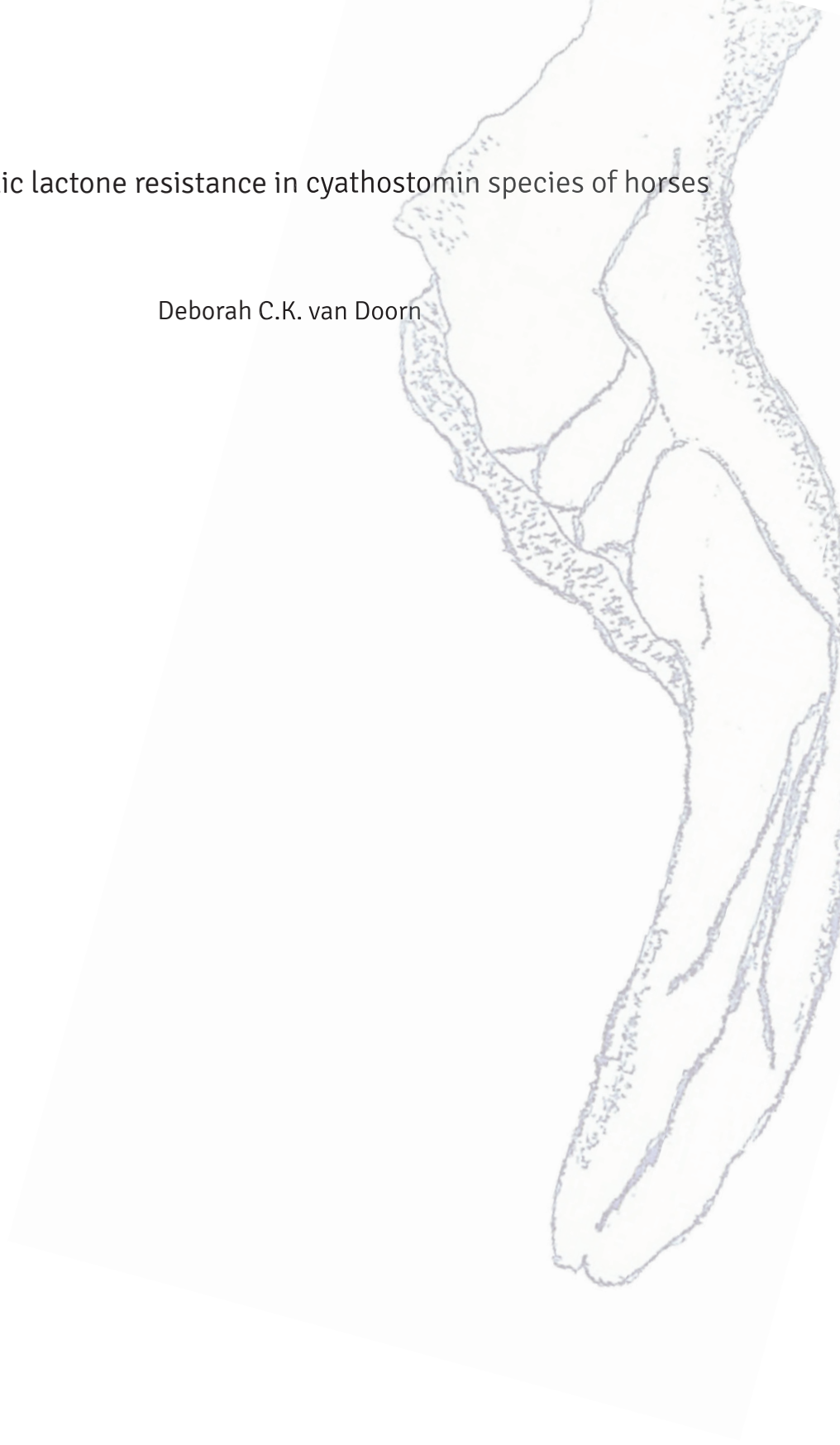
Macrocyclic lactone resistance in cyathostomin species of horses

Deborah C.K. van Doorn



Printing of this thesis was financially supported by:
Faculty of Veterinary Medicine, Utrecht University
Infection & Immunity Utrecht
Virbac Animal Health
Zoetis B.V.

Graphic design by: www.Sitestorm.nl, Deventer

Prints on inside based on line etching:
bursa of *Cylicostephanus longibursatus*
by Wim Hendrikx.

Print on cover:
Line etching and aquatint: *Cyathostomum tetracanthum*
by Wim Hendrikx.
10.5 x 11.7 cm.

ISBN/EAN: 978-90-393-6138-2

Macrocyclic lactone resistance in cyathostomin species of horses

Macrocyclisch lacton resistentie van cyathostominae soorten bij paarden

Proefschrift

ter verkrijging van de graad van doctor aan de Universiteit van Utrecht op gezag van de rector magnificus prof. dr. G.J. van der Zwaan, ingevolge het besluit van het college voor promoties in het openbaar te verdedigen op dinsdag 27 mei 2014 des middags te 12.45 uur

door

Deborah Cynthia Karolina van Doorn

geboren op 4 april 1969

te Hengelo (O)

Promotor:	Prof. dr. J.A. Wagenaar
Co-promotoren:	Dr. Ir. H.W. Ploeger
	Dr. F.N.J. Kooijman

Contents

Chapter 1	General introduction	7
Chapter 2	Searching for ivermectin resistance in Dutch horses	21
Chapter 3	Decreased Egg Reappearance Period after treatment with ivermectin and moxidectin in horses in Belgium, Italy and The Netherlands	31
Chapter 4	*Cylicocyclus* species predominate during shortened Egg Reappearance Period in horses after treatment with ivermectin and moxidectin	45
Chapter 5	Practices as performed by veterinarians before and after anthelmintics became prescription only medicines for horses in the Netherlands	69
Chapter 6	*In vitro* selection and differentiation of ivermectin resistant cyathostomin larvae	83
Chapter 7	Species composition and intra-specific variation in ivermectin susceptibility of cyathostomin larvae obtained from horses with different treatment history	103
Chapter 8	Discussion	127

Summary 146

Samenvatting 150

Dankwoord / Acknowledgements 154

Curriculum Vitae 158

Chapter 1

General introduction

General introduction and thesis outline

This thesis focuses on resistance development of Cyathostominae of horses against the macrocyclic lactones (ML). These helminths belong to the family Strongylidae of the phylum Nematoda. In horses the family Strongylidae can be divided into large strongylids (subfamily Strongylinae) and small strongylids (subfamily Cyathostominae) (Mehlhorn, 2008). In 1998 Lichtenfels et al. proposed a taxonomic classification of the Cyathostominae based on morphological characteristics of the adult worms. However, a study based on molecular analysis of the internal transcribed spacers of ribosomal DNA (Hung et al., 2000) resulted in a different taxonomic division of the Strongylidae than the one proposed by Lichtenfels et al. (1998). Subsequently, the taxonomic criteria for the subfamily Cyathostominae were re-evaluated based on both morphological characteristics as well as molecular data (Lichtenfels et al., 2002). This resulted in the adoption of a classification based on morphological criteria (Lichtenfels et al. 2008), which is followed in this thesis. According to Lichtenfels et al. (2008) the subfamily of Cyathostominae in equids consists of 14 genera among which 50 species can be identified.

Lifecycle and epidemiology of cyathostomin infections

Cyathostominae have a direct lifecycle – no intermediate host is involved – with part of the development inside the host (larval stages 3-5 (L3-L5) and adults) and part (eggs and larval stages 1-3 (L1-L3)) outside the host. Via L1 and L2 the egg will develop into the infective L3. In spring and autumn the development of eggs into L3 will take more time and in winter the development is on hold. L3 on pasture easily survive Dutch winters but embryonated eggs and L1 and L2 are far more sensitive to low temperatures. Ingested L3 penetrate the mucosa of the cecum or ventral colon and hereafter L4 develop within capsules. When the season progresses the development of an increasing proportion of L3 will become inhibited in the (sub)mucosa and these inhibited larvae can remain dormant for years. It is not known if the ability to inhibit larval development in the (sub)mucosa varies between cyathostomin species.
The strongyle population in the horse usually consists for 80-90% of inhibited early L3. After encapsulation L4 migrate towards the lumen and subsequently move to their predilection site (cecum, ventral colon or dorsal colon) where they develop into L5 and hereafter without further molting into reproducing adults. During the following patent period eggs produced by the female worms are shed with the feces.

Figure 1 presents the seasonal EPG (see textbox) pattern for set stocked horses. Egg excretion increases from January until October. If horses are turned out on pasture deposition of eggs can lead to new pasture contamination.

Textbox with Abbreviations

PPP: The Pre Patent Period is defined as the shortest time between uptake of infective larvae and shedding of eggs with the feces.

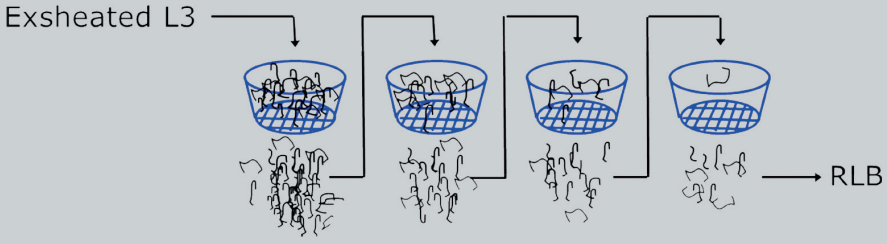
EPG: eggs per gram.

ERP: The Egg Reappearance Period is defined as the period between treatment and the reappearance of strongyle eggs in the feces. The threshold when the ERP finishes and how exactly shortened ERP should be established is not yet properly defined. The EPG threshold ranges from 'first eggs found' to 'mean EPG of 100' or 'individual EPG > 200' or 'eggs >10% of EPG at start, in individuals or as group mean'. The standard ERP is 6 weeks for pyrantel and 8 weeks for ivermectin (IVM). For moxidectin (MOX) it is 12 weeks, according to the label, and that would be the appearance of the first eggs in feces of the most susceptible age group (yearlings).

FECRT: Fecal Egg Count Reduction Test. This implies comparison of the faecal egg counts at or just before anthelmintic treatment with those 14 days after treatment using the formula FECR = 100 x (1-(EPGPost / EPGPre)), where EPGPre and EPGPost represent the faecal egg counts before and after treatment. Usually the arithmetic means of a group of at least 10 horses will be used (Coles et al., 1992), but it may also be performed for smaller groups or for individual horses, when such large groups are not available.

LMIA: is the abbreviation of Larval Migration Inhibition Assay (and is sometimes called LMIT=larval migration inhibition test). The assay is based on paralyzing of larvae by an anthelmintic and the ability of an *in vivo* resistant strain to migrate at a higher concentration of anthelmintic than a susceptible strain. The percentage of larvae that migrate is calculated.

The reiterative Larval Migration Inhibition Assay (rLMIA) is a modification of the LMIA with repeated migrations over 2 or 4 (see figure) consecutive sieves in the presence of IVM. In this way less susceptible L3 can be separated from susceptible L3 and the migration percentages for the consecutive sieves is a measure for the susceptibility of the L3. The separated L3 can be isolated and differentiated with the RLB (see below) to the species level.



Exsheathed L3 (L3 without a sheath) from one population can be separated in ivermectin susceptible and less susceptible larvae by rLMIA with either 2 or in this figure 4 consecutive sieves. The separated L3s can be differentiated to species with the RLB technique.

RLB: Reverse Line Blot. This technique was used to detect the PCR amplified IGS fragments from (a maximum of 21) individual L3. The fragments were hybridized to species-specific probes immobilized to a membrane. Positive signals are visualized by chemo-luminescence and reveal the identity of the L3. In this thesis the RLB is performed on individual larvae.

When temperatures are below 0°C eggs might freeze and no larvae will develop. When the temperature is above 7-10°C eggs develop into larvae and this process speeds up with increasing temperature. In the autumn egg excretion will drop again because adult worms only live for a limited number of months, and most newly ingested L3 will inhibit their development.

Figure 1 also presents the seasonal L3 pattern (L3 per kg grass) on pasture for set stocked horses. From the moment horses are pastured (again) in April/ May, but also when grazing contaminated pastures in winter, infective L3 will be ingested. L3 survive the winter months on pasture but as soon as the temperature rises in spring a decrease in numbers of L3 on pasture is seen. Due to the rising temperature L3 become more active. However, L3 do not feed and, therefore, they will deplete their energy reserves and die if they are not ingested by a host in time. Following pasturing of the horses in spring and renewed pasture contamination with eggs the numbers of L3 increase in summer, although many L3 may die under dry conditions because they need moisture to survive.

Under optimal laboratory conditions cyathostomin development from egg to L3 can occur in less than 7 days. Under field conditions development usually takes more time. The fastest development is seen during the summer months. In Scotland Ramsey et al. (2004) showed that infective larvae can be present on pasture within 2 weeks after eggs are deposited with the feces.

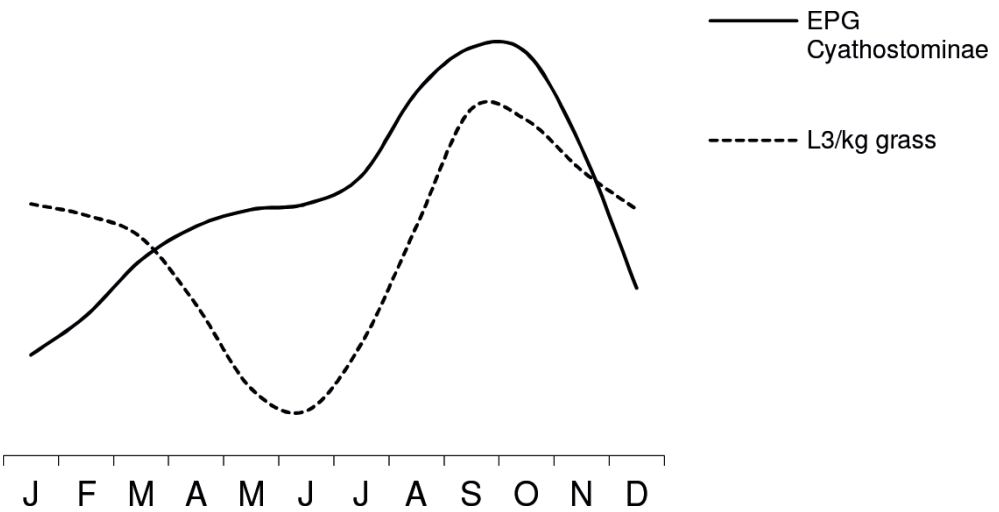


Figure 1: Seasonal pattern of fecal cyathostomin egg excretion of set stocked Shetland ponies and the pasture infestation with cyathostomin larvae (L3 per kg grass) (Mirck, 1981; Eysker, 1989).

Finally, a period that needs mentioning within lifecycle and epidemiology is the pre-patent period (PPP – see textbox). This period starts after a horse ingests infective L3 and different cyathostomin species have different PPPs. For *Gyalocephalus capitatus* a minimum PPP of 28 days was reported and for *Cylicostephanus lontgibursatus* 30 days (Tiunov, 1953 as stated in Ogbourne, 1978). Round (1969) observed a PPP of at least 5 to 6 weeks after experimental infection of cyathostomin-naïve horses, in whom PPPs are shortest. And Love and Duncan (1992) reported a shortest PPP of 53 days after single experimental infections while this was 65 days after trickle infections. The length of the PPP, as well as the egg reappearance period (ERP - see textbox), for different cyathostomin species is important for knowing how soon new eggs can be expected following ingestion of L3 or following anthelmintic treatment.

Clinical signs

The most severe form of disease caused by cyathostomins is called larval or winter cyathostominosis. This form manifests itself mainly in winter and most often horses below 4 years of age will be affected. Winter cyathostominosis is usually restricted to just one or two horses in a herd. Disease occurs when larvae develop massively and break loose of their capsules. Serious weight loss, chronic diarrhea, subcutaneous oedema and fever are the most important clinical signs (Love et al., 1999). Because adult worms may be expelled following a longer period with diarrhea, the fecal egg counts may be zero on many occasions (Reinemeyer, 1986). The L4 may be expelled as well and appear in the feces as red larvae and finding these larvae rather than finding eggs in the feces supports the clinical diagnosis of larval cyathostominosis. Massive penetration of infective larvae also can cause disease, especially in autumn.

Anthelmintics for horses

Worldwide, registered anthelmintics belonging to three drug classes are available for horses: the benzimidazoles, tetrahydropyrimidines and the macrocyclic lactones (ML). In the Netherlands, fenbendazole is currently the only registered drug from the class of benzimidazoles for treatment of horses. This drug inhibits the assembly of microtubules because it binds to -tubulin, the protein that together with -tubulin makes up these microtubules, resulting in starvation of the nematodes by intestinal disruption and inhibition of their egg production (Mehlhorn, 2008). Pyrantel (pamoate or embonate in the Netherlands) is an example of a drug from the tetrahydropyrimidines class and is an acetylcholine agonist for the nicotinic acetylcholine receptor. In helminths this leads to excitation of the muscle cells through activation of nicotinic acetylcholine receptors, resulting in a spastic paralysis (Mehlhorn, 2008).
The ML drug class can be divided in avermectins (for example ivermectin) and milbemycins (for example moxidectin). Ivermectin and moxidectin are the only two registered anthelmintics for horses in this drug class in the Netherlands. Ivermectin (IVM) has no efficacy against encapsulated larvae in the intestinal

(sub)mucosa while moxidectin (MOX) has. MOX has the longest ERP (see textbox) of these anthelmintics probably because it is more lipophilic than IVM and this contributes to a long residual effect. The mode of action of ML relies on the opening of chloride ion channels and the proposed action sites are the glutamate gated chloride channels (GluCl). Opening of these channels will lead to hyperpolarization of neurons (and muscle cells) in nematodes, causing paralysis of the parasites (Mehlhorn, 2008).

Anthelmintic resistance with emphasis on macrocyclic lactone resistance development in horses

Resistance development of strongyles and *Parascaris equorum* against anthelmintics was demonstrated with FECRT (see textbox). In the Netherlands resistance of Cyathostominae against the benzimidazoles (Boersema et al., 1991) and pyrantel (Boersema, 2000 unpublished) was shown. Resistance of *Parascaris equorum* against ML has also been reported (Boersema et al., 2002) and was shown to be associated with the frequency of treatment (van Doorn et al., 2007). For the ML indications for resistance development were found just recently. Trawford et al. (2005) reported reduced efficacy of ML against donkey cyathostomins. Von Samson-Himmelstjerna et al. (2007) reported shortening of the ERP following ML treatment, as compared to the original ERP at the time of introduction of the ML. However, it is difficult to prove that such indications reflect true resistance development if a formal FECRT (Coles et al., 1992) still shows that the ML are highly effective. Indeed, it may also reflect a shift in cyathostomin species composition or a selection for shorter cyathostomin life cycles. Lyons et al (2008, 2009, 2010) found luminal larval stages shortly after IVM or MOX treatment in necropsied horses and suggested that these larval stages showed ML resistance or, alternatively, a selection for shorter life cycles might have occurred. Despite the above considerations, evidence for ML resistance development in adult cyathostomins based on FECRT results have been found more recently (Traversa et al., 2009; Canever et al., 2013).

In vitro detection of ML resistance

Molecular detection of ML resistance is hampered by the fact that the mechanism leading to resistance is still unknown. Furthermore, the existence of over 50 species of cyathostomins in equids, of which the eggs and larvae are morphologically indistinguishable, makes it more difficult to investigate resistance development.

An *in vitro* anthelmintic detection assay would be an asset for studies on resistance. *In vitro* resistance detection models for larvae of sheep, cattle and goat helminths have been developed, such as egg hatch assays and larval migration inhibition assays (LMIA). The LMIA (see textbox) measures larval migration through a sieve in the presence of anthelmintic drugs such as IVM or MOX in a dose-dependent way. The question is to what extent *in vitro* observations on egg or larval stages can be associated with resistance observed

in vivo which often concerns the adult stages. Resistance of *Haemonchus contortus* has been detected with the FECRT and this was shown to be related to LMIA results (Gill et al., 1991). *H. contortus* L3 from resistant strains required higher ML concentrations before their migration became inhibited compared to L3 from susceptible strains. So far, only Robinson et al. (2008) compared FECRT results with LMIA data for the cyathostomins. They showed a negative correlation between FECR after IVM or MOX treatment and LMI50 values (drug concentration that results in 50% inhibition of migration). Although for cyathostomins such evidence is still very scarce, it suggests that *in vitro* observations on resistance might reflect resistance *in vivo*.

Legislative restrictions of anthelmintic usage

In July 2008 new legislation became effective in the Netherlands making anthelmintics prescription only medicines (POM). This legislation was based on a European Union (EU) directive (2001/82/EG and adapted in directive 2004/28/EG) and was implemented in the EU in 2006 (Anonymous, 2006). After 2006 several countries followed up on the EU directive. These countries were Germany, Italy, Finland, the Netherlands, Sweden and the U.K. Because Denmark already had introduced POM legislation for anthelmintics in 1999, this country may serve as an example for the effect of the POM legislation. Six to 7 years after introduction in Denmark questionnaire data showed that Danish veterinarians were very active in performing diagnostics before prescribing an anthelmintic (Nielsen et al., 2006a). The same active role of Danish veterinarians in the control of cyathostominosis was again shown recently (Nielsen et al., 2013). This differs from what has been observed in the Netherlands (van Doorn et al., 2012). As was advocated decades ago, multiple blind treatments without performing diagnostics to see whether such treatments make sense or not, are still practiced a lot. The introduced POM legislation in 2008 has been supported through several activities from Dutch experts, including an article for Dutch veterinarians (Sloet van Oldruitenborgh-Oosterbaan et al., 2009) and post academic education. These activities aimed at increasing the knowledge of veterinarians on the epidemiology of horse parasites, strengthen their involvement in parasite management, how to incorporate the new legislation in their practice and encourage them to start using diagnostics before treating horses. Veterinarians are encouraged to take a leading role in monitoring levels of egg excretion and subsequently take a more rational approach towards the usage and sales of anthelmintics.

Sustainable strategies in the control of cyathostomins

The serious situation of multi-drug resistant nematodes in sheep and the concept of refugia (van Wyk et al., 2001) became exemplary for promoting rational anthelmintic treatment of horses. In order to avoid excessive anthelmintic usage the epidemiology of cyathostomin infections should be taken into account. Furthermore, monitoring EPGs helps to identify the (adult)

horses that contaminate the environment with new parasite eggs the most. Monitoring can therefore be used as a decision tool which horses should be treated with anthelmintics and which horses do not require such treatment based on certain EPG criteria. EPG criteria depend on farm management but in general EPGs of 500 or more are considered to be high and indicative for treatment (www.parasietenwijzer.nl). The article "Frequent deworming in horses: it usually does not do any good, but it often harms" (Eysker et al., 2006 – in Dutch) marked the start of the effort to slow down resistance development in horse nematodes in the Netherlands. Development of "Parasietenwijzer" (Eysker et al., 2007; Ploeger et al., 2008), a website for veterinarians and horse owners about parasites of horses (but also other animal species), was the next step to promote rational anthelminthic usage. Next, Eysker et al. (2008) demonstrated that the costs of monitoring EPGs in horses as a decision tool for treatment could be greatly reduced by examining pooled samples. It was shown that up to 10 fecal samples, based on age clusters, could be pooled and still would produce reliable results.

Under certain management conditions anthelmintic treatment to avoid pasture contamination is unnecessary, for example for horses that are stabled year-round or horses on sand paddocks. But also in case horses have access to pastures, not all horses require frequent treatment. Comer et al. (2006) found that 84% of thoroughbred horses had less than 50 EPG in their feces at the time of a planned treatment at the end of the ERP. Therefore, on many premises the number of treatments could easily be diminished. On the other hand, on some premises regular treatment with an anthelmintic seems to be the only economically feasible option, e.g. on farms raising young horses. After anthelmintic treatment yearling horses will be the first to return to egg-shedding and egg counts will rise fastest in this age group followed by 2 and 3 year olds (Herd and Gabel, 1990; Döpfer et al., 2004). And parasite species other than the cyathostomins may require anthelmintic control as well. Nielsen et al. (2012) suggested that one annual anthelmintic treatment with a ML should be considered to avoid re-emergence of *Strongylus vulgaris*.

Monitoring of egg excretion and subsequent treatment decisions is in general targeted against the heavy egg shedders. Frequent monitoring of all horses may be considered costly and laborious by most horse owners. As mentioned above, the use of age-clustered pooled samples will reduce costs. Moreover, there is a strong correlation between multiple strongyle egg counts of the same horse (Döpfer et al., 2004; Nielsen et al., 2006b; Becher et al., 2010). Therefore, once the heavy egg excreting horses are identified, monitoring can be focused on just these horses.

Besides monitoring and only treating the high egg shedders, horse owners can take other measures to keep pasture contamination low and minimize the

number of anthelmintic treatments. Horse owners and/or stable managers may avoid heavy intake of infective cyathostomin L3 by their young horses by grazing them on clean pastures. This may involve rotational grazing and mowing of pastures, provided sufficient paddocks are available. The contribution of foals to strongyle pasture contamination will in general be minimal and foals therefore do not often require anthelmintic treatment unless targeted against patent infections of *Parascaris equorum*. Another effective measure to keep pasture contamination low would be removing all feces, containing eggs and larval stages, from pasture twice weekly. Finally, grazing horses and sheep alternatingly on pastures may help in keeping pasture contamination low for both horses and sheep (Eysker et al, 1986).

Additional epidemiological background, pathogenesis of helminth infections, monitoring of cyathostomin egg excretion and a decision tree regarding parasite control can be found on the website www.parasietenwijzer.nl, which was developed to support veterinarians and horse owners in controlling parasites on farm-level.

Scope and outline of this thesis

The aims of the thesis were to look at the current status of ML resistance and shortening of the ERP *in vivo* and to study ML resistance development in the cyathostomins of horses using *in vitro* larval migration assays. The role of veterinarians in helminth control and prudent use of anthelmintics was studied by two questionnaires with a 4-year interval.

Overall, the studies presented in this thesis were carried out to contribute in increasing the awareness of veterinarians and horse owners regarding anthelmintic resistance, the need for nematode monitoring schemes on farm-level, as well as the need for rational parasite control.

Current status of ML resistance and involvement of practitioners

In **chapter 2** a survey is described investigating *in vivo* ivermectin resistance in the Netherlands. In total on 70 farms the FECRT was performed according to the guidelines of the WAAVP (Coles et al., 1992). In **chapter 3** a study on the occurrence of shortening of the ERP with respect to both IVM and MOX in 3 European countries is described. From this study two farms with horses showing shortened ERP after ML treatment were selected and used for the study described in **chapter 4**. Following treatment of 21 horses on the selected two farms, larvae were cultured and identified to species level at several points in time for up to 56 (IVM) or 84 days (MOX) to study the temporal reappearance of different cyathostomin species after treatment. In **chapter 5** two questionnaires, with a 4 year interval, were taken among veterinarians to determine if the POM legislation of anthelmintics and supporting measures improved the prudent use of these medicines and increased the expertise of those veterinarians on nematode infections and their control in horses.

In vitro studies on susceptibility of larvae to IVM and differences between species

In **chapter 6** an *in vitro* larval migration test was developed to separate IVM susceptible from less susceptible cyathostomin larvae. We used larvae from 2 cyathostomin populations, one originating from frequently ML treated horses and one originating from horses that have never been treated. Larval migration was tested in the presence or absence of IVM over 4 consecutive sieves. Individual larvae were differentiated to species level with a reverse line blot technique to investigate possible differences in species compositions and susceptibilities between species. In **chapter 7** the *in vitro* larval migration assay, using only the maximum concentration of IVM (as described in chapter 6) and 2 sieves, was used to select less susceptible cyathostomin larvae from populations with different treatment histories, including populations very frequently exposed to ML and populations that never had been exposed to ML. Again, individual larvae were identified to species level by reverse line blot to investigate differences in susceptibility within and between cyathostomin species.

Finally, in **chapter 8** the major findings and conclusions are summarized and discussed.

References

Anonymous, 2006. Implementing Directive 2001/82/EC of the European Parliament and of the Council as regards the establishment of criteria for exempting certain veterinary medicinal products for food-producing animals from the requirement of a veterinary prescription. In: Union, E. (Ed.), vol. 2006/130/EF. Brussels.

Becher A.M., Mahling M., Nielsen M.K., Pfister K. 2010. Selective anthelmintic therapy of horses in the Federal states of Bavaria (Germany) and Salzburg (Austria): an investigation into strongyle egg shedding consistency. Vet. Parasitol. 15, 116-122.

Boersema J.H., Borgsteede F.H.M., Eysker M., Elema T.E., Gaasenbeek C.P.H., van der Burg W.P.J., 1991. The prevalence of anthelmintic resistance of horse strongyles in The Netherlands. Vet. Q. 13, 209–217.

Boersema J.H., Eysker M., Nas J.W.M., 2002. Apparent resistance of *Parascaris equorum* to macrocyclic lactones. Vet. Rec. 150, 279–281.

Canever R.J., Braga P.R.C., Boeckh A., Grycajuck M., Bier D., Molento M.B., 2013. Lack of Cyathostomin sp. reduction after anthelmintic treatment in horses in Brazil. Vet. Parasitol. 194, 35-39.

Coles G.C., Bauer C., Borgsteede F.H.M., Geerts S., Klei T.R., Taylor M.A., Waller P.J., 1992. World Association for the Advancement of Veterinary Parasitology (W.A.A.V.P.) methods for the detection of anthelmintic resistance in nematodes of veterinary importance. Vet. Parasitol. 44, 35-44.

Comer K.C., Hillyer M.H., Colest G.C., 2006. Anthelmintic use and resistance on thoroughbred training yards in the UK. Vet. Rec. 158, 596-598.

Döpfer D., Kerssens C.M., Meijer Y.G.M., Boersema J.H., Eysker M., 2004. Shedding consistency of strongyle-type eggs in Dutch boarding horses. Vet. Parasitol. 124, 249-258.

Eysker M., Jansen J., Mirck M.H., 1986. Control of strongylosis in horses by alternate grazing of horses and sheep and some aspects of the epidemiology of Strongylidae infections. Vet. Parasitol. 19, 103-115.

Eysker M. 1989. Chapter 25: Paardachtigen. In: Janssens, P.G., Vercruysse, J., Jansen, J. (Eds.) Wormen en wormziekten. Alphen aan den Rijn/Brussel, Samsom Stafleu.

Eysker M., van Doorn D.C.K., Lems S.N., Weteling A., Ploeger H.W., 2006. Vaak ontwormen bij paarden: het baat meestal niet maar het schaadt vaak wel. Tijdschr. Diergeneesk. 131, 524-530.

Eysker M., van Doorn D.C.K., Ploeger H.W., 2007. Decision tree for worm control in horses. Proc. of the 21st Int. Conf. of the WAAVP, Gent, Belgium .

Eysker M., Bakker J., Berg M. van de, Doorn D.C.K. van, Ploeger H.W., 2008. The use of age-clustered pooled faecal samples for monitoring worm control in horses. Vet. Parasitol. 151, 249-255.

Gill J.H., Redwin J.M., van Wyk J.A., Lacey E., 1991. Detection of resistance to ivermectin in *Haemonchus contortus*. Int. J. Parasitol. 2, 771–776.

Herd R.P., Gabel A.A., 1990. Reduced efficacy of anthelmintics in young compared with adult horses. Equine Vet. J. 22, 164–169.

Hung G.C., Chilton N.B., Beveridge I., Gasser R.B., 2000. A molecular systematic framework for equine strongyles based on ribosomal DNA sequence data. Int. J. Parasitol. 30, 95–103.

Lichtenfels J.R., Kharchenko V. A., Krecek R.C., Gibbons L.M., 1998. An annotated checklist by genus and species of 93 species level names for 51 recognised species of small strongyles (Nematoda: Strongyloidea: Cyathostominea) of horses, asses and zebras of the world. Vet. Parasitol. 79, 65–79.

Lichtenfels J.R., Gibbons L.M., Krecek R.C., 2002. Recommended terminology and advances in the systematics of the Cyathostominea (Nematoda: Strongyloidea) of horses. Vet. Parasitol. 107, 337–342.

Lichtenfels J.R., Kharchenko V.A., Dvojnos G.M., 2008. Illustrated identification keys to strongylid parasites (strongylidae: Nematoda) of horses, zebras and asses (Equidae). Vet. Parasitol. 156, 4-161.

Love S., Duncan J., 1992. Development of cyathostome infection of helminth naïve foals. Equine Vet J. 13, 93-98.

Love S., Murphy D., Mellor D., 1999. Pathogenicity of cyathostome infection. Vet. Parasitol. 85, 113-122.

Lyons E.T., Tolliver S.C., Ionita M., Lewellen A., Collins S.S., 2008. Field studies indicating reduced activity of ivermectin on small strongyles in horses on a farm in Central Kentucky. Parasitol. Res. 103, 209-215.

Lyons E.T., Tolliver S.C., Collins S.S., 2009. Probable reason why small strongyle EPG counts are returning 'early' after ivermectin treatment of horses on a farm in Central Kentucky. Parasitol. Res. 104, 569-574.

Lyons E.T., Tolliver S.C., Kuzmina T.A., Collins S.S., 2010. Critical tests evaluating efficacy of moxidectin against small strongyles in horses from a herd for which reduced activity had been found in field tests in Central Kentucky. Parasitol. Res. 107, 1495-1498.

Mehlhorn H. (Ed), 2008. Encyclopedia of Parasitology: A-M, Volumes 1-2, 3rd ed., Dusseldorf, Germany, Springer.

Mirck M.M.H., 1981. An investigation into the epidemiology of Strongylidae infections in the horse in The Netherlands. Vet. Q. 3, 98–100.

Nielsen M.K., Monrad J., Olsen S.N., 2006a. Prescription-only anthelmintics; a questionnaire survey of strategies for surveillance and control of equine strongyles in Denmark. Vet. Parasitol. 135: 47-55.

Nielsen M.K., Haaning, N., Olsen S.N., 2006b. Strongyle egg shedding consistency in horses on farms using selective therapy in Denmark. Vet. Parasitol. 135,333-335.

Nielsen M.K., Vidyashankar A.N., Olsen S.N., Monrad J., Thamsborg S.M., 2012. *Strongylus vulgaris* associated with usage of selective therapy on Danish horse farms-is it reemerging? Vet. Parasitol. 189, 260-266.

Nielsen M.K., Reist M., Kaplan R.M., Pfister K., van Doorn D.C.K., Becher A., 2013. Equine parasite control under prescription-only conditions in Denmark – awareness, knowledge, perception, and strategies applied. Vet. Parasitol. DOI:10.1016/j. vetpar.2013.10.016.

Ogbourne C.P., 1978. Pathogenesis of cyathostome (Trichonema) infections of the horse. A review. CAB abstracts. CIH miscellaneous publication No 5.

Ploeger H.W., van Doorn D.C.K., Nijsse E.R., Eysker M., 2008. Decision trees on the web – a parasite compendium. Trends in Parasitol. 24, 203-204.

Ramsey Y. H., Christley R. M., Matthews J. B., Hodgkinson J. E., McGoldrick J., Love S., 2004. Seasonal development of Cyathostominae larvae on pasture in a northern temperate region of the United Kingdom. Vet. Parasitol. 119, 307-318.

Reinemeyer C.R., 1986. "Small strongyles-Recent advances". The Veterinary clinics of North America. Equine practice 2(2): 281-312.

Robinson A., McArthur C., Burden F.A., Goss L., Trawford A.F., Jackson F., Matthews J.B., 2008. Use of the larval migration inhibition assay to investigate suspected macrocyclic lactone resistant cyathostomin populations. Proc. of the Int. Equine Parasite Drug Resistance Workshop. Copenhagen Denmark.

