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**Review article**

# Anthelmintic resistance in nematodes of horses

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**Abstract** – Suppressive anthelmintic treatment strategies originally designed to control *Strongylus vulgaris* in horses were extremely successful in reducing morbidity and mortality from parasitic disease. Unfortunately, this strategy has inadvertently resulted in the selection of drug-resistant cyathostomes (Cyathostominea), which are now considered the principal parasitic pathogens of horses. Resistance in the cyathostomes to benzimidazole drugs is highly prevalent throughout the world, and resistance to pyrantel appears to be increasingly common. However, there are still no reports of ivermectin resistance in nematode parasites of horses despite 20 years of use. It is unknown why resistance to ivermectin has not yet emerged, but considering that ivermectin is the single most commonly used anthelmintic in horses most parasitologists agree that resistance is inevitable. The fecal egg count reduction test is considered the gold standard for clinical diagnosis of anthelmintic resistance in horses, but diagnosis is complicated by lack of an accepted standard for the performance of this test or for the analysis and interpretation of data. Presently there is very little data available on the molecular mechanisms of anthelmintic resistance in cyathostomes; β-tubulin gene is the only anthelmintic-resistance associated gene that has been cloned. The increasingly high prevalence of anthelmintic-resistant cyathostomes must be taken into account when designing worm control programs for horses. Strategies to decelerate further selection for drug resistance thereby extending the lifetime of currently effective anthelmintics should be implemented whenever possible. Considering the nature of the equine industry in which horses often graze shared pastures with horses from diverse locations, transmission and widespread dispersal of resistant parasites is virtually assured. A proactive approach to this problem centered on understanding the molecular basis of anthelmintic resistance in cyathostomes is required if we are to expect chemical control of nematodes in horses to remain a viable element of parasite control in the future.

**cyathostome / anthelmintic / resistance / nematode / horse**

**Résumé – La résistance aux anthelmintiques chez les nématodes des chevaux.** Les stratégies des traitements anthelmintiques mises en place à l'origine pour contrôler *Strongylus vulgaris* chez les chevaux ont été extrêmement efficaces pour réduire la morbidité et la mortalité dues aux maladies

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parasitaires. Malheureusement, ces stratégies ont eu pour conséquence la sélection de cyathostomes (Cyathostominea) résistants aux anthelmintiques, qui sont désormais considérés comme les principaux agents pathogènes parasitaires du cheval. La résistance aux benzimidazoles chez les cyathostomes a une très forte prévalence à travers le monde, et la résistance au pyrantel semble de plus en plus répandue. Cependant, aucune résistance à l'ivermectine n'a été rapportée chez les nématodes parasites des chevaux malgré 20 ans d'utilisation. Les raisons pour lesquelles cette résistance n'a pas encore émergé sont inconnues, mais étant donné que l'ivermectine est l'anthelmintique le plus utilisé chez le cheval, la plupart des parasitologistes pensent que cette résistance est inévitable. Le test de réduction du nombre des oeufs dans les fèces est considéré comme le test de référence pour le diagnostic clinique de la résistance aux anthelmintiques chez les chevaux, mais l'absence de normes établies pour la réalisation de ce test ou pour l'analyse et l'interprétation des données complique le diagnostic. Actuellement il y a très peu de données sur les mécanismes moléculaires de la résistance aux anthelmintiques chez les cyathostomes ; seul le gène associé à la résistance aux anthelmintiques qui a été cloné est celui de la β-tubuline. L'augmentation de la prévalence des cyathostomes résistants aux anthelmintiques doit être prise en compte lors de la mise en place des programmes de contrôle des vers chez les chevaux. Des stratégies permettant de ralentir le phénomène de sélection de la résistance aux anthelmintiques, et par conséquent augmentant la durée de vie des anthelmintiques aujourd'hui efficaces, doivent être mises en place chaque fois que cela est possible. Considérant la nature de l'industrie équine, dans laquelle des chevaux provenant de diverses sources sont mis en pâture ensemble, la transmission et la large dispersion de parasites résistants est virtuellement assurée. Une façon active d'aborder ce problème, centrée sur la compréhension des bases moléculaires de la résistance aux anthelmintiques chez les cyathostomes, est nécessaire si nous voulons que la lutte chimique contre les nématodes des chevaux demeure pour l'avenir un élément durable du contrôle antiparasitaire.

