Challenges and opportunities for the adoption of molecular diagnostics for anthelmintic resistance

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1. Introduction - the need for resistance diagnostics

Parasitic helminths have major impacts on the health of livestock, companion animals and humans worldwide. These health impacts include significant production losses and death in livestock, as well as weight loss, anaemia and death in companion animals, and morbidity in humans. Parasite control in all of these host species relies largely on the use of anthelmintic drugs. Chemicals from the same major drug classes are utilised across all three areas; benzimidazole drugs (e.g. albendazole) are widely used for the control of gastrointestinal nematode (GIN) parasites of livestock, hookworms in companion animals, and soil-transmitted helminths (STH). Macrocyclic lactones (e.g. ivermectin, e.g. moxidectin) are used for GIN control in companion animals and onchocerciasis, lymphatic filariasis, strongyloides and scabies in humans. Pyrimidines (e.g. pyrantel) are commonly used for the control of GIN in companion animals and occasionally in humans for control of STH. Levamisole (an imidazothiazole) is used for GIN control in livestock.

As with other areas in which chemicals have been used for the control of microbes or arthropods, the use of anthelmintic drugs over many years has led to the development of drug resistance in multiple helminth species (Kaplan, 2004; Wolstenholme et al., 2004; Fairweather et al., 2020). The impact of this has been particularly severe in the livestock industries, with significant levels of resistance worldwide in GIN parasites of sheep, goats and cattle. Resistance to anthelmintics is also widespread in gastrointestinal parasites of horses (Matthews, 2014) and is increasingly reported for canines (Kopp et al., 2007; Bourguinat...
et al., 2015; Chelladurai et al., 2018; Kitchen et al., 2019). There have also been some suggestions of reduced efficacy of anthelminitics against human helminths (Osei-Atweneboana et al., 2011; Vercruysse et al., 2011; Grellen et al., 2016; Krucken et al., 2017). While there is emerging evidence to suggest that genetic change may be occurring at the population level in response to drug treatment in some human parasites (Doyle et al., 2017; Faust et al., 2019), conclusive evidence of definitive genetic changes, indicative of the emergence of resistance, is lacking.

Minimising the impact of drug resistance requires an ability to detect its presence. This allows for drug-use decisions to be made based on the knowledge of what drugs remain effective against the target helminth population. Once resistance has been detected to a particular drug or drug class, the use of alternative drugs ensures that parasitic infections can be effectively controlled and that further selection pressure is not applied by the drug for which resistance is emerging. However, despite this need for diagnostic tools to detect and monitor drug resistance, and many years of research into the molecular basis of anthelmintic resistance, no molecular-based tests are commercially-available for the diagnosis of resistance in field settings.

The development of molecular diagnostics for anthelmintic resistance has been one of the main themes behind the scientific meetings of the Consortium for Anthelmintic Resistance and Susceptibility (CARS). This group was established following discussions held at the World Association for the Advancement of Veterinary Parasitology (WAAVP) meeting in New Zealand in 2005. The group aims to promote research on the molecular mechanisms of drug resistance and drug action, and to view towards the development of molecular markers for resistance diagnosis, and to assist in the development of new anthelmintics drugs. The first meeting was held in Glasgow in 2006, with the group meeting on seven occasions since then. The most-recent meeting, in Madison, Wisconsin, in July 2019, discussed the challenges of adopting molecular diagnostics for anthelmintic resistance. The present paper reflects these discussions. The paper complements the recently-published review by Nixon et al. (2020) on the challenges of developing new anthelmintics that was also a major theme of the CARS 2019 meeting.

2. Current diagnostic tools for anthelmintic resistance, and their relative merits

Tests for anthelmintic resistance fall into three general categories:

1) **In vivo** tests measure the impact of drug treatment on the parasite population within the animal or human host. These tests generally rely on indirect measures of parasite burden before and after drug treatment in order to quantify the impact of the drug, and hence, determine if its effectiveness is reduced by drug resistance in the worm population. The most common measurement is the counting of eggs in faecal samples taken before and after drug treatment for the faecal egg count reduction test (FECRT) (Coles et al., 1992). This test is widely used across GIN parasites of livestock and is currently used for assessment of drug efficacy in terms of egg reduction rate (ERR) for human STH (WHO, 2013). Other indirect measurements of parasite burden before and after treatment include coproantigen levels for detecting drug resistance in *Fasciola hepatica* (Brockwell et al., 2013), circulating microfilaraemia after administration of macrocyclic lactone drugs to canines for control of *Dirofilaria immitis* (Geary et al., 2011), and microfilarial counts in human skin snips for detection of resistance to this same drug class in *Onchocerca volvulus* (Osei-Atweneboana et al., 2011). These indirect in vivo tests have become the industry standards.

2) **In vitro** tests measure the sensitivity of helminth eggs, larvae or occasionally adult worms, to drug exposure in laboratory-based assays. Such assays detect the phenotypic effects of drugs on various aspects of worm development, activity or viability (for example, egg hatch, larval development, or worm movement). For detection of drug resistance, they rely on resistance at a free-living larval stage being directly correlated with resistance shown by the parasitic stage in the host (generally the adult worm).

