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Anthelmintic resistance and novel control options in equine gastrointestinal nematodes

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Review

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Abstract

Control of equine nematodes has relied on benzimidazoles (BZs), tetrahydropyrimidines and macrocyclic lactones. The intensive use of anthelmintics has led to the development of anthelmintic resistance (AR) in equine cyathostomins and *Parascaris equorum*. Field studies indicate that BZ and pyrantel resistance is widespread in cyathostomins and there are also increasing reports of resistance to macrocyclic lactones in cyathostomins and *P. equorum*. The unavailability of reliable laboratory-based techniques for detecting resistance further augments the problem of nematode control in horses. The only reliable test used in horses is the fecal egg count reduction test; therefore, more focus should be given to develop and validate improved methodologies for diagnosing AR at an early stage, as well as determining the mechanisms involved in resistance development. Therefore, equine industry and researchers should devise and implement new strategies for equine worm control, such as the use of bioactive pastures or novel feed additives, and control should increasingly incorporate alternative and evidence-based parasite control strategies to limit the development of AR. This review describes the history and prevalence of AR in equine nematodes, along with recent advances in developing resistance diagnostic tests and worm control strategies in horses, as well as giving some perspective on recent research into novel control strategies.

Introduction

Gastrointestinal nematodes (GINs) are one of the major health problems of ruminants and horses throughout the world. It has been documented that GINs are responsible for significant economic losses in livestock farming systems. Strongyles (mainly cyathastomins and *Strongylus vulgaris*) and *Parascaris equorum* are the major parasites capable of causing clinical diseases in naturally infected horses (Reinemeyer and Nielsen, 2009). Effective control of parasites is essential to achieve optimum equine health, productivity and efficient breeding herd performance. Apart from clinical disease, GIN infection in many livestock species negatively affects the utilization of nutrients that can result in protein deficiency and increased amino acid demands (Coop and Holmes, 1996; Fox *et al.*, 2002). As observed in other animals, parasitism in horses causes poor body condition, distended abdomen, retarded growth, weakness and poor digestion and malabsorption especially in young horses and immunocompromised foals (Owen and Slocombe, 1985).

Control of GINs in an extensive grazing system is one of the most significant challenges in veterinary medicine (Craig, 2006). Since the discovery of anthelmintics, parasite control has relied heavily on frequent use of anthelmintics often applied round the year. The intensive use of anthelmintics has resulted in the development of resistance in benzimidazoles (BZ)s as well as macrocyclic lactones (MLs) and tetrahydropyrimidines (Peregrine *et al.*, 2014; Scott *et al.*, 2015). Anthelmintic resistance (AR) is generally defined as 'when a previously effective drug is unable to kill the parasite population while exposed to therapeutic doses (Jabbar *et al.*, 2006) or loss of sensitivity to a drug in parasitic population that was sensitive to the same drug which is thought to be genetically transmitted (Kohler, 2001)'. AR has been documented in parasites of different animal species including cattle (Lifschitz *et al.*, 2010; Geurden *et al.*, 2015), sheep and goats (Coles, 2005; Domke *et al.*, 2012) and horses (Geurden *et al.*, 2013; Wolf *et al.*, 2014; Saes *et al.*, 2016).

The emerging significance of AR demands an urgent need for the development of reliable, reproducible and standard methods/assays for its detection (Coles *et al.*, 2006). Accurate and timely detection of AR and the knowledge of the mechanism(s) involved in its development might aid to adopt the measures to slow the development of resistance. In addition, this will also help in developing new anthelmintic drugs as the control of GINs will remain at least partly dependent on anthelmintics in the foreseeable future, although adoption of complementary approaches such as bioactive diets may also play an increasing role (Taylor *et al.*, 2002). Our knowledge of the mechanisms associated with the development of AR in ruminants is much more advanced than those of horses. In addition, a number of *in vitro* AR

detection tests have been successfully used in sheep nematodes, whereas, very few of these tests are reported in horse nematodes and the results are not satisfactory, indicating that these tests require further refinement. The purpose of this article is to comprehensively review the history and current status of AR in equine GINs, to discuss the scientific aspects of development and detection of resistance and control strategies that are recommended to counter the development of AR. This article also provides further insights into key future research areas that may be considered by the equine industry and parasitology researchers for achieving sustainable parasite control in equines.

Historical hierarchy of AR in horses

Despite significant advances in the discovery of anthelmintic agents, AR has arisen as a major economic issue in animal production throughout the world, currently being most severe in parasitic nematodes of small ruminants (Kaplan *et al.*, 2004*b*). For example, in Australia, the prevalence and magnitude of resistance to all major classes of anthelmintics threatens the profitability of sheep farming (Besier and Love, 2003). This problem was initially highlighted in the mid-20th century when resistance to phenothiazine was reported in small strongyles in horses (Gibson, 1960). Thiabendazole was approved for use in horses in 1962 as a broad-spectrum anthelmintic with low toxicity; however, resistance to thiabendazole was reported in cyathostomins within few years of its discovery (Drudge *et al.*, 1964). Pyrantel (an imidazothiazole-tetrahydropyrimidine) pamoate resistance was suspected when treatment failure to equine cyathostome population occurred in 1996 (Chapman *et al.*, 1996). Subsequently, suspected ivermectin (IVM)-resistant populations of *P. equorum* were reported in 2002 (Boersema *et al.*, 2002). Currently, MLs treatment failure in cyathostomin nematodes has been observed and it is suggested that resistance to MLs is emerging primarily detected as reduced egg reappearance period (ERP) following treatment (Geurden *et al.*, 2014; Kooyman *et al.*, 2016). These patterns of resistance development highlight the need of either adopting strategies to slow down the development of resistance or hasten the discovery of new anthelmintics. Therefore, control of horse nematodes should rely on a combination of anthelmintic therapy and other management strategies to minimize the environmental contamination and reducing the exposure of animals to infection.