**cyathostome / anthelmintique / résistance / nématode / cheval**

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## 1. BACKGROUND TO THE PROBLEM

Most horse owners share a high level of concern regarding the impact of helminth disease on equine health. The importance horse owners continue to place on parasite control was recently demonstrated in the National Animal Health Monitoring System Equine '98 study (United States), where it was reported that 96.8% of all equine operations dewormed the majority of their horses during the previous 12 months, with 49.2% of horses receiving 4 or more treatments [73]. For 98.6% of equine operations, the primary reason horses were dewormed was as a general preventative measure. Such concern, combined with the availability of safe, effective, and inexpensive anthelmintics has led to a dramatic over-use of these drugs. The general fear of parasitic disease by much of the equine community often results in a 'no parasite' mentality, in which the goal is to treat frequently enough to keep fecal egg counts (FEC) near zero. This attitude is not confined to the United States, but is widespread throughout much of the world where pleasure, performance or racing horses are kept. I have dealt with horse owners that refused to skip a scheduled anthelmintic treatment even when they were shown data indicating a FEC of zero. We now find ourselves in a situation where anthelmintic resistance is an extremely important, but often overlooked issue for nematode control in horses. While drug resistance is often considered as a theoretical issue in designing rotational treatment programs, most horse owners and veterinarians have little knowledge about the true prevalence of anthelmintic resistance or the resistance status on their properties. Thus, these same individuals who refuse to skip unneeded treatments will not hesitate to unknowingly use a drug that is totally ineffective as a result of resistance.

In the 1960's, an epidemiological approach to parasite control, together with the availability of benzimidazole (BZ) anthelmintics led to recommendations for treatment of horses every 6 to 8 weeks [29]. This approach, known as the interval dose system, was designed primarily to control *Strongylus* spp. ('large strongyles') nematodes, especially the highly pathogenic *Strongylus vulgaris*. This program became widely adopted and was extremely successful in reducing morbidity and mortality from parasitic disease. Equine veterinarians whose clinical experience spanned the transition into the modern era of efficacious anti-strongyle compounds noticed a dramatic reduction in clinical cases of colic; the once common affliction of verminous colic became an unusual event [31]. By the early 1980's it was recognized that *S. vulgaris* was becoming uncommon and that cyathostomes frequently accounted for virtually 100% of the worm egg output of grazing horses [50]. Following the introduction of ivermectin in 1983, which is highly effective against migrating larval stages of *Strongylus* spp., a further and dramatic reduction in the prevalence and intensity of *S. vulgaris* occurred [47]. As a result, *S. vulgaris* is no longer considered an important cause of colic in managed horses and is uncommonly diagnosed except on farms where parasite control is severely neglected.

Unfortunately, the replacement of haphazard parasite control with the interval treatment program also profoundly affected strongyle worm populations that now is causing important problems for parasite control [46]. When the interval dose system was first implemented, cyathostomes (Cyathostominea, 'small strongyles') were considered little more than a nuisance parasite compared to the highly pathogenic *S. vulgaris* [29]. However, at about the same time of the adoption of the interval dose program came the first reports of

thiabendazole resistance in the cyathostomes [28]. Resistance to other BZ followed, and more recently pyrantel resistance has emerged. We now find ourselves in a situation where cyathostomes have developed high rates of resistance to all commonly used anthelmintics except the avermectin/milbemycins (AM) [12, 20, 88, 90, 106]. The decline of *S. vulgaris* and the rise of drug-resistant cyathostomes have changed our view of the relative importance of these nematodes; cyathostomes are now considered the principal parasitic pathogens of horses [46, 65, 95]. Despite these facts, a recent survey of Australian veterinarians found that a majority of veterinarians continue to rank *S. vulgaris* as the most important internal parasite of horses [76]. It is likely that these same misconceptions are shared by a large proportion of veterinarians worldwide.

Common clinical signs of cyathostome infections include decreased level of performance, decreased rates of growth, weight loss, colic, rough hair coat, and debilitation [96]. Infection with cyathostomes may also cause a life-threatening disease, known as larval cyathostomosis, which is characterized by severe weight loss, chronic diarrhea, and edema [70]. However, most horses show no clinical disease from cyathostomes even when heavily infected. Instead, infection usually causes a sub-clinical alteration in gastrointestinal function characterized by a mild inflammatory enteropathy [65]. This may result in alterations of intestinal microcirculation and motility leading to a protein losing enteropathy. Because most often cyathostomes are only mild pathogens, it is likely that the pathogenic potential of cyathostomes was not realized until recently because these effects were masked by the presence of large strongyles [65, 69] Another factor contributing to the problem caused by cyathostomes is the fact that immunity is slow to develop and is incomplete, thus most horses require regular anthelmintic treatment throughout their lives [54]. Additionally, most natural selection pressure on equine populations for nematode tolerance and resistance/immunity has been eliminated by the frequent application of anthelmintic treatments. The exception to this may be in some underdeveloped areas of the world where small numbers of horses are often used for agricultural purposes and kept in relative isolation. These working type horses often do not receive the same level of veterinary care, as do pleasure/performance type horses. These factors combined with the widespread and increasing prevalence of anthelmintic resistance have made cyathostomes important equine pathogens, and their importance is likely to grow as the prevalence and spectrum of anthelmintic resistance continues to increase. Currently there are three major classes of anthelmintics used to control nematodes in horses: benzimidazoles (fenbendazole, oxfendazole, oxibendazole, others), tetrahydropyrimidines (pyrantel salts), and avermectin/milbemycins (macrocyclic lactones; ivermectin and moxidectin). Piperazine and phenothiazine are additional drugs, but these are rarely used anymore. When first introduced, all of these drugs had very good to excellent efficacy against cyathostomes. However, it has now been documented worldwide that cyathostomes are commonly resistant to anthelmintics; this is gaining recognition as a serious concern in the health management of horses.