3) Molecular diagnostic tests are defined, for the purposes of this review, as being able to detect and/or analyse nucleic acid molecules (DNA or RNA), and hence, are able to directly measure genetic differences between susceptible and resistant populations. A molecular diagnostic test may aim to detect “causal” genetic differences within genes coding for: (i) drug receptors (i.e., variation that restricts the drug from physically interacting with the drug target), or (ii) various processes within the nematode that act to regulate the amount of drug that reaches the receptor (for example, genes involved in drug detoxification, drug efflux, or amphidial drug uptake) (Kotze et al., 2014). Alternatively, a molecular diagnostic test may aim to characterise sequence polymorphisms that are genetically “linked” to the causal variants within functionally relevant genes, and so act as genetic markers for resistance. Molecular tests aim to utilise DNA prepared from readily-accessible helminth life stages to define genetic changes that are directly related to drug resistance in the life stages present in the host. Molecular tests can also be used as a means to quantify parasitic species community composition (discussed further in section 5.1.). A range of different molecular testing platforms and assays are available, from whole genomes to single nucleotide variants, the choice of which is largely dependent on the scale and resolution required (see Box 1 and Table 1 of Doyle and Cotton (2019) for a comparison of approaches to assaying genome-wide diversity).

The various tests differ with respect to a number of important practical considerations:

1) **Speed:** FECRTs take >3 weeks, consisting of post-treatment sampling at 2 weeks after drug treatment, followed by an additional 1 week for larval culture in order to define species-specific resistance levels in mixed-species infections. *In vitro* assays require up to 1 week for the completion of larval development. Molecular tests can generally be performed within 2 days, and can be automated, allowing for examination of many samples in a short period of time. Molecular assays can provide information on the species composition of larval populations much more quickly than is possible with labour-intensive larval speciation by traditional microscopy techniques. When resistance-causing SNPs are known, such as in benzimidazole resistance, SNP analysis can also allow relatively high throughput, for example, by using pyrosequencing. Pyrosequencing is typically done in a 48-well plate format, and it is feasible to analyse up to 96 pooled samples per day. Furthermore, because the SNPs at codons 200 and 198 are very close in the genome, both SNPs can be analyzed in a single benzimidazole resistance assay for most species (Barrere et al., 2013; Diawara et al., 2013a; Prichard unpubl. data).

2) **Sensitivity:** the FECRT is very insensitive, and hence, is not suitable for detecting low levels of resistance (<25%) (Martin et al., 1989). Molecular tests, when the genetic changes are well characterized, can provide accurate measurements of resistance alleles even at low frequencies (for example, DNA sequencing can reliable detect variants at a frequency as low as 0.1%, dependent on the sequencing depth and error rate of the platform used), which is necessary to characterise the initial stages of resistance emergence in a population and at a time when alternative management options can be best applied (Aivaronen et al., 2020; Melville et al., 2020).

3) **Sampling:** the FECRT requires sampling of faeces from multiple animals before and after drug treatment, as well as administration of different drug treatments to separate groups of animals if multiple drugs are to be assessed. This can greatly increase the time and cost of anthelmintic resistance testing. In contrast, both *in vitro* phenotypic and molecular tests can be performed on single samples taken independently from drug treatments. One particular challenge of *in vitro* phenotyping tests is the need for very fresh live material that
Box 1
Properties of the “Ideal” Molecular Diagnostic Test.

- Detects resistance mutations in all target parasite species of relevance
- Detects resistance mutations for all drug classes that need to be considered
- Detects all mutations that contribute to the resistance phenotype for each drug
- Provides accurate quantification of resistance mutation frequencies
- Provides accurate prediction of drug sensitivity phenotype
- Detects resistance mutations present at very low frequencies, providing high sensitivity
- Highly cost effective with low cost per sample
- Rapid: either rapid pen-side test or laboratory test that takes less than 24 h
- High throughput and scalable so that it can be applied to large numbers of samples
- Simple to perform such that it does not require specialist training and can be performed in routine diagnostic laboratories, or “on-farm”
- Easily interpretable results, for both the diagnostic technician and the end-user

often has to be transported and stored under very specific conditions (for example, to prevent the hatching of nematode eggs prior to the test). This can lead to significant logistical challenges. On the other hand, molecular tests can generally be performed on parasite material stabilised with a chemical fixative (e.g. 96% ethanol; Ayana et al., 2019) or on a solid substrate (e.g. FTA cards; Doyle et al., 2019a), allowing greater flexibility of sampling, transport, and storage.

4) Use across multiple drug classes: the FECRT can inform on drug resistance across all drug classes using a similar experimental format. Some in vitro phenotypic assays are limited to single drug classes; e.g. egg hatch assays with benzimidazoles (von Samson-Himmelstjerna et al., 2009). Other in vitro tests have wider application across multiple drug classes; e.g. larval development assays with some livestock GIN species for benzimidazoles, macrocyclic lactones and imidazothiazoles (Lacey et al., 1995; Ruffell et al., 2018). However, there are a number of instances where in vitro tests are unable to detect resistance to macrocyclic lactone drugs; e.g. motility assays with livestock GIN (George et al., 2018) and migration and motility assays with D. immitis microfilariae (Maclean et al., 2017; Evans et al., 2015). Molecular tests rely on the use of known and distinct genetic markers for each of the different drug classes. Although we currently do not have candidate genetic markers for all drug classes, once these subsets of markers are established, there is potential for their use in a single assay format to detect genetic variants associated with resistance across the multiple classes.

5) Use across multiple worm species: FECRTs, accompanied by a larval culture or a molecular species identification step, are able to measure resistance to any drug class across all important GIN. Larval development assays are effective with some species (principally H. contortus), but are not effective for other species with some drugs (e.g., T. circumcincta and macrocyclic lactones; Lloyd, 1998). Molecular assays would need to be validated for each species, however, some commonalities across species may be expected, for example, mutations in the isotype 1 ß-tubulin gene have been reported to be associated with benzimidazole resistance across multiple GIN species of livestock, horses, and companion animals (Beech et al., 2011; Kitchen et al., 2019).