Prevalence of AR in equine nematodes

There is a great deal of literature available on the prevalence of AR in livestock, horses and companion animal parasitic nematodes throughout the world. Resistance to all three broad-spectrum anthelmintics, including BZs, imidothiazoles-tetrahydropyrimidines and MLs has been reported in ruminants and horses (Kaplan, 2002; Traversa *et al.*, 2009*a*, 2009*b*; Peregrine *et al.*, 2014). Generally, a single dose of anthelmintic drug should eliminate more than 95% of the parasitic nematodes and efficacy below this, certainly <90% is taken as evidence of drug resistance. However, in equine medicine, different available anthelmintic classes show different efficacy levels against cyathostomins; therefore, an arithmetic mean of <95% in fecal egg count reduction (FECR) for MLs and a cut-of value <90% for BZ and tetrahydropyrimidine anthelmintic classes is recognized as resistance to these drugs (Relf *et al.*, 2014; Stratford *et al.*, 2014).

According to previous studies in horse nematodes, BZ-resistant cyathostomins are prevalent on most of the farms in majority of the developed countries (Pook *et al.*, 2002; Kaplan *et al.*, 2004*a*; Wirtherle *et al.*, 2004; Meier and Hertzberg, 2005; Lind *et al.*, 2007; Stratford *et al.*, 2014). Pyrantel-resistant cyathostomins have also been reported in a large number of horse farms (Kaplan *et al.*, 2004*a*; Traversa *et al.*, 2009*a*, 2009*b*). Recently, Lester *et al.* (2013) reported resistance to pyrantel in South of England with approximately 87% FECR on two horse farms. In contrast, MLs demonstrate higher efficacies on almost all the farms examined (Lind *et al.*, 2007; Lester *et al.*, 2013; Stratford *et al.*, 2014). However, there have been few reports describing the various incidences of reduced efficacy of IVM in cyathostomin nematodes (Edward and Hoffmann, 2008; Lyons *et al.*, 2008*b*; Traversa *et al.*, 2009*b*). It has been suggested that reduced ERP following IVM and moxidectin treatments is an early indication of the emerging resistance to this class of anthelmintics (Geurden *et al.*, 2014; Kooyman *et al.*, 2016). The shortened ERPs following IVM and moxidectin treatments have been associated with emerging ML resistance in the fourth stage larvae (Lyons *et al.*, 2009, 2010). Similarly, there are increasing number of reports describing the reduced efficacy of MLs treatment against *P. equorum* (Stoneham and Coles, 2006; Schougaard and Nielsen, 2007; von Samson-Himmelstjerna *et al.*, 2007; Lind and Christensson, 2009). Some selected studies reporting overt AR and shortened eggs reappearance periods in small strongyles (cyathostomins) and ascarid species (*P. equorum*) in horses are summarized in Table 1.

Development of AR

Drug resistance in parasites generally results from the selection of a sub-population of parasites that can withstand the toxic effects of drugs which were previously lethal to them. The parasite population select specific genes under drug pressure that allow them to survive. These alleles are responsible for the development of resistance as a result of mutation. When the worms are treated with drugs for which resistant alleles are present, it provides them a chance to survive, leading to increased frequency of resistant worm population in the environment. The rate of resistance development is defined by the frequency of alleles coding for resistance when the worms are exposed to the drug (Gilleard and Beech, 2007; Ihler, 2010). AR is a multi-component phenomenon that involves more than single genetic change and quite often non-receptor-based mechanisms also contribute to resistance (Beech *et al.*, 2011). The quantity of anthelmintic drug used and frequency of drug exposure also impact the development of AR. Frequent use of anthelmintics exposes more generations of nematode parasites to the drug especially when pre-patent periods are shorter as compared with the parasites with longer pre-patent periods. This phenomenon is more likely associated with the development of AR (Ihler, 2010).

It has been previously suggested that many horses are being treated unnecessarily, which may expose the parasites to selection pressure (Matthews, 2014; Nielsen *et al.*, 2014*a*; Peregrine *et al.*, 2014). Selection pressure can be reduced if treatment of selected animals with only a higher FEC is practised (Nielsen *et al.*, 2014*b*). Refugia is a term used for the parasite population not exposed to the drug and it has been suggested that refugia-based parasite control approaches are important for the effective management of AR (Cornelius *et al.*, 2016), as it lowers the selection pressure on the whole population. The reversal or delaying the development of resistance to anthelmintics has been shown by maintaining the worm population in refugia in nematode parasites of small ruminants (Sissay *et al.*, 2006), and using combination therapies in preference to an annual rotation (Bartram *et al.*, 2012; Leathwick *et al.*, 2015). Therefore, treatment of selected animals and adoption of alternate parasite control strategies would help to slow down the development of drug resistance. In horse nematodes, this concept of restoring drug efficacy has been reported in limited number of studies.

Table 1. Selected studies reporting anthelmintic resistance and/or reduced egg reappearance period in equine gastrointestinal nematodes (cyathostomins and *Parascaris equorum*) from different parts of the world over the last two decades

