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Invited Review

Anthelmintic resistance in equine nematodes

Jacqueline B. Matthews *

Moredun Research Institute, Pentlands Science Park, Edinburgh, Midlothian, EH26 0PZ, UK

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ABSTRACT

Anthelmintics have been applied indiscriminately to control horse nematodes for over 40 years. Three broad-spectrum anthelmintic classes are currently registered for nematode control in horses: benzimidazoles (fenbendazole, oxibendazole), tetrahydropyrimidines (pyrantel) and macrocyclic lactones (ivermectin, moxidectin). Generally, control strategies have focused on nematode egg suppression regimens that involve the frequent application of anthelmintics to all horses at intervals based on strongyle egg reappearance periods after treatment. The widespread use of such programmes has substantially reduced clinical disease, especially that associated with large strongyle species; however, high treatment frequency has led to considerable selection pressure for anthelmintic resistance, particularly in cyathostomin species. Field studies published over the last decade indicate that benzimidazole resistance is widespread globally in cyathostomins and there are also many reports of resistance to pyrantel in these worms. Cyathostomin resistance to macrocyclic lactone compounds is emerging, principally measured as a reduction in strongyle egg reappearance time observed after treatment. Ivermectin resistance is a further concern in the small intestinal nematode, *Parascaris equorum*, an important pathogen of foals. These issues indicate that horse nematodes must now be controlled using methods less dependent on anthelmintic use and more reliant on management practices designed to reduce the force of infection in the environment. Such strategies include improved grazing management integrated with targeted anthelmintic administration involving faecal egg count (FEC)-directed treatments. The latter require that the supporting diagnostic tests available are robust and practically applicable. Recent research has focused on maximising the value of FEC analysis in horses and on optimizing protocols for anthelmintic efficacy testing. Other studies have sought to develop diagnostics that will help define levels of pre-patent infection. This review describes recent advances in each of these areas of research.

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* Tel.: +44 (0)131 445 5111.
E-mail address: jacqui.matthews@moredun.ac.uk

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1. The issue of horse nematodes

Horses worldwide are exposed to an array of gastrointestinal nematodes. Animals that graze contaminated pasture, and which are not treated with effective anthelmintics, can accumulate large numbers of worms. The most prevalent of these are members of the small strongyle group, the cyathostomins (Ogbourne, 1976; Bucknell et al., 1995; Gawor, 1995; Kuz'mina, 2012; Relf et al., 2013). When the total burden of cyathostomins is high, they can seriously compromise the health of affected individuals (Mair, 1994; Matthews, 2008; Matthews, 2008, 2014). Substantial burdens (i.e. several million) of immature cyathostomins can encyst in the large intestinal wall and it is thought that these stages can persist for years (Murphy and Love, 1997). These stages, in particular early third stage larvae (EL3), are relatively insensitive to most anthelmintics available (Monahan et al., 1996). In temperate areas of the northern hemisphere, cyathostomin larvae encyst primarily during the autumn and winter and can comprise up to 90% of the total burden (Dowdall et al., 2002). When these larvae re-emerge in large numbers from the gut wall, a fatal colitis, larval cyathostominosis, can develop (Giles et al., 1985).

Several other nematode species infect horses and other equids, but the prevalence of these species is usually lower than that of cyathostomins (Relf et al., 2013). The most important non-cyathostomin species affecting horses older than one year is *Strongylus vulgaris.* This nematode can cause non-strangulating intestinal infarction leading to severe colic and was the major parasitic threat to equine health before the advent of broad-spectrum anthelmintics, in particular, the macrocyclic lactones (Reinemeyer and Nielsen, 2009). In younger horses (i.e. those less than 2 years-old), the small intestinal ascarid, *Parascaris equorum*, can be a substantial risk, producing both respiratory and intestinal signs of disease (Cribb et al., 2006). The lungworm, *Dictyocaulus arnfieldi* (MacKay and Urquhart, 1979), and the liver fluke, *Fasciola hepatica* (Owen, 1977), can undergo life cycle development in horses and lead to clinical signs; these are a particular hazard in horses that co-graze with, or graze pastures recently populated by, more permissive hosts such as donkeys and ruminants, respectively. There are few published studies describing the factors that affect the prevalence and abundance of the various parasitic nematode species of horses. A recent publication identified that a lack of rotational grazing practices (between age groups or host species) was associated with a higher prevalence of cyathostomin egg excretion on Thoroughbred stud farms (Relf et al., 2013). In the same study, higher levels of strongyle egg shedding (i.e. >200 eggs per gram) in faeces were observed to be significantly associated with a number of factors, with a recent history of treatment with fenbendazole identified as the most significant factor. The latter observation may be linked to the fact that there is a high prevalence of benzimidazole resistance in cyathostomin populations (see below).