6) Specific knowledge requirements: the tests differ with respect to the required level of understanding of the specific changes that occur within a worm population as resistance develops. In vivo and in vitro tests are based on relatively simple observational measurements of parasite phenotypes, for example, egg numbers in faeces, egg hatch rate, and numbers of larvae able to develop to the infective stage. Molecular tests on the other hand require specific knowledge of the genetic changes that represent useful markers for diagnosis of resistance; to assay these genetic changes, knowledge of at least the gene sequence, and perhaps, whole genome sequence may be required. Initiatives such as the 50 Helminth Genomes Project (International Helminth Genomes Consortium, 2019) and databases such as WormBase ParaSite (Howe et al., 2017) provide significant advancement toward this goal. For some species, high quality genome datasets are available (Doyle et al., 2020), however, for many species these data are either not yet available or the data sufficiently poor such that the development of such assays can be confounded.

7) Cost: The costs for the different tests include labour costs involved in collecting samples in the field and their examination in the diagnostic lab, as well as cost of the equipment and consumables required for the laboratory component of the test. The FECRT is rather labour intensive, and thus expensive. For example, the cost associated with performing FECRTs (not including cost of labour on-farm) offered by a diagnostic lab in Australia is approximately $US600, which includes efficacy data for 7 different drugs or drug combinations (Tim Elliot, Invetus Pty. Ltd., personal communication). Larval development assays are somewhat cheaper than FECRTs, at an approximate cost $US450 for 3 drug classes tested against H. contortus (O’Brien 2015). Labour costs are less for molecular testing, and although they require the use of specific and often expensive laboratory equipment and expertise, molecular diagnosis is becoming routine in advanced diagnostic laboratories and most aspects of the technical requirements are common to a wide range of pathogens including bacterial, viral and fungal species. While no accurate costs are available for molecular anthelmintic resistance testing in the current absence of a commercial test, in Australia, the New South Wales Primary Industries offers realtime quantitative PCR-based diagnostic tests for various viral, bacterial or fungal pathogens at a cost of approximately $US60 per test (NSW DPI, 2019). Importantly, molecular diagnostic platforms and DNA sequencing technologies are developing at a rapid pace, and so molecular tests are likely to become even more affordable and improve in their flexibility even in the short term future.

3. Current status of anthelmintic resistance marker development for each of the major drug classes and helminth species

The degree to which the molecular mechanisms of resistance are understood, or for which resistance markers have been identified, varies considerably among the different drug classes and parasite species. Hence, the expected timeline of being able to transition a laboratory-based research tool into an assay for use as a commercial resistance diagnostic also varies between the different drug classes and worm species.

Many GIN species, particularly of livestock, have extremely high levels of sequence polymorphism which makes identifying causal
resistance mutations challenging (Gilleard and Redman, 2016; Salle et al., 2019). Nevertheless, significant progress has been made in identifying the genetic basis in resistance to several drug classes. Here, we briefly summarise the status for each of the major drug classes with a view to highlighting the degree to which there is an adequate level of knowledge to suggest that a molecular test for resistance is possible in the short-to-medium term:

1) Benzimidazoles: Resistance to broad spectrum benzimidazoles is the best understood of the various anthelmintic resistance mechanisms. There is a great deal of evidence that mutations in the isotype 1 β-tubulin gene (F200Y, E198A, E198L and F167Y) are the major determinants of resistance for many of the trichostrogyldin GIN species of ruminants. The order of prevalence overall is F200Y > F167Y > E198A and E198L, although the relative frequency and importance of each mutation can vary significantly between nematode species and geographical location (Kwa et al., 1994; Ghisi et al., 2007; von Samson Himmelstjerna et al., 2007; Redman et al., 2015; Avramenko et al., 2019). Most attempts to develop molecular tests for anthelmintic resistance have focused on these three positions in the isotype 1 β-tubulin gene, although there is some evidence that other loci and mechanisms may also be involved in benzimidazole resistance, for example, variation in the isotype-2 β-tubulin gene (Kwa et al., 1993) and increased levels of drug metabolism (Stuchlikova et al., 2018), in some resistant species.

2) Imidazothiazoles: We have a solid understanding of the target and mechanisms by which levamisole acts, and in turn, a number of candidate genetic markers for resistance second to that of the benzimidazole class. The levamisole-sensitive nicotinic acetylcholine receptor (nAChR) in C. elegans is well understood, with a pentameric structure composed of protein subunits encoded by five genes, Cel-unc-38, Cel-unc-63, Cel-unc-29, Cel-lev-1 and Cel-lev-8. Orthologues of several of these genes have been identified in H. contortus and other strongylid nematodes (Fleming et al., 1997; Culeto et al., 2004; Towers et al., 2005; Neveu et al., 2010); in some instances, reduced expression of these subunits, for example, Hco-unc-29 and Hco-unc-63, have been characterized in resistant relative to susceptible H. contortus isolates (Kopp et al., 2009; Sarai et al., 2013; Williamson et al., 2011). A screen of ~17,000 cDNA-amplified fragment length polymorphism (cDNA-AFLP) tags identified just 11 that were present in two levamisole resistant isolates but absent from two susceptible isolates (Fauvin et al., 2010). One of these cDNA-AFLP tags identified a truncated transcript of the Hco-acr-8 gene, which was suggested to act as a dominant negative allele in the resistant populations. The same truncated transcript was subsequently found in three additional resistant isolates, two from Australia and one from the US (Sarai et al., 2013; Williamson et al., 2011). An independent study revealed a 63 bp deletion leading to altered splicing of part of intron-2 in the Hco-acr-8 transcript in 12 additional levamisole resistant isolates originally derived from Zimbabwe, South Africa and the USA (Barrerre et al., 2014). Although the correlation between the presence of the truncated Hco-acr-8 transcript and the levamisole resistant phenotypes was not perfect across all the isolates examined in these studies, this work suggests that deletion mutations in the Hco-acr-8 gene are at least associated with levamisole resistance in H. contortus and warrants further validation studies to determine its suitability as a marker of levamisole resistance.