Round M.C., 1969. The prepatent period of some horse nematodes determined by experimental infection. J. Helminthol. 43, 185-192.

Sloet van Oldruitenborgh-Oosterbaan M.M., van Doorn D.C.K., Holland W., van Herten J., Ploeger H.W., Fink-Gremmels J., 2009. Antiparasitaire middelen en de receptplicht voor paarden. Tijdschr. Diergeneesk. 134, 288-295.

Traversa D., von Samson-Himmelstjerna G., Demeler J., Milillo P., Schürmann S., Barnes H., Otranto D., Perrucci S., di Regalbono A.F., Beraldo P., Boeckh A., Cobb R., 2009. Anthelmintic resistance in cyathostomin populations from horse yards in Italy, United Kingdom and Germany. Parasit. Vectors 2 Suppl. 2:S2.

Trawford A.F., Burden F.A., Hodgkinson J.E., 2005. Suspected moxidectin resistance in cyathostomes in two donkey herds at the donkey sanctuary, UK. Proc. of the 20th Int. Conf. of the WAAVP, Christchurch NZ, 16-20 Oct.

van Doorn D.C.K., Ploeger H.W., Eysker M., 2007. Resistance of *Parascaris equorum* against ivermectin due to frequent anthelmintic treatment of foals in the Netherlands. Proc. of the 21st Int. Conf. of the WAAVP, Gent, Belgium.

van Doorn D.C., Eysker, M., Kooyman, F.N., Wagenaar, J.A., Ploeger, H.W., 2012. Searching for ivermectin resistance in Dutch horses. Vet. Parasitol. 185, 355-358.

van Wyk J.A., 2001. Refugia-overlooked as perhaps the most potent factor concerning the development of anthelmintic resistance. Onderstepoort J. Vet. Res. 68, 55–67.

von Samson-Himmelstjerna G., Fritzen B., Demeler J., Schurman S., Rohn K., Schnieder T., Epe C., 2007. Cases of reduced cyathostomin egg-reappearance period and failure of *Parascaris equorum* egg count reduction following ivermectin treatment as well as survey on pyrantel efficacy on German horse farms. Vet. Parasitol. 144, 74-80.

Chapter 2

Searching for ivermectin resistance in Dutch horses

D.C.K van Doorn*, M. Eysker, F.N.J. Kooyman, J.A. Wagenaar, H.W. Ploeger

Department of Infectious Diseases and Immunology, Faculty of Veterinary Medicine, Utrecht University, Yalelaan 1, 3584 CL, Utrecht, the Netherlands

** Corresponding author: phone +31302532459*
e-mail address d.c.k.vanDoorn@uu

Published: Veterinary Parasitology 185, 355-358

2

Abstract

A study was conducted to evaluate the occurrence of resistance against, in particular, ivermectin in cyathostomins in the Netherlands. Seventy horse farms were visited between October 2007 and November 2009. In initial screening, faecal samples were collected 2 weeks after deworming with either ivermectin, moxidectin or doramectin. Pooled faecal samples from a maximum of 10 horses were examined for worm eggs using a modified McMaster technique and for worm larvae after faecal larval cultures. In total 931 horses were involved. On 15 of 70 farms eggs and/or larvae were found. On 8 of these 15 farms a FECRT with ivermectin was performed on 43 horses. Efficacy of ivermectin against cyathostomins of 93% was found in one animal on one farm. Additionally, the strategies and efforts of the horse owners to control cyathostomins, as well as risk factors for the development of macrocyclic lactone resistance were evaluated with a questionnaire. Strikingly, many responders indicated that the control of cyathostomins in horses is achieved through very frequent deworming. Fourteen percent of these owners deworm seven times per year or more. On 34% of the 70 farms treatment was repeated within the Egg Reappearance Period of a product.

Introduction

Cyathostomins are the most prevalent horse helminths, with 50 species found in equids worldwide (Lichtenfels et al., 2008). Disease may occur in horses of all ages but young horses (1 to 4 year old) are more susceptible. To control infections, management measures should be applied (e.g. pasture rotation and reducing grazing density) supported by the use of anthelmintics. Several classes of anthelmintics have been approved for use against cyathostomins in horses. Benzimidazoles were introduced as anthelmintics for horses in the 1960's and pyrantel in 1970. The first representative of the macrocyclic lactones (MLs), ivermectin, was introduced in the early 1980s and moxidectin in the middle of the 1990s. (Pro)benzimidazole resistant cyathostomins are described since the 1960s (Drudge and Elam, 1961) and in the 90s the first reports on pyrantel resistance appeared (Chapman et al., 1996). In 1994 Xiao showed that the efficacy of ML against adult cyathostomins was still 99% (Xiao et al., 1994). Worldwide, several recent studies showed shortening of the Egg Reappearance Period (ERP) (von Samson Himmelstjerna et al., 2007; Lyons et al., 2008; Molento et al., 2008) which is considered to indicate resistance (Lyons et al., 2009). Once resistance is present, spreading of the resistant cyathostomin populations is likely by transport of horses.

In the Netherlands, as anywhere else, owners and veterinary practitioners rely heavily on frequent anthelmintic treatments to avoid disease. Therefore, it is not unlikely that reduced ML efficacy also may have developed in the Netherlands. In the present study we conducted a survey to evaluate the presence of ivermectin resistant cyathostomins in Dutch horses. On the same farms, owners were questioned about their worm control practice to determine a possible association between worm control intensity and presence of ML resistance.

Materials and Methods

Selection of farms

Between October 2007 and November 2009, 159 horse farms throughout the Netherlands were selected using an internet search. Horse farmers were asked to participate in a trial to evaluate the efficacy of MLs against strongylids. A farm was included if they had three horses or more that were kept on pasture together and the last used wormer was a macrocyclic lactone. Participating farms were included for a visit if deworming fell within one of three periods (October-December period 2007 and 2009 and April-July 2008). Of the 159 approached farms 66 were visited. In addition, 30 veterinary clinics were approached to more efficiently seek farms with positive faecal egg counts after deworming. This led to four more farms that were visited. This study was not designed as a cross-sectional study as farms were not randomly selected.

Questionnaire

During the first visit, the owners were asked questions about the farm, pasture management and housing, deworming and parasitic problems. This was based on the questionnaire of Osterman-Lind et al. (2007).

Screening

Fourteen days after deworming 70 farms were visited for the first time to collect faecal samples. The samples were pooled with a maximum of 10 horses per sample (Eysker et al., 2007). On several larger farms faecal material for more than one pooled sample (2-6) was collected. In total 115 pooled faecal samples were examined. The decision on how the samples were pooled depended on how the groups of horses were maintained on pasture.
Pools consisted of 3.0 g from each individual sample and these were mixed thoroughly. A modified McMaster method with saturated NaCl solution (MAFF, 1986) was done with 3.0 g faeces from the pooled sample and 4 chambers were counted per pooled sample, resulting in a detection limit of 25 eggs per gram of faeces (EPG). For the pooled cultures each horse contributed equal amounts of faeces to a thoroughly mixed sample from which 25 g was cultured in duplicate in glass jars for 10-12 days at 21°C (Borgsteede and Hendriks, 1973). After the culture period the jars were filled with tap water and turned upside down on Petri dishes. The following day the larvae were collected, differentiated (Thienpont et al.,2003) and counted to determine the numbers of larvae per gram faeces (LPG), with an overall detection limit of 0.04 LPG (or 0.4 LPG per individual horse).

Faecal Egg Count Reduction Test (FECRT)

If screening showed a positive EPG and/or LPG the second and third visit to the farm were planned in order to perform a FECRT. The second visit was planned after the assumed ERP period had elapsed. Faeces from individual horses was examined with the McMaster technique, as well as that 25 gram of faeces from each horse was cultured as described above. Examinations were done on day 0 and day 14 after ivermectin treatment (Eqvalan® or Equimectin®, 200 µg per kg bodyweight). Bodyweight (BW) was estimated using a girth tape (Coles et al., 2006). In some cases, the weight was estimated visually by two people followed by adding 10% to the mean of both visual estimations. This was done by a trained student and the owner because we felt the girth measurement in certain breeds underestimates the actual weight. An efficacy of less than 95% for an individual animal indicated suspected resistance (Craven et al., 1998; Coles et al. 2006). We were able to perform a FECRT on 8 from the in total 15 farms with positive egg counts or larval cultures that were found within the screening.

Results

Screening and FECRT

Fifteen farms (16 pools) had a positive EPG and/or LPG 14 days after deworming. On 8 of these farms an FECRT was performed on individual animals with positive EPG's. Taken together, 931 horses were investigated within the screening procedure and an FECRT was performed on 43 horses. EPG and LPG reduction was 100% on 6 farms (Table 1). On farm 3 (Table 1) a yearling with a reduction in EPG of 93% (from 350 to 25 EPG) was found, while LPG reduction after deworming was 99.6%. The last dewormer that was used by the farmer turned out to be doramectin, a non-registered product for horses. On farm 7 a 3 year old Shetland pony stallion had a LPG of 1105 before and 5 LPG after deworming (Table 1).

FECRT was not performed on all 15 farms with positive egg counts or positive larval cultures because either EPG was too low or the farmer did not cooperate.

Table 1: Faecal Egg Count Reduction Test and larval culture counts on 43 horses from eight farms. After deworming EPG or LPG was mainly zero for most horses on a farm. Otherwise this was called individual EPG or LPG.

Farm	Number of horses on the farm	Number of FECRT horses	Before deworming				After deworming			
			Mean EPG	EPG range	Mean LPG	LPG range	Mean EPG	Mean LPG	Individual EPG	Individual LPG
1	8	5	130	25-750	108	5-450	0	0	0	1
2	40	3	300	25-700	242	10-450	0	0	-	-
3	24	7	300	50-1350	111	18-1300	3.6	0.014	25	0.1
4	55	12	250	50-1025	456	0-1640	0	0	-	-
5	110	12	444	75-1850	1094	100-3750	0	0	-	-
6	25	1	200	-	30	-	0	0	-	-
7	10	2	1450	700-2200	554	3-1105	0	2.5	0	5
8	70	1	4300	-	520	-	0	0	-	-

Questionnaire
From the 159 farms that were contacted by phone, 110 (70%) were willing to cooperate. We could not include all of the 110 farms in the screening and FECRT study because the deworming schedule not always allowed a first visit during our three sampling periods.

The mean number of animals on the 70 farms that were investigated was 61 (5-400). Half of the farms that were investigated were raising farms that stock yearlings, 2 and 3 year olds for a couple of years before they return to their owners. Five (7%) from the 70 investigated farms had more than 0.5 ha grazing land per horse available. Fifteen farms (21%) said they remove faeces from pasture regularly, 9 said they do this at least once every 2 weeks. Forty-six owners (66%) indicated that they had problems with cyathostomins in the recent past and this was based on clinical signs only. Faecal examination before considering deworming (monitoring) was routinely done on just 2 farms (3%), sometimes on 25 farms (36%) and never on 42 farms (61%).
Overall mean number of treatments was 5 times per year (range between 2 and 10 times). Anthelmintic doses were established on 2 farms (3%) by weighing with a scale. On 18 farms (26%) body weight was estimated prior to dosing. The other 50 horse caretakers (71%) and/or veterinarians apparently did not consider the weight of the horses in relation to the amount of product that had to be applied. The last treatment was performed with ivermectin on 38 farms (54%), with moxidectin on 29 (41%), and with doramectin on 2 farms (3%). On 1 farm they were not sure whether they used ivermectin or moxidectin. Twenty farms (29%) always use the same active ingredient, on 2 farms (3%) the care takers did not know which ingredients normally were used and the other 48 farms (69%) rotated active substances or thought they did. On 34% of these premises treatment was repeated within the Egg Reappearance Period of a product. Half of the 70 farms treated all the horses at the same time. Just over 50% of the farmers kept new horses in quarantine, and from these horses about 40% stayed in quarantine for more than 2 weeks.

Discussion and conclusion

To assess whether ivermectin resistance is present in the Netherlands we conducted a survey. Because we were merely interested in the occurrence of ivermectin resistance rather than actual prevalence, we included 35 raising farms in our screening procedure. In young animals strongyle eggs return sooner after deworming and egg counts get higher than in adult animals (Döpfer et al., 2004). For screening purposes the approach by using pooled samples allowed to include more farms compared to the more labour intensive FECRT. Furthermore, FECRT has shown to be an insensitive technique to detect anthelmintic resistance. Using larval cultures within the screening procedure and FECRT helps to accomplish higher sensitivity (Bello and Allen, 2009).

Within the screening procedure 15 farms were found positive, with either eggs or larvae after treatment with a ML. However, FECRT performed on 8 out of these 15 farms, did not result in proven resistance on these farms. Just one horse showed a 93% efficacy in the FECRT. Underdosing of the horses by the owners may have led to the positive faecal examinations within the screening procedure. Underdosing was excluded within the FECRT procedure, which may account for the fact that less positive FEC and/or larval cultures were found. Since both used products were found efficacious as measured with FECRT, no difference between original and generic product was found.

Although ivermectin resistance could not be demonstrated in the present study, it should be noted that shortening of the ERP after use of a ML is probably the first indication of cyathostomin resistance (Lyons et al., 2009). As the used method did not allow for registering this shortening of the ERP, developing resistance towards ivermectin might have been missed. Therefore yearly screening for developing resistance should be applied not only 2 weeks but also 4 or 5 weeks after treatment with a ML.

The results from the questionnaire were comparable with those from Osterman-Lind et al. (2007) and Von Samson-Himmelstjerna et al. (2009) and showed that horse owners/farmers were lacking knowledge on the epidemiology of cyathostomins, pasture management, and time and frequency of deworming for an efficient worm control policy. The frequency of treatment was, with an average of 5 times per year, high on most farms. One farm, for example, used moxidectin once every six weeks whereas the product is registered for use against strongylids once every 12 weeks in the Netherlands. Pasture rotation on the questioned 70 farms was always aimed at providing sufficient grass for the horses. However, none of the farmers tried to use pasture rotation as a means of worm control. Only 9 out of the 15 farms that said they remove faeces from pasture regularly did this at least once every 2 weeks. Removing faeces from pasture would lower the infection pressure enormously but probably needs to be done every week to keep the pasture sufficiently safe (Herd, 1986). Faecal examination before deworming (monitoring) was only done on 2 farms. A large majority of adult horses will have a negative EPG at the time of deworming if this is based on ERP (Comer et al., 2006). Half of the 70 visited farms were raising farms and blind treatments based on the ERP may be acceptable in young animals, in particular yearlings, considering that egg counts usually return rapidly to fairly high levels after the ERP. Considering that 34% of these 70 owners dewormed frequently within the ERP or in the winter when the horses were not on pasture, frequency of deworming could have been lower on most of these farms (Döpfer et al., 2004).

Fifty percent of the farms treated all horses present on the farm at the same time and by doing so were increasing the selection pressure for anthelmintic resistance. In a group of horses the individuals with low egg shedding should preferably be left untreated to create refugium (van Wyk, 2001). Newly introduced horses should be kept in quarantaine and after this period not turned out on pasture until a faecal check is performed. Clearly, not all horse owners did apply proper quarantine schemes.

Despite the intensive use of ML's, we were not able to detect ivermectin resistance with FECRT, apart from an apparent reduced efficacy of 93% in just one horse.

Acknowledgements

We would like to thank Eva de Vries, DVM, Jasper Simons, DVM and Muriel Paauwe, DVM, and the horse owners for their collaboration, and the Ministry of Economics, Agriculture and Innovation for their financial support.

References

Bello T.R., Allen T.M., 2009. Comparison of two fecal egg recovery techniques and larval culture for cyathostomins in horses. AJVR 70, 571-573.

Borgsteede F.H.M, Hendriks J., 1973, Een kwantitatieve methode voor het kweken en verzamelen van infectieuze larven van maagdarmwormen. Tijdschr. Diergeneeskd. 98, 280–285.

Chapman M.R., French D.D., Monahan C.M., Klei T.R., 1996. Identification and characterization of a pyrantel pamoate resistant cyathostome population. Vet. Parasitol. 66, 205-212.

Coles G.C., Jackson, F., Pomroy W.E., Prichard R.K., Von Samson-Himmelstjerna, G., Silvestre, A., Taylor, M.A., Vercruysse J., 2006. The detection of anthelmintic resistance in nematodes of veterinary importance. Vet. Parasitol. 136, 167-185.

Comer K.C., Hillyer M. H., Coles G.C., 2006. Anthelmintic use and resistance on thoroughbred training yards in the UK. Vet. Rec. 158, 596-598.

Craven J., Bjorn H., Henriksen S.A., Nansen P., Larsen M., Lendal S., 1998. Survey of anthelmintic resistance on Danish horse farms using 5 different methods of calculating faecal egg count reduction. Equine Vet. J. 30, 289-293.

Döpfer D., Kerssens C.M., Meijer Y.G.M., Boersema J.H., Eysker M., 2004. Shedding consistency of strongyle-type eggs in Dutch boarding horses. Vet. Parasitol. 124, 249-258.

Drudge J.H., Elam G., 1961. Preliminary observations on the resistance of horse strongyles to phenothiazine. J. Parasitol. 47, 38-39.

Eysker M., Bakker J., van de Berg M., van Doorn D.C.K., Ploeger H.W., 2007. The use of age-clustered pooled faecal samples for monitoring worm control in horses. Vet. Parasitol. 151, 249-255.

Herd R.P., 1986. Epidemiology and control of equine strongylosis at Newmarket. Equine Vet. J., 18, 447-452.

Lichtenfels, J.R., Kharchenko, V.A., Dvojnos, G.M., 2008. Illustrated identification keys to strongylid parasites (strongylidae: Nematoda) of horses, zebras and asses (Equidae.). Vet. Parasitol. 156, 4-161

Lyons E.T., Tolliver, S.C., Ionita, M., Lewellen, A., Collins, S.S., 2008. Field studies indicating reduced activity of ivermectin on small strongyles in horses on a farm in Central Kentucky. Parasitol. Res. 103, 209-215.

Lyons E.T., Tolliver S.C., Collins S.S., 2009. Probable reason why small strongyle EPG counts are returning 'early' after ivermectin treatment of horses on a farm in Central Kentucky. Parasitol. Res. 104, 569-574.

Ministry of Agriculture Fisheries and Food (MAFF), 1986. Manual of Veterinary Parasitological Laboratory Techniques: reference Book 418, third ed. HMSO Books, London.

Molento M.B., Antunes J., Bentes R.N., Coles, G.C., 2008. Anthelmintic resistant nematodes in Brazilian horses. Vet. Rec. 162, 384-385.

Osterman Lind E., Rautalinko E., Uggla A., Waller P., Morrison D.A., Höglund J., 2007. Parasite control practices on Swedish horse farms. Acta Vet. Scand. 49, 25.

Thienpont D., Rochettte F., VanParijs O.F.J., 2003. Diagnosing Helminthiasis by Coprological Examination, 3rd ed, Janssen Research Foundation, Belgium.

von Samson-Himmelstjerna G., Fritzen B., Demeler J., Schurmann S., Rohn K., Schnieder T., Epe C., 2007. Cases of reduced cyathostomin egg-reappearance period and failure of *Parascaris equorum* egg count reduction following ivermectin treatment as well as survey on pyrantel efficacy on German horse farms.
Vet. Parasitol. 144, 74-80.

von Samson-Himmelstjerna G., Traversa D., Demeler J., Rohn K., Milillo P., Schurmann S., Lia R., Perrucci S., Frangipane di Regalbono A., Beraldo P., Barnes H., Cobb R., Boeckh A., 2009. Effects of worm control practices examined by a combined faecal egg count and questionnaire survey on horse farms in Germany, Italy and the UK, Parasites Vectors 2, S3.

Wyk van J.A., 2001. Refugia-overlooked as perhaps the most potent factor concerning the development of anthelmintic resistance. Onderstepoort J. Vet. Res. 68, 55–67.

Xiao L., Herd R.P., Majewski G.A., 1994. Comparative efficacy of moxidectin and ivermectin against hypobiotic and encysted cyathostomes and other equine parasites. Vet. Parasitol. 53, 83-90.

Chapter 3

Decreased egg reappearance period after treatment with ivermectin and moxidectin in Belgium, Italy and the Netherlands

Thomas Geurden[1], Deborah van Doorn[2], Edwin Claerebout[3], Frans Kooyman[2], Sofie De Keersmaecker[1], Jozef Vercruysse [3], Bruno Besognet[1], Bindu Vanimisetti[1], Antonio Frangipane di Regalbono[4], Paola Beraldo[5], Angela Di Cesare[6], Donato Traversa[6]

1 Zoetis, Mercuriuslaan 20, 1930 Zaventem, Belgium
2 Department of Infectious Diseases and Immunology, Faculty of Veterinary Medicine, Utrecht University, Yalelaan 1, 3584 CL Utrecht, the Netherlands
3 Laboratory for Parasitology, Faculty of Veterinary Medicine, Ghent University, Salisburylaan 133, 9820 Merelbeke, Belgium
4 Department of Animal Medicine, Production and Health, University of Padova, Viale dell'Università 16, 35020 - Legnaro (PD), Italy
5 Faculty of Veterinary Medicine, University of Udine, Via delle Scienze 206, 33100 - Udine, Italy
6 Faculty of Veterinary Medicine, University of Teramo, Piazza A. Moro 45, 64100 - Teramo, Italy

Decreased Egg Reappearance Period after treatment with ivermectin and moxidectin in horses in Belgium, Italy and The Netherlands

Abstract

Reason for performing the study was to evaluate the occurrence of anthelmintic resistance after an oral treatment with ivermectin (IVM) or moxidectin (MOX) against gastro-intestinal Cyathostominae in naturally infected horses. Objectives were to perform a faecal egg count reduction test (FECRT) and to monitor the egg reappearance period (ERP) after treatment. A field study with a randomised complete block design for each study site was performed.

At least 10 study sites in Italy, Belgium and the Netherlands were selected and animals were allocated to one of two treatment groups based on the pre-treatment faecal egg counts (FEC). Animals were treated on Day 0 with either IVM (at 0.2 mg/kg bodyweight) or MOX (at 0.4 mg/kg bodyweight). After treatment, faecal samples were collected at least every fortnight during 56 days after treatment with IVM and during 84 days after MOX treatment. In total, 320 horses on 32 farms were examined. The FECRT on Day 14 indicated a 100% efficacy in 59 of the 64 treatment groups and >92% efficacy in the remaining 5 groups. The ERP was decreased for at least one of the anthelmintics on 17 out of 32 study sites (15 for MOX and 17 for IVM) and on 9 sites (28%) the ERP was decreased for both anthelmintics.

The present study reports a high efficacy of MOX and IVM in a FECRT 14 days after treatment, yet does indicate a shortened ERP for these treatments in more than half of the selected study sites. The results outline that a FECRT after treatment should be combined with additional efficacy evaluation at a later stage to fully understand the development of anthelmintic resistance on site.

Keywords: horse, Cyathostominae, efficacy, egg reappearance period, moxidectin, ivermectin

Introduction

Small strongyles or Cyathostominae are ubiquitous parasites of grazing horses, and treatment is often required to avoid health or animal welfare issues. Several anthelmintics are commercially available to control these infections, but the ease of use and the over-the-counter availability of cheap formulations has led to a worm management practice in which blanket treatments are preferred over targeted treatments (Relf et al., 2012; van Doorn et al., 2012a). Blanket treatments and treating more frequently than required are considered to contribute to the development of anthelmintic resistance (AR), as these treatment regimes reduce the refugia with susceptible isolates. Furthermore, other management practices contribute to the spread of resistance genes: for instance, more than half of the horse establishments in the UK receive visiting horses and only 3 out of 4 horses are actually dewormed prior to integration (Relf et al., 2012). As a result, AR in horse Cyathostominae has been reported worldwide for benzimidazoles and to a lesser extent for pyrantel. Recent studies on the efficacy of ivermectin (IVM) and moxidectin (MOX) seem to indicate that both anthelmintics are still effective against small strongyles, although treatment failures are reported in a limited number of yards and animals (Traversa et al., 2007, 2009, 2012; Slocombe et al., 2008; Canever et al., 2013; Lester et al., 2013; Stratford et al., 2013). The treatment efficacy in most studies is evaluated shortly after treatment (10-21 days), and might underestimate a potential reduction in anthelmintic efficacy. The egg reappearance period (ERP) after treatment might be an earlier indicator of reduced activity of IVM and MOX. In several studies with IVM in Kentucky (US), the shortened ERP has been related to the low efficacy of treatment against immature small strongyles. This leads to incomplete elimination of cyathostomins in the intestinal lumen or to a reduced time for maturation of the parasite, and hence to a shorter ERP (Lyons et al., 2011, 2013). The objective of the present study was to evaluate the efficacy of oral treatment by a faecal egg count reduction test and by faecal examinations up to 56 days for IVM and 84 days for MOX in Italy, Belgium and The Netherlands.

Materials and Methods

Study design

This study was designed as a field efficacy study in horses naturally infected with cyathostomins. Study sites were selected based on the history of deworming management in the previous years: all study sites had followed a strategy with either regular (at least twice a year) to frequent deworming (more than twice a year). The assessment of efficacy was based on individual faecal egg counts (FEC) before and at defined time points after treatment. The study used a randomised complete block design for each study site, with the individual animal as the experimental unit. The available animals were allocated to blocks of 2 animals defined by the pre-treatment strongyle FEC.

Within each block, animals were randomly allocated to one of the treatment groups. The horses were either treated with ivermectin (IVM: Eqvalan®oral paste at 0.2 mg/kg bodyweight)[a] or moxidectin (MOX: Equest® oral gel, at 0.4 mg/kg bodyweight)[b].

Treatment was administered on Day 0. Animals were identified using an electronic chip, and the weight was estimated using a girth tape. Any misdosing was recorded. All test animals were observed for the duration of the treatments until at least 30 minutes after the last animal was treated. Any abnormal health was recorded.

The diagnostic technique used to monitor the faecal egg excretion was a modified McMaster technique, with a sensitivity of 25 eggs per gram of faeces (epg). Laboratory personnel involved in FECs were masked to the allocation of animals to treatment groups and treatment. From the animals allocated to the IVM group and the MOX group, faecal samples were collected on Days 14, 28, 42 and 56. For the MOX treated animals, additional samples were collected on Days 70 and 84. In The Netherlands, FEC were also performed on Days 21 and 35.

Statistical analysis

The primary outcome measure was the percentage reduction in the arithmetic mean strongyle FEC on Day 14 (after) relative to Day 0 (before): (FEC D0-FEC D14)/FEC D0. A 95% CI around the efficacy was calculated using bootstrap analysis. Anthelmintic efficacy was defined as follows (Coles et al., 1992): a reduction in FEC equal to or higher than 95% with the lower 95% confidence interval (CI) >90% was considered as efficacious. Anthelmintic resistance (AR) was present if (1) the percentage reduction in FEC was less than 95% and (2) the lower 95% confidence interval (CI) was less than 90%. Suspected anthelmintic resistance (SAR) was present if (1) the percentage reduction in FEC was less than 95% or (2) the lower 95% CI was less than 90%. The secondary outcome measure was the length of the ERP. The strongylid ERP was defined as the time between treatment and the last day ≥90% efficacy was found, based on the group arithmetic mean FEC (Larsen et al., 2011).

Results

In Belgium, 100 horses were monitored on 12 different study sites (BE01-BE12) between May and September 2012. Prior to treatment the arithmetic mean FEC in both treatment groups on each study site was above the threshold of 100 epg, ranging from 113 to 1189 epg. The efficacy on Day 14 (Day 28 for BE03) was 100% on all 12 study sites, both for IVM and for MOX. On farm BE05, no faecal samples were collected after Day 56, as the efficacy was below 90% for both compounds. On 5 of the 12 sites the efficacy for both IVM and MOX was above the ERP threshold of 90% during the entire follow-up period, with efficacies

ranging from 98-100%. On the remaining 7 study sites the ERP for IVM and/or MOX was decreased. The IVM efficacy was below 90% from Day 42 on 3 farms (BE04, BE07, BE10) and on Day 56 on 1 farm (BE05). On the remaining 3 farms, the efficacy of IVM was above 90% with efficacies ranging from 92-100%. The reduction after MOX treatment was below 90% from Day 42 onwards on 3 farms (BE04, BE05 and BE10), all with reduced ERP for IVM as well, from Day 70 on 1 farm (BE03) and on Day 84 on 2 farms (BE01 and BE02). On the remaining farm, the efficacy of MOX was above 98% for 84 days. The results for the study in Belgium are presented in Table 1.

Table 1: The efficacy (with 95% confidence interval for the Day 14 efficacy) at different study days (D) after treatment with either moxidectin (MOX) or ivermectin (IVM) for the 12 Belgian (BE) study sites. The number of animals (N) on each study site is listed, as well as the minimum (Min) and maximum (Max) faecal egg count in each treatment group and the arithmetic mean faecal egg count prior to treatment (AM pre). On site BE05, no samples were collected on Day 70 and Day 84 (ND).

Site	Treatment	N	AM pre	Min	Max	D14	D28	D42	D56	D70	D84
BE01	MOX	7	586	100	1250	100	100	99	99	94	77
	IVM	6	450	50	1475	100	100	100	92		
BE02	MOX	4	319	25	925	100	100	98	100	100	69
	IVM	4	256	50	775	100	100	100	93		
BE03	MOX	4	344	125	525	ND	100	98	93	78	64
	IVM	4	556	75	1450	ND	100	100	100		
BE04	MOX	4	163	25	325	100	100	77	54	62	4
	IVM	5	150	25	325	100	93	40	0		
BE05	MOX	7	646	125	1850	100	99	45	0	ND	
	IVM	7	1189	25	5275	100	100	97	70		
BE06	MOX	4	131	50	225	100	100	100	100	100	100
	IVM	5	365	25	1125	100	100	100	100		
BE07	MOX	5	470	75	850	100	100	100	100	98	100
	IVM	6	463	50	1475	100	100	84	71		
BE08	MOX	3	317	225	450	100	100	100	100	100	100
	IVM	3	342	50	550	100	100	98	100		
BE09	MOX	2	963	250	1675	100	100	100	100	100	100
	IVM	2	225	100	350	100	100	100	100		
BE10	MOX	3	142	75	275	100	100	53	18	0	0
	IVM	3	167	25	375	100	100	85	0		
BE11	MOX	2	650	50	1250	100	100	100	100	100	100
	IVM	2	113	100	125	100	100	100	100		
BE12	MOX	4	425	150	975	100	100	100	100	100	100
	IVM	4	275	125	475	100	100	98	100		

In Italy, 100 horses were monitored on 10 different study sites (IT01-IT10) between March and April 2012. Prior to treatment the arithmetic mean FEC in both treatment groups on each study site was above the threshold of 100 epg, ranging from 220 to 1355 epg. The efficacy on Day 14 was 100% for IVM on all farms, except on one study site (IT06) with an efficacy of 96% (95% CI = 81-100%). This lower efficacy at Day 14, defined as SAR, was due to high egg shedding (275 epg) in 1 out of the 5 animals post treatment. On Days 28, 42 and 56, the efficacy of IVM on this study site was 100%, 99% and 92%, respectively. The efficacy of MOX was 100% on all study sites at Day 14. On 6 of the 10 sites in Italy the efficacy of both IVM and MOX was above the ERP threshold of 90% during the entire follow-up period, with efficacies ranging from 91-100% for both IVM and MOX. On the remaining 4 study sites (IT01, IT06, IT08 and IT10) the ERP for IVM and/or MOX was decreased. The efficacy of IVM was below the 90% ERP threshold on 3 farms on Day 56. The efficacy of MOX was below 90% on 1 study sites on Day 84 (IT06) and from Day 42 onwards on another farm (IT01). The results for the study in Italy are presented in Table 2.

Table 2: The efficacy (with 95% confidence interval for the Day 14 efficacy) at different study days (D) after treatment with either moxidectin (MOX) or ivermectin (IVM) for the 10 Italian (IT) study sites. The number of animals (N) on each study site is listed, as well as the minimum (Min) and maximum (Max) faecal egg count in each treatment group and the arithmetic mean faecal egg count prior to treatment (AM pre).

Site	Treatment	N	AM pre	Min	Max	D14	D28	D42	D56	D70	D84
IT01	MOX	5	355	200	550	100	100	86	83	90	82
	IVM	5	360	200	525	100	100	94	79		
IT02	MOX	5	235	150	400	100	100	100	100	100	100
	IVM	5	220	150	300	100	100	100	100		
IT03	MOX	5	250	125	550	100	100	100	100	100	91
	IVM	5	220	100	475	100	100	100	100		
IT04	MOX	5	680	50	1400	100	100	100	99	100	99
	IVM	5	710	75	1400	100	100	95	96		
IT05	MOX	5	420	50	1450	100	100	100	100	100	99
	IVM	5	835	50	2350	100	100	96	91		
IT06	MOX	5	1355	250	2300	100	99	100	98	93	88
	IVM	5	1220	225	2425	96 (81-100)	100	99	92		
IT07	MOX	5	265	100	575	100	100	100	100	100	96
	IVM	5	420	125	1450	100	100	100	98		
IT08	MOX	5	480	125	1000	100	100	100	100	93	100
	IVM	5	445	125	850	100	100	99	89		
IT09	MOX	5	570	125	1250	100	100	100	100	100	100
	IVM	5	530	100	1175	100	100	100	100		
IT10	MOX	5	575	150	1675	100	100	100	100	100	100
	IVM	5	385	200	575	100	100	98	83		

In The Netherlands, 120 horses were monitored on 10 different sites (NE01-NE10) between January and October 2012. Prior to treatment the arithmetic mean FEC in both treatment groups on each study site was above the threshold of 100 epg, ranging from 195 to 1075 epg. The efficacy on Day 14 was 98-100% for IVM on all farms, except on 1 study site (NE08) with an efficacy of 92% (95% CI = 76-100%). On Days 21, 28 and 35, the efficacy of IVM on this study site was 100%. The efficacy of MOX was 98-100% on all study sites at Day 14. On 4 of the 10 study sites the efficacy of both IVM and MOX was above the ERP threshold of 90% during the entire follow-up period, with efficacies ranging from 90-100% for IVM and from 93-100% for MOX. On the remaining 6 study sites the ERP for IVM and/or MOX was decreased. The reduction after IVM treatment was below 90% from Day 28 (NE02), Day 35 (NE05) and Day 42 (NE03) onwards on 1 site each and from Day 56 on 3 sites (NE01, NE07, NE08). The reduction after MOX treatment was below 90% from Day 42 onwards on 1 site (NE02) and from Day 56 on 4 sites (NE01, NE03, NE05, NE08). On the remaining farm, the efficacy of MOX was above 99% for 84 days. The results for the study in The Netherlands are presented in Table 3.

Table 3: The efficacy (with 95% confidence interval for the Day 14 efficacy) at different study days (D) after treatment with either moxidectin (MOX) or ivermectin (IVM) for the 10 study sites in The Netherlands (NE). The number of animals (N) on each study site is listed, as well as the minimum (Min) and maximum (Max) faecal egg count in each treatment group and the arithmetic mean faecal egg count prior to treatment (AM pre).