Since the 1960s, nematode control has followed interval treatment regimens involving the frequent administration of anthelmintic products at intervals based on strongyle egg reappearance periods (ERP). These periods were defined for each chemical class of compound at the time of licensing (Parry et al., 1993; Kaplan and Nielsen, 2010). Such interval treatment programmes have been successful in substantially reducing the prevalence of strongyle infections and the incidence of large strongyle-associated disease. On the flip side, these programmes have made a substantial contribution to the development of anthelmintic resistance, particularly in cyathostomin species (Kaplan, 2004). Should anthelmintic resistance levels worsen, there will be limited scope for control, as no new classes of compound appear to be under development for use in horses in the short to medium term. Based on comparative studies on sheep nematodes (Jackson and Coop, 2000), reversion to anthelmintic sensitivity is unlikely to occur once populations are measured as anthelmintic resistant by conventional means such as the faecal egg count reduction test (FECRT). For these reasons, more sustainable methods of nematode control are now required, these being based on a requirement to treat animals predisposed to larger burdens to prevent clinical disease, balanced with a need to reduce treatment frequency to preserve anthelmintic efficacy. In the last decade, regulations in the European Union (EU) require that anthelmintics be classified as prescription-only drugs. Currently, the legislation is interpreted differently across the EU, with strictest implementation in Denmark where anthelmintic administration is based on diagnostic evidence of infection (Nielsen et al., 2012). Deployment of such diagnostic-based control strategies requires that robust and practical support tools are available. Coprological analysis for nematode eggs is central to this strategy, but this method is incapable of discriminating pre-patent infection. With the extended pre-patent period of several strongyle species, there is a requirement for diagnostic tests that detect and quantify levels of immature stages. A number of antigens are under investigation as diagnostic markers for detecting pre-patent cyathostomin (McWilliam et al., 2010) and *S. vulgaris* (Andersen et al., 2013a) infections. Until these are available, FEC-directed treatments will need to be balanced with anthelmintics applied strategically to target pathogenic larvae (Matthews, 2008).

2. Anthelmintic resistance

Interval-based treatment programmes, which have been used extensively in the equine industry, will be expected to select resistance alleles within nematode populations (Kaplan and Nielsen, 2010). Resistance to the earlier registered anthelmintics, the benzimidazoles and the tetrahydropyrimidines, has been reported many times in cyathostomin populations across the world, and resistance to both of these classes in single populations is a common observation in field studies (Kaplan et al., 2004; Traversa et al., 2009; Traversa et al., 2012). As fenbendazole resistance in cyathostomins is virtually ubiquitous in many regions (Osterman Lind et al., 2007; Traversa et al., 2012; Lester et al., 2013b; Relf et al., 2014; Stratford et al., 2014b), this anthelmintic should not be recommended for use in control of these infections in these areas. Perhaps surprisingly, despite the substantial reliance on ivermectin and moxidectin for equine nematode control in the last 30 years, resistance, measured as a reduction in FEC of less than 90–95% at 14–17 days after treatment, has been reported infrequently. Nevertheless, there have now been several reports of reduced strongyle egg ERP after ivermectin or moxidectin administration in a number of countries (von Samson-Himmelstjerna et al., 2007; Molento et al., 2008; Lyons et al., 2009; Lyons et al., 2010; Rossano et al., 2010; Lyons et al., 2011; Lyons and Tolliver, 2013; Canever et al., 2013; Relf et al., 2014). Reduced ERP is believed to provide an early indicator of a shift in a nematode population's sensitivity towards resistance (Sangster, 2001) and so this provides a warning as to the likely long-term effect of macrocyclic lactone compounds in horses.