3) Macro cyclic lactones: Most of the work to date on resistance to macrocyclic lactones has been undertaken on H. contortus, where, until recently, progress on the identification of resistance loci has been hampered by a lack of good genomic resources. Consequently, many studies have focused on hypothesis-based selection of candidate genes and the investigation of genetic associations between genetic variation and/or differential expression of candidate genes in relatively small numbers of susceptible and resistant isolates. This has led to a wide range of candidate genes being implicated, including P-glycoproteins, glutamate-gated chloride (GluCl) channels, and gamma aminobutyric acid (GABA) channels. Although some encouraging results have been reported, evidence of genetic associations between these candidates and phenotypic resistance have been generally inconsistent across studies (Kotze et al., 2014). For example, a subset of SNPs within the dyf-7 gene common to multiple ivermectin-resistant isolates of H. contortus with sensory amploid head defects from five continents (Urdaneta-Marquez et al., 2014) were subsequently shown to be not predictive of resistance in other H. contortus isolates from Africa, Australia and Europe (Laing et al., 2016; Rezansoff et al., 2016; Elmahalawy et al., 2018).

The recent transition from candidate-based single (or few) gene approaches to unbiased genome-wide and genetic mapping approaches has begun to resolve some of the genetic uncertainty associated with candidate genes, and in turn, identify promising new markers linked to resistance. Genetic crosses between susceptible and resistant parental isolates of H. contortus, followed by serial backcrossing, drug-selection, and whole genome sequencing have identified a single genomic quantitative trait locus (QTL) localised on chromosome V that is associated with ivermectin resistance in two independent ivermectin-resistant isolates (MHco10 [CAVR] and MHco4 [WRS], originally derived from Australia and South Africa, respectively (Redman et al., 2012; Doyle et al., 2019b). A subsequent independent F2 mapping cross also identified the same locus in a multi-drug resistant strain derived from the southern US (MHC18 [UGA2004]) (Doyle, unpubl. data). These data are further supported by analyses of multiple ivermectin resistant H. contortus field populations in western Canada, whereby evidence of selection in the chromosome V region measured by ampiclon sequencing was observed (Rezansoff, 2018). Collectively, these data provide support for a single major ivermectin locus on H. contortus chromosome V that is of widespread importance. The ability to resolve the genetics of ivermectin resistance in H. contortus, including the identification of a single QTL, has been significantly advanced by the use of highly resolved genomic resources for H. contortus (Doyle et al., 2020), and has refocused efforts away from many previously proposed candidate genes. There are several hundred genes in the mapped Chromosome V region and finer mapping of the region is being undertaken to identify the likely single causal gene, or sufficiently tightly linked markers, for use in a molecular diagnostic test similar to that available for the isotype-1 β-tubulin markers for benzimidazole resistance.

A similar genetic cross followed by genome sequencing strategy was employed to map variation associated with multidrug resistance in a field derived strain of T. circumcincta (Choi et al., 2017). Among many genome-wide signals of differentiation between the susceptible and backcrossed populations, a number of candidate genes, and in particular a copy number variant of the drug efflux associated Tci-pgp-9, were linked to ivermectin resistance. This apparent multigenic signal was similarly observed in a genome-wide analysis of sub-optimal response to ivermectin by the filarial nematode Onchocerca volvulus, whereby multiple genes in a limited number of molecular pathways were proposed to be involved in neurotransmission, development, and stress responses were linked to regions of the genome undergoing selection in response to drug treatment (Doyle et al., 2017). These studies suggest the feasibility of developing diagnostic markers will be challenging in some contexts, and that panels of genetic markers may be needed to support diagnostics.

In contrast, the application of a whole genome approach across multiple field isolates has revealed a small number of SNPs that may be useful markers for resistance to macrocyclic lactones in the canine heartworm D. immitis (discussed in detail in section 5.4).

4) Amino aceto nitril e derivatives; laboratory-selected and field-derived monepantel-resistant isolates of H. contortus have been shown to possess numerous different mutations in the gene coding for the nACbR target of this drug (Hco-mpt-1) (Rufener et al., 2009; Bagnall...
et al., 2017; Niciura et al., 2019). These various mutations would be expected to result in the formation of truncated, and presumably non-functional, target receptors in the resistant worms. In terms of implications for design of a resistance diagnostic, causal mutations in a single gene could be seen as an advantage in terms of simplicity in requiring a focus on just a single locus. However, any test would need to account for many possible mutations across the length of this gene as more than ten different mutations have been reported to date from three isolates (Rufener et al., 2009; Bagnall et al., 2017), and it is likely that more will exist in other field-derived resistant isolates.

4. What practical information does a molecular test need to provide?

Parasite control is complex and there are an endless number of requirements one might demand of a molecular diagnostic test before it is applied in the field (see Box 1).