Species/genus of nematode	Anthelmintic(s)	Technique(s) used	Country	Reference(s)
Cyathostomins	FBZ	FECRT	USA	Bellaw *et al.* (2018)
GINs	BZ	FECRT	Nigeria	Mayaki *et al.* (2018)
Cyathostomins	FBZ	FECRT	Ethiopia	Seyoum *et al.* (2017)
Parascaris spp	FBZ, IVM, ABA	FECRT	Saudi Arabia	Alanazi *et al.* (2017)
Cyathostomins	IVM, MOX	FECRT, shortened ERP	UK	Daniels and Proudman (2016)
Cylicocyclus spp.	IVM, MOX	FECRT, shortened ERP	The Netherlands	Kooyman *et al.* (2016)
Cyathostomins	BZ	FECRT and PCR	India	Kumar *et al.* (2016)
Cyathostomins	FBZ, Piperazine	FECRT	Brazil	Saes *et al.* (2016)
P. equorum	IVM	FECRT	Australia	Beasley *et al.* (2015)
Cyathostomins, *P. equorum*	FBZ, PYR, IVM	FECRT	UK	Relf *et al.* (2014)
Cyathostomins	FBZ	FECRT	Scotland	Stratford *et al.* (2014)
Cyathostomins	FBZ, PYR, IVM	FECRT	Brazil	Canever *et al.* (2013)
Cyathostomins *P. equorum*	FBZ IVM	FECRT	France	Geurden *et al.* (2013)
Cyathostomins	FBZ, PYR	FECRT	Southern England	Lester *et al.* (2013)
P. equorum	IVM	FECRT	France	Laugier *et al.* (2012)
Cyathostomins	FBZ, PYR, IVM, MOX	FECRT	France	Traversa *et al.* (2012)
Cyathostomins	BZ	PCR (SNP at codon 167)	Ukraine	Blackhall *et al.* (2011)
Cyathostomins *P. equorum*	PYR IVM	FECRT	Finland	Näreaho *et al.* (2011)
Cyathostomins	FBZ	FECRT	USA	Rossano *et al.* (2010)
Cyathostomins	MOX (in L4)	Critical tests	USA	Lyons *et al.* (2010)
P. equorum	IVM	FECRT	Sweden	Lind and Christensson (2009)
Cyathostomins	FBZ, PYR, IVM	FECRT	Italy	Milillo *et al.* (2009)
Cyathostomins	FBZ, PYR, IVM	FECRT	Germany, Italy, UK	Traversa *et al.* (2009*a*)
P. equorum	IVM	FECRT	Italy	Veronesi *et al.* (2009)
Cyathostomins	IVM	FECRT	Australia	Edward and Hoffmann, (2008)
Cyathostomins	BZ	FECRT	Ukraine	Kuzmina and Kharchenko, (2008)
P. equorum	IVM	FECRT	Sweden	Lindgren *et al.* (2008)
Cylicostephanus calicatus, *Coronocyclus labiatus*	FBZ	FECRT and reverse line blotting	Italy	Lyons *et al.* (2008*a*)
Cyathostomins	IVM	FECRT	UK	Dudeney *et al.* (2008)
Cyathostomins, *P. equorum*	PYR IVM	FECRT	USA	Craig *et al.* (2007)
P. equorum	IVM, MOX	FECRT	Canada	Slocombe *et al.* (2007)
Cyathostomins, *P. equorum*	IVM	Reduced ERP for cyathostomins	Germany	von Samson-Himmelstjerna *et al.* (2007)
P. equorum	IVM	FECRT	Denmark	Schougaard and Nielsen (2007)
Cyathostomins	FBZ, PYR	FECRT	Sweden	Lind *et al.* (2007)
Cyathostomins	PYR	FECRT	Canada	Slocombe and Gannes (2006)
P. equorum	IVM	Necropsy findings showed *P. equorum* as cause of foal death	UK	Stoneham and Coles (2006)
Cyathostomins	BZ	EHA	Switzerland	Meier and Hertzberg (2005)
Cyathostomins	TBZ	FECRT, EHA	Germany	Wirtherle *et al.* (2004)
P. equorum	IVM	FECRT	Canada	Hearn and Peregrine (2003)
Cyathostomins		FECRT	Australia	Pook *et al.* (2002)

(*Continued*)

Table 1. (*Continued.*)

Species/genus of nematode	Anthelmintic(s)	Technique(s) used	Country	Reference(s)
	Oxibendazole, morantel			
Cyathostomins	FBZ, oxibendazole, PYR	FECRT	USA	Tarigo-Martinie *et al.* (2001)
Cyathostomins	TBZ	FECRT, EHA	Slovakia	Varady *et al.* (2000)
Cyathostomins	FBZ, PYR	FECRT, EHA	Denmark	Craven *et al.* (1999)
Cyathostomins	Oxibendazole, PYR	FECRT	USA	Chapman *et al.* (1996)
Cyathostomins	TBZ	FECRT, EHA, LDA	Norway	Ihler and Bjorn (1996)

FECRT, fecal egg count reduction test; FBZ, febendazole; PYR, pyrantel; TBZ, thiabendazole; MOX, moxidectin; BZ, benzimidazole; EHA, egg hatch assay; *P.*, *Parascaris*; LDA, larval development assay; ERP, eggs reappearance period.

Although the concept of refugia has been recommended as valuable to equine parasite control (Matthews, 2008; Kaplan and Nielsen, 2010), there is no published evidence demonstrating the effectiveness of this strategy in controlling equine parasites. However, it remains a useful working hypothesis to consider refugia as an important tool in delaying the development of resistance in equine nematodes.

Detection of AR

Apart from developing the new anthelmintics, early diagnosis of resistance is also very important to maintain the efficacy of available drugs by adopting suitable measures, for example, reduced treatment intensity and promoting the refugia, as in the future, control of helminths will remain dependent on anthelmintic chemotherapy. A range of *in vitro* and *in vivo* tests are used for measuring reduced anthelmintic efficacy in nematode populations (Coles *et al.*, 2006). These tests were generally developed for detecting AR in ruminant nematodes; however, some of these tests have been modified to use for detecting the emerging AR in equine nematodes. The benefits and drawbacks of the tests that have been utilized in equine parasitology are discussed below.

FECR test

FECR test (FECRT) is the only reliable and suitable test in detecting the reduced efficacy of currently available anthelmintics in horses. In this *in vivo* test, FECR is determined based on FEC in the same horse before and after the administration of anthelmintic, or comparison of the reduced FEC in treated with an untreated group of horses (Coles *et al.*, 1992). This test is only reliable if resistance level is higher than 25% of the total worm population (Martin *et al.*, 1989). According to the World Association for the Advancement of Veterinary Parasitology (WAAVP), a single dose of anthelmintic drug should eliminate more than 95% of the parasitic nematodes and efficacy below this, certainly <90% is taken as evidence of drug resistance. This threshold cut-off limit does not seem applicable for determining anthelmintic efficacy in horses, hence, this limit has not been applied in some other studies (Ihler, 1995). The currently available anthelmintic classes show different efficacy levels against cyathostomins (Saes *et al.*, 2016). Therefore, it was suggested to review these cut-off values particularly for some anthelmintic classes (Coles *et al.*, 2006). For example, pyrantel often reduced 95–100% FECR in susceptible worm populations (Valdez *et al.*, 1995), similarly BZ-treatment usually shows more than 95% reduction in FEC (DiPietro and Todd, 1987). Whereas MLs have been reported to reduce fecal egg count by 99% or higher (Lind *et al.*, 2007; von Samson-Himmelstjerna *et al.*, 2007). Some of the recent studies have described more precise methods for measuring anthelmintic sensitivity in horses (Lester *et al.*, 2013; Relf *et al.*, 2014; Stratford *et al.*, 2014). These authors have used an arithmetic mean FECR of >95% for MLs and a cut-of value >90% for BZ and tetrahydropyrimidine anthelmintic classes.

