *O. volvulus*. Free living nematodes display threonine at the analogous position 118, with this being

conserved across divergent species of both *Caenorhabditis* spp. and *P. pacificus*. However, at position 118 in *lev-1.2*, *H. contortus* encodes glutamic acid. Finally, the asparagine residue at analogous position 243 in *lev-1.2* was highly conserved across closely related Clade V nematodes, however, a serine residue was seen at this position in more distantly related species *P. pacificus, T. canis,* and *O. volvulus*. No species examined showed the resistance associated isoleucine residue at this position

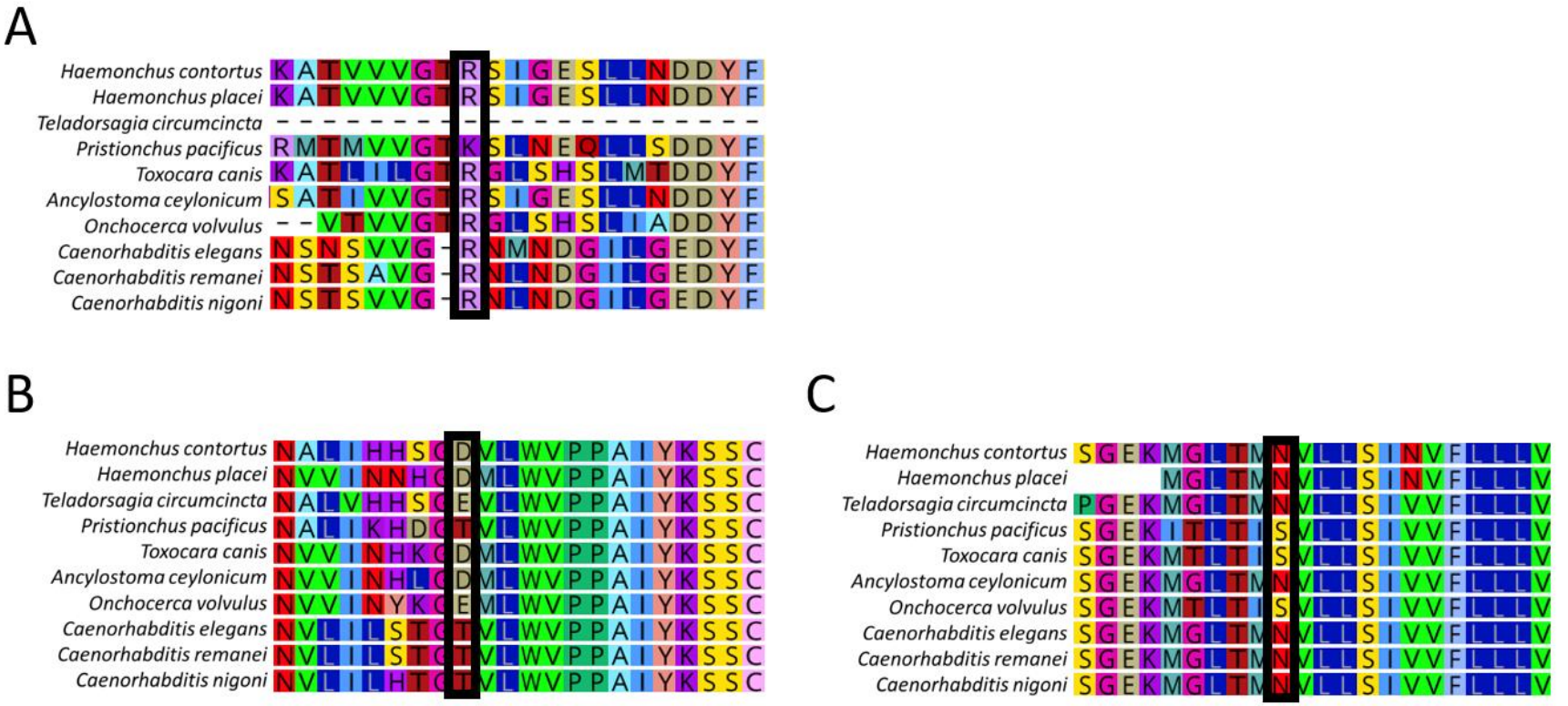

Figure 6.2: Between species diversity at LEV resistance associated non-synonymous SNP loci in LEV resistance associated genes *kdin-1, lev-1.1,* and *lev-1.2*

A: Conservation of *kdin-1* arginine 934 residue at analogous positions in homologous proteins across select *Nematoda* spp.

B: Conservation of *lev-1.1* aspartic acid 118 residue at analogous positions in homologous proteins across select *Nematoda* spp.

C: Conservation of *lev-1.2* asparagine 243 residue at analogous positions in homologous proteins across select *Nematoda* spp.

6.2.4 Primer validation

Primers were designed to amplify ~200-250 bp surrounding the SNPs of interest and associated exonic sequence for *lev-1.1, lev-1.2*, and *kdin-1*.

Following initial design, primers were used for Phusion high fidelity PCR, followed by Sanger sequencing to confirm correct target identification.

All primer sets were confirmed to amplify the correct target region, with minimal non-specific amplification (Figure 6.3). The primer set to amplify *lev-1.2*, however, showed weak amplification overall, and secondary products larger than the expected amplicon size in some individuals. This may have reflected sequence diversity at the *lev-1.2* locus, consistent with the poorer read mapping apparent in WGS data at this locus, further complicating primer design.

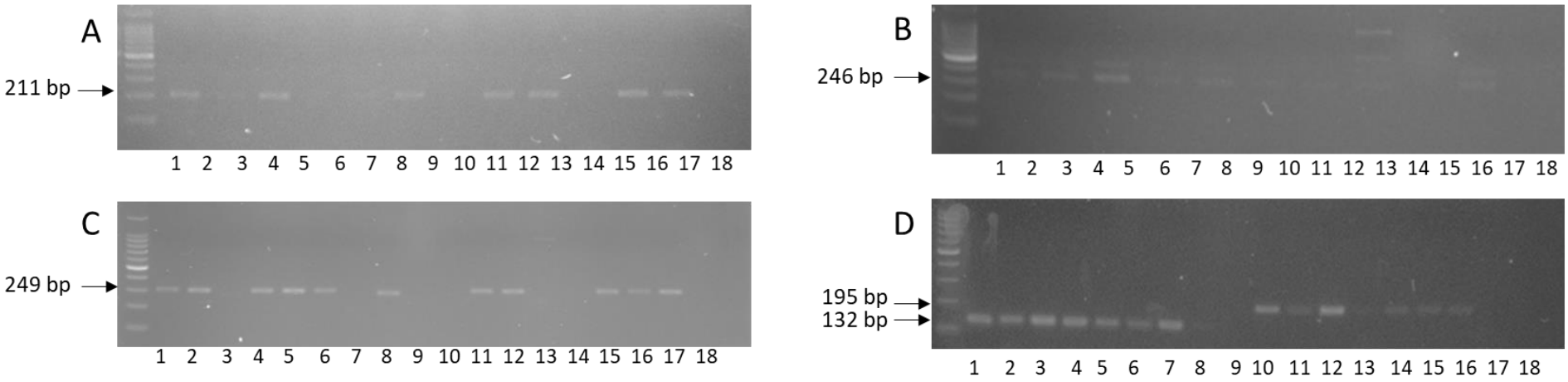

Figure 6.3: Summary of PCR validation on single L_3 for amplicon markers in *lev-1.1, lev-1.2, kdin-1, unc-29.1*, and *unc-63.*

A: Hco-lev1.1F/R. 1-8: MHco18(UGA2004). 10-17: MHco3(ISE). 9 & 18: NTC

B: Hco-lev1.2F/R. 1-8: MHco18(UGA2004). 9-16: MHco3(ISE). 17 & 18: NTC

C: Hco-kdin1F/R. 1-8: MHco18(UGA2004). 10-17: MHco3(ISE). 9 & 18: NTC

D: Hco-unc29.1F/R. 1-4: MHco18(UGA2004); 5-8 MHco3(ISE)

Hco-unc63F/R. 10-13: MHco18(UGA2004); 14-17: MHco3(ISE).

9 & 18: NTC

6.2.5 Feasibility study of single worm SNP genotyping via Sanger sequencing

Following primer design and successful target amplification, a larger Sanger sequencing experiment to test the viability of SNP genotyping using Sanger chromatograms was undertaken. Initially, *acr-8* exon 4 amplicons from 21 pre-LEV and 21 post-LEV treated MHco3/18 L_3, in addition to 10 adult males and 10 adult females from the MHco3/18 population that survived LEV treatment were selected. Good quality Sanger chromatograms were obtained from the majority of L_3 sequenced. Some degree of variation in quality across the 21 L_3 from each condition was observed, which is in line with expectations when carrying out single worm sequencing due to variations overall in quality and quantity of starting template, as also observed during single worm PCR (Chapter 4). Good quality sequencing chromatograms were obtained from adult females, but not from adult males. Base call quality surrounding the site of the S168T SNP was then evaluated, to assess whether an individual was likely to be homozygous for the S168T allele, heterozygous, or homozygous for the S168 allele. SNP identification is possible using the chromatogram and read call quality, with a high (>=30) base call quality indicating that the individual is likely homozygous at this position, whereas low/intermediate base call quality likely indicates that the individual is heterozygous at this position (Figure 6.4A, B, C, D). If a G or a C is present at the SNP position, but the base call quality is <30 this was judged as heterozygous (see Figure 6.4C). An estimate of genotype frequency of S168T in our test populations was then undertaken. A higher proportion of individuals were predicted to be RR by Sanger sequencing chromatogram analysis when compared to AS-PCR, with 25% of pre-LEV administration MHco3/18 and 68% of post LEV administration MHco3/18 individuals predicted RR (Figure 6.4E), in contrast to only 9% and 16% respectively. However, these figures more closely mirror the predicted overall allele frequency in these isolates based on WGS (35% and 70%) and presence of the S168T allele overall in AS-PCR (48% and 75% respectively).