## 2. PREVALENCE OF ANTHELMINTIC RESISTANCE IN EQUINE HELMINTHS

Anthelmintic resistance in helminths of horses appears to be confined to the cyathostomes. Over the years there have been reports of possible resistance in *Strongylus* spp. to BZ [41, 94] and pyrantel [17], however, in each case the suspected resistance was never confirmed. It is highly probable that these cases represent lack of

efficacy rather than resistance since the efficacy of pyrantel and BZ against large strongyles, especially *S. edentatus* is lower than for other strongyle species [32, 67, 69]. There are no published reports of anthelmintic resistance in any other helminths of horses.

### 2.1. Resistance to benzimidazoles

Anthelmintic resistance in cyathostomes has been widely reported throughout the world. The earliest documented cases of resistance in parasites of horses were to phenothiazine, first in the UK [43, 77] and then in the United States [27]. Soon after, resistance to thiabendazole (TBZ) was reported after only a few years of use [28]. Interestingly, clinical trials performed during 1960–1961 on a central Kentucky thoroughbred farm demonstrated that TBZ-resistant individuals were already present in this cyathostome population when TBZ was first used on this farm in 1961 [36]. This observation was confirmed when after only one year of use; the efficacy of TBZ on this farm had decreased from greater than 95% to approximately 35%. Since a large segment of this small strongyle population was already resistant to phenothiazine due to long-term use of this drug, and these trials represented the first use of TBZ in central Kentucky, it is suspected that phenothiazine 'pre-selected' for BZ resistance. This suspicion is supported by biochemical evidence, which suggests that phenothiazine acts on helminths though microtubular inhibition; the same mechanism described for BZ anthelmintics [83].

Of the three major classes of drugs, resistance to BZ is the most prevalent and widespread, with reports of resistance from over 21 countries [69]. Initially, most reports of BZ resistance were from single farm observations in isolated geographic areas. More recently, multi-farm prevalence studies in the United States and Europe have demonstrated a prevalence of resistance to BZ of 75% or greater [19, 25, 40, 51, 81, 82, 90, 101, 106]. In many areas, resistance to BZ anthelmintics is now so common that it is difficult to find populations of cyathostomes that are not BZ-resistant. With the equine cyathostomes, resistance to one member of the BZ drug class confers side resistance to other members within the same class with the exception of oxibendazole (OBZ), which remains effective against BZ-resistant worms for a limited period of time [33, 68, 89, 105]. When OBZ was used to treat horses infected with BZ-resistant, but OBZ-sensitive cyathostomes, fecal egg count reductions decreased from initial values of greater than 95% to around 80% by the second year of treatment [35, 98]. Based on these data, it appears that in horses parasitised with BZ-resistant cyathostomes, resistance to OBZ may become apparent after only 8–10 exposures to the drug. Continued treatments with OBZ resulted in further decreases in efficacy. The prevalence of OBZ resistance has not been well documented; OBZ was not included in any of the prevalence studies referenced above. However, recent studies performed in the southeastern United States using the fecal egg count reduction test suggest that the prevalence of resistance in this region is greater than 60% (Kaplan unpublished results).

### 2.2. Resistance to pyrantel

Although pyrantel has been used as an equine anthelmintic since the 1970's, it is only in recent years that reports of pyrantel-resistant cyathostomes have become common. Pyrantel-resistant cyathostomes have been reported throughout the southeastern United States [12, 71, 90, 106] as well as in Norway [51] and Denmark [19]. Since lower anthelmintic efficacies should delay the selection for resistance [3], it may be that the relatively lower efficacy of pyrantel [18, 67] has delayed the appearance of resistant worms [21]. In 1990, pyrantel tartrate began being marketed in the United

States as a daily anthelmintic for horses [23]. It now appears that the common practice of low-dose (2.64 mg/kg) daily feeding of pyrantel tartrate is one of the primary factors responsible for the increasing frequency with which pyrantel resistance is being diagnosed in the United States. Tarigo-Martinie et al. [90] reported that 2 of 10 properties tested in Georgia, USA had cyathostomes resistant to pyrantel. One of these two farms had high-level resistance (0% reduction in FEC), and this property was the only farm tested with a history of daily pyrantel use. In a more recent study also performed in Georgia, USA, the prevalence of pyrantel resistance was approximately 40% (Kaplan unpublished results). Although insufficient data is available to make direct correlations, on farms where pyrantel resistance was diagnosed there often was a history of daily pyrantel feeding. On many of these farms, it was not uncommon to find horses being fed daily pyrantel tartrate that had FEC of 300 EPG or higher. The paradox of this situation is that daily pyrantel is often selectively given to those horses believed to be most susceptible to nematode parasites and most likely to suffer from the clinical effects of cyathostome infection. Because horse owners assume that daily feeding of pyrantel will fully protect these animals from helminth infections, such horses usually get treated with single-dose anthelmintic treatments only at infrequent intervals. As a result, where pyrantel resistance occurs, the horses needing the most intensive parasite control often receive the poorest control. This is probably the clearest example of how the lack of appreciation of anthelmintic resistance by horse owners and veterinarians can lead to the mismanagement of parasite control and how drug resistance can severely impact equine health.