Similarly, there are no well-defined principles for calculating the ERP for strongyles. ERP is generally calculated in two ways: firstly, the period between anthelmintic administration to the week of first positive fecal egg count (Dudeney *et al.*, 2008; Lyons *et al.*, 2008*b*); secondly, it comprises the time period when the group mean FEC surpasses 10–20% of the group mean FEC at day 0 (von Samson-Himmelstjerna *et al.*, 2007; Larsen *et al.*, 2011). The later approach provides more conventional estimation of egg reappearance in relation to the level and spread of the egg count data sampled before treatment, and hence provides a precise measure of a population's susceptibility to anthelmintics (reviewed by Matthews, 2014). The first positive egg count approach seems imprecise, because the results then depend heavily on the pre-treatment FEC and the sensitivity of detection. So, the second approach is reasonably better, as it uses relative measures that remain useful no matter the pre-treatment FEC or the method used for FEC. For example, for ML drugs, since we expect 99.9% FECR, then a return to a group mean of 10% of pre-treatment levels seems a good definition for ERP. For other non-ML drugs, 10% can also be used, but 20% might be a better choice, since these are not nearly as effective as ML and efficacy >99% is rarely achieved. In these cases, if FECR at 14 days is only around 90%, then the relevance of ERP is questionable, as egg re-appearance cannot occur if the eggs do not disappear in the first place. In addition, waiting for the first horse to shed eggs may be biased by pre-treatment egg count levels or individual animal variability. Individual horses may be extremely higher egg shedders and may continue to shed eggs even after treatment with fully effective ML. A reduced ERP represents an early indication of changing patterns of population's susceptibility to anthelmintics, providing a warning for the possible emergence of resistance particularly to the long-term effects of MLs in horses; therefore, further research is required to measure and standardize the ERP parameters so that analysis can be made between studies.

As stated above, FECRT, currently used as 'gold standard' test is reliable only when >25% of the nematode worms in a given population are resistant (Martin *et al.*, 1989). Thus, this test may likely misdiagnose the relatively low proportion of genotypically resistant individuals in a population; therefore, *in vitro* tests or molecular techniques are urgently required for measuring AR in horses.

In vitro *tests*

Various *in vitro* tests including the egg hatch assay (EHA), larval development assay (LDA), larval migration inhibition assay (LMIA), larval motility assay, larval feeding inhibition assay and larval paralysis test have been described to detect AR in ruminant nematodes (Coles *et al*., 2006). In horses, few *in vitro* tests have been reported for detecting the relative drug sensitivity of cyathostomin nematodes in addition to FECRT. These assays include the EHA, LDA and LMIA which determine the relative sensitivity of free-living stages (eggs and larvae) to a series of drug concentrations. The protocols for these tests have been discussed previously to measure the relative sensitivity of cyathostomins to BZ, pyrantel and MLs (Ihler and Bjorn, 1996; Craven *et al*., 1999; van Doorn *et al*., 2010) but still there are discrepancies on reproducibility and reliability of these tests.

Egg hatch assay

This assay is used to measure inhibition of hatching of nematode eggs by an anthelmintic agent. The assay is not suitable for anthelmintics, which cannot penetrate the eggs, for example, IVM. It was first reported by Le Jambre (1976) and later on modified by Coles *et al*. (1992). This assay has some limitations, for example, the sensitivity of eggs to thiabendazole decreases with age; therefore, eggs should be used soon after collection (usually within 3 h) or stored under anaerobic conditions. BZ-sensitivity also decreases as embryonation progresses, so unembryonated eggs are a prerequisite for this assay (Hunt and Taylor, 1989). The EHA is capable of detecting resistance when at least 25% of the worms carry resistance genotype as has been showed previously by experimentally infecting the animals with mixtures of nematode populations with a known level of susceptibility (Martin *et al*., 1989). In horses, it is generally recommended that horses with a minimum individual egg count of 150 eggs per gram (EPG) should be included in the study (von Samson-Himmelstjerna *et al*., 2002). This test can be applied to detect BZ-resistance in cyathostomins (Coles *et al*., 2006). Previous investigations have reported a positive correlation between FECRT and EHA (Craven *et al*., 1999; Varady *et al*., 2000), but this correlation is not very strong and further research is required to validate the use of EHA in small strongyles. Standardization of fecal culture conditions for equine nematodes may further improve the suitability of this assay to detecting AR.

Larval development assay

The effects of anthelmintic drugs on the growth of parasites provide a chance to develop techniques useful for detection of resistance. In the LDA, the eggs or first-stage larvae are exposed to a range of anthelmintic concentrations incorporated into agar wells in 96-well plates containing growth medium. Methods based upon inhibition of larval development are more laborious and time consuming than for the EHA but are useful to detect resistance to all the major anthelmintic classes including MLs (Jabbar *et al*., 2006). The LDA is more sensitive than the FECRT as it identifies resistance when it is present in a worm population at levels down to 10% (Dobson *et al*., 1996). The suitability of the LDA for detection of resistance to pyrantel in livestock and horse nematodes has also been established (Ihler and Bjorn, 1996; Kotze *et al*., 1999). Currently, this test is considered as reliable, inexpensive and suitable for use in the field investigations of AR in ruminants but still not in horses. The test can also utilize first-stage larvae; therefore, there is no prerequisite for undeveloped eggs or fresh fecal samples (Coles *et al*., 1988). In equine nematodes, higher levels of variability, poor reproducibility and narrow resistance-to-susceptible ratios along with lack of significant correlation with FECRT have been reported (Pook *et al*., 2002; Tandon and Kaplan, 2004; Lind *et al*., 2005). Therefore, this test is not a reliable alternative to FECRT in horses, thus needs further improvement. There are certain limitations to using LDA with equine nematodes including lack of established cut-off values for susceptible and resistant populations and interpretation problems related to multi-species infections, so, different species of cyathostomins may show different native drug susceptibility and may require slightly different conditions for optimal development *in vitro*. In addition, the developing larvae may not show the phenotypic resistance in the LDA at equivalent levels to that of adult worms. In the future, the test can be improved by developing rapid species identification tools, *in vitro* propagating different cyathostomin species with known susceptibility profiles for use as reference strains and standardising optimal *in vitro* conditions for cyathostomin development. Given the difficulty in dealing with heterogeneous mixture of numerous species of cyathostomins, especially, lack of valid morphologic means to distinguish eggs of these species, this assay may not be as useful as it is with sheep nematodes.