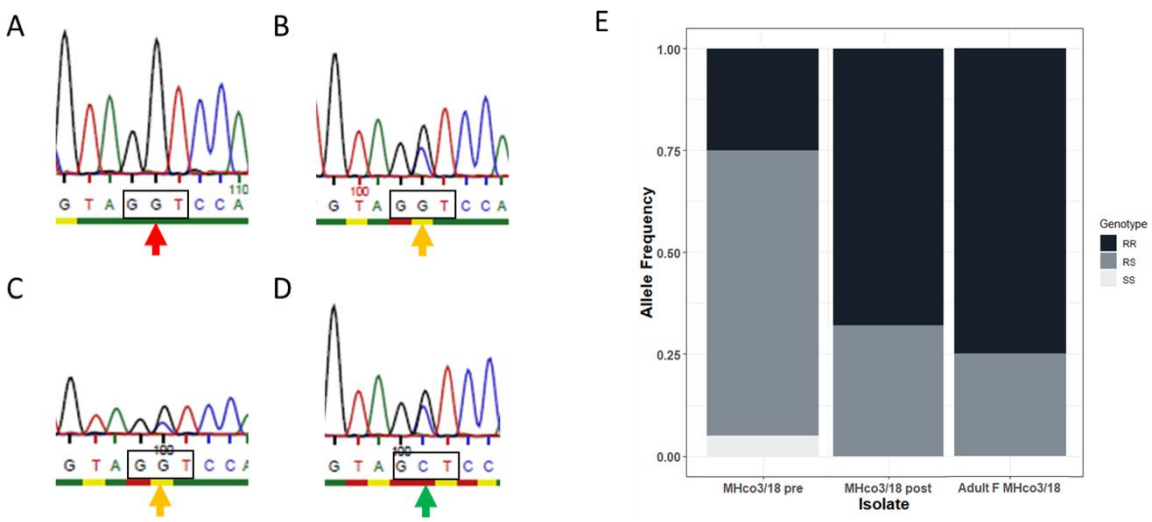

Figure 6.4: Comparison of Sanger sequencing chromatograms provided by Eurofins MWG for the detection of the S168T variant in MHco3/18 adults surviving LEV treatment.

A: Clear predicted homozygous S168T GGT allele individual showing good base call quality with no secondary peak

B: Clear predicted heterozygous S168T GGT allele individual showing poor base call quality with pronounced secondary peak corresponding to a C base

C: Predicted heterozygous S168T GGT allele individual showing poor base call quality and weak chromatogram peaks, with secondary peak corresponding to a C base

D: Predicted heterozygous individual S168T GCT allele with poor base call quality and large secondary peak corresponding to G base

E: Summary of Predicted Allele Frequency by visual analysis of Sanger-seq Chromatogram as indicated

6.2.5.1 TRACY analysis of sanger sequencing chromatographs

Next, secondary validation of genotype via chromatograms was carried out with TRACY analysis using the Pearl tool for variant calling and trace decomposition (Rausch et al., 2020; https://www.gear-genomics.com) using the MHco3(ISE) PRJEB506 reference sequence for template alignment.

TRACY analysis revealed a similar genotype pattern to that described above based on visual identification via chromatograms alone. However, TRACY analysis was found to be better able to highlight likely heterozygotes by a small margin when compared to visual identification alone (Figure 6.5A).

However, the individual MHco3/18 pre-LEV treated L_3 predicted to be homozygous S168 by visual analysis of chromatograms alone (Figure 6.4E) was likely heterozygous based on Pearl analysis. This was due to differing assessment of base call quality between the chromatograms generated by Eurofins and the .ab1 trace files analysed by TRACY Pearl (Figure 6.5B and C). In the initial visual analysis of the chromatogram, despite the presence of a secondary peak corresponding to a C nucleotide, the individual was identified as homozygous for S168 based on the base call quality of >= 30 (Figure 6.5C).

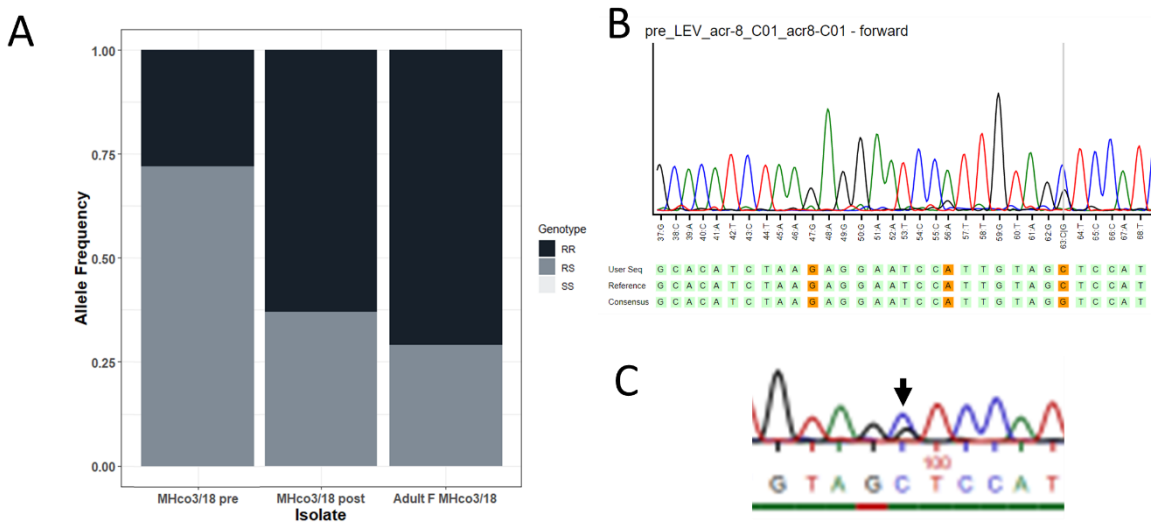

Figure 6.5: Summary of TRACY genotype results for MHco3/18, and comparison of base call chromatogram trace in Pearl and via Sanger chromatogram in individual MHco3/18 L3 identified initially as homozygous S168 via visual analysis Sanger chromatogram.

A: Summary of predicted genotype by TRACY Pearl analysis of Sanger chromatogram trace decomposition.

B: TRACY Pearl chromatogram analysis (.ab1 file input) aligned to reference sequence exon 4 *acr-8* extracted from MHco3(ISE)

C: Close-up view of predicted GCT allele homozygote base call quality and chromatogram produced by Sanger sequencing

However, both analysis methods also revealed a shortcoming in the chromatogram approach, as close visual observation of chromatogram traces showed that small secondary peaks corresponding to a C nucleotide call were visible in several individuals, but were not of sufficient strength to register as a base call conflict in either analysis pipeline (Figure 6.6). This presented the

possibility of allele amplification bias occurring with regards to the single round PCR carried out for amplification of the *acr-8* exon 4 template. This was coupled with the concomitant issue of low template abundance being the likely cause of a lack of Sanger sequences returned for the remaining markers initially assayed in the first part of this study. This also showed a significant difference in detection of the S168 allele in this approach, as 1/5 individual adult females originally assayed by AS-PCR were not identified as homozygous S168 using TRACY analysis or visual analysis of Sanger chromatograms.

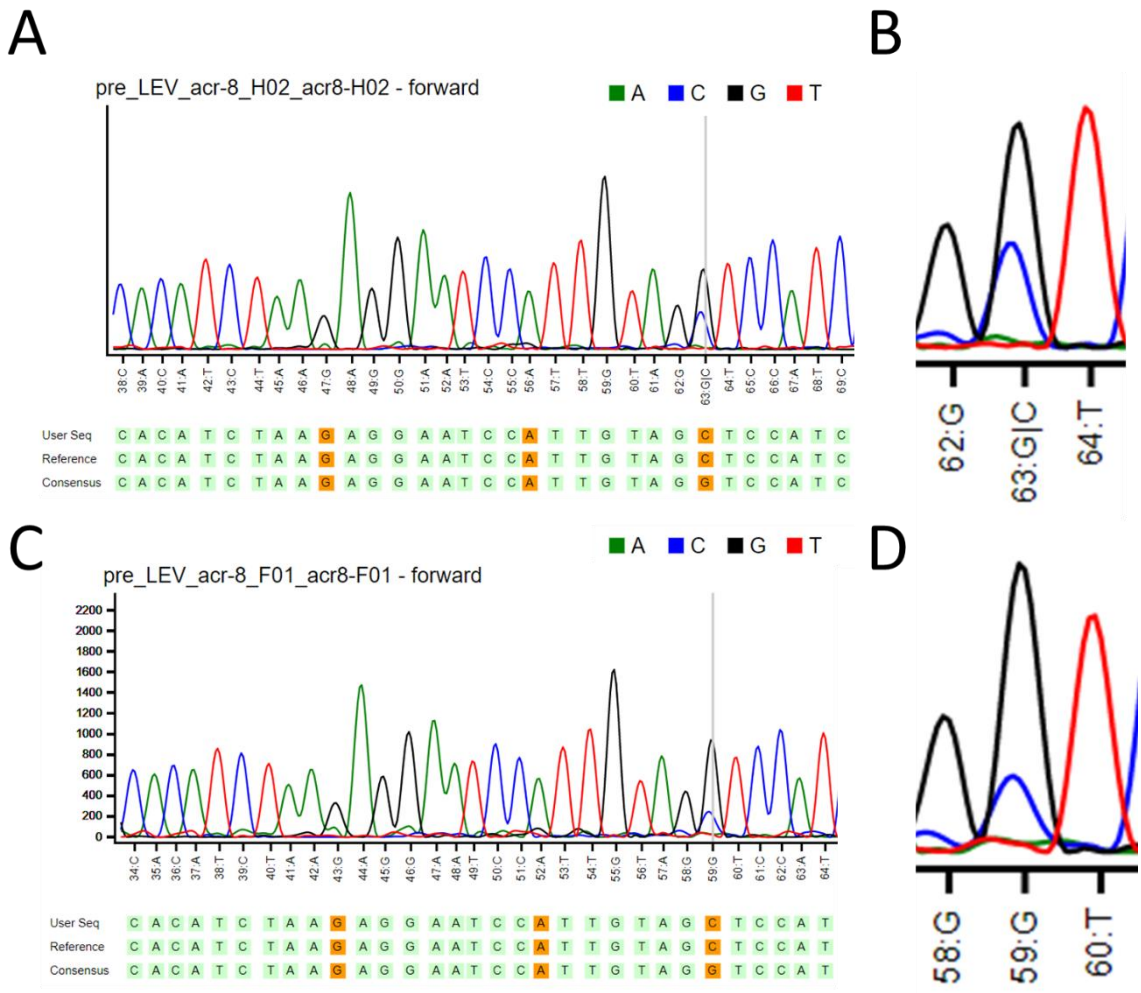

Figure 6.6: TRACY Pearl analysis showing unambiguous heterozygous individual at codon 168 (A and B; close up of S168T locus in A) vs ambiguous heterozygous individual at codon 168 (C and D; close up of S168T locus in C).

Secondary peaks corresponding to a C nucleotide are present in both individuals, but only A/B has been identified as heterozygous at this position, despite a secondary C peak also present in C/D.

6.2.5.2 Sanger sequencing of MHco3/18 adults surviving LEV administration

A second round of sequencing was then carried out for *kdin-1* and *lev-1.1* amplicons from 10 adult male and 10 adult females from the MHco3/18 population surviving LEV treatment. To correct for low template abundance, a 2-step nested PCR was undertaken to increase the availability of template overall, and to offset the potential allele bias observed in the initial round of sequencing in 2.3.