It is clear that if we require molecular diagnostic tests to fulfil all the criteria outlined in Box 1 then it is unlikely they will be deployed in the foreseeable future. However, given the limitations of current in vivo and in vitro resistance tests, molecular diagnostic tests have the potential to significantly improve drug resistance diagnostics even if they only provide incomplete information and fulfil only some of the criteria in Box 1. It is also important to bear in mind that the information that a molecular test needs to provide will vary considerably according to the nature of the application and drug-use environment in which the test is to be utilised. This means that we should not set the bar too high before attempting to implement molecular diagnostics in the field. Instead, we need to move towards an iterative approach in which molecular diagnostic tests used in research are piloted for more routine diagnostic use at a relatively early stage in their development in order to provide practical knowledge to inform further refinement and improvement. Such proof-of-concept studies should also help promote greater awareness of the value and practicality of diagnostic testing to support evidence-based parasite control.

There are situations where it may be sufficient to simply discriminate between parasite populations that are largely susceptible to a drug versus populations that have some level of resistance, with no need to accurately quantify the level of drug efficacy between 0 and 100%. In an ideal drug-use environment, identification of a worm population as being resistant, at whatever level, would allow a livestock producer to choose an alternative drug to which the helminths on the property are fully susceptible. This situation pertains, for example, in Western Canadian sheep where H. contortus is resistant to ivermectin and/or benzimidazoles on many, but not all farms, and where the newly-licensed drugs closantel and abamectin/derquantel are available and highly effective (Gilleard, unpubl. data). A diagnostic test that could be used to determine if ivermectin and benzimidazoles were still highly effective on a farm would allow their use, and hence, preserve the newer and more expensive drugs for when they are truly needed. In cattle, where resistance to some compounds among some helminth species is still relatively uncommon ( Cotter et al., 2015; Avramenko et al., 2020), the use of a molecular test to simply detect resistance may be valuable.

In addition, a molecular test that is able to discriminate between D. immitis that are susceptible or resistant to macrocyclic lactones (Ballesteros et al., 2018) would be of value (see section 5.4).

In some parts of the world, where widespread resistance to multiple drug classes occurs in small ruminants, the ability to quantify the level of resistance becomes more important. The ideal molecular test in these situations would accurately relate resistance allele frequency (or some other molecular measurement) to drug efficacy, either in terms of percentage efficacy (0–100%), or more likely in broad bands of low-medium-high-level resistance. This would allow drug-use decisions to be based firstly on whether resistance to a specific drug class existed on the property, and secondly on the degree to which the resistance had reduced the expected efficacy of the drug. In a situation where some resistance is expected towards all the major drug classes, a livestock producer may be able to choose a drug that retained a high level of expected efficacy over a drug with a very low expected efficacy. Such continued use of a drug to which some resistance already exists is not ideal as it will most-likely result in further selection pressure and increased levels of resistance to this drug, however, it will at least allow some control of parasites to be maintained in the short term. In countries where combination products are available, or settings where producers choose to administer two drugs simultaneously, knowledge on what drugs remain most effective (when some degree of resistance to all drugs is expected) will allow producers to choose the most effective drug combinations.

4.1. Molecular diagnostics to quantify species abundance as an adjunct to the FECRT

The existence of high levels of resistance to multiple drug classes (benzimidazoles, macrocyclic lactones, imidazothiazoles) in many small ruminant production systems means that for molecular tests to be useful in such environments they would need to detect resistance across multiple chemical classes. A test for a single drug class would have limited value as it would not provide any information as to what alternative drugs remain the most effective on a property. On the other hand, where resistance is more limited in scope, such that some drug classes are expected to be highly effective, the ability to use a molecular test for only a single drug class may still be of benefit. For example, with human STHs, where the focus at present is on the use of benzimidazoles, a test for just this drug class would be of benefit for detecting the emergence of resistance, informing on the need to use alternatives, and guide efforts to achieve elimination of human STHs relying on mass drug administration with just this single class of anthelmintic.