Site	Treatment	N	AM pre	Min	Max	D14	D21	D28	D35	D42	D56	D70	D84
NE01	MOX	7	650	125	1675	100	100	100	99	96	80	58	78
	IVM	6	458	100	900	100	100	100	100	95	89		
NE02	MOX	6	608	50	1650	98 (87-100)	98	100	93	71	43	0	30
	IVM	6	563	75	1250	100	100	87	73	62	41		
NE03	MOX	6	329	200	575	100	100	100	100	97	73	5	62
	IVM	6	358	150	725	100	100	100	93	76	0		
NE04	MOX	6	1075	100	3675	100	100	100	100	100	100	99	98
	IVM	6	817	100	2125	99	100	100	100	100	90		
NE05	MOX	6	583	100	1475	100	100	99	97	94	79	46	27
	IVM	6	546	125	1325	100	100	99	83	84	27		
NE06	MOX	6	288	75	675	100	100	100	100	100	98	100	95
	IVM	5	195	75	400	100	100	100	100	97	100		
NE07	MOX	7	1050	125	4025	100	100	100	100	100	99	100	100
	IVM	7	768	150	1475	100	100	100	100	99	86		
NE08	MOX	5	305	125	550	100	100	100	93	90	77	73	45
	IVM	5	265	125	500	92 (76-100)	100	100	100	96	58		
NE09	MOX	6	821	175	3000	100	100	100	100	100	100	100	100
	IVM	6	658	100	1950	100	100	100	100	99	97		
NE10	MOX	6	604	200	1700	100	96	100	100	99	100	100	93
	IVM	6	513	125	1550	100	100	100	100	98	92		

Discussion

The present study examined the egg excretion after IVM or MOX treatment on 10 to 12 study sites each in Belgium, Italy and The Netherlands. The study sites were not randomly selected but based on a history of regular to frequent deworming in previous years. As such, the results do not provide an estimate of the true prevalence of AR or decreased ERP in small strongyles in these countries.

The efficacy on Day 14 was 100% in all but 5 of the 64 treatment groups, including sites with shortened ERP. These data confirm that efficacy determination for MOX and IVM shortly after treatment provides limited information on the development of AR on site. Furthermore, in cattle it has been demonstrated that treatment with MLs and in particular MOX, might lead to a temporary suppression of the egg excretion (De Graef et al., 2012). It has been advocated before that the efficacy of anthelmintics should be evaluated routinely for each class of drugs and for MOX and IVM the post-treatment egg excretion needs to be monitored at multiple time-points after treatment. Furthermore, both biological and technical variability in faecal egg counts can greatly impact the observed anthelmintic efficacy. To accurately diagnose AR on the basis of FECRT data, levels of variability should be reduced through adequate sample size and a lower detection limit of the FEC method (Levecke et al., 2012; Vidyashankar et al., 2012). In the present study, the average number of animals in each treatment group was between 5 and 7 animals in Italy and The Netherlands, and even more limited on some study sites in Belgium due to the smaller size of horse establishments in this country. It should be noted however that this study was not limited to a FECRT and that the animals were monitored at least bi-weekly for 56 or 84 days. This high frequency of faecal screening does provide more confidence in the efficacy evaluation. Based on the current results, an additional screening around Day 35 to 42 for IVM and around Day 56 for MOX is suggested, with further sampling days if required.

A shortened ERP for both IVM and MOX was observed in these 3 countries. The ERP was decreased for at least one of the anthelmintics on 17 out of 32 study sites (53%) and on 9 sites (28%) the ERP was decreased for both anthelmintics. On some of these study sites the efficacy declined towards the end of the expected ERP, i.e. Day 56 for IVM and Day 84 for MOX, often with good efficacy 2 weeks before. Nevertheless, in each country one or multiple study sites were identified with egg shedding returning much faster as might have been expected based on previous studies or the product label claims. A similar pattern of decreased ERP was previously described on horse farms with frequent deworming in Kentucky, and has been attributed to a quicker maturation of larval stages (Lyons et al., 2009; 2010). An alternative cause could be the preferential selection by treatment of certain species in the worm

population, as the subfamily *Cyathostominae* is a diverse group of more than 50 species in equids, all with different lengths of their life cycle. In a previous study in The Netherlands, a relative increase of *Cylicocyclus leptostomum* and *Cylicocyclus ashworti* after IVM treatment on farms with a shortened ERP was observed (van Doorn et al., 2012b).

The decreased ERP observed in the present study is probably due to frequent and continuous use of MLs on the selected study sites. Avoiding the development of AR and a shortened ERP, must be obtained through environmental based approaches (including pasture management) and less frequent blanket treatments or selective individual treatments. To reduce the number of group treatments, regular monitoring of faecal egg output is required. For this purpose the use of pooled samples can be considered (Eysker et al., 2008). Several studies have indicated that the majority of the horses are actually not excreting eggs at the time of treatment and thus did not require any treatment (Comer et al., 2006; Lyons et al., 2012; Lester et al., 2013). As an alternative for adult horses, selective treatment of only those horses excreting a number of eggs exceeding a certain cut-off (e.g. more than 200 or 500 epg) can be considered (Ploeger et al., 2008). It must be noted however that a selective treatment is not recommended for foals, weanlings and yearlings (AAEP Parasite Control Guidelines, 2013). Additionally, this approach requires frequent monitoring of the egg excretion to identify those horses that have an egg excretion above the treatment threshold. The increased work load and cost of the sampling and laboratory diagnosis is often considered as a hurdle, but diagnostic tools are available to quickly identify those horses that require treatment (Ronsyn et al., 2012). A potential downside of the selective therapy approach seems to be the increased prevalence of *Strongylus vulgaris* and the health risk associated with this infection (Nielsen et al., 2012). The benefit is that the development of AR is slowed down on farms using selective therapy (Larsen et al., 2011). Next to a decreased treatment frequency, import of resistant nematodes on the site should be avoided through screening of egg excretion and treating horses in quarantine. Recent questionnaires on parasite control practices in the UK and in The Netherlands (van Doorn et al., 2012a; Relf et al., 2012) illustrate that the reality in the field deviates from best practice. In The Netherlands, 34% of the farms were found to treat within the ERP of the products, with up to 7 treatments a year. In light of the widespread evidence of fenbendazole resistance and as MOX remains the only anthelmintic with significant efficacy against encysted larval cyathostomins (Stratford et al., 2013), treatment efficacies of the MLs need to be preserved. Developing tailored treatment programs is preferred over blanket treatments, taking farm specific management conditions and the epidemiology of parasites into account. This also implies the integration of faecal samplings to identify adult horses that need to be treated and to follow-up efficacy after treatment. Furthermore, the different drug classes should be used to the best of their

efficacy range. To decrease the reliance on MOX or IVM, it has been advocated to use other anthelmintic classes, such as pyrantel (Comer et al., 2006), especially in those times of year where luminal parasite stages are present.

In conclusion, the results of the present study highlight the high efficacy of MOX and IVM 14 days after treatment, yet do indicate a shortened ERP for these treatments in more than half of the study sites.

Manufacturer's addresses

[a] Merial, Duluth, GA, US [b], Zoetis, Florham Park, NJ, US

References

American Association Equine Practitioners (AAEP) Parasite control guidelines, updated 2013. (http://www.aaep.org/parasite_control.htm)

Canever R.J., Braga P.R., Boeckh A., Grycajuck M., Bier D. and Molento M.B., 2013. Lack of Cyathostomin sp. reduction after anthelmintic treatment in horses in Brazil. Vet. Parasitol. 194, 35-39.

Coles G.C., Bauer C., Borgsteede F.H.M., Geerts S., Klei T.R., Taylor M.A., Waller, P.J., 1992. World Association for the Advancement of Veterinary Parasitology (W.A.A.V.P.) methods for the detection of anthelmintic resistance in nematodes of veterinary importance. Vet. Parasitol. 44, 35-44.

Comer K.C., Hillyer M.H., Coles, G.C., 2006. Anthelmintic use and resistance on thoroughbred training yards in the UK. Vet. Rec. 158, 596-598.

Eysker M., Bakker J., van den Berg M., van Doorn, D.C.K, Ploeger H.W., 2008. The use of age-clustered pooled faecal samples for monitoring worm control in horses. Vet. Parasitol. 151, 249-255.

De Graef J., Sarre C., Mills B.J., Mahabir S., Casaert S., De Wilde N., Van Weyenberg M., Geldhof P., Marchiondo A., Vercruysse J., Meeus P., Claerebout E., 2012. Assessing resistance against macrocyclic lactones in gastro-intestinal nematodes in cattle using the faecal egg count reduction test and the controlled efficacy test. Vet Parasitol. 189, 378-382.

Larsen M.L., Ritz C., Petersen S.L., Nielsen, M.K., 2011. Determination of ivermectin efficacy against cyathostomins and *Parascaris equorum* on horse farms using selective therapy. Vet. J. 188, 44-47.

Lester H.E., Spanton J., Stratford C.H., Bartley D.J., Morgan E.R., Hodgkinson J.E., Coumbe K., Mair T., Swan B., Lemon G., Cookson R., Matthews J.B., 2013. Anthelmintic efficacy against cyathostomins in horses in Southern England. Vet. Parasitol. 197, 189-196.

Levecke B., Dobson R.J., Speybroeck N., Vercruysse J., Charlier J., 2012. Novel insights in the faecal egg count reduction test for monitoring drug efficacy against gastrointestinal nematodes of veterinary importance. Vet. Parasitol. 188, 391-396

Lyons E.T., Tolliver S.C., Collins S.S., 2009. Probable reason why small strongyle EPG counts are returning "early" after ivermectin treatment of horses on a farm in Central Kentucky. Parasitol. Res. 104, 569-574.

Lyons E.T., Tolliver S.C., Kuzmina T.A., Collins, S.S., 2010. Critical tests evaluating efficacy of moxidectin against small strongyles in horses from a herd for which reduced activity had been found in field tests in Central Kentucky. Parasitol Res. 107, 1495-1498.

Lyons E.T., Tolliver S.C., Collins, S.S., 2011. Reduced activity of moxidectin and ivermectin on small strongyles in young horses on a farm (BC) in Central Kentucky in two field tests with notes on variable counts of eggs per gram of feces (EPGs). Parasitol. Res. 108, 1315-1319.

Lyons E.T., Tolliver S.C., Kuzmina T.A., 2012. Investigation of strongyle EPG values in horse mares relative to known age, number positive, and level of egg shedding in field studies on 26 farms in Central Kentucky (2010-2011). Parasitol. Res. 110, 2237-2245.

Lyons E.T., Tolliver S.C., 2013. Further indication of lowered activity of ivermectin on immature small strongyles in the intestinal lumen of horses on a farm in Central Kentucky. Parasitol. Res. 112, 889-891.

Nielsen M.K., Vidyashankar A.N., Olsen S.N., Monrad J., Thamsborg S.M., 2012. *Strongylus vulgaris* associated with usage of selective therapy on Danish horse farms-is it reemerging? Vet. Parasitol. 189, 260-266.

Ploeger H.W., van Doorn D.C., Nijsse E.R., Eysker, M., 2008. Decision trees on the web - a parasite compendium. Trends Parasitol. 24, 203-204.

Relf V.E., Morgan E.R., Hodgkinson J.E., Matthews, J.B., 2012. A questionnaire study on parasite control practices on UK breeding Thoroughbred studs. Equine Vet. J. 44, 466-71.

Ronsyn R., Besognet B., Geurden T., Benkhelil A., Maillard K., 2012. Evaluation of the OVATEC PLUS flotation method as a decision tool for selective anthelminthic treatment against strongyles in horses.' Proceedings of the Joint Scientific meeting of the Belgian and the Dutch Societies for Parasitology and the Belgian Society for Protozoology, Antwerp, Belgium, 2012, Oct 19.

Slocombe J.O., Coté J.F. de Gannes R.V., 2008. The persistence of benzimidazole-resistant cyathostomes on horse farms in Ontario over 10 years and the effectiveness of ivermectin and moxidectin against these resistant strains. Can. Vet. J. 49, 56-60.

Stratford C.H., Lester H.E., Pickles K.J., McGorum B.C., Matthews J.B., 2013. An investigation of anthelmintic efficacy against strongyles on equine yards in Scotland. Equine Vet. J. 46, 25-31.

Traversa D., Klei T.R., Iorio R., Paoletti B., Lia R.P., Otranto D., Sparagano O.A., Giangaspero A., 2007. Occurrence of anthelmintic resistant equine cyathostome populations in central and southern Italy. Prev. Vet. Med. 82, 314-320.

Traversa D., von Samson-Himmelstjerna G., Demeler J., Milillo P., Schürmann S., Barnes H., Otranto D., Perrucci S., di Regalbono A.F., Beraldo P., Boeckh A., Cobb, R., 2009. Anthelmintic resistance in cyathostomin populations from horse yards in Italy, United Kingdom and Germany. Parasit Vectors. 2 Suppl 2:S2.

Traversa D., Castagna G., von Samson-Himmelstjerna G., Meloni S., Bartolini R., Geurden T., Pearce M.C., Woringer E., Besognet B., Milillo P., D'Espois, M., 2012. Efficacy of major anthelmintics against horse cyathostomins in France. Vet. Parasitol. 188, 294-300.

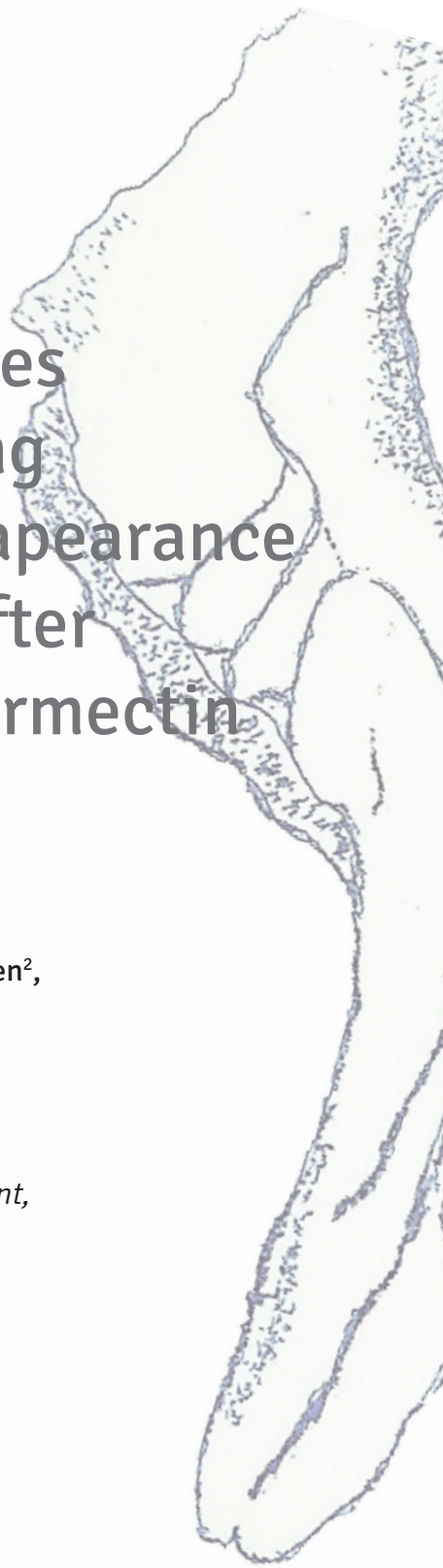
van Doorn D.C.K., Eysker M., Kooyman F.N.J., Wagenaar J.A., Ploeger H.W., 2012a. Searching for ivermectin resistance in Dutch horses. Vet. Parasitol. 185, 355-358.

van Doorn D.C.K, Kooyman F.N.J., Ploeger H.W., Eysker M., Wagenaar J.A., 2012b. Differentiation of cyathostomin species from horses with shortened Egg Reappearence Period after treatment with ivermectin. J. Eq. Vet. Science 32, S43-44 (Abstracts of the 9th ICEID Conference).

Vidyashankar A.N., Hanlon B.M., Kaplan R.M., 2012. Statistical and biological considerations in evaluating drug efficacy in equine strongyle parasites using fecal egg count data. Vet. Parasitol. 185, 45-56.

Chapter 4

Cylicocyclus species predominate during shortened Egg Reapearance Period in horses after treatment with ivermectin and moxidectin



D.C.K. van Doorn[1]*, H.W. Ploeger[1], M. Eysker[1], T. Geurden[2], J.A. Wagenaar[1], F.N.J. Kooyman[1]

1 Department of Infectious Diseases and Immunology, Faculty of Veterinary Medicine, Utrecht University, Yalelaan 1, 3584 CL Utrecht, the Netherlands
2 Zoetis - Veterinary Medicine Research and Development, Hoge Wei 10, 1930 Zaventem, Belgium

Cylicocyclus species predominate during shortened Egg Reappearance Period in horses after treatment with ivermectin and moxidectin

Abstract

The normal time interval between treatment of horses with a macrocyclic lactone (ML) and reappearance of strongylid eggs in the faeces, or 'Egg Reappearance Period (ERP)', is at its shortest 8 weeks for ivermectin (IVM), and 12 weeks for moxidectin (MOX). Nowadays it is not uncommon to have horse farms with shorter ERPs, potentially indicating the beginning of the development of drug resistance. Whether all cyasthostomin species contribute equally to a shortened ERP is not known. In the present study a Reverse Line Blot (RLB) on individual larvae cultured from excreted eggs was used on 4 farms to compare species composition before and after ML-treatment in horses with either a normal or a shortened ERP. Species composition was determined for a total of 21 horses based on differentiation of approximately 40 larvae per horse per sampling day.

The shortest ERP on each farm after IVM treatment was found to be between 28-42 days. The shortest ERP after MOX treatment was found to be between 42 and 56 days. The RLB showed that early egg shedding was dominated by species of the genus *Cylicocyclus* (*Cyc.*) (*Cyc. ashworthi*, *Cyc. insigne*, *Cyc. leptostomum* and *Cyc. nassatus*). Before treatment, species composition was generally more diverse with species from several other genera also present. Interestingly, on the farm with the shortest ERP and where all horses showed a shortened ERP more species/genera were present during early egg excretion than on other farms. It is concluded that particularly cyathostomin species within the genus *Cylicocyclus* are responsible for a shortened ERP. However, if the shortening of the ERP becomes more pronounced and wider spread within a farm, species from other genera will also be present during early egg shedding.

Keywords: Cyathostominae; Egg Reappearance Period; ERP; ivermectin; moxidectin, *Cylicocyclus*; horses

Introduction

Cyathostomins are the most prevalent horse helminths with over 50 species described in equids (Lichtenfels et al., 2008), and with between species both a varying pre-patent period (PPP) (Lyons et al., 2011) and possibly varying egg reappearance period (ERP). Cyathostomins may cause disease in horses of all ages, but is most often seen as larval cyathostominosis in young horses (Herd et al., 1986). Worldwide, the control of cyathostomin infections depends to a large extent on the use of anthelmintics.

In many countries drug resistance in equine parasites has become problematic. Today, most of the anthelmintics used belong to the macrocyclic lactones (ML's), ivermectin (IVM) and moxidectin (MOX), as these were until recently not restricted by the occurrence of anthelmintic resistance in the cyathostomins. However, recently presence of ML resistance in cyathostomin populations in horses has been suggested (Traversa et al., 2009; Canever et al., 2013).

Anthelmintic resistance can be diagnosed using the Fecal Egg Count Reduction Test (FECRT) as defined in the WAAVP guidelines (Coles et al., 1992). It is the most widely adopted technique to determine reduced efficacy of an anthelmintic by measuring the reduction in egg counts between day of treatment and 14 days later. Another technique for evaluating the efficacy of anthelmintic drugs is the critical test involving necropsy of horses which enables differentiation to species based on the morphology of adult cyathostomins (Drudge et al., 1979).

A disadvantage of only determining the FECR is that it does not take a shortening of the ERP into account. For IVM initially an ERP of between 8 and 12 weeks was observed (Borgsteede et al., 1993; Parry et al., 1993; Boersema et al., 1996, 1998). The ERP for moxidectin at introduction was found to be between 12 and 25 weeks (diPietro et al., 1997; Demeulenaere et al., 1997; Boersema et al., 1998). A shortened ERP possibly indicates developing resistance early on (Lyons et al., 2008, 2009) and has been found worldwide (diPietro et al., 1997; von Samson-Himmelstjerna et al., 2007; Lyons et al., 2008, 2010; Molento et al., 2008; Schumacher et al., 2009; Rossano et al., 2010).

Because of the large number of cyathostomin species that could be involved in resistance development, it may be interesting to know which species or genera contribute to a shortening of the ERP as different species may have varying life-cycles (Lyons et al., 2011). Unfortunately, it is not possible to monitor by morphological means which of the many species of cyathostomins contribute to a shortened ERP unless horses are necropsied sequentially. However, differentiation to species using eggs or infective larvae has become feasible molecularly (Hodgkinson et al., 2005; Traversa, et al., 2007; Cwiklinski et al., 2012), reducing the need for sequential necropsies.

Hodgkinson et al. (2005) used a PCR-ELISA technique for identification of 6 cyathostomin species to study the early strongyle egg shedding after fenbendazole (FBZ) treatment of horses. Within their study ERP per species was described, but no significant changes in species composition before and after treatment were found. Traversa et al. (2009) found 8 cyathostomin species in pre-treatment pooled egg samples whilst only *Coronocyclus* (*Cor.*) *labiatus* and *Cylicostephanus* (*Cys.*) *goldi* were detected 14 days after treatment with FBZ. Following ML treatment, three studies were published, two of which used critical tests to determine cyathostomin species. *Cylicocyclus insigne* was the

predominant species after IVM treatment of 4 yearlings and in 2010 after MOX treatment of 4 two-year old horses only L4 were identified (Lyons et al., 2009, 2010). The third study used a Reverse Line Blot (RLB) on pooled egg samples from 4 foals and 4 yearlings (Ionita et al., 2010). They found 11 cyathostomin species pre-treatment. Four weeks after IVM treatment 4 species were found to predominate: *Cylicocyclus* (Cyc.) *nassatus*, *Cys. longibursatus*, *Cylicostephanus calicatus* and *Cylicostephanus minutus*.

Studies like those mentioned above are very difficult to perform on many horses at the same time. Therefore, data on the temporal reappearance of different cyathostomin species within the original ERP following treatment are still very scarce. The aim of the present study was to estimate, in 21 horses from 4 different farms, which cyathostomin species contribute to early egg excretion following IVM or MOX treatment.

Material and Methods

Study 1
Starting in October 2011 horses (between 4 months and 4 years of age) on 2 premises were investigated for strongyle type egg shedding pre- and post-treatment. On farm 1A 11 horses and farm 1B 9 horses shedding at least 100 eggs per gram (EPG) 2-7 days before they were treated (day 0) orally with 200 µg/kg IVM (Eraquell®, VIRBAC, Cacem, Portugal). Fecal samples were collected from all individual horses according to the schedule given in Table 1.

Study 2
Between the end of January and the middle of February 2012 on another 2 premises horses shedding at least 100 eggs per gram (EPG) 2-7 days before they were orally treated (day 0) with either 200 µg/kg IVM (Eqvalan®, MERIAL, Toulouse, France) or 400 µg/kg MOX (Equest®, ZOETIS, Gerona, Spain). On farm 2A 6 horses and on farm 2B 5 horses were studied until 56 days after IVM treatment. On farm 2A 4 horses and 2B 5 horses were studied until 84 days after MOX treatment. Horses were between 4 months and 4 years of age. These 2 farms had been selected from 10 farms. Farm 2A was selected because all sampled horses showed a shortened ERP following IVM or MOX treatment, while farm 2B had horses with both regular and shortened ERP after IVM or MOX treatment. Days that fecal samples were collected on the 2 farms are given in Table 1.

Horses and cultures used for differentiation
On premise 1A and 1B L3s from a total of 6 horses were differentiated. On each farm a horse with a regular ERP and two horses with a shortened ERP were selected. At farm 2A the cultured L3s from 6 horses, all with shortened ERP of which 3 after IVM and 3 after MOX treatment, were differentiated. At farm

2B following IVM treatment, L3s from 3 horses with regular ERP and 2 with shortened ERP were differentiated. Following MOX treatment, L3s from one horse with regular ERP and 3 horses with shortened ERP were differentiated. The days on which L3s were differentiated with the RLB are presented in Table 1.

Basically, the sampling days chosen to differentiate L3s were pre-treatment and at the regular ERP days post-treatment, being 56 days for IVM and 84 days for MOX. If ERP was shortened, L3s were also differentiated at earlier days post-treatment. Details on specific days when L3s were differentiated are given in Table 1 and in Table S2. Overall, L3s were differentiated to species level for 21 horses at several points in time during the study.

Additionally, at a control farm where all sampled horses were between 1 and 4 years of age and showed normal ERPs, 4 EPG positive horses were selected to differentiate L3s from. The purpose was to compare pre-treatment species composition at farms with shortened ERP with a farm where no shortening was detected.

Fecal Egg Counts, Larval Cultures and Egg Reappearance Period

After each farm visit fecal egg counts for each horse were performed with a modified McMaster method with saturated NaCl solution with a detection limit of 50 EPG (study 1) or 25 EPG (study 2) (MAFF, 1986).

From each horse 25 g of feces and 2.5 g of saw-dust was mixed and cultured in glass jars for 7 days at 26°C. The jars were then filled with lukewarm tap water, turned upside down on petri dishes and replenished with water. L3s were harvested the next day and stored in 50 ml clean water at 4°C until further use (MAFF, 1986).

As there is still no world-wide consensus on the definition of the ERP, we used the following definition. ERP is the time after treatment at which the fecal egg count (FEC) returns to 10% or more of the pre-treatment FEC (Borgsteede et al., 1993; von Samson et al., 2007; Larsen et al, 2011). This was used for both individual horses and on farm-level.

DNA extraction, PCR amplification and Reverse Line Blot

The RLB was used for the differentiation of individual L3 instead of individual or pooled eggs because they are easier to isolate and the amount of DNA is constant in contrast to eggs where the amount of DNA depends on the development. The RLB is based on hybridization of a species specific probe with an amplified fragment of the Inter Genic Spacer (IGS) region (Traversa et al., 2007). Each individually isolated L3 was lysed in 25 µl Worm Lysis Buffer / proteinase K (van der Veer et al., 2003). The mixture was frozen for 15 minutes at -80 ºC and the L3s were lysed overnight at 56-60˚C. Proteinase K was heat

inactivated the next day at 95˚C for 15 min. Lysis was as described by van Doorn et al. (2010), except that L3s were used instead of exsheathed L3s. For a 25 µl PCR reaction 2.5 µl of lysate was used as template. Single PCRs on individual larvae were performed with CY26 (5'-GAGCTGGGTTTAGACCGTCGTGAG-3') as a forward and CY18 (5'-CTTAGACATGCATGGCTTAATC-3') as a biotinylated reverse primer in a thermal cycler using the following protocol: 10 min at 94°C and 35 cycles at 94°C for 1 min, 55°C for 1 min, and 72°C for 2 min, followed by a final extension at 72°C for 7 min. For RLB 10 µl of the IGS amplicon per lane was used for hybridization with 21 species specific probes and 1 cyathostomin catch all as described by Cwiklinski et al. (2012). If the PCR product of an individual L3 hybridizes with the catch-all probe, but with none of the 21 specific probes, the L3 was identified as "other cyathostomin". Hybridized products were detected with streptavidin-POD conjugate, followed by incubation in ECL detection reagent and exposure to Amersham Hyperfilm ECL. Approximately 40 individual L3s per culture were differentiated since only 40 samples can be processed on one mini-blot at a time. This limits the number of individual larvae that can be identified, but still allows a quantitative estimate of the species composition in each fecal sample.

Results

FEC and ERP within study 1 and study 2

The FEC means on all the sample days of the 4 farms are given in Table 1. Data for all individual horses in both studies are shown in the supplementary data (Table S1). Table 1 shows that the shortest ERP was 28 days after treatment with IVM on farm 2A. After MOX treatment the shortest ERP was 42 days also on farm 2A.

Table 1: Sampling days and arithmetic mean Fecal Egg Count (in eggs per gram) data after treatment with either IVM or MOX.

Farms	Treatment	Fecal sampling days								
		PRE	Day 14	Day 21	Day 28	Day 35	Day 42	Day 56	Day 70	Day 84
1A	IVM	446[a]	0	0	27[a]	136	223[a]	N	1318[a]	641
1B	IVM	656[a]	0	0	0	44[a]	100[a]	N	517[a]	739
2A	IVM	563[a]	0	0	75[a]	154[a]	213[a]	333[a]	N	N
2A	MOX	608[a]	13	0	0[a]	38[a]	146[a]	346	788	444[a]
2B	IVM	458[a]	0	0	0	0	25[a]	50[a]	N	N
2B	MOX	650[a]	0	0	0	7	25[a]	125[a]	275[a]	140[a]

PRE=pre-treatment; N=no sampling; [a]= individual larvae differentiated from selected horses

Differentiation results at premise 1A and 1B

In the pre-treatment samples we found 11 species (mainly belonging to *Cyathostomum* (*Cya.*) *catinatum*, *Cya. pateratum*, *Cys. goldi*, *Cyc. ashworthi*, *Cyc. leptostomum* and *Cyc. nassatus*) and 9 species (mainly *Cys. longibursatus*, *Cyc. ashworthi*, *Cyc. leptostomum*, and *Cyc. nassatus*) at premise 1A and 1B, respectively. On farm 1A the ERP was 35 days (Table 1) and for individual horses the shortest ERP was between 28 and 35 days (Table S1). On farm 1B the ERP was 42 days and for individual horses the shortest ERP was between 35 and 42 days. *Cylicocylus* spp. contributed most to earlier egg shedding after treatment (Fig.1 and Table S2). On farm 1A on day 28 after treatment the first cyathostomin species shedding eggs were *Cyc. ashworti* and *Cyc. leptostomum*. On day 35 at farm 1B *Cyc. insigne*, *Cyc. nassatus* together with *Cys. longibursatus* and *Parapoteriostomum mettami* were found. On day 42 post treatment a more diverse species composition was found on both farms, with additionally *Cya. catinatum*, *Cya. pateratum* and "other species" (not included in the panel of 21 species). For reasons unknown, some cultured larvae unfortunately did not result in useful PCR products. On one sample day from a pre-treatment culture from farm 1B, we did not gain differentiation results (Table S2).

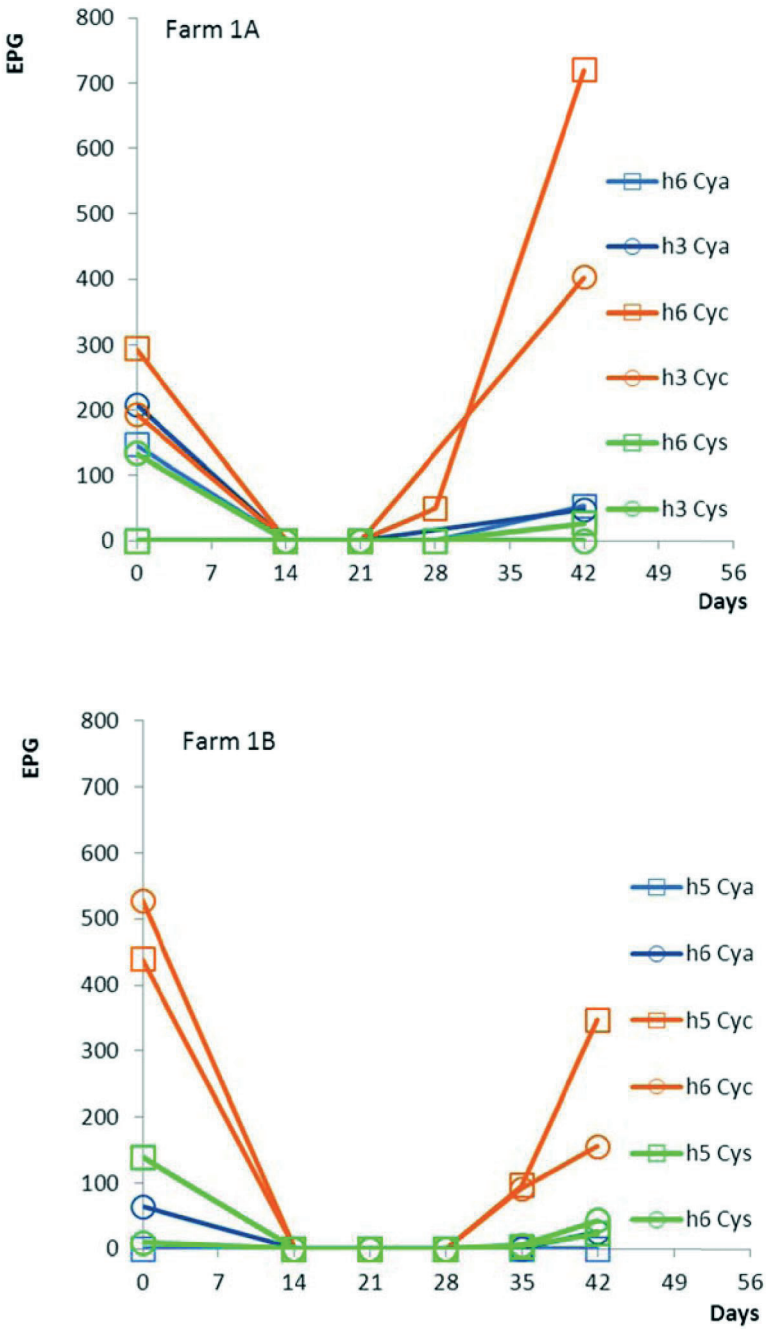


Figure 1

Fig. 1: EPG per cyathostomin genus before and after IVM treatment in 2 horses with a shortened ERP at farm 1A and 1B. *Cya*= *Cyathostomum* (blue), *Cyc* = *Cylicocyclus* (orange), *Cys* = *Cylicostephanus* (green). h# = horse ID number.

Differentiation results at farms 2A and 2B

In the pre-treatment samples of study 2 we identified 9 species (mainly *Cys. longibursatus*, *Cyc. ashworthi*, *Cyc. leptostomum* and *Cyc. nassatus*) on premise 2A and 10 identified species on 2B (mainly *Cys. longibursatus*, *Cyc. ashworthi*, *Cyc. leptostomum* and *Cyc. nassatus*). On farm 2A, the ERP after IVM treatment was 28 days and after MOX treatment it was 42 days (Table 1). On farm 2B ERP after IVM and MOX was in both cases 56 days (Table 1).

On all 4 farms the genus *Cylicocyclus* contributed most to shortened ERP after treatment with either IVM or MOX (Fig. 2, Fig. 3 and Table S2). On farm 2A *Cyc. ashworthi*, *Cyc. leptostomum*, *Cyc. nassatus* and *Cys. longibursatus* were the first species to return to egg shedding after IVM treatment. On farm 2A and 2B on day 42 and day 56 after IVM treatment *Cylicocyclus* species also predominated (Fig. 2). The cultures from farm 2A on these days contained also L3 from the genera *Cyathostomum* and *Cylicostephanus*, while on farm 2B the cultures consisted exclusively of *Cylicocyclus* species. On farm 2A mainly *Cyc. nassatus* and one "other" L3 were found 28 days after MOX treatment. The L3 from the MOX treated horses from day 42 on both farms were mainly *Cylicocyclus* spp and this was the case for the entire period until day 84 (Fig. 3). Additionally, on day 42 after MOX treatment at farm 2A L3 of the genera *Coronocyclus* and *Cylicostephanus* were found.

Differentiation results at the control farm

In the samples of the control farm we found 9 identified species (mainly belonging to *Cya. catinatum*, *Cyc. leptostomum* and *Cyc. nassatus*) given in Table S2. Overall, the same species composition was found as was found pre-treatment on the farms from studies 1 and 2.