Chromatogram analysis was sub-optimal for this part of the study, as chromatograms were found to contain significant background noise for *kdin-1* and *lev-1.1* amplicons, which made accurate identification of homozygotes and heterozygotes difficult. Therefore, the raw sequences were aligned to the *kdin-1* exon 24 and *lev-1.1* exon 4 loci, and a preliminary single worm genotype for the presence of each SNP was constructed.

Two nucleotide changes were identified leading to the D188E amino acid change in *lev-1.1*, the predicted C-A, in addition to a C-G. In addition to the predicted *kdin-1* SNP R934H, a further non-synonymous SNP was identified, R934P, at the same position. This SNP had not been previously detected, and is not present in WGS data from MHco3(ISE), MHco18(UGA2004), and MHco3/18 L_3 pre/post LEV treatment. In addition, a high degree of polymorphism was seen throughout the amplicon relative to the MHco3/18 consensus sequence to which it was aligned. R934P was identified in several individuals in which S168T was not present in raw sequencing reads (Figure 6.7). When R934P is included no adult MHco3/18 individuals surviving LEV treatment examined were found to lack a non-synonymous SNP in either *acr-8, kdin-1*, or *lev-1.1*.

These data show a majority of individuals (17/20) encode S168T. In addition, R934P is present in 8/20 individuals, whereas R934H and D118E identified in 5/20 and 4/20 individuals respectively. The synonymous variant D100D was found in 4/20 individuals, all females. The synonymous variant T933T was only found in one individual, whereas the synonymous variant E938E was found in 4/20 individuals. Two individuals were found to encode SNPs in *kdin-1* (R934H only), *lev-1.1*, and *acr-8*. The R934P variant was not identified in any individuals

encoding three SNPs. A further two individuals were found to encode four SNPs, including both S168T, and R934H, in addition to synonymous variants, which differed between each individual. Male 3 (M3) and Female 11 (F11) were predicted SS by AS-PCR, whereas Male 1 (M1) was predicted RR, showing some discrepancy between the two assays.

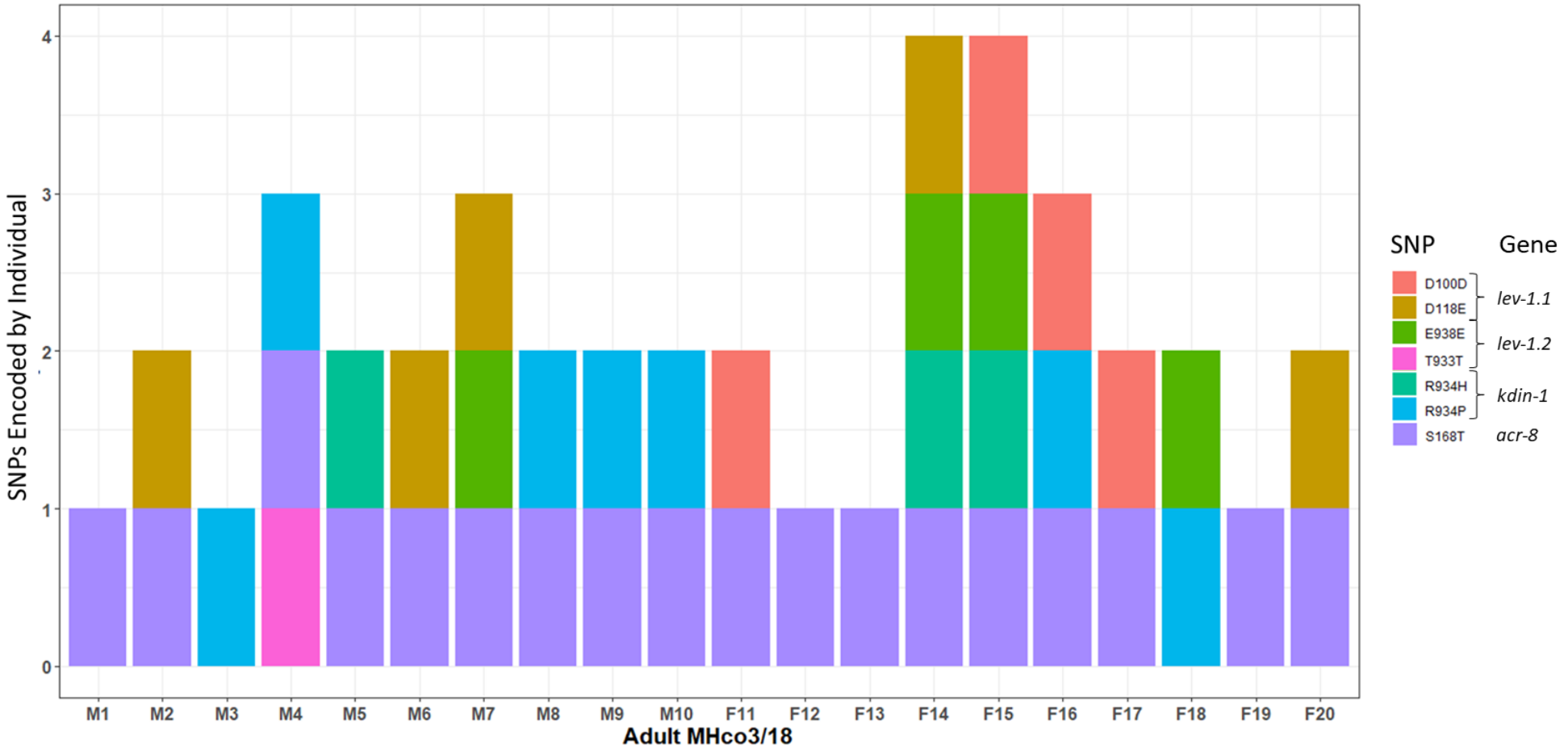

Figure 6.7: Sanger sequencing single worm genotype of MHco3/18 adults surviving LEV treatment for LEV resistance associated SNPs D100D, D118E, T933T, R934H, R934P, E938E, and S168T in *kdin-1, lev-1.1,* and *acr-8*. X axis – Adult MHco3/18. Y Axis – Number of SNPs encoded by each individual. Colours indicate the SNPs, M1-10 refer to adult male MHco3/18, F11-20 refer to adult female MHco3/18. Each colour block represents the presence of a SNP in an individual MHco3/18 adult.

If R934P is removed, two individuals with no predicted resistance associated SNPs in *lev-1.1, kdin-1* or *acr-8* amplicons are identifiable from raw sequencing, and one individual was found to encode one synonymous variant. However, examination of chromatograms of these individuals showed poor overall base call quality, and although it appeared these individuals may be heterozygous at the S168T, R934H, or D118E positions, it was not possible to confirm this with any degree of confidence.

6.3 Discussion

In this final section of the study, investigation of candidate multi-locus LEV resistance genotypes, and the early-stage development of a SNP marker panel for use with deep amplicon sequencing was undertaken. Following initial marker selection and primer design, PCR products were Sanger sequenced to confirm correct target amplification. Validated primers for *acr-8*, *lev-1.1,* and *kdin-1* were then used for Sanger sequencing of pre- and post-LEV treated MHco3/18 L_3, and post-LEV treated MHco3/18 adults recovered post-mortem following experimental infection. It was then possible to carry out preliminary genotyping using the *acr-8* primer set. However, initial results showed insufficient template quantity following a single round of PCR for amplicons generated for *kdin-1* and *lev-1.1*. A nested primer set was then generated for each of these genes, and Sanger sequencing repeated following a two-step nested PCR to improve template concentration. This led to an improvement in detection, however, background noise in sequencing data and inconsistent coverage made SNP genotyping by Sanger sequencing difficult, indicating further work will be necessary to fully exploit this approach. The presence of LEV resistance associated SNPs S168T, D118E, and R934H was then examined in the raw sequence data, and an additional SNP, R934P, was detected in *kdin-1* amplicons from MHco3/18 adults surviving LEV treatment. Examination of the presence of SNPs in *kdin-1, lev-1.1*, and *acr-8* allowed the generation of a preliminary genotype profile for adults surviving LEV treatment. S168T remained the dominant SNP, present in 17/20 individuals, however, R934P, R934H, and D188E were detected in a significant minority of individuals. However, due to sub-optimal read quality from Sanger sequencing, significant further work will be necessary to fully validate these results, and particularly to determine if R934P is a legitimate LEV resistance associated SNP, or due to sequencing error.

Following on from the identification of homozygous S168 individuals surviving LEV treatment *in vivo*, and the identification of a significant proportion of homozygous S168 in LEV resistant populations, it was necessary to explore minor LEV resistance associated SNPs. WGS data identified several SNPs of interest present in LEV resistance associated genes *lev-1.1* and *lev-1.2*. Further LEV

resistance associated SNPs were also identified in *kdin-1*, a gene which had not previously been implicated in LEV resistance (Doyle et al., 2021). These SNPs are present at a low to moderate frequency within WGS sequencing reads from MHco18(UGA2004), and MHco3/18, and were found to be absent in MHco3(ISE) WGS data. This contrasts with S168T, which was identified at a high frequency in both resistant laboratory isolates and post-LEV treated MHco3/18, and present at fixation in the Kokstad isolate. These SNPs were therefore predicted to be likely minor determinants of LEV resistance. This necessitated an alternate approach to that used to validate the S168T marker. The development of AS-PCRs for each of these SNPs was not practical for the purposes of this study, as AS-PCR optimisation and development is a time and labour-intensive undertaking, as demonstrated by the S168T AS-PCR (Chapter 4; Antonopoulos et al., 2022). Thus, it was decided to pursue a sequencing-based approach to allow for larger scale future monitoring of the presence of these SNPs within *H. contortus* populations. The eventual aim of this development process would be the integration into a Nemabiome based approach, as demonstrated for BZ resistance (Avramenko et al., 2019).

Initial efforts focused on the design and validation of primers for generation of amplicons to amplify the templates of interest. This followed on from the same approach used for amplification of the *acr-8* template (Chapter 4; Antonopoulos et al., 2022). Primer design was restricted to exonic sequence and ~20 bp of intronic sequence adjacent to the exon of interest, due to a high degree of polymorphism present within introns in *kdin-1*, *lev-1.1*, and *lev-1.2*. In addition, design for primers targeting *lev-1.2* was further complicated by poor mapping and high polymorphism in this gene. Given the presence of SNPs of interest in exons 4 and 9 of *lev-1.1*, the decision was also taken to design primer sets to amplify both of these exons for a future marker panel, although due to cost and time limitations within the current study only the amplicon fragment generated from *lev-1.1* exon 4 was included here, and was primarily selected due to better PCR performance. Although the N243I variant in *lev-1.2* shows a strong association with LEV resistance, the potential off-target amplification, and poorer overall PCR performance precluded its inclusion in this preliminary work, as further optimisation would be necessary for Sanger sequencing.