Larval migration inhibition assay

The LMIA was developed as a modification of the previously reported motility assay (Gill *et al*., 1991) to detect the sheep nematodes resistant to IVM (Kotze *et al*., 2006). Infective stage larvae (L3) are exposed to various dilutions of IVM for 48 h and then allowed to migrate through an agar/filter mesh system fitted over a receiver plate, for the next 24 h. The assay has also been standardized for detecting IVM resistance in cattle nematodes (Demeler *et al*., 2010). There is limited information available on the suitability of this assay for use in cyathostomin nematodes. The LMIA has been evaluated for identifying the cyathostomin larvae suspected of being resistant to IVM and did not evaluate the diagnostic properties of the assay (van Doorn *et al*., 2010). The authors concluded that LMIA may be used to study resistant cyathostomin populations. McArthur *et al*. (2015) has reported the ability of LMIA in discriminating the IVM sensitivity in cyathostomin populations. The EC_{50} values for L3 larvae recovered from animals that showed <95% reduction in FEC were significantly higher than the EC_{50} values for L3 population from animals with >95% reduction in egg counts. In addition, recently, Beasley *et al*. (2017) has also showed the ability of LMIA define the sensitivity of cyathostomin larvae to ML drugs. However, the authors suggested that the use of LMIA on known ML-resistant and -susceptible populations is required for further validation of its usefulness for diagnosis of AR.

In general, interpretation of all the *in vitro* tests is more complicated because of the cyathostomin species diversity present in field conditions and species can only be differentiated using molecular analysis. Furthermore, in identifying AR in field populations, it would be difficult to confirm that the different sensitivity patterns are due to resistance or the presence of different cyathostomin species, which show different susceptibility to IVM. These issues add further complications that must be considered when using *in vitro* bioassays to identify drug-resistant populations of cyathostomin nematodes. This could be tackled by coupling the FECRT data either with identifying species composition of the larval cultures obtained pre-and post-treatment (Kooyman *et al*., 2016) or molecular identification of cyathostome species using reverse line blotting (RLB) hybridization (Traversa *et al*., 2007). For example, Traversa *et al*. (2009*a*) identified eight cyathostomin species in pre-treatment fecal samples using RLB method and showed that BZ resistance was present in *Coronocyclus labiatus* and *C. goldi* species. However, lack of target sequences for many species or even that GenBank® entries are unreliable regarding the cyathostomin species may make it difficult to identify cyathostomin species. In addition, proteome-based

species identification of pathogens has already revolutionized diagnostic microbiology. Recently, Mayer-Scholl *et al.* (2016) have recently applied proteome-based matrix-assisted laser desorption/ionization time-of-flight mass spectrometry (MALDI-TOF MS) for rapid species identification of *Trichinella* spp. This was achieved by adopting a simple formic acid/acetonitrile extraction from pooled larvae and compilation of a reference database. Such an approach could also be utilized for cyathostomin species identification, as it has been revealed by the preliminary data for cyathostomin which showed distinct patterns for adult individuals of different species (Bredtmann *et al.*, 2017). However, this demands compilation of a reference database of master-spectra libraries which can only be generated with validated and correctly identified material.

Molecular techniques

Different molecular techniques have been developed for the detection of specific mutations that are associated with AR in trichostrongyloid nematodes, which include restriction enzyme digestion, direct sequencing, pyro-sequencing and diagnostic PCR. These techniques have been used to reveal a pattern of substitutions associated with BZ resistance, and the presence of specific single nucleotide polymorphisms (SNPs) in β-tubulin gene at codons 167, 198 and 200 have been reported in different trichostrongyloid species (Kwa *et al.*, 1994; Prichard, 2001; Kotze *et al.*, 2012). In cyathostomin populations, similar SNPs at codons 167 and 200 within the β-tubulin isotype-1 have been associated with BZ-resistance (Hodgkinson *et al.*, 2008; Lake *et al.*, 2009; Blackhall *et al.*, 2011); however, a polymorphism at codon 167 appears to be more common than the codon 200 polymorphism in equine cyathostomins. Currently, a few phenotypically well-defined cyathostomin populations have been studied for the mechanism of resistance at the molecular levels; thus, further research is required to assess the relative significance of these and other possible SNPs associated with BZ-resistance (von Samson-Himmelstjerna, 2012). Reliable and precise quantification of resistance-associated SNPs from samples representing different worm numbers is generally a pre-requisite for developing a reliable molecular test for routine diagnosis of resistance (von Samson-Himmelstjerna, 2006). In the case of cyathostomins, existence of more than 50 morphologically discrete species contributes considerable complications to a molecular approach for detecting AR (Lichtenfels *et al.*, 2008). In addition, association of non-specific mechanisms of AR including modified drug transporters [P-glycoprotein (P-gp)] (Raza *et al.*, 2016*a*), and altered drug metabolism (Matoušková *et al.*, 2016) that act irrespective of the drug class may further impede the development of molecular techniques. There has been very limited information available reporting the association of P-gps with AR in horse nematodes, Drogemuller *et al.* (2004) described the existence of two P-gp genes in cyathostomins and, recently, Peachey *et al.* (2017) reported that P-gps play a role in resistance to IVM in cyathostomins. The authors also described that the *P-gp-9* was transcribed at a significantly higher level in IVM-resistant larvae as compared with sensitive larvae. Janssen *et al.* (2013) studied the involvement of *Pgp-11* in the level of IVM susceptibility in *P. equorum* and observed an increased *pgp-11* mRNA expression in one putatively resistant population. This suggests that P-gps may play, at least a partial role in the observed AR in horse nematodes. Such non-specific mechanisms along with the widespread nature of IVM and BZ-resistance in horse cyathostomin populations should be considered in designing a molecular test detecting AR in cyathostomins. Furthermore, molecular markers for resistance to other anthelmintic classes in equine nematodes are still poorly understood; therefore, it has been suggested that currently FECRT may be the best available test for assessing the AR in horse nematodes (von Samson-Himmelstjerna, 2012). However, FECRT lacks sensitivity and is able to detect resistance only when more than 25% of the worms in a population are resistant, the chances of detecting the resistant worms are less when the genotypically resistant worms are low in number. Therefore, highly sensitive molecular tests are urgently required to identify AR at as early a stage as possible in horse nematodes.