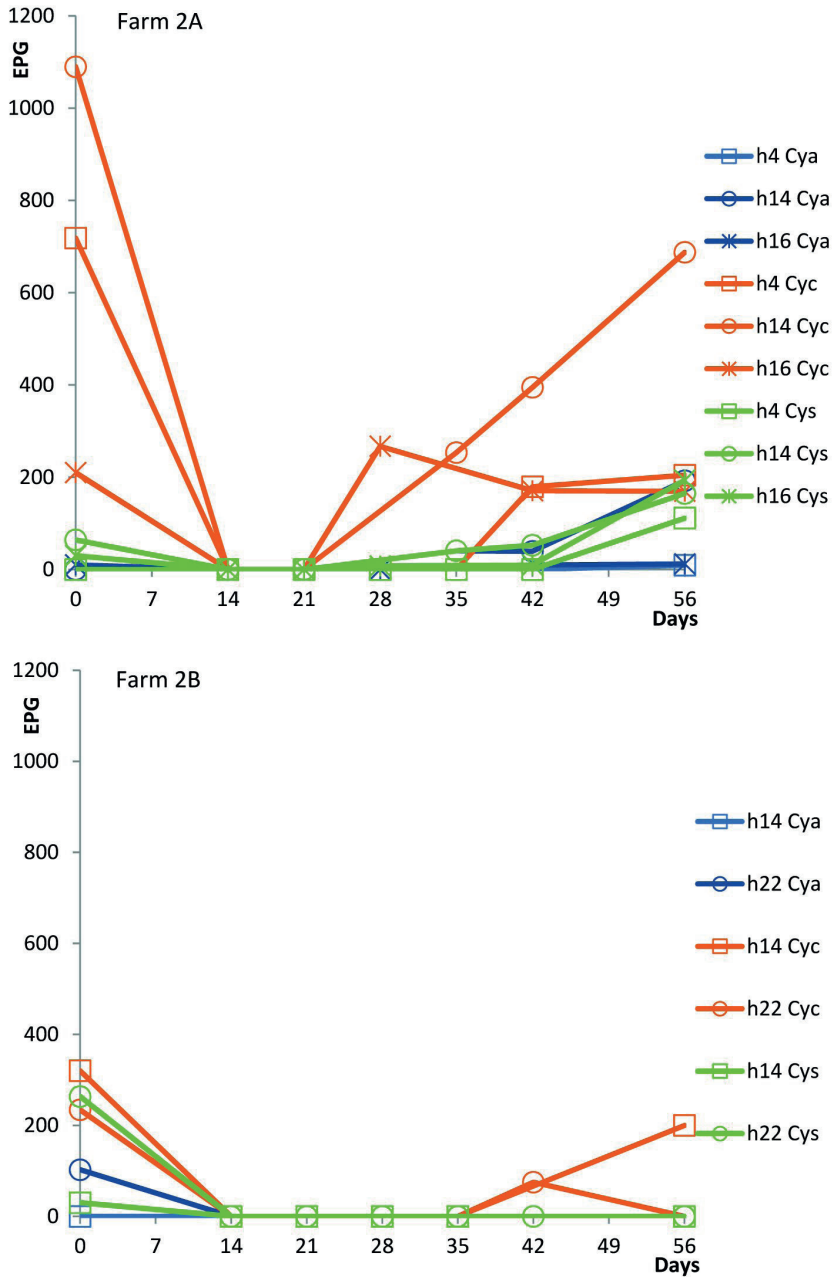


Figure 2

Fig. 2: EPG per cyathostomin genus before and after IVM treatment in horses with a shortened ERP at farm 2A (n=3) and 2B (n=2). *Cya*= *Cyathostomum* (blue), *Cyc* = *Cylicocyclus* (orange), *Cys* = *Cylicostephanus* (green). h# = horse ID number.

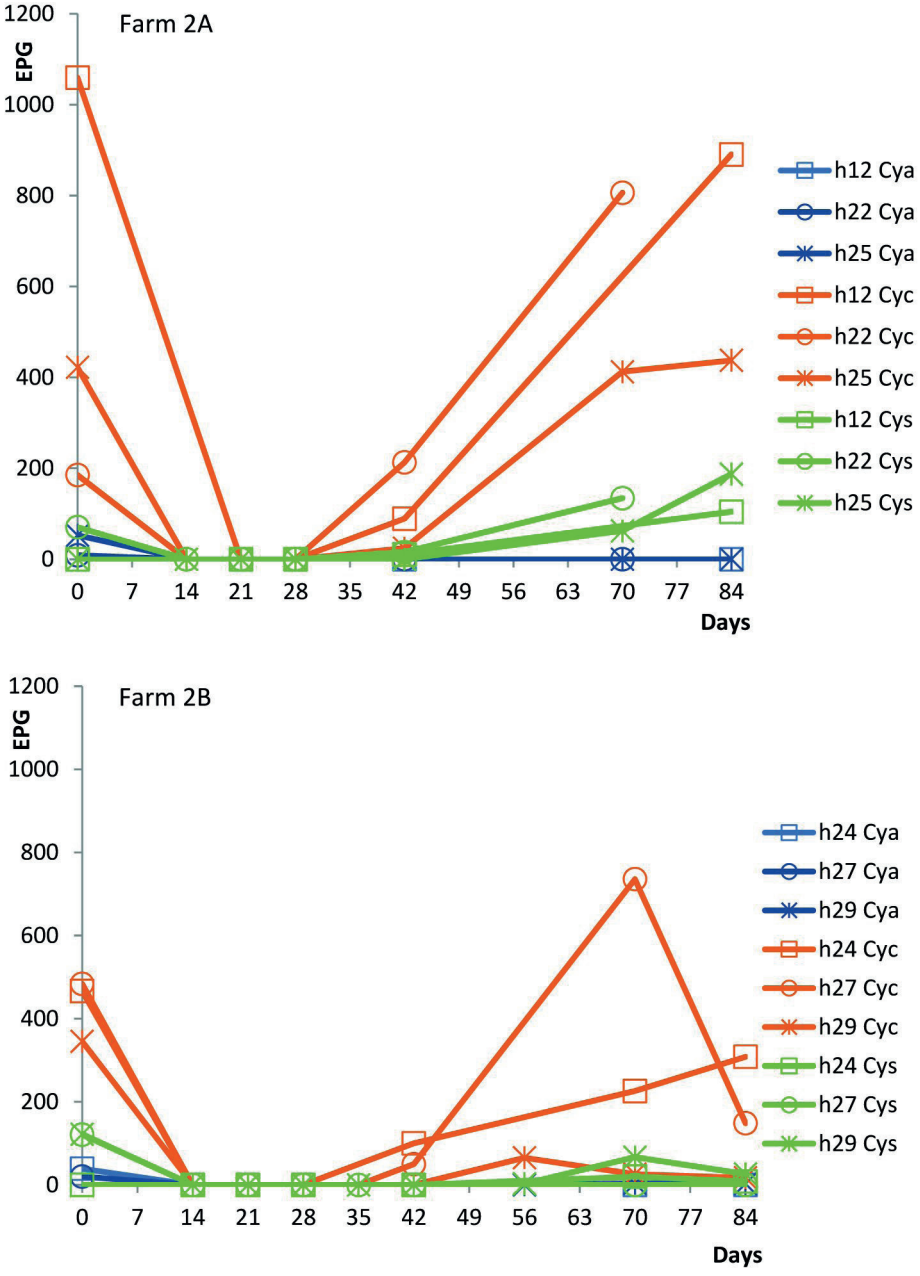


Figure 3

Fig. 3: EPG per cyathostomin genus before and after MOX treatment in horses with a shortened ERP at farm 2A (n=3) and 2B (n=3). *Cya*= *Cyathostomum* (blue), *Cyc* = *Cylicocyclus* (orange), *Cys* = *Cylicostephanus* (green). h# = horse ID number.

Discussion

Mean FEC showed a shortened ERP after IVM treatment on 4 farms to be between 28 and 42 days and a shortened ERP after MOX treatment on 2 farms to be between 42 and 56 days. The cyathostomins responsible for shortened ERP were mainly found within the genus *Cylicocyclus*.

On farm 2A all sampled horses showed a shortened ERP, while on farm 2B several horses still had a normal ERP. This might reflect lesser efficacy of ML on farm 2A compared with farm 2B. This is supported by the fact that on farm 2A more genera were present in larval cultures early after treatment than on farm 2B. Compared to results after IVM treatment, results after MOX treatment were somewhat more variable, but still *Cylicocyclus* species were predominating in horses with a shortened ERP. Interestingly, sometimes *Cylicocyclus* species that were abundant before treatment seemed to be replaced by other *Cylicocyclus* species after treatment. Our findings are generally in line with the results found by Lyons et al. (2009, 2010). They used critical tests and observed *Cylicocyclus* species to predominate in horses following IVM or MOX treatment. Further studies on more farms and in other countries will show if the genus *Cylicocyclus* is always involved in shortened ERP.

How can we explain rapid reappearance of eggs in horses within this study? At least on farms 2A and 2B egg shedding after treatment must have originated from adults that were already present as larvae at the time of treatment because horses were treated in January and February and were stabled from November until April.

A 1st explanation could be a structural shift in the cyathostomin species composition after prolonged ML usage. In the present study species composition before treatment did not differ much between farms with varying degrees of shortened ERPs. This also was the case within farms between horses with or without a shortened ERP. Moreover, on the control farm, with all horses having a regular ERP, we found the same cyathostomin species composition as on the other farms. These observations fit those from Chapman et al. (2002), who found that after 20 years of treatments with ML the diversity of cyathostomin species remained the same, without major shifts in the prevalence and ranking of species.

A 2nd explanation could be reduced susceptibility for MLs in mucosal and/or luminal L4s. Lyons et al. (2009, 2010) found luminal L4 6 days after ML treatment. This may suggest resistance developed, particularly in larval stages because L3s and L4s are partly protected by their mucosal location. Possible resistance may be found earlier in the mucosal L4s than in adult worms in the lumen.

A 3rd explanation for rapid reappearance after ML treatment might be selection for a shorter parasitic phase of the lifecycle. A shorter parasitic phase may result in less exposure to ML treatments and consequently increased survival chances. It has been observed that *Cylicocyclus* spp develop slowly in the submucosa, in eight weeks or more compared to other cyathostomin species with faster development like *Cys. longibursatus*, *Cya. catinatum*, *Cor. coronatum*, *Cys. minutus* and *Cys. calicatus* (Tiunov, 1951 referenced by Ogbourne et al., 1975, 1978). Yet, the *Cylicocyclus* spp predominate in horses with shortened ERP. So, if this explanation is true, it remains to be elucidated why in particular *Cylicocyclus* species responded to ML treatments by reducing the duration of their parasitic phase.

For now it remains difficult to explain why mainly species within the genus *Cylicocyclus* were found early after ML treatment. Perhaps their migration deeper into the submucosa of the large intestine than other species also plays a role in why species of this genus reappear earlier than species from other genera although it is difficult to envisage how.

Finally, the RLB proved to be a very useful technique to sequentially monitor species composition following ML treatment. Probes for 21 species were used whereas over 50 species of cyathostomins have been described. In 53% of the differentiated larval batches no “other” species were found and when a batch did contain “other” species this constituted between 3 and 24% of the total number of larvae. So, species specific probes for the 21 most common cyathostomin species apparently sufficed to determine the overall species composition although relative rare species may remain undetected. On the other hand, the RLB technique for use on individual larvae is not a high throughput method. Therefore, we could not apply this technique on all larval cultures from all horses involved in this study. Nonetheless, using the RLB on individual larva can provide a wealth of sequential data on species compositions without having to resort to necropsies.

Conclusion

Cylicocyclus species were present in horses shortly after ML treatment far more often than species from other genera. On the farm with the most pronounced ERP shortening, species from other genera were also found early after treatment.

Acknowledgements

We would like to thank the farm managers for their cooperation. For technical assistance we thank S. Rodenberg and B. van 't Klooster. And also C. Klaassen and C. Felix, students from HAS Dronten, and E.G. Dingboom, DVM, PhD and the students from HAS Den Bosch.

Conflict of interest

This study was co-financed by Zoetis. The interpretation of these results was not influenced by Zoetis.

References

Boersema J.H., Eysker M., Maas J., van der Aar W.M., 1996. Comparison of the reappearance of strongyle eggs on foals, yearlings and adult horses after treatment with ivermectin or pyrantel. Vet. Q. 18, 7-9.

Boersema J.H., Eysker M., van der Aar W.M., 1998. The reappearance of strongyle eggs in the faeces of horses after treatment with moxidectin. Vet. Q. 20, 15-17.

Borgsteede F.H.M., Boersma J.H., Gaasenbeek, C.P., van der Burg WP.,1993. The reappearance of eggs in faeces of horses after treatment with ivermectin. Vet. Q. 15, 24-26.

Canever R.J., Braga P.R.C., Boeckh A., Grycajuck M., Bier D., Molento M.B., 2013. Lack of Cyathostomin sp. reduction after anthelmintic treatment in horses in Brazil. Vet. Parasitol.194, 35-39.

Chapman M.R., French D.D., Klei T.R., 2002, Gastrointestinal helminths of ponies in Louisiana: a comparison of species currently prevalent with those present 20 years ago. J. Parasitol. 6, 1130-1134.

Coles G.C., Bauer C., Borgsteede F.H., Geerts S., Klei T.R., Taylor M.A., Waller, P.J., 1992. World Association for the Advancement of Veterinary Parasitology (W.A.A.V.P.) methods for the detection of anthelmintic resistance in nematodes of veterinary importance. Vet. Parasitol. 44, 35-44.

Cwiklinski K., Kooyman F.N.J., van Doorn D.C.K., Matthews J.B., Hodgkinson J.E., 2012. New insights into sequence variation in the IGS region of 21 cyathostomin species and the implication for molecular identification. Parasitol. 139, 1063-1073.

DiPietro J.A., Hutchens D.E., Lock T.F., Walker K., Paul A.J., Shipley C., Rulli D. 1997. Clinical trial of moxidectin oral gel in horses. Vet. Parasitol. 72, 167-177.

Drudge J.H., Lyons E.T., Tolliver S.C., 1979. Benzimidazole resistance of equine strongyles- critical tests of six compounds against population B. Am. J. Vet. Res. 40, 590-594.

Herd R.P., 1986. Epidemiology and control of equine strongylosis at Newmarket. Eq. Vet. J. 18, 447-452.

Hodgkinson J.E., Freeman K.L., Lichtenfels J.R., Palfreman S., Love S., Matthews J.B., 2005. Identification of strongyle eggs from anthelmintic-treated horses using a PCR-ELISA based on intergenic DNA sequences. Parasitol. Res. 95, 287-292.

Ionita M., Howe D.K., Lyons E.T., Tolliver S.C., Kaplan R.M., Mitrea I.L., Yeargan M., 2010. Use of a reverse line blot assay to survey small strongyle (Strongylida: Cyathostominae) populations in horses before and after treatment with ivermectin. Vet. Parasitol. 168, 332-337.

Larsen M.L., Ritz C., Petersen S.L., Nielsen M., 2011. Determination of ivermectin efficacy against cyathostomins and *Parascaris equorum* on horse farms using selective therapy. The Vet. J. 188, 44-47

Lichtenfels J.R., Kharchenko V.A., Dvojnos G.M., 2008. Illustrated identification keys to strongylid parasites of horses, zebras and asses. Vet. Parasitol. 156, 4-161.

Lyons E.T., Tolliver S.C., Ionita M., Lewellen A., Collins S.S., 2008. Field studies indicating reduced activity of ivermectin on small strongyles in horses on a farm in Central Kentucky. Parasitol. Res. 103, 209-215.

Lyons E.T., Tolliver S.C., Collins S.S., 2009. Probable reason why small strongyle EPG counts are returning 'early' after ivermectin treatment of horses on a farm in Central Kentucky. Parasitol. Res. 104, 569-574.

Lyons E.T., Tolliver S.C., Kuzmina T.A., Collins S.S., 2010. Critical tests evaluating efficacy of moxidectin against small strongyles in horses from a herd for which reduced activity had been found in field tests in Central Kentucky. Parasitol. Res. 107, 1495-1498.

Lyons E.T., Kuzmina T.A., Tolliver S.C., Collins S.S., 2011. Observations on development of natural infection and species composition of small strongyles in young equids in Kentucky. Parasitol. Res. 109, 1529-1535

Ministry of Agriculture Fisheries and Food (MAFF), 1986. Manual of Veterinary Parasitological Laboratory Techniques: Reference Book 418, 3rd edition, HMSO Books, London.

Molento M.B., Antunes J., Bentes R.N., Coles G.C., 2008. Anthelmintic resistant nematodes in Brazilian horses. Vet. Rec. 162, 384-385.

Ogbourne C.P., 1975. Epidemiological studies on horses infected with nematodes of the family Trichonematidae (Witenberg, 1925). Int. J. Parasitol. 5, 667-672.Ogbourne C.P., 1978. Pathogenesis of cyathostome (Trichonema) infections of the horse. A review. CIH Miscellaneous publication No. 5

Parry J.M., Fisher M.A., Grimshaw W.T.R., Jacobs D.E., 1993. Anthelmintic dosing intervals for horses: comparison of three chemical groups. Vet. Rec. 133, 346-347.

Rossano M.G., Smith A.R., Lyons E.T., 2010. Shortened strongyle-type-egg reappearance periods in naturally infected horses treated with moxidectin and failure of a larvicidal dose of fenbendazole to reduce fecal egg counts. Vet. Parasitol. 173, 349-352.

Schumacher J., Livesey L., DeGraves F., Blagburn B., Ziska M., Caldwell,M., Brock K., 2009. Efficacy of moxidectin against cyathostomins after long-term use in a large herd of draught horses with a high stocking density. Vet. Rec. 164, 652-654.

Traversa D., Iorio R., Klei T.R., Kharchenko V.A., Gawor J., Otranto D., Sparagano O.A.E., 2007. New method for simultaneous species-specific identification of equine strongyles (Nematoda, Strongylida) by reverse line blot hybridization. J. Clin. Microbiol. 45, 2937–2942.

Traversa D., Iorio R., Otranto D., Giangaspero A., Milillo P., Klei T.R., 2009. Species-specific identification of equine cyathostomes resistant to fenbendazole and susceptible to oxibendazole and moxidectin by macroarray probing. Exp. Parasitol. 121, 92-95.

Traversa D., von Samson-Himmelstjerna G., Demeler J., Milillo P., Schürmann S., Barnes H., Otranto D., Perrucci S., di Regalbono A.F., Beraldo P., Boeckh A., Cobb R., 2009. Anthelmintic resistance in cyathostomin populations from horse yards in Italy, United Kingdom and Germany. Parasit. Vectors, Sep 25; 2 Suppl. 2:S2.

van der Veer M., Kanobana K., Ploeger H.W., de Vries E., 2003. Cytochrome oxidase C subunit 1 Polymorphisms show significant differences in distribution between a laboratory maintained population and a field isolate of *Cooperia oncophora.* Vet. Parasitol. 116, 231-238.

van Doorn D.C.K., Kooyman F.N.J., Eysker M., Hodgkinson J.E., Wagenaar J.A., Ploeger H.W., 2010. *In vitro* selection and differentiation of ivermectin resistant cyathostomin larvae. Vet. Parasitol. 174, 292-299.

von Samson-Himmelstjerna G., Fritzen B., Demeler J., Schurmann S., Rohn K., Schnieder T., Epe C., 2007. Cases of reduced cyathostomin egg-reappearance period and failure of *Parascaris equorum* egg count reduction following ivermectin treatment as well as survey on pyrantel efficacy on German horse farms. Vet. Parasitol. 144, 74-80.

Supplementay data 1: EPG's study 1 (A and B) study 2 (A,B and C) and the control farm

Individual strongyle type eggs per gram of faeces for 11 individual horses at 8 sample days, pre and post treatment, on farm 1A

Farm 1A Horses	Treatment	Pre	D14	D21	D28	D35	D42	D70	D84
1	IVM	700	0	0	0	100	400	150	950
2	IVM	200	0	0	50	100	400	3950	900
3	IVM	550	0	0	50	100	250	1850	550
4	IVM	450	0	0	0	150	0	2000	1900
5	IVM	750	0	0	0	150	0	450	150
6	IVM	450	0	0	50	200	400	1400	100
7	IVM	450	0	0	50	100	150	700	500
8	IVM	750	0	0	0	200	0	1700	300
9	IVM	250	0	0	50	0	0	500	450
10	IVM	200	0	0	0	250	0	750	650
11	IVM	150	0	0	50	150	850	1050	600
mean		446	0	0	27	136	223	1318	641

the mean ERP on farm level

Individual strongyle type eggs per gram of faeces for 9 individual horses at 8 sample days, pre- and post-treatment, on farm 1B

Farm 1B Horses	Treatment	Pre	D14	D21	D28	D35	D42	D70	D84
1	IVM	900	0	0	0	0	0	50	500
2	IVM	550	0	0	0	0	0	350	700
3	IVM	500	0	0	0	0	0	1000	650
4	IVM	700	0	0	0	50	0	1250	1500
5	IVM	600	0	0	0	100	400	400	1450
6	IVM	600	0	0	0	100	250	300	100
7	IVM	900	0	0	0	50	100	450	400
8	IVM	700	0	0	0	50	100	400	1000
9	IVM	450	0	x	0	50	50	450	350
mean		656	0	0	0	44	100	517	739

the mean ERP on farm level

Individual strongyle type eggs per gram of faeces for 12 horses at 7 to 9 sample days, depending on treatment with either IVM or MOX.

Farm 2A Horses	Treatment	Pre	D14	D21	D28	D35	D42	D55	D70	D84
2	IVM	75	0	0	X	0	0	25		
4	IVM	775	0	0	0	0	200	325		
14	IVM	1250	0	0	50	375	500	1100		
15	IVM	600	0	0	0	150	175	100		
16	IVM	300	0	0	275	375	200	400		
20	IVM	375	0	0	50	25	200	50		
mean		563	0	0	75	154	213	333		
3	MOX	75	0	0	0	0	50	625	X	X
12	MOX	1100	75	0	0	75	100	425	X	1100
17	MOX	50	0	0	0	0	400	300	X	50
21	MOX	1650	0	0	0	0	0	225	X	X
22	MOX	300	0	0	X	125	300	400	1075	0
25	MOX	475	0	0	0	25	25	100	500	625
mean		608	13	0	0	38	146	346	788	444

X means no sample available/ no follow-up

the mean ERP on farm level

Individual strongyle type eggs per gram of faeces for 13 horses at 7 to 9 sample days, depending on treatment with either IVM or MOX.

Farm 2B Horses	Treatment	Pre	D14	D21	D28	D35	D42	D55	D70	D84
1	IVM	375	0	0	0	0	0	0		
6	IVM	100	0	X	X	X	X	X		
8	IVM	900	0	0	0	0	0	50		
14	IVM	350	0	0	0	0	50	200		
22	IVM	600	0	0	0	0	75	0		
28	IVM	425	0	0	0	0	0	0		
mean		458	0	0	0	0	25	50		
2	MOX	125	0	0	0	0	0	0	X	X
5	MOX	375	0	0	0	0	X	X	X	X
9	MOX	575	0	0	0	25	0	125	250	150
23	MOX	1675	0	0	0	0	0	0	0	0
24	MOX	525	0	0	0	25	100	125	275	325
27	MOX	625	0	0	0	0	50	425	750	175
29	MOX	650	0	0	0	0	0	75	100	50
mean		650	0	0	0	7	25	125	275	140

X means no sample available/ no follow-up

the mean ERP on farm level

Table S2

Supplementary data 2: Differentiation results of individual larvae with RLB on 4 farms, before and after treatment with either ivermectin or moxidectin from study 1 (Farm 1A and 1B) and study 2 (Farm 2A and 2B) and the control farm samples were from pre treatment/stock population (Pre) and from indicated days after treatment (D#). Numbers of differentiated L3 are given per species per sample date (species composition).

Farm 1A, IVM only	Horse 9, regular ERP		Horse 6, shortened ERP			Horse 3, shortened ERP	
cyathostomin species	Pre	D70	Pre	D28	D42	Pre	D42
Coronocyclus labiatus			1 (2%)				
Cyathostomum catinatum	8 (26%)		5 (11%)		2 (7%)	10 (27%)	1 (3%)
C. pateratum	1 (3%)		10 (22%)			4 (11%)	2 (6%)
Cylicostephanus goldi	1 (3%)	2 (6%)			1 (3%)	8 (22%)	
C. longibursatus	1 (3%)	33 (87%)				1 (3%)	
Cylicocyclus ashworthi	10 (32%)		9 (19%)	22 (58%)	1 (3%)	5 (14%)	
C. insigne	1 (3%)	3 (8%)					
C. leptostomum	6 (19%)		5 (11%)	16 (42%)	11 (37%)	4 (11%)	4 (13%)
C. nassatus	1 (3%)		16 (35%)		15 (50%)	4 (11%)	21 (68%)
Parapoteriostomum mettami						1 (3%)	
Poteriostomum imparidentatum	1 (3%)						
other	1 (3%)						3 (10%)
Number xL3 in RLB	31	38	46	38	30	37	31

(number %): between brackets the percentage to the total nr xL3 in RLB.

Farm 1B, IVM only	Horse 1, regular ERP		Horse 5, shortened ERP			Horse 6, shortened ERP		
cyathostomin species	Pre	D70	Pre	D35	D42	Pre	D35	D42
Cyathostomum catinatum						4 (6%)		
C. pateratum						3 (5%)		3 (10%)
Cylicostephanus goldi			1 (4%)					
C. longibursatus		27 (85%)	5 (19%)	1 (4%)	3 (6%)		2 (5%)	5 (17%)
C. minutus						1 (2%)		
Cylicocyclus ashworthi		1 (3%)	5 (19%)	1 (4%)	3 (6%)	24 (35%)	17 (51%)	2 (7%)
C. insigne		2 (6%)		1 (4%)			1 (3%)	
C. leptostomum		1 (3%)	7 (27%)	2 (8%)	6 (11%)	17 (26%)	11 (33%)	7 (24%)
C. nassatus			7 (27%)	20 (80%)	37 (70%)	17 (26%)	2 (5%)	9 (31%)
Parapoteriostomum mettami							1 (3%)	
other		1 (3%)	1 (4%)		4 (7%)			3 (10%)
Number xL3 in RLB	0	32	26	25	53	66	33	29

(number %): between brackets the percentage to the total nr xL3 in RLB.

Farm 2A, IVM	Horse 4, shortened ERP			Horse 14, shortened ERP				Horse 16, shortened ERP			
cyathostomin species	Pre	D42	D56	Pre	D35	D42	D56	Pre	D28	D42	D56
Coronocyclus coronatum								1 (3%)			
Cyathostomum catinatum			1 (3%)		4 (11%)	3 (8%)	5 (13%)	1 (3%)		1 (2%)	
C. pateratum							2 (5%)			1 (2%)	1 (3%)
Cylicostephanus goldi											
C. longibursatus			12 (34%)	1 (3%)	3 (8%)	4 (11%)	5 (13%)		1 (3%)	2 (5%)	16 (49%)
C. minutus var A				1 (3%)	1 (3%)		1 (3%)	3 (10%)			
Cylicocyclus ashworthi	1 (2%)		3 (9%)	2 (5%)	6 (16%)	5 (13%)	7 (18%)		15 (43%)	7 (17%)	
C. insigne				4 (10%)		2 (5%)	3 (8%)	1 (3%)			
C. leptostomum	1 (2%)		13 (37%)	8 (20%)	7 (19%)	5 (13%)	3 (8%)	3 (10%)	18 (51%)	8 (21%)	2 (6%)
C. nassatus	36 (88%)	26 (90%)	6 (17%)	20 (51%)	12 (32%)	18 (47%)	12 (30%)	17 (57%)	1 (3%)	20 (49%)	12 (36%)
Parapoteriostomum mettami										1 (2%)	
other	3 (7%)	3 (10%)		3 (8%)	4 (11%)	1 (3%)	2 (5%)	4 (13%)		1 (2%)	2 (6%)
Number xL3 in RLB	41	29	35	39	37	38	40	30	35	41	33

(number %): between brackets the percentage to the total nr xL3 in RLB.

Farm 2A, MOX	Horse 12 , shortened ERP			Horse 22, shortened ERP				Horse 25, shortened ERP			
	Pre	D42	D84	Pre	D35	D42	D84	Pre	D28	D42	D56
Coronocyclus coronatum			1 (5%)			2 (5%)	6 (15%)				
Cyathostomum catinatum							4 (10%)	1 (11%)			
C. pateratum				1 (3%)							
Cylicostephanus goldi							7 (18%)				
C. longibursatus		3 (11%)	2 (10%)	8 (24%)	2 (5%)	5 (13%)	1 (3%)			5 (13%)	3 (30%)
C. minutus var A											
Cylicocyclus ashworthi	1 (4%)			8 (24%)			4 (10%)	1 (11%)			1 (10%)
C. insigne	1 (4%)					6 (15%)					
C. leptostomum				2 (6%)			6 (15%)	1 (11%)		2 (5%)	
C. nassatus	24 (88%)	25 (89%)	17 (80%)	11 (32%)	27 (71%)	24 (60%)	8 (21%)	6 (67%)	35 (97%)	31 (78%)	6 (60%)
Parapoteriostomum mettami											
other	1 (4%)		1 (5%)	4 (12%)	9 (24%)	3 (7%)	3 (8%)		1 (3%)	2 (5%)	
Number xL3 in RLB	27	28	21	34	38	40	39	9	36	40	10

(number %): between brackets the percentage to the total nr xL3 in RLB.

Farm 2B, IVM	Horse 8,regular ERP		Horse 28,regular ERP		Horse 1, regular ERP	Horse 14, shortened ERP		Horse 22, shortened ERP		
cyathostomin species	Pre	D56	Pre	D56	Pre	Pre	D56	Pre	D42	D56
Coronocyclus coronatum				4 (11%)						
Cyathostomum catinatum				1 (3%)				2 (5%)		
C. pateratum	1 (3%)									
Cylicostephanus goldi			1 (3%)							
C. longibursatus	3 (8%)		22 (60%)	28 (74%)	1 (3%)	3 (9%)		17 (42%)		
C. minutus var A			2 (5%)					1 (2%)		
Cylicocyclus ashworthi	2 (5%)		8 (22%)		6 (16%)	6 (17%)	2 (6%)	3 (7%)	8 (25%)	5 (13%)
C. insigne				5 (13%)				1 (2%)		1 (3%)
C. leptostomum	1 (3%)		4 (11%)		20 (53%)	11 (31%)	2 (6%)	5 (12%)	13 (41%)	20 (51%)
C. nassatus	29 (81%)	37 (95%)			10 (26%)	15 (43%)	32 (88%)	7 (17%)	11 (34%)	13 (33%)
other		2 (5%)			1 (3%)					
Number xL3 in RLB	36	39	37	38	38	35	36	41	32	39

(number %): between brackets the percentage to the total nr xL3 in RLB.

Farm 2B, MOX	Horse 23,regular ERP		Horse 24, shortened ERP				Horse 27, shortened ERP				Horse 29 , shortened ERP			
cyathostomin species	Pre	D84	Pre	D42	D70	D84	Pre	D42	D70	D84	Pre	D55	D70	D84
Coronocyclus coronatum	1 (3%)				3 (11%)	1 (3%)			1 (2%)	5 (15%)	3 (9%)		1 (4%)	3 (8%)
Cyathostomum catinatum	1 (3%)													
C. pateratum							1 (3%)				6 (19%)		1 (4%)	2 (5%)
Cylicostephanus goldi														
C. longibursatus					2 (7%)	1 (3%)	6 (19%)				6 (19%)	1 (3%)	18 (66%)	20 (53%)
C. minutus var A		2 (17%)												
Cylicocyclus ashworthi	1 (3%)		1 (4%)	13 (35%)	5 (18%)	10 (26%)	2 (7%)	8 (21%)	12 (23%)		16 (50%)	2 (7%)		
C. insigne		1 (8%)				3 (8%)						1 (3%)	7 (26%)	12 (31%)
C. leptostomum	2 (5%)		6 (22%)	23 (62%)	14 (50%)	18 (46%)	3 (10%)	10 (26%)	18 (34%)	5 (15%)	1 (3%)	9 (29%)		
C. nassatus	32 (83%)	8 (67%)	17 (64%)	1 (3%)	4 (14%)	6 (15%)	19 (61%)	20 (53%)	22 (42%)	23 (70%)		15 (48%)		1 (3%)
Parapoteriostomum mettami														
other	1 (3%)	1 (8%)										3 (10%)		
Number xL3 in RLB	38	12	27	37	28	39	31	38	53	33	32	31	27	38

(number %): between brackets the percentage to the total nr xL3 in RLB.

Control farm	Horse 11	Horse 17	Horse 26	Horse 33
cyathostomin species	Pre	Pre	Pre	Pre
Coronocyclus coronatum	12 (32%)	2 (5%)	1 (3%)	2 (5%)
Cyathostomum catinatum	16 (43%)	17 (44%)	3 (9%)	4 (10%)
C. pateratum	7 (19%)			3 (8%)
Cylicostephanus goldi		1 (2%)		
C. longibursatus	1 (3%)	6 (15%)		
C. minutus var A			1 (3%)	
Cylicocyclus ashworthi		1 (2%)	4 (12%)	
C. leptostomum		8 (21%)	11 (32%)	
C. nassatus	1 (3%)	3 (8%)	14 (41%)	25 (64%)
other		1 (3%)		5 (13%)
Number xL3 in RLB	37	39	34	39

(number %): between brackets the percentage to the total nr xL3 in RLB.

Chapter 5

Practices as performed by veterinarians before and after anthelmintics became prescription only medicines for horses in the Netherlands

van Doorn, D.C.K., Wagenaar, J.A., Ploeger, H.W.

Abstract

Anthelmintics for horses became Prescription Only Medicines (POM) in the Netherlands in July 2008. This new regulation intended to strengthen the advisory role of veterinarians concerning parasite control. In support, several activities were undertaken by experts, notably providing guidelines for Good Veterinary Practice (GVP) with respect to the prudent use of anthelmintics. A questionnaire was used to evaluate the effect of the new legislation and GVP guidelines on the prescription of anthelmintics and the use of fecal diagnosis between 2008 and 2012. The response rate was 14% in 2008 (180 veterinarians) and 11% in 2012 (114 veterinarians). Results show that in 2012 more fecal samples were examined than in 2008 ($P<0.001$), more fecal samples were taken for monitoring purposes ($P<0.001$), more fecal samples were examined quantitatively ($P<0.001$) and more use was made of pooled fecal samples ($P<0.001$). The number of veterinarians routinely examining a fecal sample before they possibly treat increased by more than three-fold from 2008 to 2012 ($P<0.001$), whereas the number of veterinarians never examining a feces sample before treatment declined significantly ($P<0.001$). However, it was also clear that there is still room for further improvements. Specifically, continuing efforts need to be focused on improving the number of quantitatively processed fecal samples and the interpretation of fecal diagnosis results.

Keywords: Questionnaire, Veterinarians, Good Veterinary Practice, Anthelmintic use, Prescription-only-medicines, Fecal egg counts

Introduction

When in the middle of the previous century anthelmintics like dichlorvos, phenothiazine and piperazine were applied intra-gastrically through a stomach tube, veterinarians had a major role in the treatment of horses against helminth infections. Some years later anthelmintics appeared on the market that could be applied more easily. Because anthelmintics could be obtained without a prescription, veterinarians were no longer required to apply treatments and owners started treating horses themselves. Following the introduction of the broad-spectrum and very efficacious macrocyclic lactones (MLs) in the 1980's, veterinarians became even less involved in parasite management and with these drugs readily available to owners, an up-to-date knowledge from veterinarians on parasites was no longer requested for by owners (Lendal et al., 1998; O'Meara and Mulcahy, 2002). Moreover, for a long time veterinarians and pharmaceutical companies advocated regular treatments of horses based on claimed egg reappearance periods (ERP) for the available anthelmintics. Today there is widespread resistance to most anthelmintic drugs, as well as shortened ERP for the MLs.