### 2.3. Resistance to ivermectin

Ivermectin was first introduced as an equine anthelmintic in 1981 [10] and remained the only AM drug used in horses until the recent introduction of moxidectin (late 1990's). Despite being used as an equine anthelmintic for 20 years, and being the single most commonly administered anthelmintic drug [61, 64, 79], there are still no reports of resistance to ivermectin in parasites of equids. This is the only anthelmintic drug class used in horses in which there is still no resistance. In fact, FEC reductions at two weeks post treatment with ivermectin continue to be virtually 100% [19, 90, 101, 106], even though many farms have long histories of using ivermectin four to six times a year. A recent controlled efficacy study with ivermectin confirmed that ivermectin efficacy against equine nematode parasites remains extremely high (99–100%); there is no indication that ivermectin efficacy has decreased with time [55]. However, with growing reliance upon these drugs, many parasitologists suspect that resistance is inevitable [63, 86]. This is of great concern because the continued excellent efficacy of the AM drugs has led to a high level of complacency within the equine health community. Since ivermectin and moxidectin are the core drugs used in the control of parasites in horses, if (when) resistance to the AM drugs develops in parasites of horses, the clinical impact of parasitic disease would rise dramatically.

The likelihood that such resistance will develop at some future time is suggested by the high prevalence of AM-resistant *Haemonchus contortus* in sheep and goats throughout the world [91, 100, 104], and the increasingly common reports of AM-resistant parasites of cattle. Avermectin-resistant *Cooperia* spp. have now been reported in New Zealand [102, 103], the UK [16], and Argentina [1, 39]. *Haemonchus* and *Cooperia* (Trichostrongylidae) and the many species of cyathostomes (Strongylidae) are very closely related parasites belonging to the order Strongylida [37]. Recent molecular characterization of rDNA and ribosomal internal transcribed spacer sequences

suggests that genetic divergence between taxa in the Strongylida is remarkably low [13, 26]. In contrast, studies comparing mDNA sequences suggest that the genetic diversity within-populations of individual trichostrongyle species is quite large [7]. Large effective population sizes, large genetic diversity, and high gene flow in the strongylid nematode parasites of livestock and horses suggests a great opportunity for the spread of rare alleles that confer resistance to anthelmintic drugs. Considering the great similarities of the Strongylid nematodes in their genetics, biological characteristics, epidemiological features, and sensitivities to anthelmintic drugs, it seems very likely that mechanisms of anthelmintic action and resistance in these nematodes will also be very similar.

## 3. BIOLOGY OF CYATHOSTOMES: FACTORS RELATING TO THE EVOLUTION OF ANTHELMINTIC RESISTANCE

### 3.1. Species of cyathostomes reported to be resistant

More than 40 species of cyathostomes (Tribe Cyathostominea) have been described in horses [62]; however, only 12 species (*Cyathostomum* (*Cya*.) *catinatum*, *Cya. pateratum*, *Coronocyclus* (*Cor.*) *coronatus*, *Cor. labiatus*, *Cor. labratus, Cylicocyclus* (*Cyc*.) *nassatus*, *Cyc*. *leptostomus*, *Cyc. insigne*, *Cylicostephanus* (*Cys*.) *longibursatus*, *Cys. goldi*, *Cys. calicatus*, and *Cys. minutus*) are highly prevalent, comprising about 99% of total cyathostome burdens [8, 68, 74, 80, 87]. Other uncommon species not on this list will sometimes be present at greater levels than the least common of these 12, but always at relatively low prevalence and/or intensity. It is interesting to note that the relative prevalence and intensity of the most common species is quite similar throughout the world, despite the great variation in climate between study locations. Additionally, the relative prevalence and intensity of these common species has not demonstrated any noticeable changes over the past few decades despite the frequent use of anthelmintics and the increasing prevalence of resistant worms. Studies performed in Great Britain [74], the United States [80], Australia [8], and Brazil [87] spanning the period 1976 to 1999 all report the same three most abundant species; *Cys. longibursatus*, *Cya. catinatum,* and *Cyc. nassatus*. These three species frequently account for about 70–80% of the total population. A recent study performed in Louisiana USA that compared the prevalence and intensity of cyathostome species in 1981 and 1999 found no important differences in relative prevalence [11]. All 24 species present in the earlier study [93] were also found in the recent study. Additionally, the 13 most prevalent species were the same in both surveys, although ranked somewhat differently. *Cylicostephanus longibursatus* was the most prevalent species and had the greatest mean intensities in both studies. The only significant differences between these surveys were overall reductions in the intensity of infections and in the prevalence of most species in the more recent study.