Genetics and functional genomics have been playing a vital role in discovering the possible molecular mechanisms of insecticide resistance, and the availability of annotated genomes of many parasitic nematodes such as *Ascaris suum* and *H. contortus* has the potential to accelerate drug discovery in these species In contrast, there is still lack of information about equine nematodes; for example, mitochondrial genome/transcriptome sequences have been published only for *Cylicostephanus goldi* (Cwiklinski *et al.*, 2013), *P. univalense* (Jabbar *et al.*, 2014), *Triodontophorus brevicauda* (Duan *et al.*, 2015), *Strongylus equinus* (Xu *et al.*, 2015), *Oxyuris equi* (Zhang *et al.*, 2015) and *P. equorum* (Gao *et al.*, 2018). However, there is still no complete genome sequence available for any of the equine nematode species, this should be a priority area for the future research on AR in equine nematodes. The major constraints to this lack of information on genome sequences for equine nematodes are the lack of funding as well as the greater diversity in the species present. Mitochondrial genome sequence information would be helpful in differentiating the species as mitochondrial genome has been widely used as a genetic marker in the identification and differentiation of closely related species. These genome sequences once fully available along with their transcriptomic data would provide major insights into the biology of parasitic nematodes, mechanisms involved in resistance and discovering specific AR markers; therefore, the equine industry may benefit by funding the genome studies.

AR management

Rotational deworming

Researchers have suggested the possible alternative use of different anthelmintics which can be classified as slow rotation and fast rotation. Rotational deworming is still controversial and there is discrepancy between the types of rotational strategies in horses. Although limited studies are available reporting the presence of multiple resistance in equines, the idea of increased multiple resistance as a result of frequent rotational use of anthelmintics is based on studies in sheep and goats (Dash *et al.*, 1988). A recent survey study showed that most of the horse owners tend to rely heavily on the IVM in parasite control programmes round the year, with the majority preferring to follow the same plan in subsequent years (Nielsen *et al.*, 2018). The intensive use of IVM would increase the population of IVM-resistant nematodes, as has been observed for sheep parasites (Cezar *et al.*, 2010). Therefore, slow rotation of different classes of anthelmintics may be suggested (Hearn and Peregrine, 2003). Fast rotation is more commonly used in horses than slow rotation, in which anthelmintic groups are rotated at intervals of three to six times a year (reviewed by Brady and Nichols, 2009). Fast rotation between anthelmintic classes minimizes the parasite exposure to a specific class. The only definitive study that supports this theory demonstrated that fenbendazole can be used again in a herd of horses infected with resistant fenbendazole worm population following rotations of various anthelmintic classes (Brady *et al.*, 2008). In contrast, Uhlinger and Johnstone (1984) reported a lack of reversion to a susceptible state in parasite populations showing resistance to BZ despite of a 24–38 months withdrawal period.

In contrast, rotation of anthelmintics without monitoring anthelmintic efficacy by FECRT may lead to unchecked propagation of resistant worms, as resistant worms can dominate the population if drug does not kill 100% of the worms (especially in case of non-ML anthelmintics) (reviewed by Swiderski and French, 2008). However, there is little experimental evidence available because such rotational experiments are difficult to carry out and are very prolonged; the majority of the work has been performed with models, and is thus predictive. Ideally, annual rotation should give the slowest rate of accumulation of resistance genes. Therefore, it seems a reasonable strategy to adopt in practice to treating horses with MLs in first year followed by treating with a different drug next year.

Combination therapy

In parasite control programmes, intensive and repetitive use of a single class of anthelmintic are generally the well-known causes of selection for drug resistance (Kaplan, 2002; Kaplan and Nielsen, 2010). As discussed above, there is widespread resistance to BZ and pyrantel in cyathostomin populations and less commonly in *P. equorum* throughout the world, which makes most of the horse owners more reliant on using MLs. Emerging reports of reduced efficacy of MLs in equine nematodes has further compounded the problem (Traversa *et al.*, 2012; Relf *et al.*, 2014). It has been suggested that combinations of anthelmintics, especially the drugs that target the same or a similar spectrum of parasite species, may play a potential role in parasite control programmes (Scott *et al.*, 2015). Combination therapies allow the effective control of nematodes along with slowing down the development of AR (Bartram *et al.*, 2012; Kaplan *et al.*, 2014), and it may be quickly accepted for controlling the equine parasites due to higher efficacies. The different possible interactions following co-administration of two or more drugs include indifference, antagonistic, synergistic and additive/potentiative actions (Jia *et al.*, 2009). In case of anthelmintics, majority of evidence suggest that routinely used anthelmintic drugs show additive potentiative effect when co-administered (Entrocasso *et al.*, 2008; Bartram *et al.*, 2012). This additive/potentiative effect results in a higher efficacy than would be obtained by either drug using as single entity, and can be determined by the following formula described by Bartram *et al.* (2012);

$$\%\text{Efficacy}A+B = 1-[(1-\%\text{efficacy}A) \times (1-\%\text{efficacy}B)],$$

where the efficacy is expressed as proportion of the worms killed or reduction in FEC following the administration of either anthelmintic or a combination of *A* and *B*.

In horses, there is limited information available on the use of combination therapy, anthelmintic combination therapy is less frequently adapted in regions other than Australia and New Zealand, where several anthelmintic combinations are commercially available for use in horses and ruminants (Geary *et al.*, 2012). In the USA, experimental use of BZ anthelmintics combined with piperazine and other non-BZ anthelmintics has proved to be effective against BZ-resistant cyathostomins (Uhlinger and Johnstone, 1985). Kaplan *et al.* (2014) have recently reported that co-administration of oxibendazole and pyrantel shows a significantly higher efficacy in controlling cyathostomins in horses. The study showed that the reduction in FEC was significantly greater in horses given combination of both drugs (96.35%) compared with horses given either drug alone (90.03% with oxibendazole and 81.10% with pyrantel). The authors further suggested that anthelmintic combinations can considerably improve the effects of a given anthelmintic and there is clear indication that combination therapy substantially enhances the effectiveness of parasite control programmes by limiting the developing rate of AR. However, drug combinations may still lead to the development of cross-resistance to more than one anthelmintic, and may also result in selection for general mechanisms of resistance common to different drug classes, for example, drug transport proteins (P-gps and multidrug resistance proteins), as has been observed *in vitro* for *H. contortus* (Raza *et al.*, 2016*b*). Therefore, the anthelmintic combination therapy should be given thoughtful attention for controlling equine nematodes in future.