It was then decided to carry out a preliminary single worm sequencing screen using a sub section of the marker panel as further validation. This was done to determine, initially, if it would be possible to carry out preliminary genotyping of single L_3 and adult MHco3/18 using Sanger sequencing. However, due to lack of template, poor sequencing read length and base call quality was obtained for *lev-1.1* and *kdin-1*. For *acr-8* it was possible to construct a preliminary predicted genotype from Sanger sequencing for MHco3/18 adult females surviving LEV treatment, and for pre- and post-LEV treatment L_3. The genotype differed significantly from that generated by the AS-PCR, as the S168 allele was not detected at high frequency in any of these samples, and all instances of its detection were in predicted heterozygous individuals. However, due to read quality and sequence coverage, analysis was only possible in 7/10 individual MHco3/18 female adults. In addition, individuals homozygous for the S168 allele were also not detected in MHco3/18 L_3 pre- and post-LEV treatment, and where the S168 allele was present, this was in heterozygous individuals. However, in pre-LEV MHco3/18 L_3 following TRACY Pearl analysis it was only possible to get an accurate genotype in 15/21 individuals due to significant background noise present in those 6/21 individuals. It was possible to generate a more accurate genotype picture from the post-LEV MHco3/18 individuals, as 19/21 showed good quality sequencing. However, once again, no homozygous S168 individuals were identified from this sample either. This raises several issues, the first of which is potential allele bias, which presents as a distinct possibility given the relative proportions of individuals within each sample for which sequencing data was not available (6/21 and 2/21 for the pre- and post-LEV treated MHco3/18 populations respectively), which would be within the expected range of SS individuals expected within those populations (Chapter 4; Antonopoulos et al., 2022). Visual analysis of Sanger chromatograms did identify one homozygous S168 individual in the pre-LEV treated MHco3/18 sample, however, this individual also displayed a small secondary peak corresponding to a G nucleotide. Therefore, it is a distinct possibility that the S168T allele is favoured by the first round PCR reaction, which may be accounted for by increased polymorphism in the intronic sequence where the primer bind site for Hco-Intron4-F and Hco-Exon4-F are located. This likely does not present as an issue with the AS-PCR, due to the two-step nested protocol, which would account for

a lower amplification efficiency of one allele over the other by both the specificity of the second-round primers and the second round of amplification itself. There does also exist the possibility that the sensitivity of the S168T AS-PCR primer is lower than that of the S168 primer, leading to an overestimation of homozygous S168 individuals within the population. However, this is unlikely to account for the complete lack of homozygous S168T individuals observed, as the AS-PCR genotype predictions have been independently validated by both RFLP and sequencing of cloned *acr*-8 exon 4 amplicons.

The amplicon markers for *lev-1.1* and *kdin-1* were also examined initially using a single round PCR amplification. However, template quantity from a single round of PCR was not found to be sufficient. Due to this, and to take account of any potential allele bias as encountered in the case of *acr-8*, it was decided to trial a two-step nested PCR. In addition, this was also done as it would more closely mirror the standard protocol for Illumina MiSeq, where initial PCR amplifies the target of interest, followed by a second round of PCR with barcoded PCR primers (Avramenko et al., 2015; Avramenko et al., 2019; Melville et al., 2020; Francis and Slapeta, 2022). As barcoding is not necessary for Sanger sequencing, this was primarily carried out to ensure that both alleles were enriched sufficiently to be detectable within the sample. Allele bias may still lead to the presence of significantly higher peaks in certain individuals, as was observed for *acr-8*, however, this might be offset by an increase in overall template, allowing for the better detection of potential heterozygous individuals. Although primers may bind preferentially to one allele, this is likely not 100% allele-specific binding, therefore, increasing overall template quantity would potentially improve detection of the other allele in the Sanger chromatogram. However, although this improved detection significantly, there was an excess of background noise. This could in part be due to off-target amplification, particularly due to copy number variation, especially at the *lev-1* locus due to the tandem duplication present in this gene (Laing et al., 2013; Doyle et al., 2020; Doyle et al., 2021). Given the extensive evidence of multiple instances of functional duplications of AChR subunits in *H. contortus* (Laing et al., 2013; Doyle et al., 2020) this is an aspect of primer design that will need to be more closely considered in future work. There also remains the possibility that samples may have become

contaminated with *H. contortus* DNA from previous experiments and PCR purifications, and this may also have contributed to the excess background noise seen in the *kdin-1* and *lev-1.1* amplicon sequencing.

Alignment of raw Sanger sequences to their respective reference sequences indicated that the SNPs of interest may be present in several individuals within this population. It was then possible to build a preliminary multi-locus genotype for individual worms to identify further non-synonymous LEV associated SNPs that could account for the presence of SS individuals surviving LEV treatment identified previously (Chapter 4).

Analysis of the *lev-1.1* amplicon sequence detected the D100 (G-A) variant in 4/20 individuals, which was consistent with its strong association with LEV resistance in the post-treatment MHco3/18 progeny, and consistent with the significant increase detected in WGS data. The D100 (G-A) variant therefore presents as a good potential candidate marker for LEV resistance. It will be of significant interest to explore this marker further to determine if it affects expression levels of *lev-1.1* in resistant isolates, as synonymous SNPs can have a significant effect on mRNA expression (Shen et al., 2022). This could be consistent with the observation of a significantly lower level of expression of *lev-1.1* in MHco18(UGA2004) L_3. This may also occur cumulatively with synonymous variants between codons 7 and 43 of *lev-1.1*. It would, therefore, be of value in future work to examine these variants and D100 (C-T) further, either by RNA-seq or further amplicon sequencing. Future work, therefore, should also focus on improved design and optimisation of primers to amplify this locus.

Two SNPs, both of which led to a D118E coding change, were also identified in amplicon sequencing. The frequency of D118E in Sanger sequences of MHco3/18 adults surviving LEV treatment was higher than that of the WGS data for pooled F3 generation L_3 of MHco3/18, with 4/20 individuals encoding the variant. The D118E variant's potential role in resistance, however, is unclear, as despite being present in all resistant isolates examined here, and showing an increase in proportion in the post-treatment adult MHco3/18, this does did not translate to the progeny. In addition, the D188E variant was present in a high proportion of

reads from LEV susceptible farm populations. There are a number of additional synonymous SNPs identified in *lev-1.1* (Doyle et al., 2021) which also bear further investigation between codons 7-43, but which were not examined here due to the less amenable sequence for primer design. Of further interest is the observation of a glutamic acid residue at position 118 in *lev-1.2* in *H. contortus*. However, further characterisation of these proteins, and their roles in receptor assembly is necessary before any conclusions can be drawn regarding the mechanistic role of these residues.

Analysis of the *lev-1.2* N243I variant in WGS data revealed a strong association with LEV resistance, with a marked increase in proportion of reads encoding the variant following LEV treatment of MHco3/18. Farm 001 (LEV-R) showed a markedly higher proportion of reads encoding the N243I variant than MHco18(UGA2004), which is of particular interest as both populations derive from the same geographic area, however, MHco18(UGA2004) has been maintained in a laboratory (Williamson et al., 2011; Doyle et al., 2021), whereas Farm 001 has undergone repeated LEV selection in the field (Doyle et al., 2021). In addition, the N243I variant was absent from MHco3(ISE) and one LEV susceptible farm population, and present at very low frequency in another in WGS data. Amino acid sequence comparison of homologous proteins showed the asparagine residue was highly conserved at the analogous position in *H. placei, T. circumcincta, Caenorhabditis* spp. This variant is, therefore, a high priority target moving forward. Examination of previously published sequencing data from LEV resistant *T. circumcincta* for this variant would be of interest in the short term. Future work should undertake improved primer design and optimisation to better amplify the exon containing N243I.

Analysis of *kdin-1* in the MHco3/18 adult population revealed a previously unreported SNP, leading to an alternate R934P coding change at the same locus as the R934H variant identified in WGS. Analysis of pooled WGS data from MHco18(UGA2004), MHco3(ISE) and MHco3/18 pre- and post-LEV treatment also revealed a marked 5-fold increase in the number of reads encoding R934H following LEV treatment. No individuals genotyped with Sanger sequencing however, encoded R934H in isolation of S168T. Three individuals examined,

which did not display the S168T variant in Sanger sequencing, were found to encode the R934P variant. This could be of significance as it may explain the presence of SS individuals following LEV treatment. However, due to the poor overall quality of sequencing returned for these genes, and the necessity of using raw rather than clipped sequences, it is difficult to draw concrete conclusions at this stage. Furthermore, the R934P variant has not been identified in any WGS data from MHco18(UGA2004), MHco3/18, or farm isolates examined here, which further raises the possibility that this is simply an artefact of poor read quality. However, the presence of this SNP in 8/20 individuals may suggest otherwise, as if it were simply an artefact it would be less likely to be present in the same position in so many individuals. Another possibility is that this is a base-call error which should correspond to R934H, rather than a previously unreported SNP. The R934 residue is highly conserved across parasitic nematodes. In addition, R934 is predicted to lie within the cytoplasm facing KAP NTPase domain, which is thought to play a role in receptor complex assembly and neuronal growth and development (Aravind et al., 2004). Evidence of a marked increase in reads encoding the R934H variant from WGS of pooled L_3 pre/post-LEV treatment, and the detection of the R934H variant, and the R934P variant, in multiple MHco3/18 adults surviving LEV treatment is potentially a significant finding that bears further investigation. A high degree of sequence polymorphism was observed in amplicons encoding R934P, however, this may be due to poor base-call quality throughout the sequences rather than *in vivo* polymorphism. Although PCR error could theoretically account for the R934P variant, it is unlikely that this would be present in 8/20 individuals. In addition, the use of a high fidelity polymerase further reduces the likelihood of the presence of the R934P variant being due to PCR error. It is also of note that, in NGS WGS data (Doyle et al., 2021), R934H was not present, or present at very low frequency in WGS data from two LEV susceptible farms examined. The R934H variant, however, was observed in only a small proportion of NGS reads in a LEV resistant (Farm 001) field population. This suggests R934H/P could be a minor marker of LEV resistance, and may play a possible mechanistic role in receptor assembly. However, further work is necessary to fully elucidate this.