Over the last decades it became clear that the prescription-free availability of anthelmintics had led to excessive drug use. Results from many horse owner questionnaires testified to this (Lendal et al., 1998; Lloyd, 2000; Matthee et al, 2002; O'Meara and Mulcahy, 2002; Osterman-Lind, 2007; Fritzen et al., 2010; Allison et al. 2011; Relf et al., 2012; Stratford et al., 2013). Denmark was the first country in Europe recognizing the need to make anthelmintics prescription only medicines (POM) to tackle the problem of the excessive usage of these drugs. This happened in 1999 and the Danish POM legislation also does not permit prophylactic treatments and requires a diagnosis before an anthelmintic can be prescribed (Nielsen et al., 2006, 2013). Shortly after the POM introduction in Denmark a European Union (EU) directive (2001/82/EC) made veterinary products for food producing animals, including horses, POM. It took another 5 years before the directive was implemented in the EU in 2006 (Anonymous, 2006). From 2006 onwards this led to new legislation for anthelmintics in Germany, Italy, Finland, the Netherlands, Sweden and the United Kingdom. In contrast with Denmark, in the Netherlands the new legislation concentrated on making anthelmintics POM without requiring diagnosis before treatment. The legislation became effective in 2008. The regulatory change had the intention to promote prudent use of anthelmintics and to increase the involvement of veterinarians, similar to the objectives underlying the introduction of POM in Denmark in 1999 (Nielsen et al., 2006). In support several actions were taken, including an article explaining the Good Veterinary Practice (GVP) intended by the new legislation (Sloet van Oldruitenborgh-Oosterbaan et al., 2009) and setting up post academic courses, some of which were made obligatory for members of the 'Groep Geneeskunde Paard' (GGP, the Dutch group of horse veterinarians).

Because Denmark introduced the POM regulation already in 1999, it was possible to evaluate the effect of this regulatory change after about 5 years (Nielsen et al., 2006). Results showed that introduction of POM resulted in increased use of fecal samples to monitor strongyle egg counts and led to a reduced intensity of treatments.

The aim of this study was to examine the effect of the Dutch regulatory change and supportive measures on the practice of veterinarians regarding diagnostics of helminth infections and the rational use of anthelmintics. This was done by means of a questionnaire, once taken shortly after the legislation was introduced and once taken 4 years later.

Material and Methods

Questionnaires
In October 2008 a questionnaire concerning the period prior to July 2008 was sent by mail, including a return envelope, to 1265 veterinarians in production animal health and equine health. The veterinarians were selected from a list of registered practitioners from the Royal Veterinary Association of the Netherlands (KNMvD) and were potentially involved in equine health. The questionnaire included questions about deworming policies and the use of fecal samples in the diagnosis of parasite infections. In May 2012 the same questionnaire was sent to 1033 veterinarians, selected on the same criteria as in July 2008 by the KNMvD. This number was less than in 2008 because the veterinarians that were identified as non-practitioner in the first round were excluded. The questionnaires were returned anonymously. In total 34 questions were asked of which 4 were open-end questions. Some questions included an option for additional comments. If a question allowed more than one answer out of multiple choices, respondents were asked to select no more than 3 answers.

The questions were divided in 4 categories:
- general information about the responding veterinarian
- general information about the veterinary practice
- questions on anthelmintic usage
- questions concerning knowledge about horse helminths and the use of fecal diagnostics

Data analyses
Data were entered in Excel 2010. If a question was not answered, this was treated as a single missing value. Differences in proportions were analyzed according to standard procedures for two independent populations (Petrie and Watson, 2006). Independency was assumed because we did not know who the veterinarians were that filled out the questionnaires and because of the time interval of 4 years. In addition, in some cases Chi-square tests were performed if a variable was categorized into more than two categories to obtain an alternative test statistic confirming that there indeed were statistical differences between 2008 and 2012.

Results

Response rate and general data on veterinarians and practices

In 2008 214 out of 1265 veterinarians responded to the questionnaire, and 180 (14%) actually filled out the questionnaire. Out of those 180 174 indicated that they themselves practiced equine medicine and spent (on average) 46% of their time on horses. In 2012 a similar response was obtained. From 1033 veterinarians receiving the questionnaire 139 responded and 114 (11%) filled out the list of questions. Of these 114 109 indicated that they themselves practiced equine medicine, spending on average 50% of their time on horses. Table 1 presents data on the veterinarians and their practices. In 2012 a slightly higher proportion of the respondents were member of the GGP compared to the 2008 respondents ($P<0.05$). However, overall there were no obvious differences in characteristics of the respondents between both years, including the percentage of the veterinarians who indicated that they would like to become better informed on horse helminths and their control.

Table 1: Data on veterinarians and veterinary practices from the surveys of 2008 and 2012.

	2008 [2]	2012 [2]
Clinic size (fte) [1] :		
mean no. veterinarians	5.86	5.46
mean no. paraveterinarians	3.06	2.90
mean no. assistents	2.49	2.51
Graduated at Utrecht University	90%	87%
Years since graduation	16.5	16
Member of the GGP [3]	61% (107/176)	73% (82/113)
Size of practices in number of horses:		
0 – 499	31% (54/174)	23% (26/113)
500 – 999	22% (38/174)	28% (32/113)
1000 – 1499	15% (26/174)	16% (18/113)
1500 – 1999	9% (15/174)	13% (15/113)
2000 – 2499	5% (8/174)	4% (5/113)
2500 – 2999	6% (11/174)	5% (6/113)
3000 or more	13% (22/174)	10% (11/113)
Average proportion of time spent on horse medicine	46%	50%
Proportion of veterinarians spending ≥80% of time on horse medicine	36% (63/174)	36% (39/109)
Feeling a need for more information on helminths and anthelmintics	64% (111/173)	57% (64/113)

[1] All part-time and full-time personnel was added to obtain the full-time equivalent (fte) figure.
[2] Between brackets the number of veterinarians out of the total.
[3] GGP = Groep Geneeskunde Paard or the Dutch group of horse veterinarians.

Anthelmintic usage

In 2008 the 3 most frequently chosen reasons of veterinarians for selecting a specific anthelmintic for treatment were: 1) on client request; 2) vary compound within a year; and 3) price of the product (Table 2). In 2012 these reasons had shifted. The most frequently chosen reason now was to vary compounds within a year, followed by choosing a product based on anthelmintic efficacy testing and using always the same product. Notably, providing a specific anthelmintic on request of the client declined significantly, whereas choosing a product based on efficacy testing as a reason increased significantly. In both years the most prescribed anthelmintic compounds were ivermectin followed by moxidectin (Table 2).

Table 2: Reasons for choosing an anthelmintic, treatments and off-label use by veterinarians in 2008 and 2012.

	2008	2012	Difference[3]
Reason for choosing a product:			
on client request	45% (76/169)[1]	26% (29/110)[1]	P<0.01
vary compound within a year	39% (65/169)[1]	45% (49/110)[1]	ns
price of the product	33% (56/169)[1]	26% (29/110)[1]	ns
based on efficacy testing	18% (30/168)[1]	41% (45/110)[1]	P< 0.001
always the same anthelmintic	29% (48/168)[1]	28% (31/110)[1]	ns
vary compound every other year	18% (30/168)[1]	6% (7/110)[1]	P<0.01
based on formulation	8% (14/169)[1]	10% (11/110)[1]	ns
Estimated % of treatments with:			
ivermectin	53% (167)[2]	48%(102)[2]	ns
moxidectin	21% (168)[2]	19% (103)[2]	ns
pyrantel	6% (170)[2]	6%(107)[2]	ns
fenbendazole	1% (170)[2]	2%(109)[2]	ns
ivermectin/praziquantel	11% (169)[2]	6%(106)[2]	ns
moxidectin/praziquantel	8% (168)[2]	10% (107)[2]	ns
other	0	1%(109)[2]	ns
Proportion of clients treating based on age:			
foal	63% (164)[2]	60% (109)[2]	ns
yearling	19% (164)[2]	18% (109)[2]	ns
2-3 year old	13% (164)[2]	12% (109)[2]	ns
adult	12% (164)[2]	9% (109)[2]	ns
Proportion of clients treating based on time of year (all age groups):	50% (164)[2]	60% (109)[2]	ns
Using a ML off-label:	49% (80/163)[1]	58% (65/112)[1]	ns
ivermectin	40% (32/80)[1]	28% (18/65)[1]	ns
doramectin	40% (32/80)[1]	58% (38/65)[1]	P<0.05
moxidectin	21% (17/80)[1]	14% (9/65)[1]	ns
other	18% (14/80)[1]	11%(7/65)1	ns

[1] Between brackets the number of veterinarians out of the total.

[2] Number of veterinarians that answered the question.

[3] Significance of the difference between 2008 and 2012

In 2008 the veterinarians estimated that 63% of their clients routinely treated foals based on their age (Table 2). For the other age classes this was much less. No changes were observed between 2008 and 2012. Veterinarians indicated that for all age classes approximately half of their clients applied treatments depending on the time of year (Table 2). Yearlings, 2-3 year olds and adults were preferably treated in spring (April- May) and in autumn (September-October). Again, no differences were seen between 2008 and 2012.

Slightly more than half of the respondents indicated that they (sometimes) used MLs off-label with apparently no decrease in 2012 compared to 2008 (Table 2). The reasons for this off-label use varied. Veterinarians who prescribed or used MLs off-label indicated they (sometimes) used doramectin to treat horses against *Chorioptes*. The off-label use of doramectin increased from 2008 to 2012 ($p<0.05$).

Use and interpretation of fecal diagnostics

Table 3 presents results on the use of fecal samples by veterinarians. Between 2008 and 2012 the number of veterinarians making use of fecal examinations and the number of fecal examinations increased significantly as indicated by several variables. Compared to 2008, in 2012 more samples were used for monitoring and less were used to confirm a clinical diagnosis. There was no shift in fecal examinations between the various horse age classes, but there was a significant shift in season when fecal samples were examined. In 2008 most samples were examined in the autumn, whereas in 2012 most samples were examined in spring. A significant decline was observed in number of veterinarians who never examined feces before treating a horse and there was an even larger significant increase in veterinarians who routinely check the feces for worm eggs before treatment. In 2012 a higher proportion of the feces samples was examined quantitatively compared to 2008, and also more veterinarians used pooled samples at least occasionally.

Table 4 presents data about the interpretation of feces examination results by veterinarians. In 2012 significantly fewer veterinarians treated horses based on just finding a worm egg irrespective of the worm species than in 2008. In 2012 twice as many veterinarians claimed they treat only after they counted a minimum number of worm eggs than in 2008. However, they appeared not to make clear distinctions between the various horse helminths and only half of the veterinarians using a threshold for strongylus-type eggs were able to give a meaningful threshold as advocated by international experts. In 2008, 28% of the veterinarians who claimed to examine fecal samples quantitatively also said they used threshold EPGs before deciding to treat. This improved to 61% in 2012.

Table 3: The use of fecal samples by veterinarians in 2008 and 2012.

	2008	2012	Difference
In my practice fecal samples are examined:	77% (134/174)[1]	95% (105/111)[1]	P<0.001
1-100 samples a year	90% (121/134)[1]	51% (54/105)[1]	P<0.001 [4]
101-250 samples a year	9% (9/134)[1]	30% (32/105)[1]	P<0.001 [4]
251-500 samples a year	1% (2/134)[1]	15% (16/105)[1]	P<0.001 [4]
>500 samples a year	1% (2/134)[1]	3% (3/105)[1]	ns [4]
Estimated maximum number of fecal samples examined:	17,350 (134)	24,400 (105)	
Why are fecal samples examined?			
for clinical diagnosis	60% (134)[2]	26% (103)[2]	P<0.001
on client request	24% (132)[2]	34% (103)[2]	P<0.10
for monitoring	10% (133)[2]	32% (105)[2]	P<0.001
for drug efficacy testing	10% (132)[2]	9% (105)[2]	ns
Proportion of fecal samples analyzed in:			
own laboratory	69% (135)[2]	83% (101)[2]	P<0.05
veterinary diagnostic lab	31% (135)[2]	17% (101)[2]	P<0.05
Fecal samples taken from:			
foals	10% (127)[2]	7% (104)[2]	ns
yearling	15% (127)[2]	13% (104)[2]	ns
2-3 year olds	25% (127)[2]	21% (104)[2]	ns
adults	50% (127)[2]	59% (104)[2]	ns
Proportion of fecal samples examined in: [3]			
winter (Dec-Feb)	8% (113)[2]	6% (98)[2]	ns [5]
spring (Mar-May)	23% (113)[2]	41% (98)[2]	P<0.01 [5]
summer (Jun-Aug)	31% (113)[2]	29% (98)[2]	ns [5]
autumn (Sep-Nov)	38% (113)[2]	25% (98)[2]	P<0.05 [5]
Do you examine a fecal sample before treatment?			
never	28% (47/170)[1]	10% (11/111)[1]	P<0.001 [6]
yes, routinely	21% (36/170)[1]	69% (77/111)[1]	P<0.001 [6]
yes, if there is a clinical suspicion	68% (116/170)[1]	42% (47/111)[1]	P<0.001 [6]
Proportion of fecal samples examined:			
quantitatively	33% (125)[2]	62% (82)[2]	P<0.001 [7]
qualitatively	66% (125)[2]	38% (82)[2]	P<0.001 [7]
otherwise	1% (125)[2]	0% (82)[2]	ns
Do you examine pooled samples?	44% (40/91)[1]	78% (74/95)[1]	P<0.001

[1] Between brackets is the number of veterinarians out of the total.
[2] Number of veterinarians that answered the question.
[3] Veterinarians gave the three months in which the most fecal samples were examined. Subsequently, for each season the total score of the respective three months was added and calculated as a percentage of the overall total score for all twelve months as an estimate in which season the most fecal samples were examined.
[4] Comparison by 2x4 chi-square gave χ^2=46.8, P<0.001.
[5] Comparison by 2x4 chi-square gave χ^2=8.1, P<0.05.
[6] Comparison by 2x3 chi-square gave χ^2=56.2, P<0.001.
[7] Comparison by 2x2 chi-square gave χ^2=16.4, P<0.001.

Table 4: Interpretation of the fecal examination result by veterinarians in 2008 and 2012.

	2008	2012	Difference
I treat if one worm egg is found irrespective of worm species	57% (67/117)[1]	28% (29/104)[1]	P<0.001
I only treat if I find eggs from a particular species:	38% (44/117)[1]	44% (46/104)[1]	ns
Strongylus-type eggs	70% (28/40)[1]	48% (22/46)[1]	P<0.05
Parascaris equorum eggs	83% (33/40)[1]	74% (34/46)[1]	ns
Strongyloides westeri eggs	60% (24/40)[1]	47% (21/45)[1]	ns
Tapeworm eggs	68% (27/40)[1]	71% (32/45)[1]	ns
I only treat if a minimum number of eggs have been counted	25% (28/113)[1]	50% (52/104)[1]	P<0.001
No. of veterinarians giving a threshold for:			
Strongylus-type eggs	79% (22/28)	83% (43/52)	ns
range of EPGs	5-500	50-500	
No. providing a meaningful threshold [3]	41% (9/22)	51% (22/43)	ns
Parascaris equorum eggs [2]	61% (17/28)	56% (29/52)	ns
range of EPGs	5-500	1-400	
Strongyloides westeri eggs	71% (20/28)	54% (28/52)	ns
range of EPGs	1-2000	1-2000	
No. providing a correct threshold [3]	5% (1/20)	10% (5/52)	ns
Tapeworm eggs [2]	4% (1/28)	37% (19/52)	P<0.01
range of EPGs	1-500	1-710	

[1] Between brackets is the number of veterinarians out of the total.

[2] For roundworms and tapeworms there are no specific EPG thresholds communicated.

[3] Meaningful thresholds as advocated by a large number of scientists over the last decade.

Discussion

In 2008 the Dutch government changed the legislation of anthelmintics from freely available into POM. This is similar to the current legislation in Denmark, Finland, Germany, Italy and Sweden (Hinney et al., 2011; Nielsen et al, 2013) and following the European directive (2001/82/EC). However, unlike in Denmark, performing diagnostics before treatment is not part of the current legislation in the Netherlands. The Dutch legislation made veterinarians responsible for the prescription of anthelmintics. The aim of the Dutch legislation was to strengthen the advisory role of veterinarians concerning parasite control in food producing animals, including horses, and to support a rational use of anthelmintics. Their expected role of rational prescribers and distributors was acknowledged by many veterinarians, but how they were supposed to achieve this was not immediately clear to them as became obvious by questions from veterinarians in the field. Subsequently, the Dutch government asked experts to clarify the intention of the new legislation. Sloet van Oldruitenborgh-Oosterbaan et al (2009) therefore provided guidelines for GVP with respect to the prudent use of anthelmintics for horses in the Netherlands. These guidelines were supported by equine internal medicine specialists, veterinary parasitologists as well as legal advisors. It was, therefore, relevant to evaluate the effect of the changed legislation on both the role of the veterinarian in helminth control and the use of fecal diagnostics and anthelmintic usage in horses.

The response rate for both years with 14% in 2008 and 11% in 2012 is low. However, a considerable (but unknown) proportion of the veterinarians was probably not practicing equine medicine and therefore did not return the questionnaire. Considering only the members of the GGP in 2012, the response rate was 25%, about twice as high as the overall 11%. Another factor may have been that most practices employ several veterinarians, usually for different animal groups but in large horse practices also more than one horse veterinarian. It might well be that in such cases only one in the entire practice responded to the questionnaire. Finally, sending the veterinarians a paper questionnaire without an incentive to cooperate and without contacting them otherwise probably resulted in a larger proportion of non-responders. Nielsen et al. (2006), who also questioned veterinarians, got a response rate of 51%, but they had prior phone contact and gave reminders every two weeks for about two months. From a different perspective, based on the sizes of the respondent's practices the numbers of horses covered ranged, conservatively estimated, between 100,000 to 200,000 in both 2008 and 2012. With approximately 400,000 horses in the Netherlands this appears to be a considerable coverage of the total horse population. Overall, the respondents in both years appeared comparable for all variables listed in Table 1. Consequently, it is thought that the comparison between 2008 and 2012 is valid and produced data

representative for the entire Dutch population of veterinarians attending horses.

With respect to the usage of anthelmintics several differences were observed from 2008 to 2012. In 2012 fewer veterinarians indicated they chose a specific anthelmintic on the request of the client. Also, more often an anthelmintic was chosen because it had been found efficacious. In itself these changes seem to indicate an increased conscious involvement of the veterinarian in the choice of product. However, in both years the MLs were the products of choice, which is not surprising as there is more drug resistance to the products from the other anthelmintic groups. More interestingly, the number of veterinarians routinely examining a fecal sample prior to possible treatment increased by more than three-fold from 2008 to 2012, whereas the number of veterinarians never examining fecal samples before (possible) treatment declined significantly. These results do suggest that veterinarians increasingly apply anthelmintics when based on more rational grounds. Nonetheless, there appears to be room for further improvement, especially since it is still allowed to sell anthelmintics for horses to owners for one year in advance.

No large changes were observed in the off-label use of anthelmintics. On the contrary, the off-label use of doramectin, a product not registered for horses in the Netherlands, increased significantly from 2008 to 2012. Particularly in 2012 doramectin was mainly used to treat chorioptic mite infections. The substantial off-label use of doramectin was also shown by Fritzen et al. (2010).

As the use of anthelmintics as such was put under the POM legislation in 2008, supporting activities from experts focused on putting the POM legislation within the context of Good Veterinary Practice as intended by the government (Sloet van Oldruitenborgh-Oosterbaan et al., 2009). One of the major aspects in this respect concerns the use of fecal samples to monitor helminth infection and in particular cyathostomin infection. Results clearly showed that in 2012 more fecal samples were examined than in 2008, more fecal samples were taken for monitoring purposes, more fecal samples were examined quantitatively and more use was made of pooled fecal samples (Eysker et al., 2008). Also, though borderline significant, more fecal samples were examined on request by clients. This confirms findings from Nielsen et al. (2006) and may suggest that, apart from veterinarians, horse owners also show increased awareness regarding the prudent use of anthelmintics. Interestingly, relatively more feces samples were examined in spring in 2012 compared to 2008. This suggests that more horses are examined before they are turned out after the winter to evaluate the risk of new pasture contamination and hence an evaluation of the need to treat before pasturing. On the other hand, the (optimistically) estimated processed number of fecal samples in 2012 was ca. 25,000 samples and compared to the total number of ca. 400,000 horses this appears to be a small number of

diagnostics performed. Also, although the use of fecal samples has increased from 2008 to 2012, the interpretation of the results of those fecal examinations still leaves room for substantial improvement. Although veterinarians claim they do examine at least part of the samples quantitatively, about 40% of those veterinarians appeared not to use a threshold EPG before deciding to treat in 2012. Moreover, many of the veterinarians who did claim to use threshold EPGs could not provide meaningful threshold values. Also, threshold values were given for parasites such as roundworms and tapeworms, for which no threshold values were ever indicated by experts in the Netherlands. These findings corroborate with the fact that in 2012 as many veterinarians expressed a desire to become better informed on horse helminths and their control as in 2008.

Overall, given the fact that the current legislation only made anthelmintics POM, without further restrictions regarding amounts of drugs allowed per prescription and without requiring diagnostic testing before allowing treatment, some significant improvements in the prudent use of anthelmintics were accomplished. Clearly, more use is made of fecal diagnosis in the control of helminth infections in horses and treatments appear to be increasingly based on fecal diagnosis. However, there is still room for further improvements. Specifically, continuing efforts need to be focused on improving the number of quantitatively processed fecal samples and the interpretation of fecal diagnosis results.

Acknowledgments

We thank the ministry of Economische zaken, Landbouw & Innovatie, and especially Mrs. Sparnaaij, for their financial support. We thank Dick Heederik from the Institute of Risk Assessment Sciences (Utrecht University) for his support with the questionnaire design. We thank our colleagues from the division of Clinical Infectiology for their help with the distribution. We would like to thank all our respondents for filling in the questionnaire.

References

Allison K., Taylor N.M., Wilsmore A.J., Garforth C., 2011. Equine anthelmintics: survey of the patterns of use, beliefs and attitudes among horse owners in the UK. Vet. Rec. 168:483 doi:10.1136/vr.d731.

Anonymous, 2006. Implementing Directive 2001/82/EC of the European Parliament and of the Council as regards the establishment of criteria for exempting certain veterinary medicinal products for food-producing animals from the requirement of a veterinary prescription. In: Union, E. (Ed.), vol. 2006/130/EF. Brussels.

Eysker M., Bakker J., van de Berg, van Doorn, D.C.K., Ploeger H.W., 2008. The use of age-clustered pooled faecal samples for monitoring worm control in horses. Vet. Parasitol. 151, 249-255.

Fritzen, B., Rohn, K., Schnieder, T., von Samson-Himmelstjerna G., 2010. Endoparasite control management on horse farms - lessons from worm prevalence and questionnaire data. Equine Vet. J. 42, 79-83.

Hinney B., Wirtherle N.C., Kyule M., Miethe N., Zessin K.H., Clausen P.H., 2011. A questionnaire survey on helminth control on horse farms in Brandenburg, Germany and the assessment of risks caused by different kinds of management. Parasitol. Res. 109, 1625-1635.

Lendal S., Larsen M. M., Bjùrn H., Craven J., Chriel M., Olsen S.N., 1998. A questionnaire survey on nematode control practices on horse farms in Denmark and the existence of risk factors for the development of anthelmintic resistance. Vet. Parasitol. 78, 49-63.

Lloyd S., Smith J., Connan R.M., Hatcher M.A., Hedges T.R., Humphrey D.J., Jones, 2000. Parasite control methods used by horse owners: factors predisposing to the development of anthelmintic resistance in nematodes. Vet. Rec. 146, 487-92.

Matthee S., Dreyer F.H., Hoffmann W.A., van Niekerk F.E., 2002. An introductory survey of helminth control practices in South Africa and anthelmintic resistance on Thoroughbred stud farms in the Western Cape Province. J.S. Afr. Vet. Assoc. 73, 195-200.

Nielsen M.K., Monrad J., Olsen S.N., 2006 Prescription-only anthelmintics; a questionnaire survey of strategies for surveillance and control of equine strongyles in Denmark. Vet. Parasitol. 135, 47-55.

Nielsen M.K., Reist M., Kaplan R.M., Pfister K., van Doorn D.C.K., Becher A., 2013. Equine parasite control under prescription-only conditions in Denmark – awareness, knowledge, perception, and strategies applied. Vet. Parasitol. DOI:10.1016/j.vetpar.2013.10.016.

O'Meara B., Mulcahy G., 2002. A survey of helminth control practices in equine establishments in Ireland. Vet. Parasitol. 109, 101–110.

Osterman Lind E., Rautalinko E., Uggla A., Waller P., Morrison D.A., Höglund J., 2007. Parasite control practices on Swedish horse farms. Acta Vet. Scan. 49: 25.

Petrie A., Watson P., 2006. Statistics for veterinary and animal science. Second edition. London, Blackwell publishing.

Relf V.E., Morgan E.R., Hodgkinson J.E., Matthews J.B., 2012. A questionnaire study on parasite control practices on UK breeding Thoroughbred studs. Equine Vet. J. 44, 466-71.

Sloet van Oldruitenborgh-Oosterbaan M.M., van Doorn D.C.K., Holland W., van Herten J., Ploeger H.W., Fink-Gremmels J., 2009. Antiparasitaire middelen en de receptplicht voor paarden. Tijdschr. Diergeneeskd. 134, 288-295.

Stratford C.H., Lester H.E., Morgan E.R., Pickles K.J., Relf V.,McGorum B.C., Matthews J.B., 2013. A questionnaire study of equine gastrointestinal parasite control in Scotland. Equine Vet. J. 46, 25-31.

Chapter 6

In vitro selection and differentiation of ivermectin resistant cyathostomin larvae

D.C.K. van Doorn[1], F.N.J. Kooyman[1], M. Eysker[1], J.E. Hodgkinson[2], J.A. Wagenaar[1], H.W. Ploeger[1]

1 Department of Infectious Diseases and Immunology, Faculty of Veterinary Medicine, Utrecht University, Yalelaan 1, 3584 CL Utrecht, the Netherlands
2 Department of Comparative Molecular Medicine, School of Veterinary Science, University of Liverpool, L69 7ZJ, UK

** Corresponding author: phone +31302532459*
e-mail address d.c.k.vanDoorn@uu

Published: Veterinary Parasitology 174 (2010), 292-299.

Abstract

Cyathostomins are considered to be the primary helminth pathogen of horses and macrocyclic lactones (ML) are the most frequently used anthelmintics. Therefore, ML resistance is a serious threat for the control of these parasites.

In the present study ivermectin resistant cyathostomin L3 were *in vitro* selected, using a reiterative larval migration inhibition assay (rLMIA) and differentiated by reverse line blot (RLB). Larvae were obtained from two populations, one from a never treated, free-roaming horse population in the nature reserve Oostvaardersplassen (OVP) and the other from regularly ivermectin-treated ponies of Utrecht University (UU). In the rLMIA the proportion of larvae that migrated increased with each passage, demonstrating that the applied procedure indeed selects for larvae the least susceptible for ivermectin. This was further supported by the fact that glutamate addition to this procedure reversed the selection effect, which also suggests that glutamategated chloride channels (GluCls) play a role in the ivermectin resistance of the selected L3.

In both populations the predominant species were *Cyathostomum catinatum*, *Cylicostephanus longibursatus* and *Cylicocyclus nassatus*. After *in vitro* selection in the rLMIA in the presence of ivermectin the predominant species became *C. catinatum* in both larval populations, while *C. nassatus* disappeared in the never treated OVP larval population but not in the regularly ivermectin-exposed UU population.

It is concluded that the rLMIA and RLB can be used to study anthelmintic resistance in cyathostomin populations and to study differences and changes in species composition between populations with different anthelmintic exposure histories.

Keywords: Cyathostomins, glutamate gated chloride channel, Ivermectin, Larval Migration Inhibition Assay, Resistance, Reverse Line Blot

Introduction

Cyathostomins are the most prevalent pathogenic helminths in horses (Love et al. 1999). Predominantly young horses are affected. Clinical signs include weight loss, diarrhea and edema. Larval cyathostominosis may even result in death. Horse owners tend to rely heavily on anthelmintics to control these parasites.

As resistance of cyathostomins against benzimidazoles and pyrantel is common, worm control in horses strongly relies on the efficacy of the macrocyclic lactones (Boersema et al., 1991, Kaplan, 2002). However, recently resistance against ML has also been discovered. With a faecal egg count reduction test (FECRT), reduced ML efficacy has been shown in donkeys in the United Kingdom (Trawford et al., 2005). Other authors reported shortening of the egg reappearance period (ERP) (von Samson Himmelstjerna et al., 2007, Lyons et al., 2008, Molento et al., 2008) and this shortening of the ERP should be considered as a sign of developing resistance (Lyons et al., 2009). This resistance is a threat for controlling cyathostomins in horses.

The FECRT is still the method of choice to confirm anthelmintic resistance, but it does not allow detection of early development of ivermectin resistance in cyathostomin populations. As the molecular mechanisms leading to ML resistance in cyathostomins are unknown, there are currently no options for molecular detection of resistance.

Ivermectin belongs to the class of ML and glutamate-gated chloride channels (GluCls) are considered to be the major site of action (Wolstenholme and Rogers, 2005). Binding of ivermectin to GluCl of nematodes was first described for *Caenorhabditis elegans* (Cully et al., 1994). GluCl is a family with many members and not all of them are able to bind ivermectin (Wolstenholme and Rogers, 2005). Mutations in one member, avr-14, caused decreased binding of ivermectin in *Cooperia oncophora* and this was correlated with ivermectin resistance (Njue and Prichard, 2004). Introducing the same mutation in GluCls of susceptible *Haemonchus contortus* also caused decreased ivermectin binding (McCavera et al., 2009). Not much is known about GluCls in cyathostomins. Only one α and ß subunits of GluCl from the cyathostomin *Cylicocyclus nassatus* were cloned and expressed (Tandon et al., 2006). They were able to demonstrate ivermectin binding to the GluCl-α subunit, but not to the GluCl-ß subunit. Glutamate is the physiological ligand for the GluCl and decreased affinity for ivermectin goes together with decreased affinity for glutamate (Njue and Prichrad, 2004). Furthermore, the presence of glutamate increases the affinity for ivermectin without affecting maximum binding (Forrester et al., 2002), suggesting that the binding sites for both ligands are located in each other's vicinity.

Molecular detection of resistance is also hampered by the existence of 50 species of cyathostomins (Lichtenfels et al., 2008) of which eggs and larvae cannot be differentiated morphologically. Therefore, the availability of techniques that enable detection of resistance and the differentiation of the infective larvae (L3) would be useful. The larval migration inhibition assay (LMIA), where L3s migrate in the presence of different concentrations of ML is such a technique. Robinson et al (2008) used this method and showed a dose response curve for exsheathed L3 migrating in different concentrations of ivermectin and moxidectin. They also found slightly lower LD50's for susceptible

than for resistant populations. With respect to species differentiation, the Reverse Line Blot assay (RLB) has proven to be a reliable molecular technique (Kaufhold et al., 1994) which has also been applied for differentiating 13 cyathostomin species (Traversa et al., 2007). The RLB is based on hybridization of a species specific probe with an amplified fragment of the inter genic spacer (IGS) region.

The aims of this study were to select ivermectin resistant L3 by a modified LMIA and to differentiate the selected L3 individually by a modification of the reverse line blot.

Materials and Methods

Parasites
Two cyathostomin populations were included. An ivermectin naive population was obtained from Konik ponies grazing the nature reserve Oostvaardersplassen (OVP) in The Netherlands. This reserve was formed in the 1970s and the horses were introduced before ivermectin became available on the Dutch market. Since their introduction in OVP the horses were never treated with anthelmintics and therefore harbour a cyathostomin population never exposed to ML. The larvae were cultured from freshly dropped faeces collected in September 2008, April 2009 and October 2009. The second cyathostomin population was obtained from Shetland ponies from the Faculty of Veterinary Medicine, Utrecht University (UU). Until 2006 they were year round routinely treated (approximately four times per year) with ML and since 2006 were treated with ML based on faecal egg counts (FEC). The on average treatment per horse between 2006 and April 2009 is once per year with ivermectin. The faeces for larval culture were collected rectally in April 2009.

All faecal samples were cultured in glass jars with a plastic lid, at approximately 21°C (room temperature). These pooled cultures contained faeces from 5 to 10 horses. After 10 days the jars were completely filled with lukewarm tap water and turned upside down on Petri dishes, replenished with tap water. The next day the third stage larvae (L3) were recovered and stored for future use at 4°C.

Larval Migration Inhibition Assay (LMIA)
In the LMIA, ML paralyses the exsheathed larvae (xL3) and inhibit migration through a sieve. The method is based on Robinson et al. (2008). L3 were exsheathed with 0.1% hypochlorite the day before LMIA and viable xL3 were collected in a Baermann glass overnight. A known number of larvae were pre-incubated (2h, 37°C in the dark) in PBS containing different concentrations of ivermectin (Sigma-Aldrich) from a stock solution in dimethylsulfoxide (DMSO) with an end concentration of 1% DMSO. After pre-incubation the larvae were allowed to migrate in the same solution through a sieve (cell strainer 40 µm,

Becton Dickinson) for 1 h at 37°C in the dark. For reasons of accuracy numbers of xL3s applied to the sieve were such that each fraction after migration contained at least 50 larvae. xL3 that migrated through the sieve were fixed and stained in iodine, counted using a dissection microscope and, when appropriate, isolated individually and stored at -80ºC until analysis by reverse line blot (RLB).

Reiterative LMIA (rLMIA)

For *in vitro* selection of ivermectin resistant larvae a rLMIA, using four consecutive sieves, was employed. The principle is based on the selection of the least susceptible larvae by repeated migration in the presence of ivermectin. Larvae from a population homogenous for ivermectin susceptibility will all have an equal chance on migration at a given ivermectin concentration. Larvae that have migrated through the first sieve will have the same chance of migrating through a second sieve. But if the population is heterogeneous for ivermectin susceptibility, however, the proportion of larvae that migrate through each consecutive sieve will increase because with each passage the least susceptible L3 are selected. As a control for adaptation we performed the LMIA with only one sieve, but at different times after the pre-incubation. One batch was allowed to migrate directly after 2 h of pre-incubation, at the same time as the first migration of the rLMIA. Another batch was allowed to migrate after 5 h of pre-incubation, at the same time as the fourth migration of the rLMIA. Pre-incubation and migration conditions were performed as described above for the LMIA. Two concentrations of ivermectin were used, 0.24 or 30 µg/ml, next to a control without ivermectin. The 0.24 µg/ml ivermectin concentration was used because it is comparable to the therapeutic dose (200 mg/kg body weight), and 30 µg/ml was the maximum concentration that dissolves in 1% DMSO. For determining the involvement of GluCls in the ivermectin susceptibility of xL3s, L-glutamic acid was added, when appropriate, to the pre-incubation solution from a stock solution of 10 mM in PBS. For calculating the migration percentage per sieve, the larvae that did not pass a sieve were collected and together with the larvae that migrated through the fourth sieves, they were stained, counted and isolated as described for LMIA.

Lysis of individual xL3

Each individually isolated xL3 was lysed in 25 µl proteinase K/Worm Lysis Buffer (WLB) (4.5 U/ml proteinase K, 50 mM KCl, 10 mM tris pH 8.3, 2.5 mM MgCl2, 0.45% NP-40, 0.45% tween-20 and 0.01% gelatin). The mixture was frozen for 15 min at -80°C and the xL3s were lysed overnight at 56-60°C. Proteinase K was heat inactivated the next day at 95°C for 15 min.