Selective therapy

Since AR has been established worldwide, a new pharmacological drug class has not been introduced for the equine industry since the introduction of IVM in the early 1980s and it remains uncertain when new drugs with different modes of action will be available for use in horses. Over the past two decades, veterinary parasitologists have recommended to adopt a reduced intensity of anthelmintic treatment to retard the development of AR. The recommended strategy of 'selective therapy' (targeted treatment) has been successfully applied for the control of trichostrongyle infection in small ruminants (Kaplan *et al.*, 2004*b*). Selective strategy is based on screening of the animals with a suitable parasite-related measure and then selection of the animals for anthelmintic treatment that exceed a predetermined threshold value.

In horses, the criterion of the targeted therapy is FECs from all horses in a given herd, treating only the horses with a higher FEC than the predetermined cut-off value (Nielsen *et al.*, 2014*a*). Questionnaire-based survey studies in various countries showed that a large proportion of horse owners do not implement this recommendation (Matthee *et al.*, 2002*a*; Relf *et al.*, 2012). However, Danish legislation of anthelmintics as prescription-only medicine disallowed random prophylactic treatments and appear to have strong effects on selective therapy (Nielsen *et al.*, 2006). The parasite populations are very unevenly distributed over a herd of hosts, and in horses, this pattern is quite obvious with strongyle FECs, where it has been shown that some horses are shedding the large majority of strongyle eggs within the population (Lester *et al.*, 2018). Based on this phenomenon, equine parasitologists have devised the 20/80 rule for horse strongyle egg counts which means in a given herd, approximately 20% of the horses shed about 80% of the total number of eggs within the population (Kaplan and Nielsen, 2010; Relf *et al.*, 2013). Therefore, this raises a possibility that targeted treatment of the higher egg shedder horses and leaving the lower shedders untreated may result in satisfactory reduction in overall FECs despite using significantly fewer treatments. It has been suggested that in adult horses, treatment of horses with 200 EPG of feces using an anthelmintic drug with 99% efficacy will lead to an overall egg count reduction of 95% on herd level (Kaplan and Nielsen, 2010). However, this predetermined cut-off value would vary with geography, season, breed and age of the horses; therefore, cut-off values should be predetermined for each herd accordingly.

Selective therapy may reduce the treatment intensity in addition to leaving a part of parasite population unexposed. The reduced intensity of anthelmintic drugs and maintenance of refugia decrease the selection pressure on parasite population which may slow the development of AR (Sissay *et al.*, 2006). Although no such evidence is observed for equine parasites, findings of the sheep parasite studies may help to implement this strategy in equine parasites to counter the development of resistance. It has been previously documented that overall cyathostomin egg shedding can be controlled by treating half of the adult horse population. In addition, economical calculations suggested that the selective approach was cost-effective, when compared with

treating all horses a fixed number of times in a year (Duncan and Love, 1991). However, it remains unknown whether such an approach would also provide effective control over other important parasites such as ascarids, large strongyles and tapeworms (Nielsen *et al.*, 2014*a*). A significant association has been found between prevalence of *S. vulgaris* and selective therapy which was particularly observed in parasite population of foals and young horses (Nielsen *et al.*, 2012). *Strongylus vulgaris* is more pathogenic compared with cyathostomins; therefore, control of this parasite should also be considered while implementing selective therapy as this may lead to potential health risks in untreated horses. In addition, processing large numbers of FECs is another drawback of the selective therapy. It may be even difficult to collect numerous fecal samples on large horse farms; in addition, it does not seem cost-effective when a single use of anthelmintic is cheaper than the fecal analysis (Nielsen, 2012). Therefore, further work is required to evaluate the potential health risks accompanied with selective therapy, and to assess the usefulness of this technique in delaying the development of AR in equine GINs.

Pasture management strategies

Traditionally, free-living developmental stages of equine parasites were the major focus of parasite control programmes due to lack of safe and efficacious anthelmintic drugs, and pasture management was the major tool for modulating parasite burden. Later on, availability of broad-spectrum anthelmintics such as IVM precluded the importance of pasture management and free-living stages. However, emerging AR stresses the need to revisit the pasture management strategies including non-chemical-based approaches for controlling parasites in ruminants and equines. Fecal analysis should be performed routinely to monitor the status of parasites in the herd. Additionally, adapting good management strategies such as rotational grazing within and between species, avoidance of overstocking, avoid feeding on the ground, regular removal of feces and strict quarantine measures before introduction of new horses to the pastures are recommended (Brady and Nichols, 2009). Pasture hygiene has been recognized as one of the most effective methods to control horse parasites (Herd, 1986; Matthee *et al.*, 2002*b*). Ideally, feces should be removed from the pasture regularly but practically this seems laborious, time consuming and unacceptable by most of the owners. In addition, multispecies grazing also results in reducing the parasite burden, but there is limited information available in equine parasitology and most of the guidelines are acquired from ruminant studies (Nielsen, 2012). These practices should be employed in addition to the currently available therapies aiming to reduce the frequency of anthelmintic treatment for controlling equine parasites.