Ivermectin resistance measured as low FEC reduction after treatment has been reported with regularity in *P. equorum* populations (Boersema et al., 2002; Hearn and Peregrine, 2003; Stoneham and Coles, 2006; Craig et al., 2007; Schougaard and Nielsen, 2007; von Samson-Himmelstjerna et al., 2007; Reinemeyer, 2012). These findings are unsurprising given the excessively frequent use of ivermectin in foals on stud farms. Control of ivermectin resistant *P. equorum* populations can theoretically be achieved using tetrahydropyrimidine or benzimidazole compounds; however, ivermectin resistant *P. equorum* populations have been shown to exhibit resistance to tetrahydropyrimidines as well (Reinemeyer, 2012). Benzimidazole resistance in *P. equorum* has not yet been published in the literature, but there is now anecdotal evidence of a lack of efficacy of this compound on stud farms in the UK (Matthews, unpublished observations). These reports highlight the threat of multi-class resistance in this nematode species and is a major concern for stud farmers given the potential pathogenicity of this parasite in foals.

All of the aforementioned issues highlight the risk of multi-class resistance in equine nematode populations and the complexity of patterns of infection and resistance that will need to be dealt with in the field. As anthelmintic choice now needs to be more evidence based, the tools that inform on levels of infection and anthelmintic efficacy need to be robust.

3. Tools for monitoring infection and detecting anthelmintic resistance

3.1. Faecal egg count analysis

The FEC test is a relatively easy method in which the number of strongyle and *P. equorum* eggs in equine faeces can be estimated at a specific point in time. A number of FEC techniques exist and these differ in sensitivity, speed of generation of results and the level of expertise required to perform the test. In all cases, it is essential that good practice be followed at each stage: from collection of samples at the yard or farm, to processing and analysis in the laboratory. In this way, examination of representative samples should provide a reasonable estimation of the level of egg excretion in each individual. Several studies, published in the last few years, have highlighted several factors that affect the accuracy of FEC analysis in horses and how these factors might impact the outcome of efficacy testing (Nielsen et al., 2010; Vidyashankar et al., 2012; Lester and Matthews, 2014). Generally, FEC test output is affected by differences in egg shedding at individual level (Denwood et al., 2012), the over-dispersion of nematode eggs in faeces (Lester et al., 2012), the non-uniform distribution of nematode eggs in suspension (Vidyashankar et al., 2012), the type of FEC method used (Lester and Matthews, 2014) and by sampling and storage practices (Nielsen et al., 2010). A number of recommendations have come out of these studies and are as follows. Studies on strongyle egg hatching and larval development suggest that faecal samples should be collected as fresh as possible (at most < within 12 h), and then refrigerated (Nielsen et al., 2010). Nematode eggs are unevenly distributed throughout equine faeces and it is therefore important to ensure that several samples are taken from different parts of the dung heap at sampling and that thorough mixing be performed before taking further sub-samples for counting (Denwood et al., 2012; Lester and Matthews, 2014). If samples need to be stored for any period, anaerobic storage is advised (Nielsen et al., 2010). As horses tend to have lower egg per gram values than other species, such as sheep, FEC methods with a higher sensitivity should be used. For example, when egg detection limits are higher (for example, when using the modified McMaster method with a 25 or 50 multiplication factor), the test will not be sufficiently sensitive to changes in egg abundance below or around the detection limit and, as a consequence, false negative results are more likely (Lester and Matthews, 2014). This is particularly relevant when assessing anthelmintic efficacy, as post treatment counts are more likely to be low. A test with a low egg detection limit (i.e. one with a lower/no multiplication factor for conversion of number of eggs) is more sensitive and will provide a more accurate estimation of eggs per gram.