Synonymous variants in *kdin-1* which showed a strong association with LEV resistance in the LEV selected MHco3/18 population were also examined. These lie adjacent to the locus of the R934H variant, but do not appear to be linked SNPs to the R934H variant. The E938 (G-A) variant showed a sharp decrease following LEV treatment, but was identified in 5/20 individual MHco3/18 adults surviving LEV treatment. Its association with LEV resistance is also inconsistent, as it was detected in a high proportion of both LEV resistant and susceptible laboratory and field isolates. The T933 (G-A) variant also showed inconsistent results outside of MHco3/18, with a high proportion of reads encoding the variant in both resistant and susceptible and farm isolates, despite a pronounced increase in MHco3/18 populations post treatment. This suggests that these SNPs will likely not serve as useful markers of LEV resistance. They may, however, be involved in an isolate- or geographic-specific altered expression mechanism which may be related to the role of KDIN-1 in receptor assembly, neuroplasticity, and oxygen sensing (Li et al., 2020; Serobyan et al., 2020).

The individual worm multi-locus genotypes for MHco3/18 adults surviving LEV treatment are consistent with the increase in variants in both *acr-8* and *kdin-1* following LEV treatment in the F3 progeny. It will also be of interest in future to determine if the individuals encoding the R934H variant are homozygous at this position, as unfortunately Sanger sequencing was not able to resolve this question.

Some of the issues encountered in this part of the study, which could benefit from further improvement, were the poor abundance of template, the significant background noise in sequencing reads, and the potential issues of contamination. Further work could also help elucidating the genotype of MHco3/18 adults surviving LEV treatment with chromatogram analysis, which was not possible at the time of writing. In addition, WGS of several of the individual MHco3/18 adults surviving LEV treatment may also be of value. This would allow a more accurate genotype to be generated for all of the resistance associated SNPs identified by Doyle et al. (2022), which was beyond the scope of this study. One of the principal ways in which poor template abundance and background noise can be addressed is by moving to next generation sequencing

technology, such as Illumina Miseq, which offers significantly more depth of sequencing than Sanger and provides haplotypic data rather than consensus (Abel and Duncavage, 2013). This allows both the inclusion of a lower overall template input, and facilitates the identification of variants (Midha et al., 2019), in addition to numerous tools available for this purpose (Roca et al., 2019). This would then allow for a more accurate prediction of the genotype of individual worms, as significant read depth would be available, which would also offset a degree of the potential allele bias seen herein.

6.3.1 Concluding remarks

In the final part of this study, single worm amplicon sequencing to detect additional LEV resistance associated SNPs was undertaken. Genotyping via chromatogram trace deconstruction was possible for some individuals, but results differed from that predicted by AS-PCR. In addition, template abundance issues, and background sequencing noise complicated confident identification of homozygous or heterozygous individuals. It was, however, possible to tentatively confirm the detection of R934H and D100 (C-T) variants within our test population, in addition to the possible presence of a previously unreported variant, R934P. Although further validation is necessary to confirm the relationship of these SNPs to S168T, and to further examine the N243I variant in *lev-1.2.*, the results generated herein confirm correct target amplification, and the potential for future use of a next-generation sequencing based population level approach for resistance associated SNP detection.

7 Discussion

7.1 General discussion

7.1.1 Background and Knowledge at the Outset of the Study

At the outset of the current study the knowledge and understanding of the field regarding molecular markers of LEV resistance centred on several mechanisms related to up or down-regulation of certain AChR subunits (Sarai et al., 2013; Sarai et al., 2014; Raza et al., 2016), the expression of various truncated transcripts of key AChR subunits (Neveu et al., 2007; Neveu et al., 2010; Fauvin et al., 2010; Williamson et al., 2011; Boulin et al., 2011), and a variable length deletion in intron 2 of *acr-8* (Barrere et al., 2014). Furthermore, at the outset of the current study, no molecular assays existed for the detection of LEV resistance in *H. contortus*, nor any other GIN (Kotze et al., 2016). Therefore, the main objectives of the study were to validate putative molecular markers of LEV resistance, determine their efficacy, and develop a proof-of-concept molecular diagnostic assay for the detection of LEV resistance in *H. contortus*.

7.1.2 Summary of final results

During the course of the study a variety of data encompassing population level data, individual worm genotypes, and optimisation of molecular assays was produced. This is summarised below in Table 7.1.

Table 7.1: Summary of final results covering all genotype data using PCR, qPCR, RFLP, AS-PCR, LEC-LAMP and Sanger sequencing to detect the presence of LEV resistance associated genetic markers in *H. contortus*

Assay	Data Type	Resistance Marker	Populations Tested	Summary of Results	Further Comments
Size discrimination PCR	Individual *H. contortus* L_3 genotype	Intron 2 deletion in *acr-8*	MHco18(UGA2004), MHco3(ISE)	90% of MHco18(UGA2004) and 64% of MHco3(ISE) are homozygous for the deletion.	Covered in chapter 3. These data suggest that the intron 2 deletion in *acr-8* would not serve as a diagnostic marker of LEV resistance due to a high proportion of homozygous deletion individuals in the susceptible MHco3(ISE) population.
qPCR	Comparative quantification of transcripts in pooled adult and L_3 *H. contortus* cDNA	Expression levels of *acr-8* and *lev-1.1*	Pooled adult and L_3 MHco18(UGA2004), MHco3(ISE)	MHco18(UGA2004) adults show a significantly lower expression of *lev-1.1* compared to MHco3(ISE) adults. MHco18(UGA2004) L_3 show a significantly higher expression of *lev-1.1* and a significantly lower expression of *acr-8* compared to MHco3(ISE) L_3.	Covered in chapter 3. These data suggest a life-cycle stage specific expression pattern of resistance associated AChR subunits
RFLP	Individual *H. contortus* L_3 genotype	S168T (*acr-8)*	MHco18(UGA2004), MHco3(ISE), MHco3/18 (untreated)	75% of MHco18(UGA2004) and ~50% of MHco3/18 encoded the S168T variant, 0% of	Covered in Chapter 4. These data suggest that S168T could serve as a marker of LEV

				MHco3(ISE) encoded the S168T variant. A high proportion of MHco3/18 were of ambiguous genotype.	resistance, as it is present at a high proportion in LEV resistant isolate populations and absent in LEV susceptible MHco3(ISE). These data also suggest the RFLP is not optimal for accurately determining genotype.
AS-PCR	Individual *H. contortus* L_3 genotype	S168T (*acr-8)*	MHco18(UGA2004), MHco3(ISE), MHco3/18 (pre-LEV treatment), MHco3/18 (post-LEV treatment), MHco4(WRS), MHco10(CAVR), LEV-S farm isolate (USA), LEV-R farm isolate (USE)	94% of MHco18(UGA2004), 58% of pre-LEV treatment MHco3/18, 75% of MHco3/18 post-LEV treatment, and 80% of LEV-R farm population (USA) encoded the S168T variant, whereas 0% of the MHco3(ISE), MHco4(WRS), MHco10(CAVR) and the LEV-S farm population (USA) encoded the S168T variant. The proportion of homozygous S168T individuals also increased following LEV treatment in MHco3/18.	Covered in chapter 4. These data suggest that S168T serves as a robust marker of LEV resistance in *H. contortus*. It is present in a high proportion of LEV resistant isolates, and absent in all susceptible isolates examined. Its presence is strongly associated with a LEV resistant phenotype in field isolates from the USA. A relatively large sub-population of homozygous S168 individuals were observed in LEV resistant isolates.
AS-PCR	Individual *H. contortus* adult genotype	S168T (*acr-8)*	MHco3/18 (adults collected post-mortem following LEV treatment)	5/9 individuals were found to be homozygous for the S168T variant, 2/9 heterozygous, and 2/9 homozygous S168.	Covered in chapter 4. These data suggest that homozygous S168 individuals may be able to survive LEV treatment. This also suggests LEV resistance may be multigenic.
LEC-LAMP	Discrimination of cloned alleles	S168T (*acr-8)*	Cloned S168T allele from homozygous resistant MHco18(UGA2004) from a single L_3 Cloned S168 allele from homozygous susceptible	S168 probe accurately discriminates between the S168 and S168T alleles. S168T probe requires further optimisation as overall fluorescence is weak.	Covered in chapter 5. These data suggest that LEC-LAMP would serve as a suitable assay for the detection of the S168T, and serves as a proof-of-concept demonstration of the

			MHco3(ISE) from a single L_3		technology, but further optimisation is required.
Sanger Sequencing	Individual *H. contortus* adult genotype	S168T (*acr-8)*, R934H/R934P (*kdin-1)*, D118E/D100D (*lev-1.1*)	MHco3/18 (adults collected post-mortem following LEV treatment)	17/20 individuals encode the S168T variant, 8/20 individuals encode the R934P variant, 4/20 individuals encode the D100D variant. Of the 3/10 individuals without the S168T variant 2/10 encoded only the R934P variant, and 1/20 encoded the R934P and the D100D variants.	Covered in chapter 6. These data suggest that S168T constitutes the major marker of LEV resistance, and that the R934P variant may account for SS individuals identified by AS-PCR. However, R934P has not been observed in WGS data and requires further investigation.

7.1.3 Putative Marker Validation

Initial efforts to validate putative molecular markers of LEV resistance centred on *acr-8* (HCON_00151270). This was primarily due to its location at the centre of the major locus under LEV selection (Doyle et al., 2021) and a significant body of evidence in the literature suggesting a key role for this gene in LEV resistant *H. contortus* (Williamson et al., 2011; Martin et al., 2012; Barrere et al., 2014; Blanchard et al., 2018), albeit without a clearly resolved mechanism. The presence of a truncated transcript of *acr-8*, termed *acr-8b*, the expression of which was correlated with a resistant phenotype (Williamson et al., 2011), and the variable length deletion in intron 2 of *acr-8* (Barrere et al., 2014) were therefore seen to be the most likely candidates as putative markers of LEV resistance. A secondary QTL was also identified on Chromosome IV following LEV selection of the MHco3/18 genetic cross, which included *lev-1.1* (HCON_00107700) (Doyle et al., 2021), and this gene was therefore a secondary target for investigation.