Alternative measures for sustainable parasite control

Future control efforts for equine helminths may require a focus on discovering natural parasiticide drugs from plant origin, or other dietary additives such as probiotics. This has been a quite popular area of research in ruminants, but the use of plant (leaves, seed and/or other parts) extracts has gained little attention in equine parasitology research. There are limited studies available reporting the (mainly *in vitro*) efficacy of plant-based parasiticide agents in equine GINs. For example, Rakhshandehroo *et al.* (2017) tested the anthelmintic activity of different plant extracts on *P. equorum* larval viability (inhibition of whip-like larval movement). The findings showed that all concentrations (50, 75, 100 and 125 mg mL^{-1}) of *A. dracunculus* (tarragon) and *M. pulegium* (squaw mint/pudding grass) extracts were lethal against larvae while only higher concentrations of *Z. multiflora* (100 and 125 mg mL^{-1}) showed toxic effects on larval motility. On the other hand, extracts from *E. camadulensis* (red river gum tree) and *A. sativum* (garlic) showed very little effects on larval viability. Similarly, Procyanidin A2, a bioactive compound from an Australian plant *Alectryon oleifolius* showed significant anthelmintic efficacy by completely inhibiting development of cyathostomin egg to third stage larvae at concentrations as low as 50 $\mu g\ mL^{-1}$ and having an IC_{50} value of 12.6 $\mu g\ mL^{-1}$ (Payne *et al.*, 2013; Payne *et al.*, 2018). Other novel anthelmintic candidates, such as Cry5B protein derived from *Bacillus thuringiensis*, have also recently been shown to have direct anthelmintic activity against cyathostomes (Hu *et al.*, 2018). Peachey *et al.* (2015) reported that hydro-alcoholic extracts of plants from Ethiopian (*Acacia nilotica*, *Cucumis prophetarum* and *Rumex abyssinicus*) and the UK [*Allium sativum* (garlic), *Chenopodium album* and *Zingiber officinale* (ginger)] showed significant anthelmintic activity in EHA and larval migration inhibition test in equine strongyle nematodes. The EC-50 values ranged from 0.18 to 2.3 mg mL^{-1}, and the authors suggested that these plants have the potential as anthelmintic forages or feed supplements in equines. In addition, methanol extracts of *Diospyros anisandra* (bark and leaves) and *Petiveria alliacea* (stems and leaves) also showed potential anthelmintic effects by inhibiting the egg hatching in cyathostomins at much lower concentrations [>90% egg hatch inhibition at and above 37.5 $\mu g\ mL^{-1}$ for *D. anisandra* (both bark and leaves) and at 75 $\mu g\ mL^{-1}$ for *P alliacea* (both stems and leaves)] (Flota-Burgos *et al.*, 2017). The effects of *D. anisandra* extracts were largely due to its ovicidal activity, whereas in the *P. alliacea* extracts, it was due to L1 larval hatch failure. These studies were conducted *in vitro*, and the effects of these plants remain to be confirmed through *in vivo* studies. Recently, Collas *at el.* (2017) have investigated the efficacy of a short-term consumption of tannin-rich sainfoin (*Onobrychis viciifolia*) through *in vitro* and *in vivo* experiments in naturally infected horses. The *in vivo* experiments showed that a tannin-rich diet with 70% DM sainfoin pellets resulted in a lower rate of strongyle larval development. Similarly, addition of sainfoin pellets (29%) to feces reduced the strongyle egg development into infective larvae by 82%, suggesting that such bioactive forages may have the ability to disrupt the infection dynamics of strongyle nematodes. Moreover, other strategies may be applied to improve equine health in the face of drug-resistant nematodes. For example, given that helminth infection in horses has been shown to significantly disrupt the commensal gut microbiota (Clark *et al.*, 2018; Peachey *et al.*, 2018), probiotic dietary additives that aim to restore microbiome homoeostasis may play a role in alleviating the negative effects of infection, as has been proposed for a variety of pathogens in different animal production systems (Markowiak and Śliżewska, 2018).

In vivo, controlled infection studies are inherently difficult to perform in horses due to expenses, ethical and logistical issues; therefore, a focus of the equine parasitology research community should be to develop effective models for *in vivo* testing of novel anthelmintic agents. In addition to measurement of fecal egg counts in naturally infected animals treated with novel plant extracts or grazed on bioactive forages, adoption of model laboratory systems such as rabbits to mimic the horse gastrointestinal environment may be a worthwhile alternative.

Furthermore, strategies for the biological control of parasites have been given considerable attention, but no such technology is available at commercial levels for use in most parts of the world. For example, the predacious fungus *Duddingtonia flagrans* has potential antiparasitic activity, and is able to survive passage through the herbivore digestive tract. After oral administration, *D. falgrans* has potential effects on growth and survival of strongyle larvae in the pasture environment (Larsen, 2000). The fungus would be more valuable in controlling resistant parasite

populations by reducing the resistant larvae in pasture environment.

Conclusions and future directions

The widespread resistance to BZs and pyrantel in equine cyathostomins along with the emerging significance of ML resistance in *P. equorum*, suggests that our ability to control equine parasites with anthelmintics is being significantly compromised which emphasises the need to revisit the control strategies. In addition, many researchers find it difficult to define AR because there is no set standard for equine parasiticide drugs. Furthermore, no reliable tests other than the FECRT are available for diagnosing AR in equine parasites. There is limited information available on the usefulness of *in vitro* assays for detecting AR in cyathostomins. In addition, presence of multi-species populations of cyathostomin nematodes further complicates the interpretation of *in vitro* assays. This makes the detection of AR difficult since it may have an important impact on resistance ratio, as proportions of different species may affect the drug sensitivity patterns of the whole population (Matthews *et al.*, 2012). Some of the major reasons for lack of knowledge about AR in equine nematodes are few research groups researching equine nematodes globally as well as limited research funds as compared with ruminants which take the privilege of being the food animals and are considered important for food security. Therefore, in future equine nematode research, following points may be considered for reducing the AR and designing sustainable parasite control programme:

(1) Generating large-scale datasets about epidemiological patterns of AR and its impact on equine health from different geographical locations worldwide.
(2) Establishing a governing body to set anthelmintic standards and guidelines for standardizing FECRTs with cyathostomins and *P. equorum*, so the findings can be compared across the regions.
(3) Development and implementation of sensitive diagnostic tools capable of detecting resistance at an early stage, which is the pre-requisite of parasite control programmes. High-throughput molecular detection assays should be a future goal since these techniques could detect genotypic resistance beforehand.
(4) The equine industry may benefit by funding the research to investigate the resistance and discovering newer anthelmintics or dietary additives that restore a healthy gut in the face of helminth infection, especially from natural resources.
(5) One of the key priorities should be the development of genomic datasets and their accompanying transcriptomic data for cyathostomins and *P. equorum* which is essential to provide major insights into the biology of these nematodes.

In summary, a combination of sustainable approaches including maintenance of susceptible equine parasite populations and adoption of regular parasite surveillance by horse owners and the equine veterinarians as well as FEC-directed use of anthelmintics may be considered to prolong the efficacy of currently available anthelmintics until new drugs are available for treating horse parasites.

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