Of the 12 common species listed above, 10 and 8 of these have been shown to be resistant to BZ [9, 34, 68, 88, 92] and pyrantel, [12, 71] respectively. Additional species reported to be resistant to BZ include *Cyc. brevicapsulatus*, and *Petrovinema poculatus* [9, 88]. This list is most likely incomplete since most studies use fecal egg count reduction tests to establish the presence of resistant worms. Few reports have actually identified those species that are resistant. These data strongly suggest that most if not all cyathostome species have the genetic diversity necessary to respond successfully to selection pressure from anthelmintics. Thus, the potential for cyathostomes to develop anthelmintic resistance to the AM

drugs and any newly developed drugs appears to be high.

### 3.2. Issues related to the emergence of ivermectin resistance

The issues surrounding the question of whether AM resistance will develop in cyathostomes has been reviewed in detail by Sangster [86]. However, a brief discussion of some of the important issues relating to this topic is warranted here. There are a number of minor biological differences between the Trichostrongylidae and Cyathostominea that affect the stage-specific efficacy of ivermectin. In ruminant hosts, all stages of trichostrongyle nematodes including the arrested mucosal larval stages are killed with high efficacy by ivermectin. Any survivors of treatment are, therefore, the sole contributors of genetic material onto the pasture. The same is not true for the cyathostomes. Cyathostomes remain in an encysted/arrested state in the intestinal mucosa of horses for extended periods of time following infection. This period of arrest can last for months or more, resulting in an infection dynamic where the majority of cyathostomes infecting a horse are often the mucosal larval stages. Unlike in ruminant hosts, IVM does not penetrate these cysts and therefore does not kill these mucosal stages. Because the mucosal stages do not 'see' the drug and are not selected by treatment, they serve as refugia. It is now believed that one of the major factors affecting the rate of selection of anthelmintic resistance is the size of this unselected population/refugia [86, 99]. The large mucosal refugia of horses will greatly slow the selection process and it will take many more treatments to reach resistant gene frequencies high enough to produce the phenotypic expression of treatment failure. This may be especially true with a drug such as ivermectin which has $>99.9\%$ efficacy against adult lumenal stages. Lower rates of selection are, therefore, at least partly responsible for the continued excellent efficacy of ivermectin against cyathostomes.

However, there could also be some important genetic differences that are responsible for the slow development of avermectin resistance in cyathostomes as compared to *H. contortus* and other trichostrongyle nematodes. A potentially important factor is the means of inheritance of the resistance trait. Inheritance of resistance to levamisole, BZ and ivermectin in *H. contortus* and *Trichostrongylus colubriformis*, varies between drugs and parasites; incomplete dominant, complete dominant, incomplete recessive, sex-linked recessive, and autosomal recessive inheritance have all been reported [24]. In *H. contortus*, ivermectin resistance has been shown to be inherited as a completely dominant trait [24, 60]. Computer modeling has demonstrated that resistance evolves fastest when it is inherited as dominant trait, more slowly when co-dominant, and slowest when it is recessive [4]. This fact partly explains the rapid and widespread development of ivermectin resistance in *H. contortus*. It must be kept in mind though that the pattern of inheritance of a trait can only be determined after that trait is expressed phenotypically. Therefore, until ivermectin resistance is demonstrated in cyathostomes, there is no way to predict or determine what the pattern of inheritance will be. If inheritance of the ivermectin resistance trait in cyathostomes is recessive, then this would also greatly decrease the rate of the selection process toward resistance.

A third important factor may be the number of genes involved in conferring the resistance trait. In *Caenorhabditis elegans*, simultaneous mutation of three genes encoding glutamate-gated chloride channel (GIuCl) alpha-type subunits confers high-level resistance to ivermectin. However, mutating any two of these GluCl genes confers modest or no resistance [22]. A model was proposed in which ivermectin sensitivity in *C. elegans* is mediated by genes affecting parallel genetic pathways defined by the

family of GluCl genes, with further modulation of drug sensitivity by several genes that alter the structure of the nematode nervous system. In *H. contortus*, a precise model for avermectin resistance has not yet been developed but available data suggests that more than one gene may be involved [78]. It is quite possible that between species differences in the number of different GluCl subunit genes, or other as yet unidentified genes may be important in determining how quickly resistance can evolve. In cyathostomes, a model of avermectin resistance in which drug resistance is mediated by multiple simultaneous mutations in several different parallel pathways could account for the slow (unapparent) development of avermectin resistance in these parasites. It should be kept in mind, however, that the mutations shown to confer ivermectin resistance in *C. elegans* can produce resistance ratios of several thousand. In contrast, parasitic nematodes that are resistant to ivermectin demonstrate resistance ratios of less than 100. Therefore, while many similarities probably exist between the parasitic and non-parasitic nematodes in terms of resistance mechanisms, it cannot be assumed that they will be the same.