3.2. Measuring anthelmintic resistance

In evidence-based parasite control programmes, anthelmintic efficacy should be tested on a regular basis. The FECRT is the commonest method for assessing efficacy *in vivo* and for monitoring prevalence of anthelmintic resistance in the field. The most widely published method is that recommended by the World Association for the Advancement of Veterinary Parasitology (WAAVP; Coles et al., 1992). This method calculates efficacy based on the arithmetic mean reduction in FEC observed between Day 0 and Days 14–17 after treatment. The WAAVP guidelines were originally designed for small ruminants and there exist no universally agreed cut-off limits for determining efficacy for anthelmintic classes in horses; these values vary among published reports. This absence of a unanimous definition for equine FECRT methodology makes it challenging to draw comparisons between studies. Moreover, inherent variability observed in equine FEC datasets (associated with the factors outlined above) can complicate the outcome the test (Denwood et al., 2010; Lester et al., 2013b; Stratford et al., 2014b). Recently published studies have attempted to define more accurate methods for calculating anthelmintic efficacy in horses (Vidyashankar et al., 2007; Kaplan and Nielsen, 2010; Vidyashankar et al., 2012; Lester et al., 2013b; Relf et al., 2014; Stratford et al., 2014b). In these, an arithmetic mean FECR of >95% was set for macrocyclic lactone anthelmintics, whilst a threshold of >90% was set for efficacy for benzimidazole and tetrahydropyrimidine anthelmintics. To give an indication of the data range inherent in these datasets, 95% lower confidence limits (LCL) were calculated (Vidyashankar et al., 2007; Lester et al., 2013b; Relf et al., 2014). In terms of the 95% LCL selected for classifying resistance, this varied depending on the class of anthelmintic tested with the percentage reduction threshold used for classifying resistance to macrocyclic lactones set at 90% and, for benzimidazoles and tetrahydropyrimidines, 80%. These cut-offs have been selected to reflect original efficacy levels reported in anthelmintic-sensitive strongyle populations soon after the products were registered for use in horses (Cornwell and Jones, 1969; Colglazier et al., 1977; Xiao et al., 1994). Maximum likelihood models, based on the negative binomial distribution for estimating FEC reduction (Torgerson et al., 2005), and Markov Chain Monte Carlo methods (Denwood et al., 2010) have also been suggested to account for the highly aggregated distribution inherent in equine FEC data. A limitation in these methodologies is that they require the ability to use advanced statistical programmes such as R. Recently, though, a web-interface has been developed to enable researchers to enter FEC datasets online, together with the detection limit of the FEC method used to generate the counts. This interface estimates the percentage of FEC reduction using Bayesian hierarchical models via Markov Chain Monte Carlo sampling (http://www.math.uzh.ch/as/index.php?id=calc, Torgerson et al., 2014) and now provides access for the layperson to more robust methods of computing efficacy.

Similar to the issues encountered with the FECRT, there are no well-defined guidelines on how to calculate and interpret strongyle ERP datasets in horses. In the main, two methods have been used: one, defined as the week of the first positive strongyle FEC after anthelmintic administration (Dudeney et al., 2008; Lyons et al., 2008; Molento et al., 2008), and the other, defined when the group arithmetic mean FEC exceeds 10% of the group arithmetic mean FEC at Day 0 (Borgsteede et al., 1993; Jacobs et al., 1995; Boersema et al., 1996; Mercier et al., 2001; Tarigo-Martinie et al., 2001; von Samson-Himmelstjerna et al., 2007; Larsen et al., 2011). The second method gives a more conservative estimate of egg reappearance with respect to the level and spread of the FEC data sampled prior to treatment and so gives a more accurate measure a population's sensitivity to anthelmintic. More research is warranted and measurement of the ERP parameter needs to be standardized so that analysis can be made between studies.