7.1.3.1 Altered expression levels of key AChR subunits

Laboratory validation, however, of these putative markers yielded unexpected results. Initially, qPCR was undertaken to quantify the expression of *acr-8* and *lev-1.1* in resistant isolates. The observed up-regulation of *lev-1.1* and concurrent down-regulation of *acr-8* in MHco18(UGA2004) L_3 relative to MHco3(ISE) L_3 may reflect a synergistic interaction between *acr-8* and *lev-1.1* in resistance, particularly as LEV-1 is not essential for AChR functionality (Boulin et al., 2011) and is thought to lack a signal peptide for membrane insertion (Laing et al., 2013). Furthermore, re-analysis of RNA-seq data (Laing et al., 2013) revealed a strong life-cycle stage specific skew for these subunits, with *acr-8* showing low expression in the L_3, and *lev-1.1* showing its highest expression in that life-cycle stage. Due to the difficulty of reliably culturing *H. contortus* past the L_4 stage (Stringfellow, 1986; Sonibare et al., 2011; Gutierrez-Amezquita et al., 2017), the majority of work on AChR subunit composition *in vivo* has been carried out the free-living larval stages, and adults collected *post-mortem* (Sarai

et al., 2013; Sarai et al., 2014; Raza et al., 2016). Given the wide variation seen in expression levels of various AChR subunits and ancillary proteins across life cycle stages, it remains distinctly possible that *H. contortus* AChR subunit composition varies greatly throughout its life-cycle, and between isolates. For example, the LEV resistant Wallangra 2003 (WAL) isolate, derived from an Australian field population (Love et al., 2003), showed markedly different expression patterns of several key AChR subunits across different life-cycle stages. Altered expression levels, relative to the LEV susceptible Kirby isolate, of certain resistance associated subunits, such as *acr-8* and *unc-63,* was observed in resistant WAL larvae, but this change in expression was not seen in adults of the same isolate (Sarai et al., 2013). In a subsequent study the WAL isolate was separated into sub-populations based on resistance to LEV, assessed by LDA. Expression levels of key AChR subunits were then compared in populations of L_3 that had been exposed to increasing concentrations of LEV during development. This again revealed some marked differences in expression, with the sub-population showing the lowest LEV resistance exhibiting up-regulation of *unc-63b*, *unc-38,* and *acr-16,* expression changes which were not seen in the more highly LEV-resistant sub-populations (Sarai et al., 2014). It is of note that the ACR-16 AChR is a homomeric ligand gated ion channel which is antagonised by LEV in Clade V nematodes (Raymond et al., 2000; Boulin et al., 2011; Kaji et al., 2020). Thus, this may represent an induced, but not highly effective, response to LEV by shifting receptor composition to the homomeric ACR-16 variety at the neuromuscular junction. The ancillary protein RIC-3 has been shown to be necessary for the ACR-16 receptor assembly (Boulin et al., 2008), however, this subunit was down-regulated in the least LEV resistant sub-population of the WAL isolate. As LEV resistance increased, down-regulation of *acr-8*, and *unc-63* were then observed. However, at the highest level of LEV resistance, *unc-63b* was also down-regulated. The *acr-8b* transcript was not detectable in any of the populations WAL examined, nor was *lev-1.1* found to be significantly altered in any of the WAL sub-populations examined (Sarai et al., 2014).

The WAL isolate showed a distinctive plateau of LEV susceptibility in LDAs, indicative of a highly resistant sub-population. Given the observation of ~30% of MHco18(UGA2004) L_3, and a similar proportion of LEV resistant US field isolate L_3

were homozygous for S168T (Chapter 4; Antonopoulos et al., 2022), these could also represent a highly resistant sub-population, albeit these US populations, overall, displayed a higher level of resistance than WAL (based on DrenchRite assays which are expected to be comparable to the LDAs used in Sarai et al., 2014). In addition, DrenchRite assays revealed two phenotypes emerging from LEV resistant farm isolates in the USA. A population level rightward shift in the dose response, and the presence of a highly resistant sub-population which develop to L_3 even in the presence of LEV concentrations of 25-50 μM (Ray Kaplan, personal communication). The latter highly resistant sub-population, could therefore, represent the 20-30% homozygous S168T population seen in these isolates. This could also represent a sub-population where multiple LEV resistance mechanisms co-occur, leading to a synergistic increase in LEV resistance. Taken together with ~5-25% of L_3 found to be homozygous for S168 in MHco18(UGA2004) and Farm 001 (LEV-R) respectively, it remains possible that altered expression levels of certain subunits may account for some resistance, or tolerance to LEV, in homozygous S168 L_3. As S168T was not known when these studies were carried out, it would be of great interest to genotype these populations for the presence of S168T.

7.1.3.2 Cold-sensitivity of LEV resistant L_3

Another aspect related to altered subunit expression patterns that bears further investigation, is the observed cold sensitivity of LEV resistant L_3. This has primarily been observed when LEV resistant L_3 were previously stored in liquid nitrogen. When subsequently used in an experimental infection, despite establishing and coming to patency, egg counts were negligible post-LEV treatment (Eileen Devaney; Roz Laing; David Bartley, personal communication). The mechanism underlying this apparent fitness costs are not known, but it is possible that exposure to extreme temperatures, such as those below freezing, may activate a stress-response which is perturbed in some way by certain LEV resistance mechanisms affecting the same pathways. It is also possible that certain environmental parameters, such as temperature, and/or potentially nutrient availability, may trigger altered expression patterns of subunits prior to establishment of infection which could affect sensitivity to LEV. For example,

there is some evidence in *C. elegans* that activity levels in response to temperature can regulate the structure of synaptic connections in larval stages (Zhao and Nonet, 2000). In addition, in *C. elegans*, thermotaxis is an essential homeostatic mechanism (Garrity et al., 2010), and heat stress has been shown to sensitise the AChR, whereas hypothermia can block cholinergic transmission (Kalinnikova et all., 2016). Thus, there may be significant environmental factors patterning AChR subunit expression which could merit further exploration. This is particularly relevant if underdosing is occurring in the flock (Stafford et al., 2009; Besier et al., 2010), as this may be adding a further selective pressure, which operates synergistically with environmental parameters driving a LEV tolerant L_3 phenotype to emerge. The spread of *H. contortus* further and further north in the context of climate change (Salle et al., 2019), and the increased occurrence of extreme weather conditions in warmer regions, may also contribute to this, by selecting for increased neural plasticity to better deal with temperature and nutrient stresses. It is also of interest to consider the intron 2 deletion in *acr-8* regarding the role of cold sensitivity in LEV resistant L_3. Although the intron 2 deletion has been shown not to constitute an effective marker of LEV resistance (Chapter 4; Baltrusis et al., 2021), an interesting aspect of the deletion is its potential role in the expression and regulation of a cuticle collagen gene (HCON_00151260). The deletion is present between exons 1 and 2 of this gene, which itself lies within intron 2 of *acr-8* (HCON_00151270). Given the importance of the cuticle in nematode physiology in terms of both barrier and locomotion functions (Page and Johnstone, 2007), and evidence of increased permeability of the cuticle to LEV when certain collagens are lost in *C. elegans* (Sandhu et al., 2021), this requires further investigation. Initially, it would be of value to examine its expression across different life cycle stages by re-analysis of available RNA-seq data, followed by RNAi silencing to explore the role of HCON_00151260, in a manner similar to that described by Blanchard et al. (2018). It may also be of value to examine its role in environmental tolerance and locomotion by examining RNAi knockdown and LMIA in response to various temperatures and other environmental stimuli. Preliminary experiments optimising an LMIA in the presence of LEV in conjunction with exposure of L_3 to various temperatures (ambient, 4^{o}C, -20^{o}C, liquid N) were also carried out during the course of this study (Watson and Antonopoulos et al., unpublished). Larvae

from these LMIA experiments have been archived, and future work could examine the genotype of migrated and non-migrated L_3 relative to several of the markers and mechanisms discussed herein.

7.1.3.3 Role of host physiology

One final aspect that also bears consideration is the role of host physiology, and immunology, on these mechanisms. There is significant evidence of the immunomodulatory activity of *H. contortus* during blood-feeding obligate parasitic (L_4-adult) stages (Wang et al., 2019b; Hildersley et al., 2021). This, coupled with widely reported differences in tolerance of certain sheep breeds to *H. contortus* infection (Toscano et al., 2019; Albuquerque et al., 2019; Cruz Tamayo et al., 2021; Weaver et al., 2021), bears further investigation. Given the role of many LEV resistance associated genes in motility (Aceves et al., 1970; Boulin et al., 2011; Martin et al., 2012) and environmental sensing (Garrity et al., 2010; Kalinnikova et al., 2016) in *H. contortus* and closely related nematodes, alterations in AChR composition may occur in response to environmental stimuli. This mechanism may, thus, operate synergistically to the emergence of a LEV resistant/tolerant phenotype when drug pressure is applied.

7.1.3.4 Truncated transcripts and their role in resistance

Regarding the role of truncated transcripts in LEV resistance, a strong correlation with a resistant phenotype has been reported in some isolates (Boulin et al., 2011; Williamson et al., 2011; Barrere et al., 2014). Furthermore, the preferential integration of UNC-63b into AChR in heterologous functional reconstitution experiments (Boulin et al., 2011) raises the possibility of this mechanism playing a significant role in LEV resistance. However, this may be isolate and/or life-cycle stage specific, as no up-regulation of *unc-63b* was seen, for example, in any stage of the highly LEV resistant LV isolate. The truncated *unc-63b* transcript was also down-regulated in L_3 of the LEV resistant LW isolate, and it was significantly up-regulated in the moderately LEV resistant WAL adult females (Sarai et al., 2013). The potential isolate specificity of this response may explain the lack of selection seen at this locus following LEV selection of the MHco3/18 genetic cross (Doyle et al., 2021). Though the *unc-63b* transcript

has not previously been detected in some isolates of *Haemonchus*, such as the MHco18(UGA2004) population (Williamson et al., 2011), it has been detected in other species of parasitic nematodes (Whittaker et al., 2017). Its role in LEV resistance, therefore, bears further investigation.

Returning to the role of *acr-8b*, detection of the truncated transcript in MHco3(ISE) L_3 was unexpected. This presented the second instance of its detection in the L_3 of a LEV susceptible isolate, following initial detection in the CRA isolate (Barrere et al., 2014). Multiple splice variants were observed in MHco3(ISE), in addition to a lack of detectable expression in adults (Chapter 3). This, therefore, precluded the use of *acr-8b* as a diagnostic marker of LEV resistance, leaving the actual role of this transcript in the development of the *H. contortus* AChR unclear at the time of writing. It may perform a regulatory function, and following a similar protocol to that for *unc-63b* (Boulin et al., 2011), would be of great value to determine if ACR-8b preferentially integrates into the *H. contortus* AChR. Lack of observable detection in adults also points to a life-cycle stage specific expression pattern and, given the overall low expression levels of *acr-8* in L_3, this transcript may represent a developmental, regulatory, or receptor assembly mechanism in the transition from free-living to parasitic stages in *H. contortus*. In *C. elegans*, there is evidence of a truncated insulin receptor isoform modulating signalling via sequestration of insulin peptides, with a potential role in the quiescent *dauer* larvae and insulin signalling in response to environmental cues (Martinez et al., 2020). Thus, truncated transcripts of AChR may contribute, alongside altered subunit expression patterns in general, to tolerance mechanisms (Sarai et al., 2013; Sarai et al., 2014; Sarai et al., 2015; Raza et al., 2016).