## 4. MECHANISMS OF RESISTANCE IN EQUINE CYATHOSTOMES

The biochemistry and molecular biology of BZ resistance in trichostrongyle nematodes of ruminants has received much attention over the years [59, 84]. Benzimidazole resistance in *H. contortus* results from selection on both the isotype-I and isotype-II β-tubulin genes [6, 56, 66]. In *H. contortus* and *Ostertagia* (*Teladorsagia*) *circumcincta*, BZ resistance has been linked to a single Phe to Tyr mutation at amino acid 200 in the isotype-I β-tubulin [38, 57, 58]. Additionally, BZ-resistant populations of *H. contortus* have been identified with a Phe at position 200, but with a Tyr or His at position 167 [78].

Though it has been more than 35 years since the first report of BZ resistance in equine cyathostomes, little has been done to determine the molecular mechanisms involved. The only anthelmintic-associated gene that has been fully cloned and studied to date is the β-tubulin gene from *Cyc. nassatus* [52, 75]. Pape et al. [75] reported the full genomic organization of a gene that corresponds to the isotype-I gene of *H. contortus*; the full-length gene is 2652 bp in size and is organized into nine exons and eight introns. They were unable to demonstrate the presence of an isotype-II gene. Kaplan et al. [52] reported only cDNA sequences, but demonstrated the presence of both isotype-I and isotype-II β-tubulin genes. β-tubulin is known to be a highly conserved protein, and in the case of the isotype-I gene, it was demonstrated that *Cyc. nassatus* and *H. contortus* share greater than 98% protein sequence identity.

As mentioned previously, cyathostomes resistant to a broad range of BZ anthelmintics remain sensitive to oxibendazole for a limited period of time. The mechanism of this differential sensitivity remains unknown. We recently examined this issue by looking for differences in β-tubulin sequence and isotype expression that may be responsible for this differential drug susceptibility. We cloned and sequenced β-tubulin gene fragments from both *Cyc. nassatus* and *Cya. catinatum* in oxibendazole-resistant (OBZ-R), oxibendazole-sensitive/fenbendazole- resistant (OBZ-S/FBZ-R), and fenbendazole-sensitive (FBZ-S) cyathostome populations [53]. In all worms examined, we identified clones of both isotypes, although isotype-1 was more common. All of the OBZ-R worms had a Tyr at position 167 and Phe at position 200; all of the FBZ-R/OBZ-S worms had a Phe to Tyr mutation at either position 167 or 200, but never at both sites; and half of the FBZ-S worms had a mutation at one of these positions but not the

other. Based on these data, there does not appear to be any clear associations between differential sensitivity to FBZ or OBZ and β-tubulin sequence or isotype expression. Further work investigating this issue is in progress. An allele-specific PCR assay has recently been developed that can discriminate the TAC/TTC (Phe200Tyr) mutation in cyathostome β-tubulin [85]. This assay is capable of detecting a single larva-equivalent making it useful for assessing allele frequencies in sensitive and resistant populations. However, the Phe167Tyr is also a common mutation and it appears that all BZ-resistant cyathostomes have either the 167 or 200 mutations, but not both. Therefore, to be truly useful, a PCR assay will need to test for both mutations. Other than the β-tubulin data referenced above, there have been no published reports on the molecular basis of anthelmintic resistance in cyathostomes.

## 5. CRITERIA FOR DIAGNOSIS OF ANTHELMINTIC RESISTANCE IN HORSES

Critical and controlled efficacy tests [30] offer the highest level of accuracy in establishing the presence of resistant worms in horses. These methods also permit the identification of resistant species. However, necropsy of the test animals is required restricting these tests to a research setting and making this approach unusable in an on-farm situation. In vitro assays have potential usefulness, but have not been adequately tested and validated in horses to be routinely used with accuracy [20]. Further complicating interpretation of data from in vitro assays is the presence of multiple cyathostome species, the majority of which have larvae that are morphologically indistinguishable, and a lack of available sensitive and resistant reference strains. Molecular assays are currently unavailable, although this approach must be a future goal since these tests can detect resistance (genotypic) prior to therapeutic failure (phenotypic). Because of the shortcomings of these approaches, the fecal egg count reduction test (FECRT) is considered the gold standard for clinical diagnosis of anthelmintic resistance. Unfortunately, interpretation of data from FECRT in horses often can be difficult, and cutoffs for establishing resistance have not been standardized. Given that there is no practical means to confirm the presence of resistance on privately-owned horse farms, the FECRT despite its own shortcomings (does not quantify resistance, but only leads to a strong suspicion of resistance) remains the standard used in declaring the presence of resistance on the farm.

The World Association for the Advancement of Veterinary Parasitology (WAAVP) has published recommendations for standardizing procedures used for the detection of anthelmintic resistance in nematodes of veterinary importance [15]. However, these recommendations concentrate mostly on methods for detecting resistance in nematodes of sheep and goats. When using the FECRT in sheep and goats, resistance was defined as a reduction in fecal egg counts of less than 95% with a lower confidence limit (LCL) of less than 90%. If only one of these two criteria is met then resistance is suspected. These recommendations have become fairly well accepted as a standard for detecting anthelmintic resistance in sheep and goats.