There is considerable potential for molecular tests in surveillance for anthelmintic resistance. Although it is critical that the emergence of resistance in different regions is detected at an early stage, surveillance is currently limited to a relatively small number of ad hoc research projects. Molecular tests are much more suited to large scale surveillance than efficacy-based or in vitro tests as the former can utilise robotics and high throughput sequencing technologies to increase workflow. In addition, the tools to implement more routine surveillance for resistance emergence are already available in some cases. A case also can be made that we are similarly placed in other cases such as such benzimidazole resistance in Ancylostoma caninum in dogs (Kitchen et al., 2019) and, as discussed above, levamisole resistance in H. contortus in small ruminants (Barrere et al., 2014). Further, molecular tests such as DNA sequencing generate information-rich data that can be used to monitor genetic change over time, for example, during mass drug administration, to ensure parasite populations respond predictably to drug pressure, or that if resistance does emerge, the mode of selection (for example, soft or hard genetic sweeps (Doyle and Cotton, 2019)) and, as discussed above, levamisole resistance in H. contortus in small ruminants (Barrere et al., 2014). Further, molecular tests such as DNA sequencing generate information-rich data that can be used to monitor genetic change over time, for example, during mass drug administration, to ensure parasite populations respond predictably to drug pressure, or that if resistance does emerge, the mode of selection (for example, soft or hard genetic sweeps (Doyle and Cotton, 2019)) and, as discussed above, levamisole resistance in H. contortus in small ruminants (Barrere et al., 2014). Further, molecular tests such as DNA sequencing generate information-rich data that can be used to monitor genetic change over time, for example, during mass drug administration, to ensure parasite populations respond predictably to drug pressure, or that if resistance does emerge, the mode of selection (for example, soft or hard genetic sweeps (Doyle and Cotton, 2019)) and, as discussed above, levamisole resistance in H. contortus in small ruminants (Barrere et al., 2014). Further, molecular tests such as DNA sequencing generate information-rich data that can be used to monitor genetic change over time, for example, during mass drug administration, to ensure parasite populations respond predictably to drug pressure, or that if resistance does emerge, the mode of selection (for example, soft or hard genetic sweeps (Doyle and Cotton, 2019)) and, as discussed above, levamisole resistance in H. contortus in small ruminants (Barrere et al., 2014). Further, molecular tests such as DNA sequencing generate information-rich data that can be used to monitor genetic change over time, for example, during mass drug administration, to ensure parasite populations respond predictably to drug pressure, or that if resistance does emerge, the mode of selection (for example, soft or hard genetic sweeps (Doyle and Cotton, 2019)) and, as discussed above, levamisole resistance in H. contortus in small ruminants (Barrere et al., 2014). Further, molecular tests such as DNA sequencing generate information-rich data that can be used to monitor genetic change over time, for example, during mass drug administration, to ensure parasite populations respond predictably to drug pressure, or that if resistance does emerge, the mode of selection (for example, soft or hard genetic sweeps (Doyle and Cotton, 2019)) and, as discussed above, levamisole resistance in H. contortus in small ruminants (Barrere et al., 2014). Further, molecular tests such as DNA sequencing generate information-rich data that can be used to monitor genetic change over time, for example, during mass drug administration, to ensure parasite populations respond predictably to drug pressure, or that if resistance does emerge, the mode of selection (for example, soft or hard genetic sweeps (Doyle and Cotton, 2019)) and, as discussed above, levamisole resistance in H. contortus in small ruminants (Barrere et al., 2014). Further, molecular tests such as DNA sequencing generate information-rich data that can be used to monitor genetic change over time, for example, during mass drug administration, to ensure parasite populations respond predictably to drug pressure, or that if resistance does emerge, the mode of selection (for example, soft or hard genetic sweeps (Doyle and Cotton, 2019)) and, as discussed above, levamisole resistance in H. contortus in small ruminants (Barrere et al., 2014).

5. What might be a good starting point for field use of molecular diagnostics?

It is critical that molecular diagnostic tests are not expected to be a panacea for all the current challenges around anthelmintic resistance diagnostics, or for a single test to be equally applicable in all situations (see Box 1). Instead, there is a need for a stepwise approach in which molecular diagnostics are introduced in a targeted way to provide specific value in particular situations. If this can be achieved then there will be an increasing number of proof-of-concept examples to allow a culture change in attitudes towards the value of molecular diagnostics in anthelmintic resistance management. A number of potential examples are discussed below. These are not intended to be prescriptive, nor exhaustive, but aim to illustrate how molecular diagnostic tests could provide value in specific situations.

5.1. Molecular diagnostics to quantify species abundance as an adjunct to the FECRT
Routine testing of anthelmintic drug efficacy is undertaken by pre- and post-treatment faecal egg counts as part of FECRTs. A major limiting factor to accuracy and interpretability of these tests is the lack of information on the species present pre- and post-treatment. This can be achieved, at least to the genus level by culture to L3 and morphological examination, but this is a time consuming and specialist task, and hence is costly and rarely performed. However, there are now several technologies such as LAMP assays (Rashwan et al., 2017a), multiplex quantitative PCR (Roebor et al., 2017; Höglund et al., 2013; McNally et al., 2013) or ITS-2 rDNA nematodei sequencing (Avramenko et al., 2015, 2017, 2018; Redman et al., 2019) which allow species quantitation on either larvae or eggs to be reliably performed. The application of these in a more routine diagnostic setting could provide significant value in resistance diagnosis. For example, if pre-treatment species diversity is reduced to one or two species post-treatment, this not only confirms that resistance is present, but also identifies the resistant parasite species, as illustrated recently by McIntyre et al. (2018). Such knowledge on the identity of species showing resistance in a mixed-species population may inform on alternative drug choice or management strategies. The converse is also true; if there is little or no change in species diversity post-treatment, this may suggest that resistance exists in all species detected, or alternatively, it may suggest that an apparent treatment failure is due to factors other than resistance, such as underdosing. Another example is in the use of the narrow spectrum drug closantel, which is specific for the blood-feeding H. contortus but has no activity against the other GIN species. Species quantification in this case can determine whether apparent treatment failure is due to the presence of drug resistant H. contortus or simply the presence of other non-target worm species.

2) Detection of benzimidazole resistance in ovine GIN species in specific regions

The molecular mechanism of resistance is best understood for benzimidazoles, and therefore, this provides a potential opportunity to apply molecular diagnostics tests of benzimidazole resistance both as proof-of-concept and in a commercial setting. In many countries and/or regions, benzimidazole resistance is at an advanced stage for multiple species of small ruminant trichostrongylid GINs (Kaplan and Vidyashankar, 2012), and consequently, a diagnostic test specifically for benzimidazole resistance is likely to be of little operational value or commercial interest. However, in some regions of the world, benzimidazole resistance is still at an early stage for several important ovine GIN species and therefore, a benzimidazole resistance-only diagnostic molecular test would be of significant practical and potentially commercial value. For example, control of the trichostrongylid nematode Nematothorax battus, which is a major cause of diarrhoea and mortality in young lambs in the UK, is primarily through the use of benzimidazoles due to their higher efficacy than the macrocyclic lactones (Abbott et al., 2012). Although benzimidazole resistance has been detected in N. battus (Mitchell et al., 2011; Morrison et al., 2014), a recent survey of UK farms found only 5 out of 170 farms tested contained the resistance-associated F200Y β-tubulin variant at a frequency greater than 20% (Melville et al., 2020). Importantly, resistance to the other drug classes has yet to be reported in this parasite species. In this situation, a molecular diagnostic test limited to just benzimidazole resistance would enable an evidence-based approach in deciding whether to use the first choice benzimidazole or to switch to a second choice drug, and therefore, maximise parasite control and avoid selection for higher levels of resistance alleles on specific farms.