Amplification of inter genic spacer (IGS) fragment

With RLB we were able to detect the amplified IGS from a single individual xL3. Conditions and primers for amplification of the IGS fragment were as described

for adult worms by Traversa et al. (2007), except that we used 2.5 µl lysate of individual xL3 per 25 µl PCR reaction. Primers were 5' biotinylated as described by Traversa et al. (2007).

Reverse line blot (RLB) hybridization

The RLB for species specific hybridization of IGS amplicons from cyathostomins is a modification of the method of Traversa et al. (2007). Probes specific for *Coronocyclus coronatus*, *C. labiatus*, *C. labratus*, *Cylicostephanus goldi*, *C. longibursatus*, *C. minutus*, *Cylicocyclus ashworthi*, *C. insigne*, *C. leptostomum* and *C. nassatus* were based on IGS sequences (Kaye et al.1998) and are given by Traversa et al. (2007). We confirmed the specificity of the probes for the common species (*C. goldi*, *C. insigne*, *C. labratus*, *C. longibursatus* and *C. nassatus*) by hybridisation with PCR products from morphologically differentiated adults (Lichtenfels et al., 2008). Probes specific for *Cyathostomum catinatum* (CATD5, CGACTAGGCGTACATCATA), *C. pateratum* (PAT5, CATACAGTTGTAACATTCTCG) and *Cylicostephanus calicatus* (CAL2, ACATGCAACACCCTGTTCAA) were designed by us, because we could not achieve specific hybridization for these species with the probes described by Traversa et al. (2007). For that, we aligned the contemplated probes (see Supplementary data) with recently corrected IGS sequences for *C. catinatum* (accession no. HM142927) and *C. calicatus* (accession no. HM142928), and a new *C. pateratum* consensus sequence. Specificity of the three newly designed probes was confirmed by hybridisation with PCR products from morphologically differentiated adults (Lichtenfels et al., 2008) of these three species. Specificity of the probes for *C. ashworthi*, *C. leptostomum* and *C. nassatus* was confirmed by alignment with new consensus sequences showing a greater level of intraspecific variation (Hodgkinson unpublished data). Alignments of all specific probes and IGS sequences are given as Supplementary data with the exception of the IGS sequences from *C. labratus* (not present in any database) and *C. labiatus* (too different from the others for a meaningful alignment). Ten microlitres of the IGS amplicon per lane was used for hybridization. A mixture of PCR products of xL3s were used as positive controls for each hybridization. Hybridized products were detected with streptavidin-POD conjugate (Roche), followed by incubation in ECL detection reagent and exposure to Amersham Hyperfilm ECL (both GE Healthcare).

Statistics

Where appropriate, differences in means between groups or treatments were statistically analysed using general variance of analysis techniques (Student's t, one-way ANOVA, GLM) using the package SPSS 16.0 for Windows. Changes in population composition were tested using χ^2. Tests were done after appropriate transformation of data to ensure homogeneity of variance across groups. All differences, and where appropriate interaction terms, were tested two-tailed and were considered significant when $p < 0.05$.

Results

Dose dependent inhibition of migration

In the LMIA a dose response effect ($p<0.001$) was found for both populations over a wide range of ivermectin concentrations (Fig. 1). There was also a significant difference between the UU and the OVP population ($p<0.01$), while the interaction between ivermectin concentration and larval population was not significant. The concentration that inhibits 50% of the larvae from migrating (IC50) was 0.031 and 0.025 µg/ml ivermectin for the OVP and UU population, respectively.

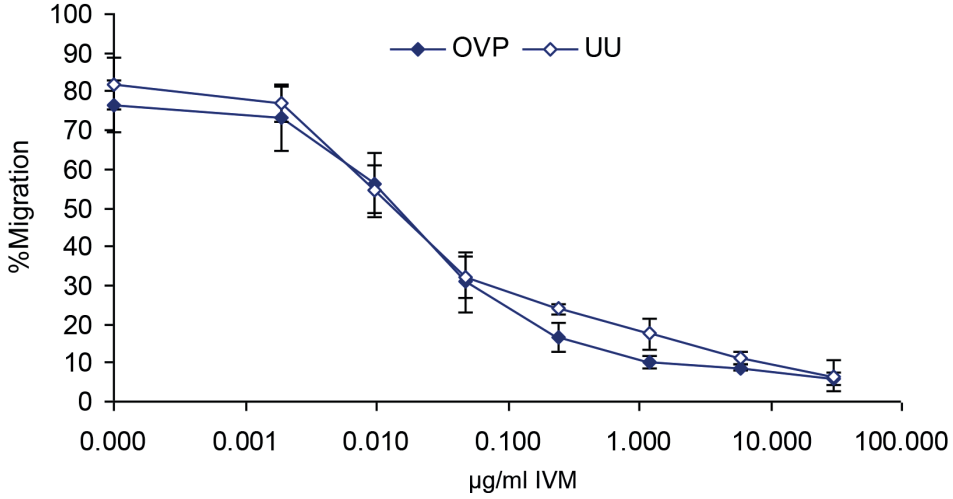


Fig. 1. Effect of different ivermectin concentrations on migration of xL3 in the LMIA. For each larval population (OVP and UU) two migration assays were done, both in duplicates. Results are given as the mean (± SD).

Selection of ivermectin resistant xL3

In the rLMIA we determined the migration percentage through four consecutive sieves in the presence of 0, 0.24 or 30 µg/ml ivermectin. The proportion migrated larvae increased with each passage in both populations (Fig. 2). The proportion migrated larvae after the fourth sieve in the presence of ivermectin almost equalled that without ivermectin. The overall migration percentage through all four sieves combined was for the OVP population 46%, 0.6% and 0.2% for 0, 0.24 and 30 µg/ml ivermectin, respectively. For the UU populations it was 64%, 4.1% and 0.9%, respectively. There was no increase in migration percentage in the absence of ivermectin, ruling out selection for motility as such. The prolonged pre-incubation step did not result in a change in the migration percentage over the first sieve (data not shown), ruling out adaptation as a cause for the increasing migration over consecutive sieves.

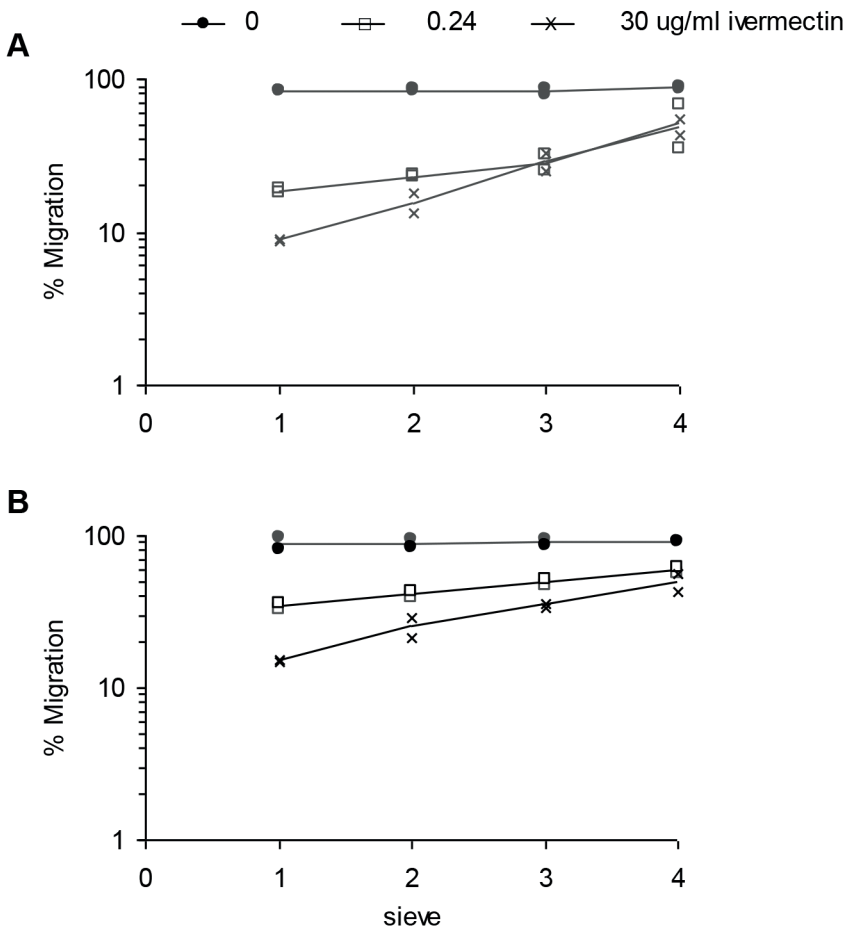


Fig. 2. Migration percentage through four consecutive sieves in the rLMIA. xL3s from OVP, culture II (A) and UU, culture I (B) were allowed to migrate in the presence or absence of ivermectin The percentage migration through each sieve was determined by duplicate measurements. The mean (line) and duplicate values are given (●, □, X)

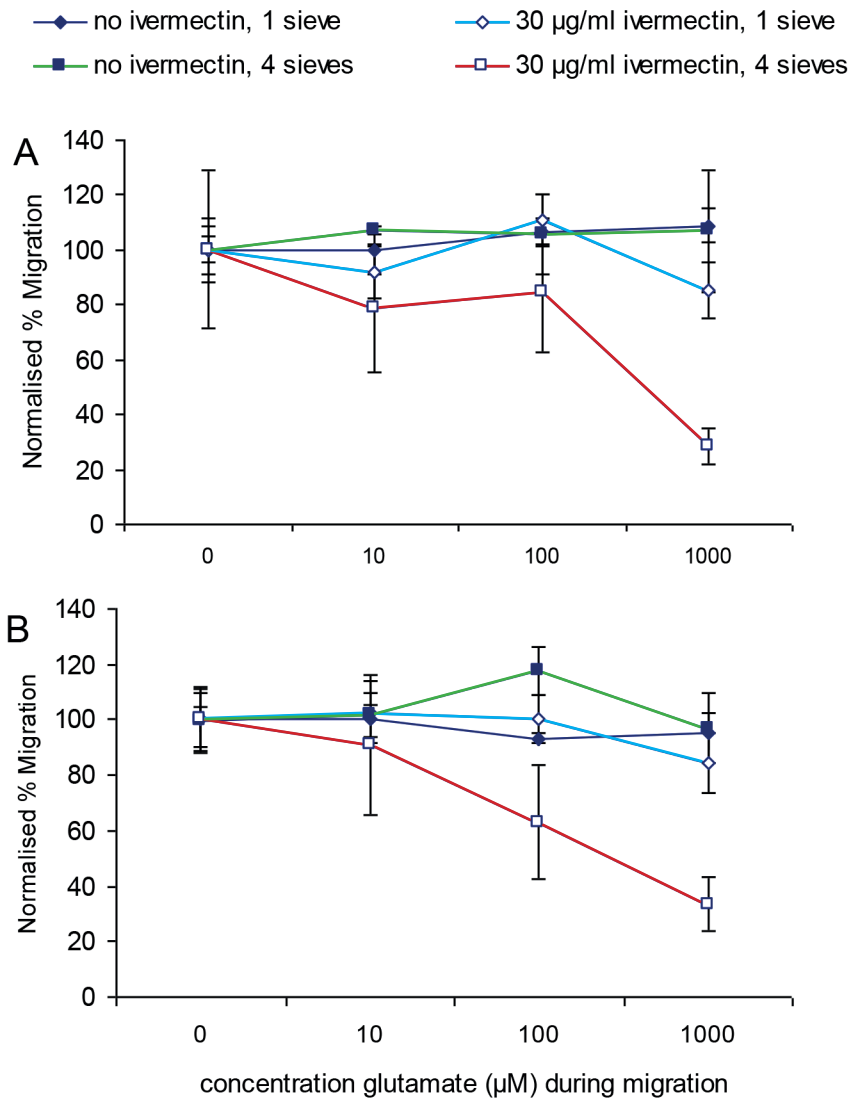


Fig. 3. The effect of different glutamate concentrations on larval migration in the absence or presence of ivermectin. xL3's from population OVP, culture III (A) and population UU, culture I (B) were allowed to migrate through 1 (control) or four consecutive sieves in the presence of glutamate and ivermectin in the concentrations as indicated. Migration percentage was normalized to 100% for the given ivermectin concentration and number of sieves without glutamate. Each data point is the mean (± SD) of two independent migration assays and each assay was performed in duplicate. No significant difference was detected between both larval populations. The main effects of glutamate concentration and the combination of number of sieves and ivermectin concentration were both significant at $p<0.001$. Also, the interaction between both main effects was highly significant ($p<0.001$).

Selection of ivermectin resistant xL3 is reduced in the presence of glutamate

Exsheathed L3s were allowed to migrate in the presence or absence of glutamate and/or ivermectin. Glutamate by itself did not result in a change in migration in the absence of ivermectin (Fig. 3). The same was true for the combination of glutamate and ivermectin when the migration of the initially predominantly susceptible larvae fraction was measured (migration through the first sieve). However, when larvae were allowed to migrate over four consecutive sieves in the presence of both ivermectin and glutamate the migration percentage became substantially reduced, suggesting that glutamate increased binding of ivermectin in resistant xL3. The effect was similar for the OVP (Fig. 3A) and UU population (Fig. 3B).

Differentiation of ivermectin resistant xL3

Three cultures from the OVP population and one from UU population were analysed with RLB before and after rLMIA (Table 1). In the absence of ivermectin no significant differences were found between the fraction not subjected to LMIA and the fraction obtained after the rLMIA, indicating that migration itself was not selective. All 13 species for which a specific probe was present were found. The three cultures of OVP showed comparable species composition and the three most abundant species in both the OVP

Table 1: Species composition in percentages before and after selection by reiterative LMIA and differentiated by RLB. xL3 from OVP (3 cultures) and UU (one culture) were selected in rLMIA with different concentrations of ivermectin and/or glutamate as follows: fraction not subjected to LMIA (X), selected without ivermectin (0), with 0.24 µg/ml ivermectin (0.24), with 30 µg/ml ivermectin (30) or with 30 µg/ml ivermectin and 1 mM glutamate (30G). Random aliquots from each fraction were analyzed by RLB in the numbers as indicated on the bottom line.

	OVP											UU			
	culture I, Sept 2008				culture II, April 2009				culture III, Oct 2009			culture I, April 2009			
	x	0	0.24	30	x	0	0.24	30	x	30	30G	x	0	0.24	30
Cyathostomum catinatum	5	12	17	74	18	25	51	89	38	89	79	31	35	45	76
C. pateratum	5	6		5	3	6		2	15	5	5	3			
Coronocyclus coronatus	10		6		8										
C. labiatus					2							3			
C. labratus							3								
Cylicostephanus calicatus						3	3	2					3		
C. goldi	5			5			3	2	3	5	11	6	15	11	5
C. longibursatus	25	18	44	11	11	6	34	6	9		5	11	12	3	3
C. minutus					9	16			6			8	3	11	
Cylicocyclus ashworthi	10	18	6			9	3		3						
C. insigne													3		
C. leptostomum	5	6													
C. nassatus	5	18			36	31	3		12			33	29	29	11
cyathostome + / specific -	30	24	28	5	14	3			15			6		3	5
Number XL3 in RLB	20	17	18	19	66	32	35	63	34	19	19	36	34	38	38

and UU population were *C. catinatum*, *C. longibursatus* and *C. nassatus*. We analysed from both populations the cultures collected at the same period (April 2009). The species other than *C. catinatum*, *C. longibursatus* and *C. nassatus* were grouped together as other cyathostomins (Fig. 4), because prevalence of these species was generally too low to be analysed separately. *In vitro* selection for resistance resulted in a changed species composition in both the OVP and UU population ($p<0.001$).

Before the rLMIA 18% of the OVP population was *C. catinatum* and after selection it increased to 51% and 89% in the presence of 0.24 or 30 µg/ml ivermectin, respectively. Within this population, *C. nassatus* appeared to be very susceptible to ivermectin. Before the rLMIA, the relative frequency was 36% and this decreased to 3 and 0% after selection with 0.24 or 30 µg/ml ivermectin, respectively. The species composition of the UU population also changed after *in vitro* selection. The relative frequency of *C. catinatum* increased from 35% before to 45% and 76% after selection with 0.24 and 30 µg/ml ivermectin, respectively, with changes not being significantly different from those observed in the OVP population. However, the relative frequency of *C. nassatus* in the UU population did not change after selection with 0.24 µg/ml ivermectin, while after selection with 30 µg/ml *C. nassatus* still made up 11 % of the population. These changes for *C .nassatus* did differ significantly from those seen in the OVP population ($p<0.001$).

The relative frequency of one species is influenced by the abundance of other species. Therefore, the migration percentage per species before and after selection was calculated (Fig. 5). The migration percentage in the absence of ivermectin was approximately 60% for all species. The percentage migrated *C. catinatum* was for both populations 1-2% in both the 0.24 and the 30 µg/ml ivermectin concentration, respectively. Hardly any selection for *C. catinatum* occurred between 0.24 and 30 µg/ml ivermectin. For the other species the migration percentages after selection with 30 µg/ml ivermectin were lower and ranged from 0% to 0.12% and from 0.24% to 0.42% for the OVP and UU population, respectively. Including glutamate in the reiterative LMIA decreased the migration (Fig. 3), but no influence on the species composition was observed (Table 1).

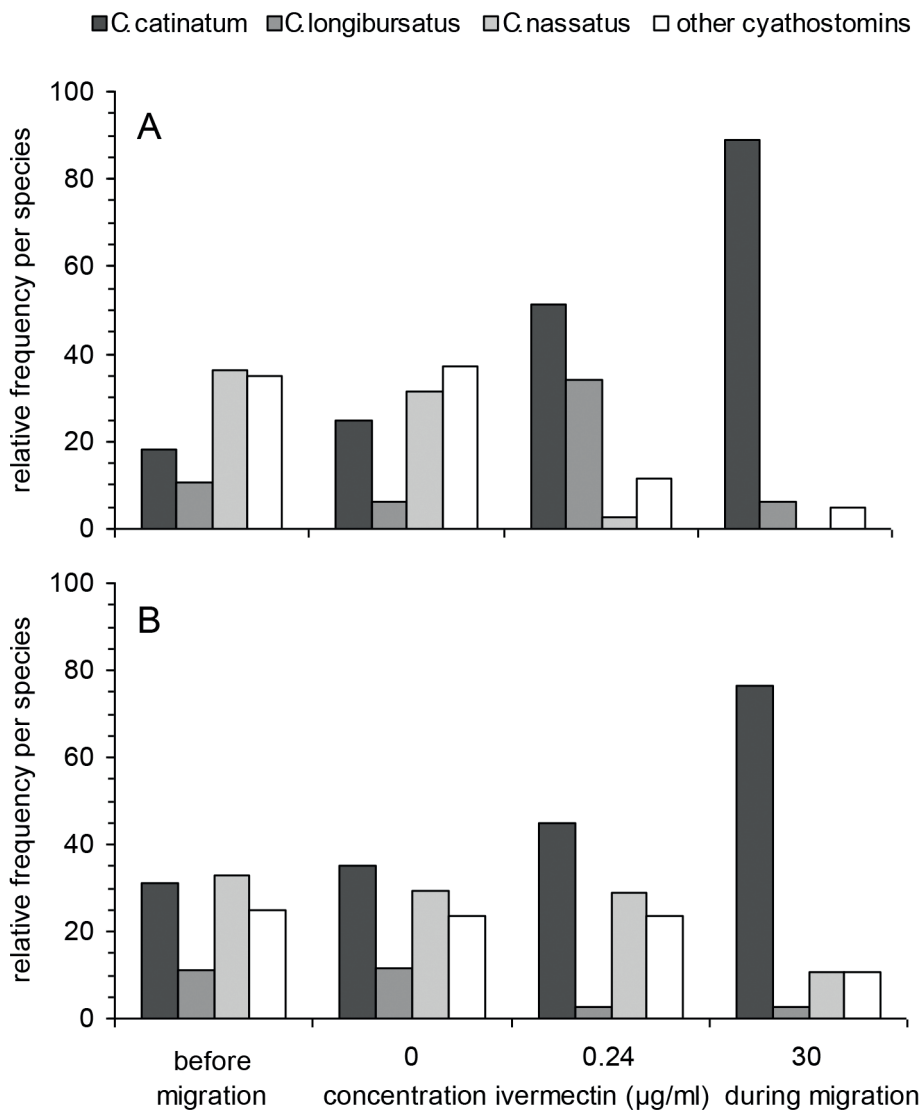


Fig. 4. Species composition of cyathostomins before and after selection by reiterative LMIA and differentiated by RLB. xL3s from population OVP, culture II (A) and UU, culture I (B) were allowed to migrate in rLMIA with ivermectin concentrations as indicated. Randomly picked xL3 were differentiated by RLB. The three most common species (*Cyathostomum catinatum*, *Cylicostephanus longibursatus* and *Cylicocyclus nassatus*) are indicated separately, all other cyathostomins are combined. For total number of xL3 subjected to RLB and composition of "other cyathostomins", see Table 1.

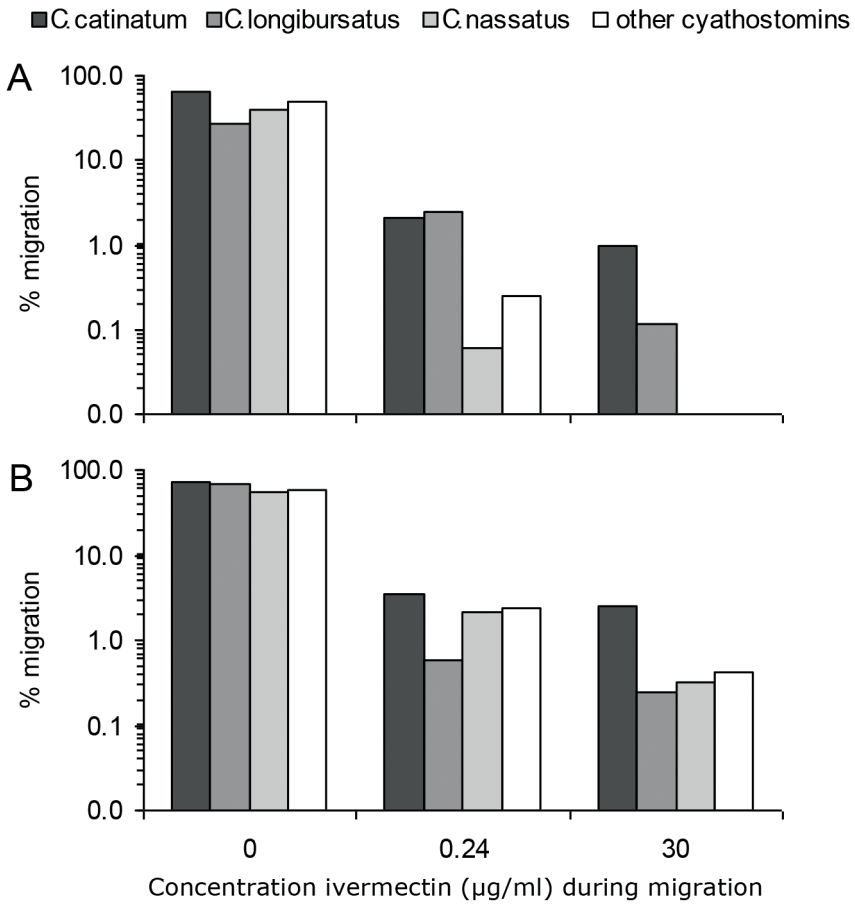


Fig. 5. Percentage migration per cyathostomin species through four sieves of the OVP (A) and UU (B) population in 0, 0.24 or 30 µg/ml ivermectin. Data were calculated from the rLMIA results as shown in Table 1 and based on actual numbers of larvae as used in the assays.

Discussion

We selected and differentiated ivermectin resistant cyathostomin xL3 by the combined use of rLMIA and RLB. This demonstrated a large difference in susceptibility between species and it allows studying the molecular mechanisms of ivermectin resistance.

Re-applying the migrated xL3 on a consecutive sieve demonstrated that the proportion of larvae that migrated in the presence of ivermectin increased with each consecutive sieve. The migration percentage through the 4th sieve in the presence of ivermectin approached that of migration without ivermectin. We found no increase in the proportion of larvae that migrated in the absence of ivermectin and prolonged pre-incubations had no influence on the migration percentage, suggesting that selection for adaptation and motility as such did not occur. Therefore, the results indicate that the observed increase in migration through consecutive sieves in the presence of ivermectin indeed reflects selection for xL3 that are non-responsive or resistant for ivermectin.

Glutamate is the ligand for glutamate-gated chloride channels (GluCls) and binding to these GluCls is considered to be the principle working mechanism of ivermectin. Ivermectin concentrations used in the rLMIA were relatively high (0.24 and 30 µg/ml), having the risk of not selecting for the relevant GluCls, but for other receptors with lower affinity for ivermectin than GluCls, like GABA. However, in the rLMIA we did find an effect of glutamate on ivermectin susceptibility. Glutamate increases the affinity of ivermectin for GluCls (Forrester et al., 2002 and Wolstenholme and Rogers, 2005), offering an explanation for the increased effect of ivermectin on resistant xL3 in the presence of glutamate as indicated by the decreased migration found in the present study. This effect makes it likely that GluCls also play a role in this *in vitro* selection procedure.

Larvae are not adults and even when GluCls are involved in ivermectin resistance in both stages, the effect may differ. For example, xL3 do not feed and therefore ivermectin that binds to GluCls located in the pharynx (if they are present) will probably not strongly affect the motility of those larvae, but may kill an adult worm. Whatever the mechanism, the LMIA for ML susceptible cyathostomin populations gave different results than for resistant ones (Robinson et al., 2008), indicating that at least some effect involving motility is similar in xL3's and adults. In the more thoroughly studied *H. contortus* it was found that a correlation between *in vitro* LMIA results and *in vivo* resistance depended on the way the strains were selected (Gill and Lacey, 1998). When sub-therapeutic doses were used for selection, there was no correlation. However, when strains were isolated in the field with therapeutic doses there was a good correlation between motility as measured in the LMIA and observed

in vivo resistance. Although it remains to be demonstrated that different behavior in larval migration reflects *in vivo* ML resistance in cyathostomins, the LMIA has been identified as a promising diagnostic tool (McArthur et al., 2009; Kaplan, 2009). This is supported by the present results. Modifying the LMIA to an rLMIA increases the detection of resistant larvae while at the same time the resistance of the population as a whole can be quantified as increase in migration percentage. The rLMIA also offers the possibility to study pre-existing resistant variants, because there is no new generation necessary for selection, meaning no recombination due to sexual reproduction. rLMIA also offers the possibility to isolate individual xL3 for differentiation, allowing examination of possible cyathostomin species differences.

We differentiated the selected xL3 by RLB. Initially, we tried the probes as described by Traversa et al. (2007). However, by applying morphologically differentiated adult worms to the RLB we could not reproduce the specificity of the RLB for all species. Therefore, the authors re-sequenced the IGS from 6 species, aligned with all known IGS sequences and designed 3 new probes specific for these new sequences (see Supplementary data). These probes were found to be specific, although we found that some species cross-hybridized with probes from another species. For example, *C. longibursatus* cross-hybridizes with the CATD5 probe of *C. catinatum*. This was no surprise, because there were no nucleotide differences in *C. longibursatus* with the CATD5 probe. However, *C. catinatum* shows eight nucleotide differences with the *C. longibursatus* probe (LON) and does therefore not cross-hybridize, making it still possible to differentiate individual L3 of *C. longibursatus* from *C. catinatum*. Furthermore, the identification of a subset of larvae identified by RLB as *C. catinatum* was confirmed by PCR-RFLP (Gasser et al., 1996). The differentiation between *C. nassatus* and *C. minutus* was more complicated and could not be made with absolute certainty, in part due to the lack of a morphologically identified *C. minutus* adult as a positive control. Adults, morphologically confirmed as *C. nassatus* hybridized only with the *C. nassatus* specific probe (NAS2). However, some xL3 reacted with the *C. minutus* specific probe only (MIN1), while others reacted with both the MIN1 and NAS2 probe. An explanation for this inconsistency might be the presence of cryptic species within *C. minutus*. Hung et al. (1999) studied internal transcribed spacer sequences and found more differences within the *C. minutus* species as between the morphologically distinct species *C. goldi* and *C. longibursatus*. They suggested the existence of at least 2 species within the *C. minutus* complex.

Differentiation of xL3 from both the OVP and UU populations demonstrated that *C. catinatum*, *C. nassatus* and *C. longibursatus* are the most abundant species, as has been found elsewhere (Gasser et al., 2004). After *in vitro* selection *C. catinatum* is by far the most prevalent species in both populations. This suggests that, also in a never treated population resistant larvae are

present in low numbers. This was earlier suggested by Young et al. (1999) for cyathostomins, without differentiating them. So despite *C. catinatum* being the least susceptible species in the rLMIA the majority of the larvae were still susceptible. *C. nassatus* declined in numbers after selection and it even disappeared completely from the OVP population. Interestingly, the life cycle of *C. nassatus* is much shorter than that of *C. catinatum*, because *C. nassatus* seems to lack the possibility to inhibit its development unlike *C. catinatum* (Ogbourne, 1975). It might be that a longer cycle increases the selection pressure upon the parasite when exposed to anthelmintics, resulting in higher numbers of *C. catinatum* resistant larvae compared with *C. nassatus*, although this can not explain the high numbers of resistant *C. catinatum* in the never treated OVP population. For a proper evaluation why some species appear to be more prone for developing anthelmintic resistance than others, more information is required about the entire life-history traits of each species influencing their refugia status in time as well as their inherent susceptibility to certain drugs.

For all four "species" (*C. catinatum*, *C. longibursatus*, *C. nassatus* and "others"), the migration percentage was higher in the UU population, the population that received regular anthelmintic treatment. Whether anthelmintic treatment is the cause of this difference has to be determined by analysis of more populations with different levels of anthelmintic resistance.

Finally, the successful selection, isolation and differentiation of susceptible and resistant xL3 offer the possibility to study resistance mechanisms in greater detail. Gene variants correlated with ivermectin resistance in xL3 can then be investigated in adults. *C. catinatum* is an obvious candidate to start with in such studies, because it is an abundant species with a relatively high percentage of resistant xL3 within both studied larval populations. *C. nassatus* is also interesting, because of the apparently large difference in ivermectin susceptibility of this species between the OVP and UU larval populations. Because of the demonstrated effect of glutamate on larval migration in the presence of ivermectin, it appears reasonable to initially focus on the role of GluCls in ML resistance of the cyathostomins (Tandon et al., 2006).

Acknowledgements

We would like to thank DVM Bernard and DVM Spierenburg, working at the Department of Equine Sciences, Utrecht University and their ponies for providing us with faecal samples.

References

Boersema J.H., Borgsteede F.H.M., Eysker M., Elema T.E., Gaasenbeek C.P.H., van der Burg W.P.J., 1991. The prevalence of anthelmintic resistance of horse strongyles in The Netherlands. Vet. Q. 13: 209–217.

Cully D.F., Vassilatis D.K., Liu K.K., Paress P.S., van der Ploeg L.H.T., Schaeffer J.M., Arena J.P., 1994. Cloning of an avermectin-sensitive glutamate-gated chloride-channel from *Caenorhabditis elegans*. Nature 371, 707-711.

Forrester S.G., Prichard R.K., Beech R.N., 2002. A glutamate-gated chloride channel subunit from *Haemonchus contortus*: Expression in a mammalian cell line, ligand binding and modulation of anthelmintics binding by glutamate, Biochem. Pharmacol. 63, 1061-1068.

Gasser R.B., Stevenson L.A., Chilton N.B., Nansen P., Bucknell D.G., Beveridge I., 1996. Species markers for equine strongyles detected by PCR RFLP. Mol. Cell. Probes 10, 371-378.

Gasser R.B., Hung G.-C, Chilton N.B., Beveridge I., 2004. Advances in developing molecular diagnostic tools for strongyloid nematodes of equids: fundamental and applied implications. Mol. Cell. Probes 18, 3-16.

Gill J.H., Lacey E., 1998. Avermectin/milbemycin resistance in trichostrongyloid nematodes, Int. J. Parasitol. 28, 863-877.

Hung G.-C., Chilton N.B., Beveridge I., Zhu X.Q., Lichtenfels J.R., Gasser R.B., 1999. Molecular evidence for cryptic species within *Cylicostephanus minutus* (Nematoda:Strongylidae), Int. J. Parasitol. 29, 285-291.

Kaplan R.M., 2002, Anthelmintic resistance in nematode of horses, Vet. Res. 33, 491-507.

Kaplan R.M., 2009. Evaluation of a larval migration inhibition assay in equine cyathostomin nematodes, Proc. 22nd Int. Conf. WAAVP Calgary Can, 9-13 Aug.

Kaufhold A., Podbielski A., Baumgarten G., Blokpoel M., Top J., Schouls L., 1994. Rapid typing of group A streptococci by the use of DNA amplification and non-radioactive allele-specific oligonucleotide probes. FEMS Microbiol. Lett. 119, 19-25.

Kaye J.N., Love S., Lichtenfels J.R., McKeand J.B., 1998. Comparative sequence analysis of the intergenic spacer region of cyathostome species. Int. J. Parasitol. 28, 831-836.

Lichtenfels J.R., Kharchenko V.A., Dvojnos G.M., 2008. Illustrated identification keys to strongylid parasites (strongylidae: Nematoda) of horses, zebras and asses (Equidae.) Vet. Parasitol. 156, 4-161.

Love S., Murphy D., Mellor D., 1999. Pathogenicity of cyathostome infection. Vet. Parasitol. 85, 113-122.

Lyons E.T., Tolliver S.C., Ionita M., Lewellen A., Collins S.S., 2008. Field studies indicating reduced activity of ivermectin on small strongyles in horses on a farm in Central Kentucky. Parasitol. Res. 103, 209-215.

Lyons E.T., Tolliver S.C., Collins, S.S., 2009. Probable reason why small strongyle EPG counts are returning 'early' after ivermectin treatment of horses on a farm in Central Kentucky. Parasitol. Res. 104, 569-574.

McArthur C., Robinson A., Hodgkinson J.E., Matthews J., 2009. A larval migration assay for assessing macrocyclic lactone sensitivity in equine cyathostomin populations. Proc. 22nd Int. Conf. WAAVP Calgary Can, 9-13 Aug.

McCavera S., Rogers A.T., Yates D.M., Woods D.J., Wolstenholme A.J., 2009. An Ivermectin-sensitive glutamate-gated chloride channel from the parasitic nematode *Haemonchus contortus*. Mol. Pharmacol. 75, 1347-1355.

Molento M.B., Antunes J., Bentes R.N., Coles, G.C., 2008. Anthelmintic resistant nematodes in Brazilian horses. Vet. Rec. 162, 384-385.

Njue A.I., Prichard R.K., 2004. Genetic variability of glutamate-gated chloride channel genes in ivermectin-susceptible and – resistant strains of *Cooperia oncophora*. Parasitology 129, 741-751.

Ogbourne, C.P., 1975. Epidemiological studies on horses infected with nematodes of the family Trichonematidae (Witenberg, 1925). Int. J. Parasitol. 5, 667-672.

Robinson A., McArthur C., Burden F.A., Goss L., Trawford A.F., Jackson F., Matthews J.B., 2008. Use of the larval migration inhibition assay to investigate suspected macrocyclic lactone resistant cyathostomin populations. Proc. of the Int. Equine Parasite Drug Resistance Workshop. Copenhagen Denmark.

Tandon R., LePage K.T., Kaplan R.M., 2006. Cloning and characterization of genes encoding and ß subunits of glutamate-gated chloride channel protein in *Cylicocyclus nassatus*. Mol. Biochem. Parasitol. 150, 46-55.