On the issue of using the FECRT in horses, the WAAVP publication makes only brief mention of circumstances specific to performing and interpreting results of this test. Reduction in fecal egg counts of less than 90% was said to be indicative of BZ resistance [5], but no explanation was provided on the 90% cutoff and no recommendations were made for any other anthelmintics. It was acknowledged that oftentimes only small groups of horses are available for testing, and untreated control groups may not be practical. However, no

recommendations were made as to the number of horses that should be included in each treatment group or how the data should be analyzed. A review of methods used in recent anthelmintic resistance prevalence studies in horses demonstrates that the WAAVP recommendations are not being widely accepted or utilized by current researchers. In some reports the WAAVP standard for sheep and goats was used (< 95% reduction, LCL < 90%) [19, 51], in others the WAAVP standard for BZ resistance in horses was used (< 90% reduction) [82, 101], and in other studies treatment was categorized as effective (worms sensitive) if FEC reduction was > 90%, equivocal (suspected resistance) if the FEC reduction was between 80 and 90%, and ineffective (resistant) if the FEC was reduced by < 80% [81, 90, 106]. In these studies, the same standard for declaring resistance was used for all drugs tested. Furthermore, there is no standard on the number of horses that should be tested per drug-treatment group or per farm, what the minimum pre-treatment FEC should be for inclusion in the analysis, what the sensitivity of detection for determining FEC should be, or whether an untreated control group needs to be included. Recently, Dargatz et al. [21] suggested that individual horse FEC data should be transformed by angular transformation prior to calculating group means so that the data approximates a normal distribution. Using this method, he suggested that a level of 95% reduction be set for BZ and AM drugs, but 90% be used for pyrantel (and morantel).

These differences in data interpretation are not merely academic. Disparities in the standards used for declaring resistance will have a direct impact on the prevalence of resistance that is reported. Craven [19] reported a prevalence of resistance to pyrantel on Danish horse farms of 20%. However, if the 90% cutoff was used, prevalence decreased to 6.7%, and if the 80% cutoff was used, pyrantel resistance would not have been detected. With pyrantel resistance appearing to be on the rise and ivermectin resistance looming in the future, it is essential that equine parasitologists establish international standards for the performance and analysis of FECRT in horses. Because numbers of horses available for testing on most farms is relatively small, variability in the data set is usually large. Although it is desirable to use a stringent threshold for determining the presence of resistance in order to avoid the misdiagnosis of resistant parasites as susceptible, it is also important to be conservative in declaring resistance when variability in data is large.

Considering the differences between drugs in their efficacy against susceptible cyathostomes, a universal standard is probably not advisable. Instead, different cutoffs should be established for each drug. For BZ, the 90% reduction level previously recommended is probably a fair cutoff for declaring resistance. However, this measure is probably not adequate for ivermectin and pyrantel. Treatment with ivermectin consistently gives virtually 100% reduction in FEC at two weeks post-treatment. This high efficacy makes the appearance of any eggs in the feces following ivermectin treatment a cause for concern. Therefore, a definition of resistance for ivermectin of < 95% reduction with LCL < 90% may be too conservative and a more stringent definition is warranted. On the other hand, pyrantel efficacy against cyathostomes is quite variable. Unlike ivermectin, efficacy of pyrantel against cyathostomes is not very high; even when first introduced, percentage efficacies for pyrantel were only in the low to mid nineties [18, 67]. In a recent study, we reported a prevalence of resistance for pyrantel of 20% using a conservative definition (< 80% reduction in FEC) [90]. Had we chosen a more stringent definition, the results would have been dramatically different. On the 10 farms studied, percent reductions varied from 0 to 100% between farms, and on only

three farms was the percent reduction > 95%. We also noticed a large variation in response among horses on the same farms. This was especially apparent when pyrantel efficacy was tested in yearlings. It has been previously reported that anthelmintic efficacy is reduced in yearlings compared to adults [48, 49]. This age-related and overall variability in drug efficacy needs to be considered when assigning definitions for resistance and suggests that a conservative definition for resistance to pyrantel is required.

## 6. MANAGEMENT ISSUES AND FUTURE IMPLICATIONS RELATING TO ANTHELMINTIC RESISTANCE IN HORSES

The increasingly high prevalence of anthelmintic-resistant cyathostomes must be taken into account when designing worm control programs for horses. It is strongly recommended that prior to using a BZ drug or pyrantel, veterinarians perform a FECRT to rule out the presence of drug-resistant worms on that property. Additionally, strategies to decelerate further selection for drug resistance thereby extending the lifetime of currently effective anthelmintics should be implemented whenever possible. This goal can best be achieved using epidemiological principles of nematode control [47]. Properly timed anthelmintic treatments (will vary depending upon drug used and geographic region) combined with sound pasture management/hygiene and good horse husbandry can be effective both in reducing the number of drug treatments required, and in reducing environmental contamination with cyathostome eggs and larvae. These will decrease the selection for drug resistance while also minimizing levels of infection in horses.