3.3. Measuring pre-patent infections

Because of the pathogenicity of immature cyathostomin and *S. vulgaris* larvae, diagnostic tests that detect and quantify levels of these stages are required. A number of antigens are under investigation as diagnostic markers for detecting pre-patent cyathostomin (Dowdall et al., 2002; Dowdall et al., 2004; McWilliam et al., 2010) and *S. vulgaris (*Andersen et al., 2013a) infections. A cyathostomin ELISA is being developed, which is based on measurement of levels of serum IgG(T) specific to two antigens present in early and late third stage larvae and developing fourth stage larvae (Dowdall et al., 2002; Dowdall et al., 2004; McWilliam et al., 2010). IgG(T) levels specific to these antigens have been shown to increase to 20 and 25 kDa complexes in native extracts of larvae (Dowdall et al., 2004) and to recombinant versions of proteins present within these complexes (McWilliam et al., 2010) within 5–6 weeks of a primary cyathostomin infection. The recombinant proteins have now been evaluated in an indirect ELISA format as a cocktail of antigens spanning nine common cyathostomin species and this cocktail is currently under assessment as to its utility in informing on cyathostomin encysted larval burden. A number of *S. vulgaris* antigens that are targets of antibody responses in infected horses have been described, but most of these have not been well characterised in terms of their specificity or their predictive value in informing on larval burdens (Andersen et al., 2013b). Recently, a *S. vulgaris* antigen, SvSXP, was identified as a possible diagnostic marker (Andersen et al., 2013a). Similar to the cyathostomin proteins described above, this antigen was identified by immunoscreening a larval stage complimentary DNA library using rabbit serum raised against adult worm excretory/secretory products. Immunoblotting experiments and preliminary ELISA analysis indicate that serum IgG(T) responses to this protein have potential as diagnostic markers of pre-patent infection (Andersen et al., 2013a). Until these tests are developed further and become commercially available, FEC-directed treatments will need to be balanced with anthelmintic applied strategically to target pathogenic larvae for both types of infections.

4. Sustainable control

Clearly, there is a real need for better management of equine nematodes, with improvement in anthelmintic use decisions the cornerstone of improved programmes that aim to avoid unnecessary or ineffective treatments. The practice of FEC-directed treatments can be highly effective in reducing anthelmintic administration frequency in horses because egg excretion is highly over dispersed amongst individuals (Relf et al., 2013; Lester et al., 2013b). A commonly quoted dogma is that 20% of the equine population excretes 80% of the parasite burden into the environment (Matthews, 2008) and recent studies have demonstrated that, in well-managed populations, the actual percentage of horses responsible for 80% excretion is lower than 20% (Relf et al., 2013; Lester et al., 2013b). In FEC-directed treatment programmes, high shedders (for example, horses excreting more than 200 eggs per gram [EPG] in faeces) are targeted with anthelmintics, whilst those identified as shedding negligible to moderate levels of eggs are left untreated (Duncan and Love, 1991). In this way, anthelmintic treatments are reduced at the same time as pasture contamination is lowered. It is assumed that nematodes in untreated horses will act as a source of 'refugia' (van Wyk, 2001) and that the progeny of these will act to dilute resistant alleles in offspring derived from worms that survive in horses administered with anthelmintic. There have been no quantitative studies that substantiate these principles in horses; nevertheless, the delivery of fewer anthelmintic treatments over time and the requirement to monitor nematode egg excretion profiles within populations provides the basis for more responsible control programmes. Due to the long pre-patent period of a number of strongyle worm species and, because severe disease can be caused by the larval stages of some of these species, FEC-directed treatments need to be balanced with a requirement to treat stages of nematodes that are undetectable by FEC analysis. In the absence of diagnostic tests that allow estimation of immature larvae, treatments with larvicidal anthelmintics (i.e. moxidectin) are recommended at specific times of year (Matthews, 2008; Hertzberg et al., 2014). The future availability of diagnostics that allow estimation of burden of pre-patent stages will enable specific targeting of individuals with larvicidal anthelmintics, as these tests can be used to identify horses estimated to harbour above a certain threshold of larval numbers. Such targeting should facilitate further reductions in anthelmintic usage at those times of year when larvicidal treatments are indicated.