Similarly, a diagnostic test specifically for benzimidazole resistance may be useful for sheep GIN in western Canada, where benzimidazole resistance is widespread for H. contortus but is still relatively rare for the other major species of concern such as T. circumcincta and T. colubriformis (Gilleard, unpub. data). In this case, a diagnostic test would enable this drug class to still be used effectively in many flocks, in conjunction with a second drug against H. contortus such as closantel, as and when required, and so preserve the use of other drug classes until needed. This would both minimize treatment costs and reduce the selection pressure being applied, and so slow the emergence of resistance to the newer anthelmintic drug classes.

3) Benzimidazole resistance in cattle nematodes

Cattle producers have relied primarily on macrocyclic lactones for GIN control for many years (Sutherland and Leathwick, 2011). However, macrocyclic lactone resistance is increasingly common in several bovine GIN species such as Cooperia oncophora, Cooperia punctata and H. placei in many countries, with a number of reports also describing its presence in the highly pathogenic nematode Ostertagia ostertagi (Gasbarre 2014; Geurden et al., 2015; Ramos et al., 2016). Because of this situation, cattle producers are now more interested in alternative drug choices such as benzimidazoles. Although, benzimidazole resistance is emerging in O. ostertagi in New Zealand and Australia, available evidence suggests it is still at an early stage for bovine GIN species in many regions (Sutherland and Bullen, 2015; Cotter et al., 2015; Waghorn et al., 2016; Avramenko et al., 2020). For example, in North America, benzimidazole resistance appears to be at a very early stage of emergence in all the major cattle nematodes (Chaudhry et al., 2014; Avramenko et al., 2020). A recent study of calves sourced from 38 different stocker herds from Arkansas and Oklahoma used deep amplification sequencing to detect the F200Y variant in a number of trichostrongylid nematode species (C. oncophora, C. punctata, O. ostertagi, H. placei, H. contortus, and T. axei). The F200Y variant was present in a small minority of herds and at very low frequency for all species (~5%) except for T. axei where this variant was found in 4 out of the 5 herds for which this parasite species was detected, and was at a frequency of 57.4% in one case. Further, the isolate 1 β-tubulin codon 167, 198 or 200 benzimidazole-resistance associated mutations were not found in O. ostertagi and C. oncophora populations in any of the 43 beef herds sampled across Canada using an allele frequency threshold of 0.1% (Avramenko et al., 2020). Consequently, in this situation, a specific molecular diagnostic test for benzimidazole resistance could provide value in a number of different ways. These include routine surveillance for the emergence of benzimidazole resistance as the use of this drug class increases, quarantine screening of purchased cattle to ensure that benzimidazole-resistant parasites are not brought onto a farm, and to allow the selection of an alternative drug, such as levamisole, if benzimidazole resistance alleles are already present. Additionally, the molecular tests could be used to detect the presence of benzimidazole-resistant H. contortus in cattle (possibly resulting from the flow of this species between small ruminants and cattle).

4) Macrocyclic lactone resistance in D. immitis

As described above, resistance to macrocyclic lactones in small and large ruminants is widespread in some parts of the world, possibly reducing the usefulness of resistance diagnostics (Rose et al., 2015; Geurden et al., 2015; Ramos et al., 2016). However, the situation is different with respect to the canine heartworm, D. immitis, where resistance to this drug class is considered to be an emerging issue (Bourginat et al., 2011; Geary et al., 2011; Pulaski et al., 2014; Moorhead et al., 2017). Resistance to heartworm prevents (all of which, so far, are macrocyclic lactones) is recognized as a serious issue because heartworm infection can be lethal. Bourginat et al. (2015) investigated genetic markers for resistance using a whole-genome approach across four susceptible D. immitis populations and four ‘loss of efficacy’ populations, and identified 186 potential SNP markers for resistance, with a subset of 42 SNPs being most promising for resistance diagnosis. The number of useful SNPs was narrowed further by Bourginat et al. (2017) through analysis of a further ten field isolates. More recently, Ballesteros et al. (2018) provided some
clinical validation for these markers by measuring their association with the ability to reduce microfilaraemia by treatment of infected dogs with moxidectin. They described a 2 SNP model as a potential marker for resistance, and hence provided a basis for further clinical validation of this as a useful resistance diagnostic.

Given the pathogenicity of heartworm infection in dogs, alongside the evidence to date pointing to a useful SNP-based molecular marker for resistance, it would seem appropriate that this model be seen as one in which development of a molecular assay should be prioritised. Such a diagnostic test would face fewer obstacles than those outlined in section 4, above with respect to livestock as the heartworm test would need to apply to only one parasite species and one drug class, with the microfilariae being very easy to sample from infected dogs.