Veterinarians should also be monitoring FEC of horses on a regular basis. Data from regularly scheduled FEC will help measure the effectiveness of a worm control program and identify those horses that do not need frequent treatments. Such monitoring will reduce the total number of treatments given, thereby increasing the percentage of the cyathostome population in refugia, and will also identify those horses that are highly susceptible to cyathostomes and require extra attention. Importantly, routine performance of FEC gets the veterinarian more involved in the health management of the stable, and increases the likelihood that emerging anthelmintic resistance will be detected in a timely manner. On many of the farms we recently examined for anthelmintic resistance (Kaplan, unpublished results), more than 50% of the horses had no strongyle eggs in the feces although all horses were overdue for deworming based on the farms normal treatment schedule. All of these farms had a history of using ivermectin +/– moxidectin at frequent intervals. The message here is clear: many horses are dewormed too frequently and the AM drugs are heavily relied upon for nematode control. Frequent use of anthelmintics in an attempt to keep FEC near zero is not a sustainable approach and may actually increase the risk of disease from cyathostomes by impairing the development of acquired immunity [46, 72]. Many equine parasitologists agree that horses do not need to be dewormed until the herd mean FEC are 100–300 EPG [97].

The common practice of rotating drugs with each treatment does not appear to slow the development of resistance, [98] and may actually increase the rate at which resistance develops by selecting for resistance to more than one drug simultaneously. When more than one anthelmintic class is effective, an effort should be made to perform annual (slow) rotation [14]. A single anthelmintic should be used for an entire year, and a second drug used the next. Although a slow rotation is generally accepted as the best approach for delaying resistance,

recent computer models that examined the effects of various worm control strategies on anthelmintic resistance suggest that the most effective approach for delaying the selection for resistance is to treat simultaneously with two chemically distinct anthelmintics. Although expensive and not routinely practiced, this approach deserves further attention in light of the current situation where only two chemical classes remain effective on most farms.

It is also advisable to always use the correct dose, and to institute quarantine treatment measures for new or visiting horses. The common practice of "treatment upon arrival" may actually accelerate the spread of resistance if the treated horse is infected with worms resistant to that drug. In such instances, horses will shed pure-resistant eggs for several weeks following treatment. Furthermore, most available drug treatments do not kill the mucosal larval stages, which usually are much more numerous than the lumenal adults. Therefore, even if a new arrival is treated with an effective drug that kills all adult worms infecting that horse, over the next few weeks as the mucosal larval worms mature into adults, a new round of egg shedding will occur. Because these eggs will be derived from the population of worms brought by the horse to its new location, any drug-resistant worms infecting that horse will rapidly contaminate the new environment with drug-resistant infective larvae. For these reasons, long-term additions should be treated upon arrival with a larvicidal drug (moxidectin, fenbendazole double dose $\times 5$ days) to remove as much of the total worm burden as possible. The efficacy of the larvicidal fenbendazole regimen against benzimidazole-resistant cyathostomes has not been established, but long-term benzimidazole treatment has been documented to overcome resistance in gastrointestinal nematodes of sheep [2, 45]. However, it is the experience of the author that most horses treated with larvicidal doses of fenbendazole continue to shed cyathostome eggs following treatment. For this reason, if fenbendazole is used for larvicidal therapy, a single dose of ivermectin or moxidectin should also be administered at the end of the 5-day regimen to remove remaining lumenal worms. Short-term additions (less than six weeks) can be treated with a single dose of ivermectin since the egg reappearance period following ivermectin treatment is six to eight weeks and ivermectin continues to demonstrate virtually 100% efficacy against lumenal cyathostomes.

## 7. CONCLUDING REMARKS

The importance of cyathostomes continues to increase, because (1) extensive reliance on drug treatment has led to the development of resistance to all classes of available anthelmintics except the avermectin/milbemycins and (2) the market for anthelmintics in host species that are plagued by resistance (horses, sheep, goats) is perceived by the pharmaceutical industry as being too small to sustain a discovery program [42]. It is extremely unlikely, therefore, that new anthelmintics with novel modes of action will be developed and marketed in the foreseeable future [44]. Because reversion to susceptibility does not appear to occur, the aim of resistance control must be to delay the accumulation of resistance genes [86]. While this can be achieved (at unknown levels) by following published recommendations, currently there are no means to measure this accumulation of resistance genes. Since virtually nothing is known about cyathostome genes involved in anthelmintic resistance, gaining basic knowledge in this area is a critical need. Without such knowledge, genotypic diagnosis of anthelmintic resistance will not become possible, leaving phenotypic detection of resistance (treatment failure) as the only alternative. Considering the nature of the equine industry in which horses

often graze shared pastures with horses from diverse locations, transmission and widespread dispersal of resistant parasites is virtually assured. A proactive approach to this problem centered on understanding the molecular basis of anthelmintic resistance in cyathostomes is required if we are to expect chemical control of nematodes in horses to remain a viable element of parasite control in the future.

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