7.1.4 Potential major marker of LEV resistance: S168T

Based on the inconsistent expression of *acr-8b*, and the high proportion of fully susceptible individuals encoding the *acr-8* deletion, the study then focused on identifying an alternative marker in the chromosome V locus under LEV selection (Doyle et al., 2021). The S168T variant in *acr-8* was extensively validated with RFLP and AS-PCR and found to constitute a robust marker of LEV resistance.

Non-synonymous SNPs have previously been identified in *acr-8*, L200P and K457T, however, these were not consistently and exclusively present in LEV resistant isolates (Sarai et al., 2013) and were not at high frequency in the genetic cross. There also remains the distinct possibility that S168T may also constitute a mechanism of LEV resistance. Preliminary protein modelling predicted S168T to lie in the extracellular domain, and therefore could play a role in ligand binding, due to conformational changes in the tertiary structure of the protein. A serine-threonine amino acid change does not represent a significant shift in amino acid property, with both showing relatively similar structure, as threonine contains a methyl substitution in place of hydrogen on the β-carbon relative to serine. In addition, both are hydrophilic and polar uncharged, therefore, unlikely to lead to complete destabilisation of tertiary structure due to changes in solubility or electrostatic interactions. Nevertheless, serine-threonine substitution in the *katG* gene is sufficient to confer isoniazid resistance in *Mycobacterium tuberculosis* (Ramaswamy et al., 2003), albeit via a significantly different mechanism as *katG* encodes a catalse-peroxidase responsible for activating the pro-drug form of isoniazid (Cade et al., 2010). Thus, S168T may theoretically function by altering drug binding, as the extracellular interface between ACR-8 and an adjacent subunit is thought to form an allosteric binding site for LEV (Martin et al., 2012). The serine-threonine change may, therefore, be sufficient to alter the binding efficiency of LEV, but without significant detriment to the ACh sensitivity of the receptor. It would also be of interest to determine the ACh mediated conductance of the S168T ACR-8 receptor, in addition to its conductance in the presence of LEV. The essential nature of ACh signalling to nematode motility (Aceves et al., 1970; Garrity et al., 2010) could suggest that significant changes in protein structure may lead to a change in neuronal function. This could also explain why the observed amino acid change is relatively minor, and is not predicted to significantly alter tertiary structure, as this may be lethal or confer a serious fitness cost to the parasite. Given the key role for AChR signalling in chemotaxis and temperature related stress-responses in *C. elegans* (Garrity et al., 2010; Kalinnikova et al., 2016), alterations to the amino acid composition of a key subunit such as ACR-8 may also have knock on effects in other aspects of parasite physiology. The development of the S168T AS-PCR, therefore, could facilitate a better understanding of how allele copy

number relates to ACR-8 function, environmental sensing, locomotion, and acetylcholine signalling to elucidate further its role in fitness cost *in vivo*.

7.1.5 Further potential minor markers of LEV resistance

Following the observation of the presence of homozygous S168 individual MHco3/18 adults surviving LEV treatment, single worm amplicon sequencing was undertaken to elucidate the genotype of these individuals at moderate frequency LEV resistance associated SNPs D100 (C-T) in *lev-1.1* (HCON_00107700) and R934H in *kdin-1* (HCON_00107560). It was observed that these SNPs were not present independently of S168T in any of the 20 individual adults assayed. An additional SNP, not previously identified in WGS data, R934P, was detected in *kdin-1*, and was present in all three individual adult MHco3/18 in which S168T was not detected by Sanger sequencing. It is difficult to ascertain with certainty if this SNP is a base-call error or genuinely present within the population, due to the overall poor quality of the sequencing, however its presence in 8/20 individuals suggests further investigation is needed. The role of R934H is also of significant interest for future exploration. This is due in part to a clear increase in allele frequency seen following LEV treatment of the MHco3/18 population, and a complete absence in the MHco3(ISE) isolate, coupled with the observation of a high number of individuals encoding this variant in preliminary amplicon sequencing. The KDIN-1 arginine-histidine amino acid substitution encoded by R934H does not represent a significant change in amino acid property. Both amino acids are basic and polar positive. Arginine and histidine do, however, display differing secondary structure. Arginine possesses a positively charged guanidino group, whereas histidine possesses an imidazole group, which can serve as a general acid or base depending on protonation. This difference in secondary structure is greater than that seen in the serine-threonine substitution encoded by S168T. The arginine-proline change however, represents a significant shift in amino acid properties. Proline is a cyclic and non-polar amino acid, and these properties can lead to the disruption of α-helical structures, particularly in transmembrane helices, and in soluble proteins (Li et al., 1996). The presence, therefore, of a proline residue in place of an arginine at position 934 in the KAP P-loop domain may lead to a significant perturbation of function, potentially

conferring a fitness cost related to neuronal growth and development (Aravind et al., 2004), O_2 sensing and neuroplasticity (Li et al., 2020), transcriptional activation and stress response (Serobyan et al., 2020). This may also confer LEV resistance by altering the assembly and conformation of AChR, as *kdin-1* has been observed to co-localise with AChR at neuromuscular junctions in rats, and a proline at this position may also disturb normal receptor assembly (Luo et al., 2005). This variant may have escaped detection thus far in WGS data due to this allele not mapping well due to polymorphism and poor sequencing quality. There also exists the possibility it may confer a large fitness cost or a reproductive disadvantage in the post-treatment progeny. The large effective population sizes, of *H. contortus* (Redman et al., 2012), coupled with the high degree of polymorphism (Laing et al., 2013; Doyle et al., 2020), should, however, ensure that this variant remains present in populations at least at very low frequency. The highly motile nature of males migrating in search of more stationary females, and the polyandrous mating behaviour of females may explain how a mechanism in which altered motility and cholinergic muscle contraction could elicit its effects (Redman et al., 2008; Gilleard and Redman, 2016), as males may move slower, respond to stimuli slower, and thus be less likely to pass on the variant. It may also be the case that, for this fitness cost to become apparent, the individual must be homozygous for R934P, with only heterozygous individuals viable to mate, further diluting the variant in the population. More extensive analysis of post-treatment populations of LEV resistant adult *H. contortus* would therefore, be of great interest to elucidate this further. This could be examined, for example, through detailed *in silico* protein modelling to better understand the overall structure and function of *H. contortus* KDIN-1, followed by heterologous functional reconstitution in the presence of AChR subunits such as ACR-8.

In addition to R934H, the synonymous D100 (C-T) variant in *lev-1.1* was also investigated as a potential minor marker of LEV resistance. The variant was detected in 4/20 individual adult MHco3/18, and was found to be strongly associated with LEV resistance in both WGS data and amplicon sequencing. It did not, however, occur in isolation of S168T. The only other SNP occurring in isolation of S168T was the E938 (G-A) variant in *kdin-1*, detected in one

individual, but was found at a high frequency in LEV susceptible field isolate WGS data. The mechanistic role of D100 (C-T) in LEV resistance is unclear. Recent evidence in yeast suggests that synonymous mutations can also lead to deleterious effects on the organism, leading to alterations in mRNA, for example (Shen et al., 2022). Although only a preliminary finding in a distantly related organism, this should be further investigated in *H. contortus*, due to the association of a number of synonymous SNPs in *lev-1.1* with LEV resistance in MHco3/18 (Doyle et al., 2021). The D100 (C-T) variant could therefore, play a mechanistic role in altered expression of *lev-1.1*, which was seen in both MHco18(UGA2004) L_3 (Chapter 3), and in several other isolates (Sarai et al., 2013; Sarai et al., 2015). In addition, multiple synonymous LEV resistance associated SNPs lie between codons 7-43 of *lev-1.1*, which should be integrated into further analysis. Further analysis of SNPs likely to affect expression lying within the 3' and 5' UTR should also be examined in future work.

Analysis of a further non-synonymous variant identified in *lev-1*.2 (HCON_00107690), N243I, was also present in WGS data at moderate frequency in all resistant isolates. In WGS data, the N243I variant was either absent or present at very low (<5% of reads) frequency in all susceptible isolates (Chapter 6). As off-target amplification and weak amplification were apparent, future work should prioritise on optimisation of PCR for future amplicon sequencing. It is also of particular interest to determine if N243I occurs in homozygous S168 individuals. Expression of *lev-1.2* in RNA-seq data shows a strong skew towards the L_4, with moderate expression also seen in the adult male and L_3 (Chapter 3). This subunit may, therefore, play a more active role during the parasitic life-cycle stages, an aspect that could be explored by WGS of individual adult MHco3/18, or by amplicon sequencing at this locus for individuals previously genotyped by S168T AS-PCR. Heterologous functional reconstitution of the *H. contortus* AChR in the presence of LEV-1.2, or RNAi of *lev-1.2*, may also be of interest to elucidate any mechanistic role it may have in receptor assembly or cholinergic signalling.

7.1.6 Development of molecular diagnostics for LEV resistance

Finally, the second aim of this study was the development of a proof-of-concept molecular diagnostic assay for the detection of LEV resistance in *H. contortus*, based on the translation of the identified and elucidated markers. Both at the outset of the current study, and at the time of writing, this remains of critical importance to the sheep farming sector (Kotze et al., 2016; Kotze et al., 2020). The spread of multi-drug resistance severely threatens the sustainable use of anthelmintics (Kotze et al., 2016; Kotze et al., 2020; Kaplan, 2020; Arsenopoulos et al., 2021; Charlier et al., 2022), and leads to welfare issues and production losses worldwide (Miller et al., 2012; Emery et al., 2016; Besier et al., 2016b; Salle et al., 2019). Therefore, it is critical to maintain the efficacy of LEV, which remains an important treatment option. Initially, a laboratory based "gold-standard" proof-of-concept RFLP and then AS-PCR were developed, to facilitate genotyping of individual L_3, both for validation purposes of the marker, and for future use in resistance monitoring in research laboratory settings. Following this, the use of emerging point-of-care amenable LAMP technologies were explored, with the aim of moving resistance detection away from the FECRT towards fast, accurate, and affordable molecular testing (Kotze et al., 2020). Finally, the future use of next-generation sequencing was also taken into account, with the aim of developing multi-functional assays as a valid alternative to the well-established FECRT.