5) Benzimidazole resistance and SNPs in β-tubulin genes in human STH

The World Health Organization (WHO), national Ministries of Health, and a number of Non-Governmental Organizations (NGOs) and Foundations have implemented mass drug administration (MDA) programs to reduce the morbidity of STH in humans around the world. Typically, this involves treatment of school-aged children (SAC) and pre-SAC once a year, or more often, with either albendazole or mebendazole at standard dose rates of 400 mg or 500 mg (single dose per person), respectively, as these anthelmintics are moderately effective (Moser et al., 2017) and are donated (ABZ by GlaxoSmithKline; MB by Johnson & Johnson). In 2017, approximately 750 million people were treated with albendazole and mebendazole donations. These anthelmintics reduce morbidity and transmission, but people become reinfected and treatment is needed in successive years. The large amount of these drugs being used to treat human STH, and other parasitic infections, and the need to use these same drugs repeatedly is likely to impose selection pressure for resistance in STH (Vercryusse et al., 2011).

The principal STHs being targeted by these MDA programmes are Ascaris lumbricoides, the hookworms (Necator americanus, Ancylostoma duodenale and A. ceylonicum), and Trichuris trichiura. There are a number of studies that suggest a decline in the efficacy of benzimidazole drugs (e.g., Moser et al., 2017; Soukhathammavong et al., 2012; Humphries et al., 2017; Krucken et al., 2017; Vlaminck et al., 2019). So far, there have only been a limited number of studies that have investigated SNPs in β-tubulin in STH species, and these molecular investigations have been on small numbers of samples from Kenya, Haiti, Panama (Diawara et al., 2013a, 2013b), and Tanzania (Albonico et al., 2004; Diawara et al., 2013b; Schwenkenbecher et al., 2007) using either pyrosequencing, Sanger sequencing and realtime PCR. More recently, methods have also been established to detect SNPs in STH β-tubulin using LAMP assays (Rashwan et al., 2016, 2017b); while LAMP assays are highly sensitive for detecting the presence of mutations and do not require expensive sequencing equipment, the throughput of samples is limited due to a lack of automation.

These various studies have detected SNPs, most commonly at codon 200 in T. trichiura and N. americanus and less commonly at codon 167 or 198, in these species. The only significant association between treatment response and mutant SNP frequency was at codons 200 and 198 in T. trichiura (Diawara et al., 2013a). A SNP in codon 167 in A. lumbricoides was observed commonly but there was no correlation with drug efficacy, and in fact, efficacy was uniformly high against this species. It was concluded that this SNP was not informative for predicting response to treatment with benzimidazoles in A. lumbricoides (Diawara et al., 2013a). However, as mentioned above, sample sizes were small and further studies, in which egg count reduction is adequately determined, on larger sample sizes, are needed to establish whether these SNPs in the particular β-tubulin genes analyzed, can be predictive of anthelmintic resistance in human STHs. One of the limitations with linking β-tubulin genes in human STH is that, unlike nematode infections in animals, experimental infections from parasites that survive treatment cannot be established, for ethical reasons, so that isolates that are truly refractory to treatment can be genetically analyzed.

While the β-tubulin genes that have been analyzed so far in human STH seem most similar to the isotype 1 gene used for detection of benzimidazole resistance in animal Trichostrongylid nematodes and the ben-1 gene in C. elegans (all Clade V nematodes), T. trichiura (Clade II) and A. lumbricoides (Clade III) are phylogenetically quite different from the veterinary nematodes where benzimidazole resistance has been most studied. High throughput sequencing of populations of human STHs that are susceptible or refractory to drug treatment is needed to determine which β-tubulin gene(s) cause resistance, or whether resistance may be caused by other genes.

A degree of uncertainty still exists around the use of molecular tests to detect resistance to benzimidazole drugs in human STH, and the degree of ‘readiness’ to implement molecular testing is therefore much less than for the other examples highlighted here. However, despite this, we suggest that the development of benzimidazole resistance tests for human STH warrants urgent attention because of the opportunity to establish sensitive molecular diagnostics before resistance impacts on the usefulness of these drugs.

6. Conclusions

It is clear that there is a need for sensitive diagnostic tools to detect anthelmintic resistance as it emerges in field settings across livestock, companion animals and humans. This will allow for drug-use decisions (most importantly, the use of alternative drugs and combinations) to be made in order to ensure that the impact of any emerging resistance is minimised. The FECRT is the only diagnostic currently used in the field, however, it suffers from a lack of sensitivity, high costs, and labour-intensive sampling procedures, and hence is not used widely. In vitro phenotypic tests remain as laboratory tools only and currently lack utility across different drug classes and parasite species. Although molecular tests are currently used as research tools, they offer significant advantages in terms of sensitivity, cost, sampling procedures and speed that make them ideal for use in diagnosing resistance in field settings.

We argue that there is sufficient evidence of the effectiveness of molecular tests in research settings for them to begin to be developed as field diagnostics. The precise way in which a test would be deployed will differ between regions due to differences in the composition and complexity of the parasite communities, the extent to which resistance has developed, and the number of alternative drugs available. An early step would be their use in species identification and/or quantification as an adjunct to the FECRT, with further steps able to be taken in the short term to introduce molecular tests into specific parasite/drug class interactions in small and large ruminant industries. While such prototype diagnostic tests will not satisfy many of the criteria required of an ‘ideal’ molecular diagnostic, this needs to be balanced against the lack of sensitivity, cost and sampling issues that affect the usefulness of the FECRT. Other early steps in the introduction of molecular tests into parasite management could be their use with canine heartworm and human STH. The development and use of prototype molecular tests in managing specific parasite/drug class resistances in the field will provide valuable information on practical aspects of test performance. In this way, the use of such prototypes will provide direction for the further development of improved molecular anthelmintic resistance diagnostics.

Declaration of competing interest

The authors declare no conflict of interest with regard to the present manuscript.

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