Traversa D., Ioro R., Klei, T.R., Kharchenko V.A., Gawor J., Otranto D., Sparagano O.A.E., 2007. New method for simultaneous species-specific identification of Equine Strongyles (Nematoda, Strongylida) by reverse line blot hybridization. J. Clin. Microbiol. 9, 2937-2942.

Trawford A.F., Burden F.A., Hodgkinson J.E., 2005. Suspected moxidectin resistance in cyathostomes in two donkey herds at the donkey sanctuary, UK. Proc. of the 20th Int. Conf. of the WAAVP, Christchurch NZ, 16-20 Oct.

Von Samson-Himmelstjerna G., Fritzen B., Demeler J., Schurman S., Rohn K., Schnieder T., Epe C., 2007. Cases of reduced cyathostomin egg-reappearance period and failure of *Parascaris equorum* egg count reduction following ivermectin treatment as well as survey on pyrantel efficacy on German horse farms. Vet. Parasitol. 144, 74-80.

Wolstenholme A.J., Rogers A.T., 2005. Glutamate-gated chloride channels and the mode of action of the avermectin/milbemycin anthelmintics. Parasitology 131, 85-95.

Young K.E., Garza V., Snowden K., Dobson R.J., Powell D., Craig T.M., 1999. Parasite diversity and anthelmintic resistance in two herds of horses. Vet.Parasitol. 85, 205-214.

Supplementary data

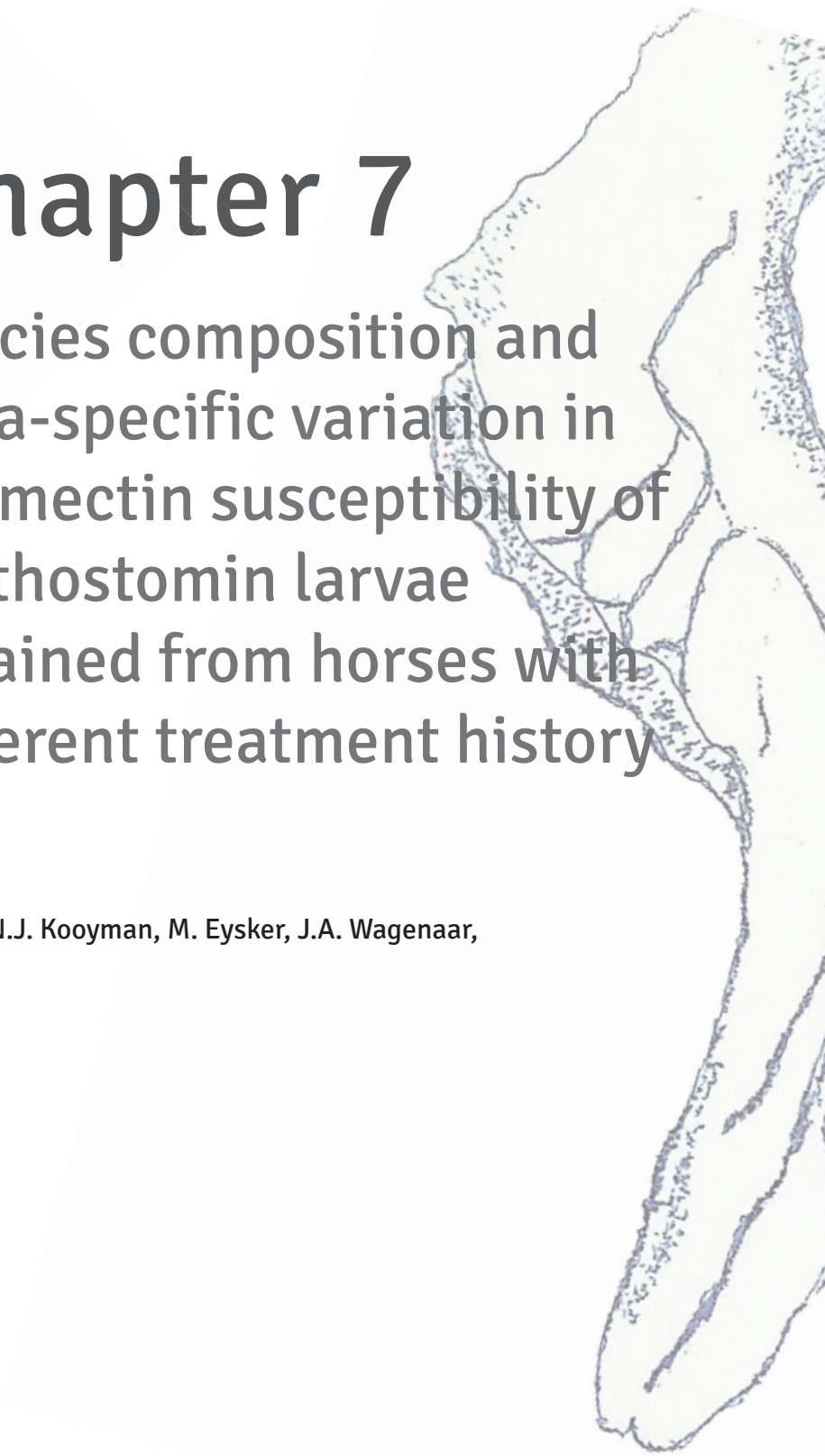
Alignment of IGS sequences from cyathostomins with species specific and catch-all RLB probes.
Names of the species and accession numbers are given in front of the sequences, references are given at the end
Probes and the corresponding homologous sequences are shaded.

http://dx.doi.org/10.1016/j.vetpar.2010.08.021

Chapter 7

Species composition and intra-specific variation in ivermectin susceptibility of cyathostomin larvae obtained from horses with different treatment history

D.C.K. van Doorn*, F.N.J. Kooyman, M. Eysker, J.A. Wagenaar, H.W. Ploeger



Abstract

In a previous study, using two different cyathostomin populations, we demonstrated that *in vitro* susceptibility of the larvae to ivermectin varied for different cyathostomin species as measured in a reiterative larval migration assay (rLMIA) combined with a reverse line blot (RLB) to identify larvae to species level. Furthermore the susceptibility of the larvae, at least for some cyathostomin species, seemed to be related with previous ML-treatments of the horses from which they were obtained. The intra-specific variation in IVM susceptibility was not studied.
Therefore, the present study was performed to study this intra-specific variation in ivermectin susceptibility and to examine the variation in outcome in the assays using 8 worm populations with different histories of ML-exposure.
Larvae were obtained from 8 horse premises, 2 free-roaming (FR) horse populations and 6 regularly treated (T) populations. FR1 was never ML treated and FR2 was not treated since 2008, T1 was selectively treated since 2006 and T2-T6 were frequently ML-treated horse populations.
In all 8 populations the dominant species were *Cyathostomum catinatum*, *Cylicocyclus nassatus*, *Cylicostephanus longibursatus* and *Cyathostomum pateratum*. After *in vitro* separating the less susceptible larvae by rLMIA in the presence of ivermectin over 2 consecutive sieves the most prevalent species were *Cya. catinatum* and *Cys. longibursatus*. The combined rLMIA RLB demonstrated a higher migration percentage for the second sieve compared with the first indicating separation of larvae with lower susceptibility. The highest migration percentage for the 2nd sieve was found for *Cya. catinatum*. Overall, a significantly lower migration percentage for the second sieve was observed for *Cya. catinatum* in the free-roaming horses (FR) compared with the treated horses (T).
It is concluded that within cyathostomin species there is heterogeneity with respect to *in vitro* ivermectin susceptibility. This was also found in larvae obtained from free-roaming horses (FR1 and FR2). In *Cya. catinatum*, the observed heterogeneity was found to be related to previous ML treatments.

Keywords: Cyathostominae, Ivermectin, Larval Migration Inhibition Assay, Anthelmintic resistance, Reverse Line Blot

Introduction

Cyathostomins are considered to be one of the primary helminth pathogens of young horses. Control of cyathostomins relies heavily on the use of anthelmintics. Of these, the macrocyclic lactones (ML) are the most widely used, because there is widespread resistance to the benzimidazoles, at least in Northwest Europe. Recently, however, development of ML resistance has been noticed (Lyons et al., 2009).

Detecting ML resistance *in vivo* still relies on the Fecal Egg Count Reduction Test (FECRT) since the molecular mechanisms leading to ML resistance in cyathostomins are unknown. Furthermore, some 50 cyathostomin species are known (Lichtenfels et al., 2008). The FECRT cannot distinguish between these different cyathostomin species, whereas there may be differences between species in susceptibility to the ML.

In a previous *in vitro* study we demonstrated that separation in ivermectin (IVM) susceptible and unsusceptible larvae was possible by using a reiterative larval migration inhibition assay (rLMIA) with four sieves (van Doorn et al, 2010). This was done in 2 cyathostomin populations, one never treated and another regularly treated with IVM. In both populations the dominant species, identified by reverse line blot (RLB), were *Cyathostomum catinatum*, *Cylicocyclus nassatus* and *Cylicostephanus longibursatus*. After *in vitro* separation in the rLMIA in the presence of IVM the predominant species became *Cya. catinatum* in both populations, while *Cyc. nassatus* disappeared in the population which was not treated before but not in the regularly IVM treated population. This suggested that the level of susceptibility measured in these *in vitro* assays may depend on the level of earlier exposure to ML. However, no observations were made on actual selection for decreased IVM susceptibility within species, the intra-specific variation.

The aim of this study was to further examine the variability in cyathostomin species composition and to detect intra-specific variation in IVM susceptibility in cyathostomin larvae obtained from horses differing in ML treatment history.

Material and methods

Origin of cyathostomin populations
Horse premises in the Netherlands with free roaming (FR) or different ML treatment schemes (T) were approached. In total 8 premises (2 FR and 6 T) agreed to cooperate. All premises had 10 or more horses that were 3 years or older and all horses had access to pasture. Horses were sampled in autumn of either 2010 or 2011. Horses on 2 premises (FR1 and one FR2) were also sampled at another time of the year (summer or winter). Horses were sampled at least 8 weeks after IVM or 12 weeks after moxidectin treatment. Ten fresh fecal droppings were collected and only the samples with positive egg counts were cultured.

Below, each of the 8 horse populations is described in more detail.
FR1: Free-roaming Konik horses in the nature reserve Oostvaardersplassen. These horses never received treatment. They were introduced into the reserve in the beginning of the nineteen-eighties and this was before IVM introduction on the Dutch market and therefore harbours a cyathostomin population never exposed to ML.
FR2: Free roaming Exmoor ponies were introduced in a dune area in 2008 and have never been dewormed since. Treatment history before introduction to the area is unknown.
T1: A population of adult Shetland ponies was frequently treated with ML until 2006. After 2006, ponies were more restrictively treated on average once a year with either IVM or moxidectin.
T2-T6: Various stables (for boarding, training, raising or breeding) where all horses are treated at least 4 times a year with an ML.

Larval cultures
Fecal samples were examined for egg counts (FEC) with a modified McMaster method with a sensitivity of 50 eggs per gram. Positive samples were cultured in glass jars with a plastic lid and incubated at 25°C for 10 days. The jars were then filled with lukewarm tap water, turned upside down on petri dishes and replenished with water. L3 were harvested the next day by draining them into Baermann glasses so they could be collected from the bottom and stored in 20 ml clean water at 4°C until further use (MAFF, 1986). The presence or absence of *Strongylus vulgaris* was scored by microscopic inspection of all batches of cultured larvae.

Reiterative Larval Migration Inhibition Assay
rLMIA was performed as was described by van Doorn et al. (2010) using 2 sieves instead of 4. Results from van Doorn et al. (2010) indicated that using 2 sieves would not result in a significant loss of selection power, while it substantially reduced the amount of work involved. The rLMIA was performed within a month after sampling the horses. The rLMIA is based on ML paralyzing exsheathed L3 (xL3) thereby inhibiting their migration through sieves. If a L3 isolate is homogenous with respect to IVM susceptibility, no separation into susceptible and less susceptible larvae can occur. If a population is heterogeneous with respect to IVM susceptibility, meaning the presence of both susceptible and less susceptible L3, the proportion of larvae that migrate through a second sieve will be higher than that migrated through the first sieve. This gives the opportunity to separate susceptible from less susceptible L3.

A known number of xL3 were pre-incubated in PBS (2h, 37°C in the dark) with IVM (30 µg/ml) or without IVM (assay control), both in dimethylsulfoxide (DMSO) with an end concentration of 1%. After pre-incubation, the larvae were allowed to migrate in the same solution through 1 or 2 consecutive sieves

(cell strainer 40 µm) for 1 hour per migration at 37°C in the dark. The xL3 that migrated through 1 (S1) or 2 sieves (S1 followed by S2) were stained with iodine and counted. From these, a maximum of 80 xL3 were collected and stored individually in 2 µl staining solution at -80°C until further use. Larval migration data were normalized by standardizing the larval migration percentage in the absence of IVM at 100%. Migration percentages used for analyses were adjusted for a small fraction (5%) of immobile xL3 that by accident passed through the sieve. This fraction was estimated from migration assays with xL3 that were killed by overnight incubations in 0.2% NaN3. The rLMIA was performed in duplicate and these duplicates were pooled before the xL3 were individually differentiated by RLB.

DNA extraction, PCR amplification and RLB hybridization
Each individually isolated L3 was lysed in 25 µl proteinase K /Worm Lysis Buffer (van der Veer et al., 2003). The mixture was frozen for 15 minutes at -80ºC and the xL3 were lysed overnight at 56-60˚C. Proteinase K was heat inactivated the next day at 95˚C for 15 min.

For PCR amplification of the Inter Genic Spacer (IGS) region the forward primer CY26 (5'-GAGCTGGGTTTAGACCGTCGTGAG-3') and the biotinylated reverse primer CY4 (5'-CGGTACAAAAAGACTTCTACTCG-3') was used. For a 25 µl PCR reaction 2.5 µl genomic DNA was used.

Single PCRs on individual larvae were performed in a thermal cycler using the following protocol: 10 min at 94°C and 35 cycles at 94°C for 1 min, 55°C for 1 min, and 72°C for 2 min, followed by a final extension at 72°C for 7 min.
The RLB is based on hybridization of a species specific probe with an amplified fragment of the IGS region (Kaye et. al., 1998, Hodgkinson et. al., 2001 and 2005, Traversa et. al., 2007). Ten µl of the IGS amplicon per lane was used for hybridization. Hybridized products were detected with streptavidin-POD conjugate, followed by incubation in ECL detection reagent an exposure to Amersham Hyperfilm ECL.

Probes for the following 13 species were used: *Coronocyclus coronatus*, *Coronocyclus labiatus*, *Coronocyclus labratus*, *Cya. catinatum*, *Cyathostomum pateratum*, *Cylicocyclus ashworthi*, *Cylicocyclus insigne*, *Cylicocyclus leptostomum*, *Cyc. nassatus*, *Cylicostephanus calicatus*, *Cylicostephanus goldi*, Cys. longibursatus, *Cylicostephanus minutus* (van Doorn et al., 2010).
A catch-all probe, of which the targeted sequence is conserved across species, was included in all analyses. Larvae of which the PCR product hybridized with the catch-all probe, but with none of the species specific probes were considered "other cyathostomin".

With the RLB only 40 samples can be processed on one mini-blot at a time. This limits the number of individual larvae that can be identified. Van Doorn et al. (2010) showed that species composition determined on larvae from the original stock before processing in the rLMIA does not differ from the composition determined from larvae processed through the rLMIA in absence of IVM. Therefore, where possible the differentiations of the original stock were combined with those after migration without IVM (assay control). For the number of larvae involved see supplementary data (Table S1).

Confirming reproducibility of the rLMIA and RLB

The rLMIA and RLB on the larvae from population T2 were repeated to confirm reproducibility of both techniques. Population T2 was chosen because it had one of the most varied species compositions and large numbers of larvae were available. This 2nd analysis of T2 took place 6 weeks after the 1st analysis.

Confirming rLMIA and RLB results from premises FR1 and FR2

As it was not possible to identify other horse populations that were (almost) never treated, populations FR1 and FR2 were sampled a second time (FR1b and FR2b) at a different time of the year. Results of this second sampling were used to support the observations from the first sampling thereby making interpretation of migration percentages more solid.

Analyses and Statistics

Differences between the 1st and 2nd migration percentages from the same population were tested by Wilcoxon Signed Rank Test for paired samples. Differences in overall mean migration percentages (for S1 and S2) for FR versus T populations were tested with the Mann Whitney U Test. The same was done for differences in migration percentages after subtracting the migration percentage through S1 from that through S2. The latter aided in a closer evaluation of the heterogeneity in susceptibility of the tested populations (see supplementary data S2). The difference between migration percentage for S2 and S1 may comparatively tell something about either the fraction of less susceptible larvae present in the original population and/or the degree of susceptibility of those larvae. Tests were performed within SPSS version 20.

Results

Species composition

Table 1 shows the species composition in each of the 8 sampled cyathostomin populations. *Cya. catinatum* was present in all 8 populations and, generally, was one of the dominant species. *Cys. longibursatus* was present in all cyathostomin populations and in 7 of these was one of the more abundant species. *Cyc. nassatus* was the most abundant species in T1 and T5.

The species *Cys. minutus* was found in none of the examined populations. Of the other 12 species, *Cyc. leptostomum* and *Cor. labratus* were only found in the never treated population (FR1). For all other cyathostomin species, no conspicuous differences could be observed between FR and T populations. Larvae of *S. vulgaris* were found only in cultures from the 2 free-roaming horse populations FR1 and FR2.

Table 1. Species compositions (in %) of larval cultures from 8 horse populations with different ML treatment history. FR =free roaming/not treated; T = ML treated. The bottom row presents the actual numbers of differentiated xL3 in the RLB.

Species	FR1	FR2	T1	T2	T3	T4	T5	T6
Coronocyclus coronatus	6			3				
Coronocyclus labiatus	1				2			
Coronocyclus labratus	1							
Cyathostomum catinatum	35	50	7	30	23	27	18	69
Cyathostomum pateratum	5			19	33	1	9	2
Cylicocyclus ashworthi		1		5			7	
Cylicocyclus insigne	2		1	11			5	
Cylicocyclus leptostomum	2							
Cylicocyclus nassatus	7	5	68	13	4	4	28	
Cylicostephanus calicatus	4	3	7	1	1		4	
Cylicostephanus goldi	6	13		1	3	5	8	7
Cylicostephanus longibursatus	22	26	3	16	25	57	19	15
Other cyathostomin	9	2	14		9	6	2	7
Nr. of differentiated xL3 in RLB	101	101	88	98	127	78	84	112

Reproducibility of rLMIA in the presence of IVM followed by RLB

To confirm the reproducibility of the rLMIA combined with RLB, larvae from the same culture of T2 were subjected to rLMIA - RLB a 2nd time. The migration percentages for all cyathostomin xL3 were around 0.5% for the 1st migration in the presence of IVM. For the migration through the 2nd sieve these numbers were 54% and 61.5% for the 1st and 2nd assay, respectively. After differentiation by RLB 7 species were found. Numbers of xL3 of only 2 species were sufficient to compare the migration percentage between the repeats. Table 2 shows for both *Cya. catinatum* and *Cya. pateratum* an increased migration percentage from the 1st to the subsequent 2nd sieve. Given the relatively low numbers of larvae that were identified by RLB, both duplicates produced a reasonably similar result. All numbers of larvae applied to the sieves and migrated through the sieves as well as the numbers of larvae differentiated in the RLB and the resulting migration percentages of the duplicate assays are given in Table S1 of the supplementary data.

Table 2. Repeated rLMIA performed with xL3 from population T2 (in duplo a and b). Migration percentages for sieve 1 (S1) and sieve 2 (S2) are given for *Cyathostomum catinatum* and *Cyathostomum pateratum* in presence of 30 µg/ml IVM.

	S1		S2	
	a	b	a	b
Cyathostomum catinatum	3.3	2.9	75.2	90.9
Cyathostomum pateratum	0	5.8	58.8	38.2

Separation of undifferentiated xL3

In the rLMIA we determined the migration percentages of the xL3 for 1 or 2 consecutive sieves in the presence of 30 µg/ml IVM. The migration percentage for undifferentiated xL3 was significantly (p= 0.01) higher for the 2nd sieve compared to the 1st when all species were taken together (Fig. 1). The migration percentages from both FR populations versus all T populations together showed no statistical difference, not over the 1st nor the 2nd sieve.

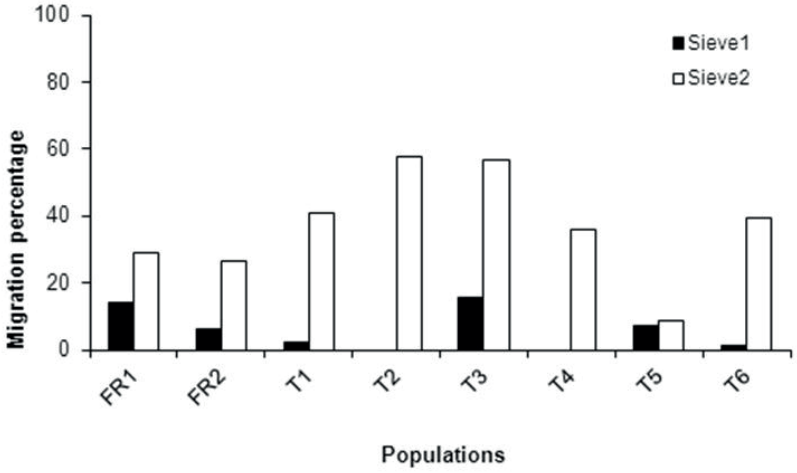


Fig. 1. The migration percentage of undifferentiated cyathostomin xL3 for sieve 1 (black bars) and sieve 2 (open bars) in the rLMIA in the presence of 30 µg/ml IVM for each horse population. FR = free roaming/not treated; T = ML treated.

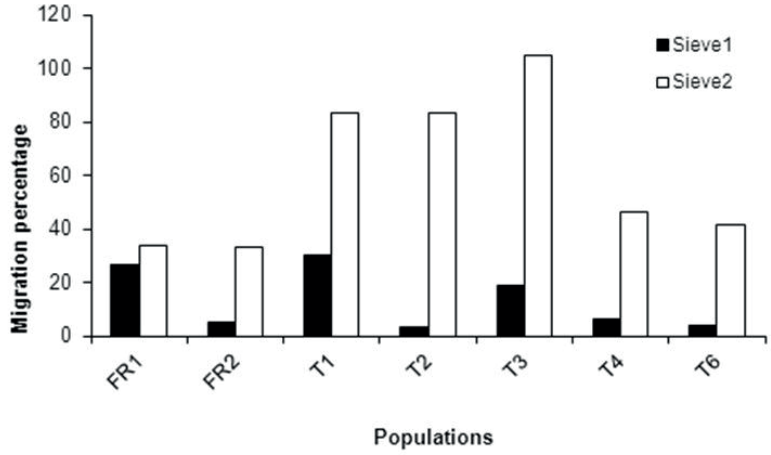


Fig. 2. The migration percentage of *Cyathostomum catinatum* xL3 for sieve 1 (black bars) and sieve 2 (open bars) in the rLMIA in the presence of 30 µg/ml IVM for each horse population. FR = free roaming/not treated; T = ML treated. Migration percentage in T5 is not shown, because the number of differentiated *Cya. catinatum* larvae was less than 5.

Table 3. Numbers of differentiated L3, number of L3 in rLMIA and migration % in rLMIA for the 1st and 2nd sieve for the total applied L3, total migrated L3 and migrated *Cya. catinatum* in the FR and T populations.

		number of differentiated L3 by RLB			number L3 rLMIA		migration % rLMIA	
	L3	before migration	1st sieve in IVM	1st +2nd in IVM	1st sieve in IVM	1st +2nd in IVM	1st sieve in IVM	2nd sieve in IVM
FR1a	total applied	-	-	-	534	10680	-	-
	total migrated	36	34	36	90	490	14	29
	migrated *Cya. catinatum*	11	20	24	53	327	27	33
FR1b	total applied	-	-	-	1240	12400	-	-
	total migrated	39	40	39	85	352	4	46
	migrated *Cya. catinatum*	32	32	31	68	280	5	46
FR2a	total applied	-	-	-	870	8700	-	-
	total migrated	32	28	43	69	152	6	27
	migrated *Cya. catinatum*	17	13	24	32	85	5	33
FR2b	total applied	-	-	-	334	6680	-	-
	total migrated	32	58	70	31	137	10	18
	migrated *Cya. catinatum*	8	12	17	6	33	6	22
T1	total applied	-	-	-	1450	26100	-	-
	total migrated	24	37	38	96	543	2	41
	migrated *Cya. catinatum*	2	12	23	31	329	30	83
T2a	total applied	-	-	-	1668	16680	-	-
	total migrated	39	38	40	64	266	0	54
	migrated *Cya. catinatum*	11	19	25	34	190	3	75
T2b	total applied	-	-	-	1792	17820	-	-
	total migrated	39	38	40	92	385	1	61
	migrated *Cya. catinatum*	11	19	25	36	214	3	91
T3	total applied	-	-	-	962	19480	-	-
	total migrated	51	38	39	187	1614	16	57
	migrated *Cya. catinatum*	12	10	18	49	745	19	105
T4	total applied	-	-	-	544	5420	-	-
	total migrated	30	18	31	26	60	0	36
	migrated *Cya. catinatum*	7	9	19	13	37	6	46
T6	total applied	-	-	-	734	7340	-	-
	total migrated	35	35	37	40	87	1	40
	migrated *Cya. catinatum*	23	30	33	34	78	4	42

Intra-specific variation in IVM susceptibility

To adequately evaluate whether there is variation in IVM susceptibility within species associated with previous treatments with ML, sufficient numbers of larvae should be available for analysis. Only for *Cya. catinatum* at least 5 larvae were differentiated in all rLMIA fractions of 7 out of the 8 examined populations (Table 3). When all populations were taken together the migration percentage for *Cya. catinatum* for the 2nd sieve was higher than for the 1st sieve (Fig. 2). This was statistically significant with a p value of 0.02.

The *Cya. catinatum* migration percentage for FR1a and FR2a were compared with the *Cya. catinatum* migration percentage of the T populations (except T5 with too few larvae identified as *Cya. catinatum*). The T populations showed significantly higher migration percentages for the 2nd sieve (p=0.05). No significant difference in migration percentage for the 1st sieve for these FR versus T populations was found.

FR populations were repeated in a different season since we only had access to these two FR populations. Overall, the repeats of FR1 (a or b) and FR2 (a or b) showed reasonably comparable migration percentages for *Cya. catinatum* in 30 µg/ml IVM. For FR1a and FR1b percentages were 26.8 % and 5.2 % for the 1st sieve, and 33.5% and 46.1% for the 2nd sieve. For FR2a and FR2b migration the respective percentages were 4.8 % and 5.6 % for the 1st sieve and 33.4% and 22.1% for the 2nd sieve. The difference in migration percentage between 2nd sieve and 1st sieve including the data from the repeated FR populations (including FR1b and FR2b) remained significantly (P=0.05) higher in the T populations than in those FR populations (Table 4).

Table 4: *Cya. catinum* migration percentage for sieve 1 (M% S1) and sieve 2 (M% S2) and M% S2 M% S1 for FR and T populations.

Populations	M % S1	M % S2 *	M% S2 - M% S1 **
FR1a	27	33	6
FR2a	5	46	41
FR2a	5	33	28
FR2b	6	22	16
mean FR	*10.75*	*33.5*	*22.75*
T1	30	83	53
T2	3	75	72
T3	19	105	86
T4	6	46	40
T5	4	42	38
mean T	*12.4*	*70.2*	*57.8*

***** significant difference (p=0.035) for M% S2 between the 4 FR and 5 T populations.
****** significant difference (p=0.050) for the difference M% S2 - M% S1 between the 4 FR and 5 T populations.

Discussion

It appears to be possible to separate larvae that are the least susceptible to IVM from more susceptible larvae *in vitro* using the rLMIA. This principle, although with four sieves, was described in a previous study (van Doorn et al., 2010) in which an overall increase in migration percentage of cyathostomin larvae over consecutive sieves in the presence of IVM was shown. Within the present study we looked at differences in the species composition and migration percentage of separate species in populations with different ML-exposure histories.

A varied cyathostomin species composition was found in each of the sampled horse populations. In population FR1, the never treated horses in a nature reserve, the highest number of species were identified. In population T6, the most frequently treated population, the number of species found was lowest. *S. vulgaris* was only found in cultures from the 2 free-roaming populations (FR1 and FR2). Frequent anthelmintic treatments may reduce the number of cyathostomin and *Strongylus* species in a population. This is in agreement with Nielsen et.al (2012) who found more *S. vulgaris* positive horses when the interval between ML treatments was 6 months or longer. However, in our study the numbers of actually identified larvae in the RLB were small with sometimes just one larva identified of a certain species. Therefore results should be interpreted cautiously. *Cya. catinatum* and *Cys. longibursatus* dominated in all 8 populations, except T1 and T5 where *Cyc. nassatus* made up 68% or 28% of the population. The T3 population was dominated by *Cya. pateratum*. The basic epidemiology for single cyathostomin species is mostly unknown. It remains, therefore, difficult to suggest what species to expect, especially in relation to horse populations with different treatment histories. Further it is worth mentioning that we sampled the frequently treated horses shortly after the ERP of the anthelmintic passed. This means that we perhaps mainly sampled eggs from species that returned first after ML treatment. This bias is not unwanted since we use this *in vitro* system to separate larvae that, for whatever reason, are less susceptible for ML from susceptible larvae. Nonetheless, it will be interesting to study the epidemiology of separate species more closely and over longer periods, for which the RLB for example provides a useful tool as it can identify L3 at the species level.

Cys. minutus is a very common species in The Netherlands (Eysker et al. 1986) so we expected to identify these. However, we did not find *Cys. minutus* in any of the populations before or after rLMIA with the RLB. It was found that the CY4 primer site is not conserved in *Cys. minutus* and *Cor. coronatus* (Cwiklinski et al., 2012). *Cys. minutus* and *Cor. coronatus* were therefore not efficiently amplified and were missed in the identification. In our on-going studies we adjusted these primers.

Analysis showed that the increase in migration of *Cya. catinatum* from the 1st to the 2nd sieve was significant. *Cya. catinatum* in the free roaming populations (FR1 and FR2) showed a lower migration percentage over the 2nd sieve in the presence of IVM than the treated populations. This demonstrates intra-specific IVM susceptibility to be more diverse in the T populations. This suggests a relation between frequency of ML treatments and variation in *in vitro* IVM susceptible L3. Within population T3, for example, all of the *Cya. catinatum* L3 migrated through the 2nd sieve in the presence of IVM. A similar analysis could not be done for the other species, because for most species numbers of identified larvae were less than 5.

Interestingly, van Doorn et al. (2010) also noticed for the FR1 population a higher occurrence of *Cya. catinatum* following *in vitro* migration in the presence of IVM. At that time it could not be determined if this was due to variation in IVM susceptibility within the *Cya. catinatum* xL3 subpopulation. Within the present study, migration over 2 sieves in the presence of a high concentration of IVM (30 µg/ml) could be followed for each species separately. Results showed that the FR1 *Cya. catinatum* subpopulation also contained larvae that were less susceptible. This is remarkable as this is found in the free-roaming horse population that was never treated. This supports observations of pre-existing ML unsusceptible isolates as reported by Young et al. (1999).

To help understand the migration patterns through S1 and S2 in terms of susceptibility of the larvae, we created a model based on some simple assumptions (Figure S2A in the supplementary data). Fully susceptible L3 are assumed not to migrate through S1. Further, we chose 3 degrees of unsusceptibility, with either 10%, 50% or 100% of the less susceptible larvae migrating through each sieve. Then, Fig. S2A gives the migration percentage for S1 and subsequently S2 as a function of the fraction of less susceptible L3 in a population. It shows that, the migration percentage for the 2nd sieve is always higher than for the 1st sieve, unless the fraction of less susceptible larvae in the population is 100%. This is seen irrespective of the degree of unsusceptibility of the larvae present in the population. Combining this with the difference in migration percentage between S2 and S1 one may obtain an indication how large the fraction of less susceptible larvae was in the original larval population as well as their average degree of unsusceptibilty (Fig. S2B). For our FR and T populations we found that the migration percentage for S1 was low and for S2 the migration percentage was higher for the T than for the FR populations. The same applied for the difference between migration percentage for S2 versus S1. Overall, this suggests that the fractions of less susceptible larvae were low in both FR and T populations, but that the degree of unsusceptibility of those larvae was higher in the T than in the FR populations.

It remains to be seen if unsusceptible cyathostomin L3 will develop into resistant adults. The genes that are expressed in L3 are likely different from those in adults. Furthermore, their role can be different. For example, glutamate-gated chloride channel proteins expressed in the esophagus will cause IVM susceptibility in adults, but will not be of influence on L3 because L3 do not feed. On the other hand in L3 from gastrointestinal nematodes from ruminants, where they were able to use monospecific isolates, less migration of *in vivo* resistant isolates was seen. This was found for *Cooperia oncophora* and *Ostertagia ostertagi* (Demeler et al. 2010). No species differentiations after *in vitro* assays in horses were performed in the past. Because there are 50 cyathostomin species with different L3 susceptibility for IVM (van Doorn et al., 2010) this can be one of the reasons that as yet no relationship between *in vivo* resistance in adult worms and *in vitro* unsusceptibility of L3 has been found in cyathostomins.

Our *in vitro* selection method offers the opportunity to study molecular differences between *in vitro* separated ML susceptible and less susceptible L3 from the same cyathostomin species. The advantage of studying these larvae is that they share the same genetic background and, since there is no sexual reproduction involved, the pheno- and genotype of the same individual or generation can be studied.
In conclusion, results indicated that larvae can be *in vitro* separated in IVM susceptible and less susceptible larvae and that this *in vitro* reduced susceptibility appears to be related to anthelmintic treatment history on the horse premises.

Acknowledgments

We thank our students M.F. Haikens, M. Guliker, S.J. van der Woude and M.A. Colen for their assistance. We would like to thank J. Griekspoor and P. Boelens (Dutch Forestry Commission) for giving us the opportunity to sample the never treated horse population in the Oostvaardersplassen and B. Veerkamp, DVM from DAP IJmond-Noord for his help with the other free roaming horse herd. We also thank the owners of the other premises for their cooperation.

References

Cwiklinski K., Kooyman F.N.J., van Doorn D.C.K., Matthews J.B., Hodgkinson J.E., 2012. New insights into sequence variation in the IGS region of 21 cyathostomin species and the implication for molecular identification. Parasitol. 139, 1063-1073

Demeler J., Küttler U., von Samson-Himmelstjerna G., 2010. Adaptation and evaluation of three different *in vitro* tests for the detection of resistance to anthelmintics in gastro intestinal nematodes of cattle. Vet. Parasitol. 170, 61-70.

Eysker M., Jansen J., Mirck M.H., 1986. Control of strongylosis in horses by alternate grazing of horses and sheep and some other aspects of the epidemiology of strongylidae infections. Vet. Parasitol. 19, 103-112.

Hodgkinson J.E., Love S., Lichtenfels J.R., Palfreman S., Ramsey Y.H., Matthews, J.B., 2001. Evaluation of the specificity of five oligoprobes for identification of cyathostomin species from horses. Int. J. for Parasitol. 31, 197-204.

Hodgkinson J.E., Freeman K.L., Lichtenfels J.R., Palfreman S., Love S. and Matthews J.B., 2005. Identification of strongyle eggs from anthelmintic-treated horses using a PCR-ELISA based on intergenic DNA sequences. Parasitol. Res. 95, 287-292.

Kaye J.N., Love S., Lichtenfels J.R., McKeand J.B., 1998. Comparative sequence analysis of the intergenic spacer region of cyathostome species. Int. J. for Parasitol. 28, 831-836.