Targeted treatment programmes have had variable uptake across regions and countries and between different types of management systems, with poorer uptake on Thoroughbred stud farms (Relf et al., 2013; Robert et al., 2014) and better uptake by the leisure horse sector (Lester et al., 2013a; Stratford et al., 2014a). Where targeted treatment programmes have been followed, large reductions in anthelmintic use have resulted (Lester et al., 2013a). The challenge now lies in disseminating these programmes further. Recent data from the USA indicates that stud farm owners, for example, are only willing to change to more evidence-based control measures if they are assured that such approaches would prevent anthelmintic resistance and decrease health risks significantly (Robert et al., 2014). Further research is required to provide such evidence, particularly as one recent study indicated an apparent increase in the prevalence of *S. vulgar*is infections on farms where reduced anthelmintic treatment intensity had been implemented over several years (Nielsen et al., 2012).

Despite a gradual move towards more evidence-based anthelmintic use, the prevalence of resistance in some populations is very high, with resistance reported to every class available (Trawford and Burden, 2012). Thus, it is imperative that, alongside implementation of these strategies, other methods of control are used so that there is not sole reliance on the use of anthelmintics to break the nematode transmission cycle. One option is to reduce contamination of grazing by removal of faeces from pasture. In 1986, Herd (Herd, 1986) demonstrated that pasture management, via the removal of faeces twice a week alone, was more effective than anthelmintic therapy in reducing pasture levels of strongyle larvae. This study was performed before the widespread use of moxidectin, which has a prolonged egg suppressive effect. More recently, studies undertaken on a UK donkey sanctuary confirmed the effectiveness of faecal removal from pasture in reducing nematode transmission (Corbett et al., 2014). These studies confirmed that twice-weekly removal of faeces from pasture significantly reduced the number of strongyle eggs shed in faeces from groups of co-grazed donkeys, thus verifying this practice was a useful management tool to further reduce use of anthelmintics. Faecal removal is recommended at intervals frequent enough to prevent third stage larvae developing and translating onto pasture and this interval has been measured as approximately two weeks in warm temperate conditions (Ramsey et al., 2004), but development may be more rapid in tropical and sub-tropical regions. Alternated grazing with ruminants will also decrease levels of strongyle contamination on pasture over time, but care must be taken to monitor for helminths that can be transmitted between sheep and horses (in particular, *F. hepatica*). Further research is required in these areas to provide quantitative evidence on the utility of these control methods and to provide baseline values on which to build practical recommendations.

5. Final conclusions

The lack of benzimidazole and tetrahydropyrimidine efficacy measured in equine nematode populations across the world, along with clear indications of emerging resistance against ivermectin and moxidectin, emphasises the need for fundamental changes in the way that nematodes are managed in horses. The macrocyclic lactone anthelmintics have the major market share globally and until recently, serious consideration had not been given to protecting efficacy of these compounds by implementing control programmes that use these medicines in a more targeted manner. Although targeted programmes are being used in some regions, the advantages of these programmes need further dissemination to ensure further uptake. Many horses are still subjected to regular blanket anthelmintic treatments with no attention paid to efficacy and a lack of uptake of evidence-based strategies may be due to the perceived complexity involved in integrating these methods in practice. It is essential that barriers be broken down so that those involved in prescribing and administering anthelmintics have confidence in delivering evidence-based protocols. This requires that stakeholders have easy access to up-to-date knowledge, as well as to robust diagnostic tests required to support decision-making. The gap between research findings and implementation at farm level is a continuing challenge for scientists working in equine parasitology. One piece of evidence to start reducing this gap is that initial feedback from horse owners indicates that by using targeted treatment strategies, considerably fewer anthelmintic treatments are applied (Lester et al., 2013a; Hertzberg et al., 2014). Furthermore, that this can lead to substantial financial savings (Lester et al., 2013a). There is still a requirement for better tools that will inform evidence-based parasite control, but new tests are on the horizon and these will need to be priced and marketed properly to ensure that they are used across the equine industry. By moving to evidence-based parasite management built on best practice (i.e. limiting use of anthelmintics and ensuring correct dose rates), combined with targeted grazing management and exploiting available diagnostic tools, the efficacy of the currently effective anthelmintics might be prolonged until new chemotherapeutics are discovered. This is paramount because reversion to anthelmintic susceptibility, in all probability, will not occur in nematode populations once they have become resistant.

Conflict of interest

The authors declared that there is no conflict of interest.

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