7.1.6.1 S168T AS-PCR

The development of the AS-PCR allowed for the demonstration of an accurate and fast laboratory based assay that was capable of identifying, with a high degree of specificity, the individual genotype of L_3. This is a significant development, as it shows resistance conferred by the S168T allele is not a recessive mechanism and is likely multi-genic. Previous studies examining molecular markers of LEV resistance had, to date, either focused on qPCR-based (dos Santos et al., 2019), or digital-droplet PCR-based (Baltrusis et al., 2021) allele quantification at a population level. Although valuable, these approaches are not capable of offering the required resolution, at the single worm level, to determine the zygosity of the individual, which is of particular importance when

considering the proposed recessive nature of LEV resistance (Dobson et al., 1996; Sangster, 1996). Furthermore, the demonstration of the amenability to the inclusion of deoxyinosine bases for highly polymorphic and divergent *H. contortus* isolates also represents a step forward within the field of helminth molecular diagnostics, the approach having previously been described mainly for virology (Wang et al., 2000). This assay, which has been successfully validated on a number of different isolates, offers significant translational potential. In the immediate term, the S168T AS-PCR could be integrated with existing AS-PCR protocols for BZ resistance SNPs (Mohanraj et al., 2017; Baltrusis et al., 2018). This would facilitate the genotyping of individual worms for multiple resistance SNPs and build a clear understanding of the genotype of multi-drug resistant populations. This can contribute to the longitudinal tracing of resistance alleles through the population across generations. It would also be applicable to determine the genotype frequency of S168T in LEV resistant isolates from other geographic regions, as the primary focus here was on US field isolates (Williamson et al., 2011; Doyle et al., 2021). This mirrors work already being undertaken with regard to BZ resistance in Sudan (Mohammedsalih et al., 2020) and Brazil (Fávero et al., 2020) and would help determine whether S168T is a global major marker of LEV resistance. In addition, there is further immediate translational potential integrating the AS-PCR into Nemabiome. The two-step nested PCR protocol for template enrichment, in addition to extensive validation of this primer set, makes the first-round products ideally suited for use with next-generation sequencing, as first demonstrated for BZ resistance (Avramenko et al., 2019). However, Illumina Miseq sequencing remains cost-prohibitive for single worm sequencing, and thus, there is still a valuable role for AS-PCR in research settings, and for use in surveillance in LMICs where sequencing is not available, or needs to be sent abroad (Sadlowski et al., 2021). In addition, several recent studies have made use of the combination of portable PCR machinery with Nanopore MinION sequencing to deliver a point-of-care/point-of-need amenable next generation sequencing platform that can fit within a suitcase for waterborne bacterial hazard monitoring (Acharya et al., 2020), and wheat-rust drug resistance surveillance (Radhakrishnan et al., 2019). Thus, these protocols could be adapted and modified, offering a faster and more field-based alternative to Nemabiome.

7.1.7 S168T LAMP

The optimisation of an established “gold-standard” proof-of-concept PCR based molecular assay then facilitated the exploration of emerging point-of-care amenable isothermal amplification technologies. Although AS-LAMP has been widely demonstrated for SNP genotyping (Yongkiettrakul et al., 2017; Chahar et al., 2017; Mohon et al., 2018; Malparteda-Cardenas et al., 2018; Malparteda-Cardenas et al., 2019), this technique was not judged to be suitable for the purposes of this study. Although AS-LAMP has been successfully demonstrated for E198A detection in *H. contortus* (Tuersong et al., 2020), this type of assay is much less amenable to multiplexing. Aside from the significant difficulties encountered when optimising a multiplex AS-LAMP for BZ resistance detection (Costa-Junior et al., 2022), amplification detection in a multiplexed AS-LAMP will not be SNP specific, as amplification of any of the SNPs will produce a positive result. In practice, an individual AS-LAMP reaction can be SNP specific, however, as all end-point detection methods demonstrated to date for AS-LAMP measure amplification, then this precludes the multiplexing of BZ and LEV resistance in a single assay. A multi-tube assay would, therefore, lead to a higher overall cost, and complexity of the assay (Kotze et al., 2020). Thus, LAMP technology which was more amenable to SNP specific detection was explored. Two novel emerging technologies were considered, ProofMan (Chen et al., 2020; Ding et al., 2021) and LEC-LAMP (Higgins and Smith, 2020). Although operating under relatively similar methodologies, LEC-LAMP yielded the best results due to more sequence specific SNP detection (Chapter 6). It was possible, in collaboration with the research group first describing this technique, to demonstrate SNP specific discrimination between the S168T and S168 alleles. Simultaneous detection of each allele was successfully demonstrated in a single tube assay, which offers the potential for future development of quantification of resistance alleles within a population. The isothermal nature of LAMP has the added benefit of facilitating a one-tube assay for multiplex PCR. Integration of multiple region-specific LAMP primer sets has previously been demonstrated for Dengue virus, integrating up to 14 primer sets within a single tube assay (Lopez-Jimena et al., 2018). This approach would account for sequence variation in field isolates, integrating multiple isolate/region specific primer sets in a single

assay. The sequence specificity of the LEC-LAMP probe cleavage arm further adds to the feasibility of this approach for a global SNP-genotyping approach.

7.1.7.1 POC end-point detection

Following on from this foundation, the translation of end-point detection to a lateral-flow platform was also explored. This proof-of-concept demonstration would represent the first instance of point-of-care amenable SNP specific detection of an anthelmintic resistance marker in *H. contortus*. Although issues were encountered with non-specific detection of the S168T allele, the assay was capable of the specific detection of *H. contortus acr-8*. Future work should now prioritise validating this primer set against closely related nematodes to ensure no cross-reactivity occurs (Melville et al., 2014). This is particularly important for those GIN species potentially present in mixed co-infections, as, for example, an analogous serine-threonine substitution is also present in LEV resistant *T. circumcincta* (Choi et al., 2017). Regarding increasing the specificity of the lateral-flow detection of S168T, the use of a dual-labelled oligo would represent a high priority for future work.

In the medium term, LEC-LAMP also has the potential to be integrated into a portable next-generation sequencing protocol. LAMP has been extensively demonstrated for field-based amplicon sequencing for resistance associated SNP detection in combination with Nanopore MinION sequencing for human eukaryotic parasites and other pathogens (Imai et al., 2017; Imai et al., 2018; Runtuwene et al., 2018). However, the S168T LEC-LAMP assay is in a much earlier stage of development than the S168T AS-PCR, which has been validated on both laboratory and field isolates. Therefore, the immediate goal should be to carry out preliminary validation of the LEC-LAMP primer set to amplify the *acr-8* exon 4 fragment in further laboratory and field isolates. Further development of the AS-PCR and LEC-LAMP assays, and combination with emerging portable next-generation sequencing technologies and point-of-care amenable end-point detection, has the capability to offer on farm species identification and quantification of resistance alleles within the population.

7.1.8 Translation to the field

To translate the developments of this study to a final diagnostic to be used in the field there are a number of key considerations moving forward. First and foremost is the ultimate diagnostic target of any test. In order to be able to replace the FECRT it is necessary to eliminate any complex and time consuming culturing, thus, the eggs shed in faeces constitute the ultimate target for any test. However, this comes with a number of complexities which will be considered in future work. Eggs yield significantly less genetic material than L_3, particularly due to their overall smaller size, and there is also the issue of extracting the eggs from the faeces which has been considered by several studies (Doyle et al., 2019; Khangembam et al., 2021; Francis and Slapeta, 2022). However, to date, there is not yet a fully developed, standardised, and optimised faecal egg extraction technique for molecular diagnostics. However, the field is moving in this direction, and future work will need to consider the groundwork laid by Doyle et al. (2019), as this remains one of the principal barriers to widespread adoption of molecular diagnostics within veterinary parasitology.

Finally, it is also necessary to consider that most GIN infections in the field will be mixed species co-infections. Any diagnostic test that seeks to replace the FECRT must therefore, be amenable to multiplexing with multiple species and/or drug resistances (Kotze et al., 2020). The selection of techniques undertaken in this study was thus patterned by this consideration. Although, for example, AS-PCR is not particularly amenable to multiplexing, when compared to other technologies, it was nonetheless a necessary development for the validation of the S168T marker, and the primers designed therein are also amenable to use within a Nemabiome protocol. The selection of the LEC-LAMP technology, however, was particularly influenced by this consideration as this technology allows for multiplexing with relative ease, as has been preliminarily demonstrated herein. Lastly, the early stage development of the marker panel for major and minor markers of LEV resistance is envisaged as the first stage towards developing an integrated multi-species Nemabiome sequencing protocol for large scale resistance surveillance across multiple species of GIN. However,

significant further development is necessary to realise this, but the foundation has been laid in this, and other studies.

7.2 Concluding remarks

During the course of this study, a number of putative LEV resistance mechanisms and molecular markers were explored. Examination of altered expression patterns of AChR subunits showed an up-regulation of *lev-1.1* in LEV resistant L_3. Extensive use of single worm PCR also demonstrated that the intron 2 deletion in *acr-8* is not a viable marker of LEV resistance, providing evidence that significant open questions still remain regarding the mechanistic role of *acr-8b* splice variants. An alternate marker was therefore sought, with the S168T variant in *acr-8* identified and assayed by RFLP and AS-PCR. The AS-PCR assay was then optimised, which allowed extensive single-worm genotyping to be carried out on LEV resistant and susceptible laboratory and field isolates, confirming that S168T represents a viable diagnostic molecular marker of LEV resistance. Early-stage validation of a marker panel for the detection of LEV resistance associated SNPs was trialled, and strong candidate markers were identified in *kdin-1, lev-1.1,* and *lev-1.2*. Emerging isothermal amplification technologies were also examined, with LEC-LAMP showing SNP specific detection of S168T. Finally, the amenability of LEC-LAMP for point-of-care testing on lateral-flow platforms was also explored. The results of this study have clearly advanced the field of anthelmintic resistance and molecular diagnostics in veterinary medicine by demonstrating the first validated molecular marker of LEV resistance in *H. contortus*, S168T, and preliminary validation of further candidate molecular markers. This study, furthermore, has laid the foundation for the development of molecular diagnostic assays for both laboratory and point-of-care detection of LEV resistance in *H. contortus*.

Appendices

Appendix 1: Rcode.docx

R code used for visualisation of data

Appendix 2: GenomicLandscapeDoyleSNPs.xlsx

SNPs of interest identified from WGS analysis, described in Doyle et al. (2021) – provided with author permission

Appendix 3: FPKMbetatubulin.pdf

FPKM Expression of β-tubulin in MHco3(ISE)

Appendix 4: DissociationCurvesFemaleAdults.pdf

acr-8 detection in adult female *H. contortus*

Appendix 5: IndelSharedSequence51bp.pdf

Alignment showing shared 51 bp deletion sequence

Appendix 6: FullGenotypes.docx

Full genotype results by batch and isolate for RFLP and AS-PCR

Appendix 7: ProofManLECLAMPoptimisation.docx

Supplementary data from optimisation of LEC-LAMP primers, single worm LEC-LAMP, and probes temperature tests for S, R1, R2, R3, R4, R5

Appendix 8: Pre-print of paper (open access): AlleleSpecificPCRMajorMarker.pdf

Antonopoulos, A., et al. (2022) “Allele specific PCR for a major marker of levamisole resistance in *Haemonchus contortus*. Can also be accessed at: https://www.biorxiv.org/content/10.1101/2022.04.08.487639v1.full

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