Lichtenfels J.R., Kharchenko V.A., Dvojnos G.M., 2008. Illustrated identification keys to strongylid parasites of horses, zebras and asses. Vet. Parasitol. 156, 4-161.

Lyons E.T., Tolliver S.C., Collins S.S., 2009. Probable reason why small strongyle EPG counts are returning 'early' after ivermectin treatment of horses on a farm in Central Kentucky. Parasitol. Res. 104, 569-574.

MAFF, 1986. Manual of Veterinary Parasitological Laboratory Techniques. ADAS, HMSO, UK.

Nielsen M.K., Vidyashankar A.N., Olsen S.N., Monrad J., Thamsborg S.M., 2012. *Strongylus vulgaris* associated with usage of selective therapy on Danish horse farms-is it reemerging? Vet. Parasitol. 189, 260-266.

Traversa D., Iorio R., Klei T.R., Kharchenko V.A., Gawor J., Otranto D., Sparagano O.A.E., 2007. New method for simultaneous species-specific identification of equine strongyles (Nematoda, Strongylida) by Reverse Line Blot Hybridization. J. Clin. Microbiol. 45, 2937–2942.

van der Veer M, Kanobana K., Ploeger H.W., de Vries E. 2003. Cytochrome oxidase C subunit 1 Polymorphisms show significant differences in distribution between a laboratory maintained population and a field isolate of *Cooperia oncophora*. Vet. Parasitol. 116, 231-238.

van Doorn D.C.K., Kooyman F.N.J., Eysker M., Hodgkinson J.E., Wagenaar J.A., Ploeger H.W., 2010. *In vitro* selection and differentiation of ivermectin resistant cyathostomin larvae. Vet. Parasitol. 174, 292-299.

Young K.E., Garza V., Snowden K., Dobson R.J., Powell D., Craig T.M., 1999. Parasite diversity and anthelmintic resistance in two herds of horses. Vet. Parasitol. 85, 205–214.

Table S1. Numbers of xL3 selected by rLMIA for ivermectin resistance and differentiated by RLB.

FR1a		number of differentiated xL3 by RLB				number of (migrated) xL3 in rLMIA				migration percentages in rLMIA	
	before migratio	1st, -IVM	1st+2nd, -IVM	1st, +IVM	1st+2nd, +IVM	1st, -IVM	1st+2nd, -IVM	1st, +IVM	1st+2nd, +IVM	1st, +IVM	2nd, +IVM
number L3s applied to sieve(s)	-	-	-	-	-	267	267	534	10680	-	-
r L3s migrated through sieve(s)	-	-	-	-	-	242	200	90	490	-	-
L3s per species Cor. coronatus	4		2	3		14	12	8	0		
Cor. labiatus		1				2	2				
Cor. labratus		1		1		2	2	3			
Cya. catinatum	11	12	13	20	24	86	71	53	327	27	33
Cya. pateratum	2	1	2	2	1	12	10	5	14	18	10
Cyc. ashworthi											
Cyc. insigne		2				5	4				
Cyc. leptostomum		1	1			5	4				
Cyc. nassatus	3	1	3		1	17	14		14		
Cys. calicatus	1	1	2	1	3	10	8	3	41	9	93
Cys. goldi	4	1	1	3	2	14	12	8	27	24	16
Cys. longibursatus	9	6	7	3	4	53	44	8	54	2	38
other cyathostomin species	2	5	2	1	1	22	18	3	14	1	27
all cyathostomins	**36**	**32**	**33**	**34**	**36**	**242**	**200**	**90**	**490**	**14**	**29**

FR1b		number of differentiated xL3 by RLB				number of migrated xL3 in rLMIA				migration percentages in rLMIA	
	before migratio	1st, -IVM	1st+2nd, -IVM	1st, +IVM	1st+2nd, +IVM	1st, -IVM	1st+2nd, -IVM	1st, +IVM	1st+2nd, +IVM	1st, +IVM	2nd, +IVM
number L3s applied to sieve(s)	-	-	-	-	-	310	310	1240	12400	-	-
r L3s migrated through sieve(s)	-	-	-	-	-	184	154	85	352	-	-
L3s per species Cor. coronatus											
Cor. labiatus				1				2			
Cor. labratus			1			2	2				
Cya. catinatum	32	29	25	32	31	133	111	68	280	5	46
Cya. pateratum		2	4	1	1	9	8	2	9	0	48
Cyc. ashworthi											
Cyc. insigne				1				2			
Cyc. leptostomum											
Cyc. nassatus		1	3		1	7	6		9		
Cys. calicatus											
Cys. goldi	1	1	1	1	2	5	4	2	18	4	102
Cys. longibursatus	6	6	5	3	3	26	21	6	27	0	48
other cyathostomin species		1		1	2	2		9			
all cyathostomins	**39**	**39**	**40**	**40**	**39**	**184**	**154**	**85**	**352**	**4**	**46**

FR2a		number of differentiated xL3 by RLB				number of migrated xL3 in rLMIA				migration percentages in rLMIA	
	before migratio	1st, -IVM	1st+2nd, -IVM	1st, +IVM	1st+2nd, +IVM	1st, -IVM	1st+2nd, -IVM	1st, +IVM	1st+2nd, +IVM	1st, +IVM	2nd, +IVM
number L3s applied to sieve(s)	-	-	-	-	-	218	218	870	8700	-	-
r L3s migrated through sieve(s)	-	-	-	-	-	126	88	69	152	-	-
L3s per species Cor. coronatus											
Cor. labiatus											
Cor. labratus											
Cya. catinatum	17	17	17	13	24	64	44	32	85	5	33
Cya. pateratum					1				4		
Cyc. ashworthi	1					1	1				
Cyc. insigne											
Cyc. leptostomum											
Cyc. nassatus	2		3			6	4				
Cys. calicatus		2	1	4	4	4	3	10	14	63	15
Cys. goldi	4	4	5		5	16	11		18		
Cys. longibursatus	7	9	10	10	8	32	23	25	28	12	10
other cyathostomin species	1		1	1	1	2	2	2	4	18	15
all cyathostomins	**32**	**32**	**37**	**28**	**43**	**126**	**88**	**69**	**152**	**6**	**27**

FR2b		number of differentiated xL3 by RLB				number of migrated xL3 in rLMIA				migration percentages in rLMIA	
	before migratio	1st, -IVM	1st+2nd, -IVM	1st, +IVM	1st+2nd, +IVM	1st, -IVM	1st+2nd, -IVM	1st, +IVM	1st+2nd, +IVM	1st, +IVM	2nd, +IVM
number L3s applied to sieve(s)	-	-	-	-	-	167	167	334	6680	-	-
r L3s migrated through sieve(s)	-	-	-	-	-	87	87	31	137	-	-
L3s per species Cor. coronatus											
Cor. labiatus											
Cor. labratus											
Cya. catinatum	8	9	9	12	17	23	23	6	33	6	22
Cya. pateratum			1			1	1				
Cyc. ashworthi											
Cyc. insigne											
Cyc. leptostomum											
Cyc. nassatus	1	2	2	3	2	4	4	2	4	12	8
Cys. calicatus											
Cys. goldi	14	15	16	31	37	39	39	17	72	14	18
Cys. longibursatus	8	7	7	12	11	19	19	6	22	9	13
other cyathostomin species	1	1	1		3	3	3		6		
all cyathostomins	**32**	**34**	**36**	**58**	**70**	**87**	**87**	**31**	**137**	**10**	**18**

T1		number of differentiated xL3 by RLB				number of migrated xL3 in rLMIA				migration percentages in rLMIA	
	before migratio	1st, -IVM	1st+2nd, -IVM	1st, +IVM	1st+2nd, +IVM	1st, -IVM	1st+2nd, -IVM	1st, +IVM	1st+2nd, +IVM	1st, +IVM	2nd, +IVM
number L3s applied to sieve(s)	-	-	-	-	-	726	726	1450	26100	-	-
r L3s migrated through sieve(s)	-	-	-	-	-	680	474	96	544	-	-
L3s per species Cor. coronatus											
Cor. labiatus											
Cor. labratus											
Cya. catinatum	2	1	3	12	23	46	32	31	329	30	83
Cya. pateratum				1	5			3	71		
Cyc. ashworthi											
Cyc. insigne		1				8	5				
Cyc. leptostomum											
Cyc. nassatus	18	19	23	11	6	463	323	29	86	0	18
Cys. calicatus	2	3	1	3		46	32	8			
Cys. goldi				1	2			3	29		
Cys. longibursatus	1		2	1		23	16	3			
other cyathostomin species	1	5	6	8	2	93	65	21	29	6	4
all cyathostomins	**24**	**29**	**35**	**37**	**38**	**679**	**473**	**96**	**543**	**2**	**41**

T2a		number of differentiated xL3 by RLB				number of migrated xL3 in rLMIA				migration percentages in rLMIA	
	before migratio	1st, -IVM	1st+2nd, -IVM	1st, +IVM	1st+2nd, +IVM	1st, -IVM	1st+2nd, -IVM	1st, +IVM	1st+2nd, +IVM	1st, +IVM	2nd, +IVM
number L3s applied to sieve(s)	-	-	-	-	-	417	417	1668	16680	-	-
r L3s migrated through sieve(s)	-	-	-	-	-	282	208	64	266	-	-
L3s per species Cor. coronatus		2	1	1		9	6	2			
Cor. labiatus											
Cor. labratus											
Cya. catinatum	11	6	12	19	25	83	61	34	190	3	75
Cya. pateratum	8	4	7	7	10	55	40	8	38	0	59
Cyc. ashworthi		3	2			14	11				
Cyc. insigne	5	1	5	5	2	32	23	8	15	0	19
Cyc. leptostomum											
Cyc. nassatus	7		6	2	1	37	27	5	8		
Cys. calicatus	1					3	2				
Cys. goldi		1			1	3	2		8		
Cys. longibursatus	7	4	5	4	1	46	34	7	8	0	9
other cyathostomin species											
all cyathostomins	**39**	**21**	**38**	**38**	**40**	**282**	**207**	**64**	**266**	**0**	**54**

T2b		number of differentiated xL3 by RLB				number of migrated xL3 in rLMIA				migration percentages in rLMIA	
	before migratio	1st, -IVM	1st+2nd, -IVM	1st, +IVM	1st+2nd, +IVM	1st, -IVM	1st+2nd, -IVM	1st, +IVM	1st+2nd, +IVM	1st, +IVM	2nd, +IVM
number L3s applied to sieve(s)	-	-	-	-	-	445	445	1792	17820	-	-
r L3s migrated through sieve(s)	-	-	-	-	-	337	220	92	385	-	-
L3s per species Cor. coronatus		2	1	1		10	7	3	11		
Cor. labiatus											
Cor. labratus											
Cya. catinatum	11	6	12	19	25	100	65	36	214	3	91
Cya. pateratum	8	4	7	7	10	65	43	31	86	6	38
Cyc. ashworthi		3	2			17	11				
Cyc. insigne	5	1	5	5	2	38	25	8	21	0	38
Cyc. leptostomum											
Cyc. nassatus	7		6	2	1	45	29			0	0
Cys. calicatus	1					3	2				
Cys. goldi		1			1	3	2	8	11		
Cys. longibursatus	7	4	5	4	1	55	36	8	43	0	84
other cyathostomin species											
all cyathostomins	**39**	**21**	**38**	**38**	**40**	**337**	**220**	**92**	**385**	**1**	**61**

T3		number of differentiated xL3 by RLB				number of migrated xL3 in rLMIA				migration percentages in rLMIA	
	before migratio	1st, -IVM	1st+2nd, -IVM	1st, +IVM	1st+2nd, +IVM	1st, -IVM	1st+2nd, -IVM	1st, +IVM	1st+2nd, +IVM	1st, +IVM	2nd, +IVM
number L3s applied to sieve(s)	-	-	-	-	-	481	481	962	19480	-	-
r L3s migrated through sieve(s)	-	-	-	-	-	471	337	187	1614	-	-
L3s per species Cor. coronatus		1	1	2		7	5	10			
Cor. labiatus											
Cor. labratus	1	1	3			19	13				
Cya. catinatum	12	9	8	10	18	4	3				
Cya. pateratum	16	16	10	12	10	108	77	49	745	19	105
Cyc. ashworthi						156	111	59	414	15	45
Cyc. insigne											
Cyc. leptostomum											
Cyc. nassatus	1	1	3			19	13				
Cys. calicatus	1					4	3				
Cys. goldi	1	1	2	3	4	15	11	15	166	47	76
Cys. longibursatus	15	8	9	11	6	119	85	54	248	19	27
other cyathostomin species	5	2	5			45	32				
all cyathostomins	**51**	**38**	**38**	**38**	**39**	**471**	**337**	**187**	**1614**	**16**	**57**

T4		number of differentiated xL3 by RLB				number of migrated L3 in rLMIA				migration percentages in rLMIA	
	before migratio	1st, -IVM	1st+2nd, -IVM	1st, +IVM	1st+2nd, +IVM	1st, -IVM	1st+2nd, -IVM	1st, +IVM	1st+2nd, +IVM	1st, +IVM	2nd, +IVM
number L3s applied to sieve(s)	-	-	-	-	-	136	136	544	5420	-	-
r L3s migrated through sieve(s)	-	-	-	-	-	97	54	26	60	-	-
L3s per species Cor. coronatus											
Cor. labiatus					2				4		
Cor. labratus											
Cya. catinatum	7	3	11	9	19	26	15	13	37	6	46
Cya. pateratum		1				1	1				
Cyc. ashworthi											
Cyc. insigne											
Cyc. leptostomum											
Cyc. nassatus		3		1		4	2	1			
Cys. calicatus											
Cys. goldi	3		1	3	4	5	3	4	8	16	26
Cys. longibursatus	16	12	16	4	6	55	30	6	12	0	30
other cyathostomin species	4	1		1		6	3	1			
all cyathostomins	**30**	**20**	**28**	**18**	**31**	**97**	**54**	**26**	**60**	**0**	**36**

T5		number of differentiated L3 by RLB				number of migrated xL3 in rLMIA				migration percentages in rLMIA	
	before migratio	1st, -IVM	1st+2nd, -IVM	1st, +IVM	1st+2nd, +IVM	1st, -IVM	1st+2nd, -IVM	1st, +IVM	1st+2nd, +IVM	1st, +IVM	2nd, +IVM
number L3s applied to sieve(s)	-	-	-	-	-	116	116	464	4640	-	-
r L3s migrated through sieve(s)	-	-	-	-	-	86	63	45	48	-	-
L3s per species Cor. coronatus											
Cor. labiatus											
Cor. labratus											
Cya. catinatum	6	3	7	4	7	15	11	5	12	2	27
Cya. pateratum	4	4		4		8	6	5			
Cyc. ashworthi		2	2	2	1	6	4	3	2	5	3
Cyc. insigne	3				1	4	3		2		
Cyc. leptostomum											
Cyc. nassatus	5	11	9	8	8	24	18	10	14	5	13
Cys. calicatus	1	1	2	2		4	3	3			
Cys. goldi	3		2	6	6	7	5	8	10	22	13
Cys. longibursatus	4	6	7	8	4	16	12	10	7	10	3
other cyathostomin species	1	1		1	1	2	1	1	2	11	13
all cyathostomins	**27**	**28**	**29**	**35**	**28**	**86**	**63**	**45**	**48**	**7**	**9**

T6		number of differentiated xL3 by RLB				number of migrated xL3 in rLMIA				migration percentages in rLMIA	
	before migratio	1st, -IVM	1st+2nd, -IVM	1st, +IVM	1st+2nd, +IVM	1st, -IVM	1st+2nd, -IVM	1st, +IVM	1st+2nd, +IVM	1st, +IVM	2nd, +IVM
number L3s applied to sieve(s)	-	-	-	-	-	185,5	185,5	734	7340	-	-
r L3s migrated through sieve(s)	-	-	-	-	-	109	52	40	87	-	-
L3s per species Cor. coronatus											
Cor. labiatus											
Cor. labratus											
Cya. catinatum	23	32	31	30	33	74	35	34	78	4	42
Cya. pateratum			1	1		3	1	1			
Cyc. ashworthi											
Cyc. insigne											
Cyc. leptostomum				1				1			
Cyc. nassatus											
Cys. calicatus											
Cys. goldi	3				1	8	4		2		
Cys. longibursatus	6	5	8	3	3	16	8	3	7	0	37
other cyathostomin species	3					8	4				
all cyathostomins	**35**	**37**	**40**	**35**	**37**	**109**	**52**	**40**	**87**	**1**	**40**

Figures S2 Migration model for rLMIA

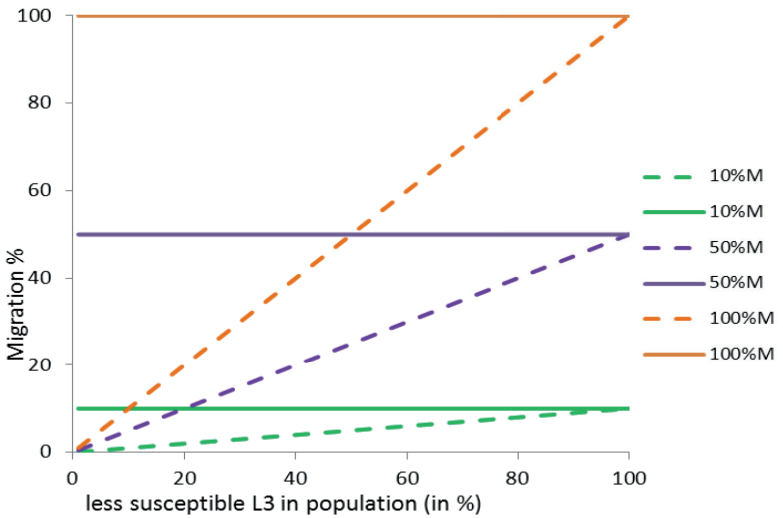


Fig. S2A: Migration percentage as a function of the fraction of less susceptible L3 in the population

1. Degree of unsusceptibility is defined in terms of percentage of larvae that are able to migrate:
   10%M - 10% of the less susceptible larvae migrate (green); 50%M - 50% migrate (purple); 100%M - 100% migrate (orange)
   The latter (100%M) implies that all less susceptible larvae indeed are fully unsusceptible.
2. Dashed line - migration percentage for sieve 1; Straight line - migration percentage for sieve 2.

Fig. S2A shows:
The migration percentage for the 2nd sieve is always higher than for the 1st sieve, unless the fraction of less susceptible larvae in the population is 100%. The migration percentage for sieve 2 depends on the degree of unsusceptibility, but does not depend on the fraction of the less susceptible L3 in a population.

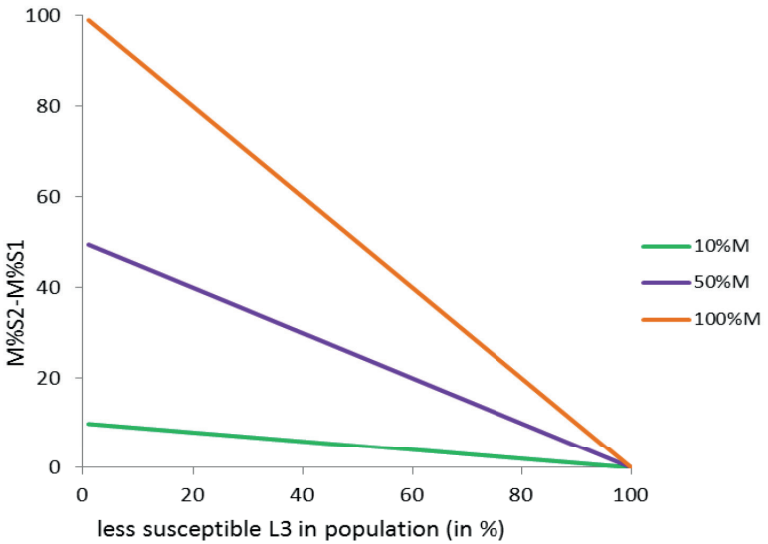


Fig. S2B: The difference between migration percentage for sieve 2 (M% S2) and that for sieve 1 (M% S1) as function of less susceptible L3 in the population

Degree of unsusceptibility is defined in terms of percentage of larvae that are able to migrate (see above Fig. S2A).

Fig. S2B shows:

The difference between migration percentage of S2 and S1 (M%S2-M%S1) can differentiate between the 2 ways that populations can become less susceptible: 1- An increase in the fraction of less susceptible larvae in population, will decrease the M%S2-M%S1.
2- An increase in the degree of unsusceptibility in the less suceptible larvae , will increase the M%S2-M%S1.

M%S2-M%S1 decreases with increasing fraction of less susceptible larvae for all degrees of unsusceptibility of those larvae. However, this should be interpreted in combination with the observed migration percentage for S2. If this is low, this indicates that the fraction of less susceptible larvae possessed a low degree of unsusceptibility. Consequently, the difference between M%S2 and M%S1 will not be high either.

Chapter 8

Discussion

Aims of the thesis

Worldwide, cyathostomins are considered the most prevalent and pathogenic helminths of young (1-3 years) horses. Control of these nematode parasites relies heavily on the use of anthelmintics, in particular the macrocyclic lactones (MLs) because widespread resistance in cyathostomins occurs against the benzimidazoles (BZ) and in some parts of the world also against pyrantel. Given the extensive use of MLs, it was to be expected that resistance would develop against these drugs. Therefore, this thesis focused on several aspects of the occurrence of ML resistance in cyathostomins and the use of the MLs in practice. More specifically, the first aim of the studies in this thesis was to investigate the current status of ML resistance of cyathostomins in horses. This was performed by fecal egg count reduction test (FECRT) in the Netherlands and 2 other European countries. In addition, a larval migration assay was used to study ML susceptibility of the cyathostomins *in vitro*, including differences between species of cyathostomins. The second aim was to study the occurrence of shortened egg reappearance period (ERP) after ML usage through counting of eggs at weekly or biweekly intervals. Shortened ERP and some aspects on the nature of the shortened ERP were studied by differentiation of the larvae obtained before and after ML treatments. Finally, the third aim was to study changes in the practice of veterinarians regarding helminth control after the introduction of prescription-only-medicine (POM) and post academic courses. Therefore, 2 questionnaires with a 4 year time interval were taken. The studies presented in this thesis were carried out to contribute to increasing awareness of veterinarians and horse owners regarding ML resistance as well as the need for rational parasite control.

Main results

In the Netherlands ML resistance as measured with FECRT was suspected on 1 farm and shortening of ERP was found on farms that frequently treated with MLs (**chapters 2 and 3**). Shortening of ERP was found in all 3 investigated countries. The combined results indicated that the MLs are still largely effective in removing adult cyathostomin burdens, but that on many farms the ERP has been shortened suggesting loss of efficacy of the MLs. Eggs that appeared within the regular ERP belonged mostly to the genus *Cylicocyclus* (**chapter 4**). With the reiterative larval migration inhibition assay (rLMIA) it was shown that the proportion of larvae that migrated through consecutive sieves in the presence of ivermectin (IVM) increased with each passage, demonstrating that the applied procedure selects for the least IVM susceptible larvae. This selection process could be inhibited by adding L-glutamate to the IVM solution, which suggested that glutamate-gated chloride channels (GluCls) possibly play a role in ivermectin resistance (**chapter 6**). The predominant species in our *in vitro* studies before selection were *Cyathostomum catinatum*, *Cylicostephanus longibursatus* and *Cylicocyclus nassatus*. After *in vitro* selection in the rLMIA

in the presence of IVM the predominant species became *Cya. catinatum* and it was shown that migration percentages were related to treatment frequency (**chapter 7**). Apart from the above mentioned occurrence of resistance and the involvement of different species as such, the way anthelmintics are applied in the field is important in how fast resistance may develop. It is generally accepted that prudent use of anthelmintics can slow down the development of resistance. Therefore, the role veterinarians have in the control of cyathostomin infections is important to consider. To strengthen this role, anthelmintics were made prescription only medicines (POM) in the Netherlands in 2008. After the introduction of POM and offering post academic education veterinarians were shown to be more involved in diagnostics and prudent use of anthelmintics (**chapter 5**).
Several aspects of these results will be discussed further in this final chapter and this is done to reflect on resistance development of cyathostomin species, diagnosis of anthelmintic resistance and to put the results into a more practical perspective.

Relation between use of anthelmintics and resistance
During the last decades it was noticed that in some helminth species more individuals survived anthelmintic treatment than at the initial introduction of the drug. This anthelmintic resistance develops either through selection of pre-existing resistance alleles in the parasite populations or through mutations in genes or alleles occurring during the use of the anthelmintics. Selection of anthelmintic resistance occurs when survivors of treatment make up the next generation, a process that will be enhanced when frequent systematic treatments are applied. Such frequent anthelmintic treatments (at regular intervals) are often given at or within the expected time of reappearance of eggs in the feces (Reinecke, 1980). However, single treatments can also select strongly for resistance when they are performed in 'no refuge' situations. These occur when treatments are given while no free-living stages occur on pasture and all stages in the host are affected by the drug. Such 'strategic' treatments (Reinecke, 1980) in dry seasons are probably the main reason why anthelmintic resistance initially was prominent in sheep in the Southern Hemisphere. Van Wyk et al. (2001) discussed the important role of refugium (the population that is not under selection pressure by an anthelmintic, for example the larvae on pasture) on the speed of resistance development. Another possible reason for selection for anthelmintic resistance is underdosing and this should therefore be avoided by using weighing scales or girth tapes.

In general, the order of introduction of anthelmintic drugs to the veterinary market were phenothiazine, piperazine compounds, benzimidazoles (from this class onwards called broad spectrum or modern anthelmintics), imidathiazoles, macrocyclic lactones, octadepsipeptides (emodepside), aminoacetonitrile derivatives (monepantel) and spiroindoles (derquantel but only with the ML abamectin). Resistance against anthelmintics always developed and was most often shown approximately a decade after introduction (Waller, 2006).

The speed of development of resistance depends on the number of genes that are involved and whether the resistant variants were already present before the anthelmintic was used for the first time. In the 1960's resistance of cyathostomins against thiabendazol, a member of the BZ drug class, occurred very soon after its introduction for control in horse helminths. After its first use on a farm resistance was already found and this suggested pre-existing resistant variants. This was probably due to resistance development against phenothiazine that caused cross resistance against thiabendazol (Drudge et al., 1990) since in both cases the modes of action depends on tubulin binding.

An initial explanation for quick development of BZ resistance in *Haemonchus contortus* was that only one gene was involved. A single nucleotide polymorphism (SNP), which resulted in an amino acid change in the isotype-1 -tubulin polypeptide in *Haemonchus contortus* causing BZ resistance, was already present in low frequencies in the founding susceptible strain (Kwa et al. 1994). As was shown by Eysker et al. (1983) *in vivo* selection with fenbendazole of *H. contortus* resulted in resistance after only one generation. Therefore, this also appeared to be an example of resistance development where there was only one gene involved and the resistant variant was pre-existing. Later on, it was found that other amino acid changes within the isotype-1 -tubulin also contributed to BZ resistance (von Samson-Himmelstjerna et al., 2009). In cyathostomins comparison of isotype-1 -tubulin in BZ-susceptible versus BZ-resistant isolates showed consistent differences at amino acids 167 and 200 (Hodgkinson et al., 2008).

In the second currently licensed anthelmintic drug class (imidathiazoles) for treatment of cyathostomins, resistance against pyrantel was found to be related to frequent treatments (e.g. daily dosing in the US and Canada) (Kaplan, 2002). The resistance mechanism of cyathostomins against pyrantel is not well understood. Resistance of *Oesophagostomum dentatum* against pyrantel was found to be associated with a modification of the target nicotinic receptor properties (Robertson et al, 2000). Also in a pyrantel resistant isolate of the dog helminth *Ancylostoma caninum* significantly lower levels for three pyrantel receptor subunits were found than in an isolate with low-level resistance (Kopp et al., 2009). Reduced transcription of the mRNA coding for nicotinic acetylcholine receptor subunits that form the pyrantel-sensitive receptors was thought to be the cause.

For the third licensed class, the ML, some of the resistance mechanisms are known. MLs act by binding to GluCls and therefore resistance against MLs can be achieved by changes in these channel proteins (McCavera et al., 2007). However, multiple GluCl genes are present (Wolstenholme, 2011) and multiple mutations are needed to induce resistance. In *Caenorhabditis elegans* three simultaneously mutated genes encoding GluCl type subunits confer resistance

to IVM, but one or two mutated genes showed hardly any effect (Dent et al., 2000). Therefore, it seemed likely that resistance against MLs would be difficult to develop. However, Coles et al., (2005) only needed three generations to turn IVM susceptible *H. contortus* into IVM resistant worms. Prichard (2007) discussed involvement of ABC transporters and beta-tubulin in ML resistance. MLs are substrates for ABC transporters and these transporters may regulate drug concentration in the parasite. There is evidence that MLs select for certain alleles of P-glycoprotein and overexpression of some P-glycoproteins in nematodes is induced. Therefore multiple genes must be included in ML resistance.

In the free-living nematode *C. elegans* an avermectin resistant strain that has never been in contact with MLs was found. This strain possessed a deletion in the GluCl coding gene GLC-1 (Ghosh et al., 2012). Ghosh et al. stated that not all individuals with the deletion were resistant and therefore also demonstrated the multigenic nature of IVM resistance in *C. elegans*. Young et al. (1999) found a small part of cyathostomin larvae to be completely ML unsusceptible with a larval development assay (LDA). This was in accordance with rLMIA results in our studies in which unsusceptible cyathostomin larvae still migrated in very high concentrations of IVM (**chapter 6 and 7**). These results suggest that a sub-population of larvae was not susceptible for IVM. L3 from premises that did not treat with anthelmintics seemed more susceptible to IVM than L3 from premises that frequently treated with ML. The fraction of susceptible L3 did not seem to be larger in horses from the never treated compared with the frequently treated premises. This again suggests that a part of the population is a pre-existing resistant variant.

IVM was introduced to the equine market at the beginning of the 1980's. Selection of cyathostomins proceeded slowly although IVM had a high efficacy against strongyle luminal stages. Approximately 15 years after IVM came onto the market refugia decreased further with the introduction of moxidectin (MOX), because MOX has an (partial) efficacy against inhibited larvae and IVM does not (Xiao et al., 1994; Monahan et al., 1995; Boersema et al., 1998; Vercruysse et al., 1998). The fact that MOX has a very long ERP and treatments with MOX often have been given within the ERP might have sped up the resistance development against the ML drug class. On intensive horse premises in many countries, including the Netherlands, control of cyathostomins was mainly performed through frequent blind anthelmintic treatments. Mainly the MLs were used during the last decades because resistance of cyathostomins against the 2 other groups of anthelmintics has spread (Boersema et al, 1991; Chapman et al., 1996). Based on results from FECRT 14 days after treatment, IVM resistance against cyathostomins was suspected in Brazil on one out of 11 farms where frequent anthelmintic treatments were applied (Canever et al., 2013). The shortening of ERP is probably an indication of the onset of ML

resistance development in larval stages or selection for shorter lifecycles (Lyons et al., 2009; 2013). When MLs would lose their efficacy, also indicated by shortened ERP (von Samson-Himmelstjerna et al. 2007; Molento et al, 2008), this would seriously hamper the options to control cyathostomins by use of drugs. The three newly introduced drug classes mentioned earlier (emodepside, monepantel and derquantel) are not registered for use as an anthelmintic in horses but the first is registered for cats and the last two for sheep. Further delay of development of ML-resistance in cyathostomins should be a priority because no new groups of anthelmintics are under development for horses. In general, we should preserve all available anthelmintic products through a more rational use.

The relative contribution of certain species to resistance

Treatments with MLs contributed to the rise of cyathostomins as pathogenic species. Before the introduction of MLs, *Strongylus vulgaris* was considered to be the most pathogenic parasite in horses. Since the introduction of IVM, of the large strongylids, particularly *Strongylus vulgaris* has noticeably decreased in prevalence. Parallel, (larval) cyathostominosis was noticed more and this became the most important nematode infection in horses worldwide. The basic epidemiology of the cyathostomins is known (**chapter 1**). However, the cyathostomins form a complex group of 14 genera and 50 different species (Lichtenfels et al., 2008). There still is debate about the taxonomic classification of the different species and not much is known about differences in life cycles of those species nor about possible differences between species with respect to their development of anthelmintic resistance.

Hung et al. (2000) performed phylogenetic analysis of 30 species of equine strongylids. Lichtenfels (2002) acknowledged the molecular approach in classifying cyathostomins (Kaye et al., 1998; Hung et al., 2000; Hodgkinson et al., 2001, 2003, 2005) and Lichtenfels et al. (2008) partially included this knowledge in his morphological division. The *Cylicocyclus* (Cyc.) species that we found in our studies (*Cyc. ashworthi*, *Cyc. insigne*, *Cyc. leptostomum*, *Cyc. nassatus*), group together in one clade (monophyletic group) based on analyses of the internal transcribed spacer (ITS)-1 and ITS-2 (Hung et al., 2000). No support for the classification of *Cylicostephanus* or *Coronocyclus* as a genus was found, because they were polyphyletic in all dendrograms. In our RLB the larvae were differentiated to species level, but in the final results they were grouped together per genus (**chapter 4**). Based on phylogeny this grouping seemed justified because the abundant *Cylicocyclus* (*Cyc.*) species always grouped together in the dendrogram, except *Cyc. ultrajectinus*, but we did not find this species. From the genus *Cylicostephanus* (*Cys.*) we almost exclusively found *Cys. longibursatus* and from the genus *Coronocyclus* (*Cor.*) we almost exclusively found *Cor. coronatus*. Furthermore, Lichtenfels et al. (2008) suggest that cyathostomins belong to one clade but results of Hung et al. (2000) did not

support this. Phylogenetic analysis suggested that the genera *Triodontophorus*, *Craterostomum* and *Oesophadontus* belong to the Cyathostominae instead of the Strongylinae (Hung et al., 2000). Overall, it had little consequences for results in the current thesis.

In horses with shortened ERP cyathostomin species belonging to the genus *Cylicocyclus* appeared earlier than other species. Several reasons may explain the observed results. Perhaps the deeper migration of *Cylicocyclus* species into the submucosa of the large intestine is of influence on selection of this genus for shortened ERP. *Cylicocyclus* spp develop slowly in the submucosa, in eight weeks or more, which is long compared to cyathostomin species from other genera like *Cys. longibursatus*, *Cyathostomum* (*Cya.*) *catinatum*, *Cor. coronatum*, *Cys. minutus* and *Cys. calicatus* (Tiunov, 1951 referenced by Ogbourne, 1978). ML treatments during the development in the submucosa of *Cylicocyclus* spp. might select for either resistant larvae or for re-activated larvae with shorter lifecycles. Maybe the long mucosal development time is compensated by a short luminal time, minimizing contact with MLs, and this could be a drug evading strategy. Another reason could have been that the use of MLs has induced a shift in species composition so that the *Cylicocyclus* species became more abundant. However, in **chapters 6 and 7** no difference in species composition was seen before treatment in the larval cultures of horses with or without a shortened ERP. This suggests that many years of ML use did not select for those cyathostomin species or genera with shortest ERP. This is in agreement with Chapman et al. (2002) who did not find a shift in the cyathostomin species composition after 20 years of ML usage.

Field studies always involve mixed infections and no horses infected with a single species of cyathostomins have been investigated as far as we know. The pre patent period (PPP) for mixed infections was determined in the past through fecal egg counts and the shortest were estimated to be 5 to 6 weeks. Also sequential necropsies were performed on foals after natural infection and although the PPP by definition is the period between infection and egg shedding, and with necropsies no egg shedding is measured, indications for species specific PPP were derived for some species (Ogbourne, 1978; Lyons et al, 2011). Speculating a bit, a long development time to adults increases the chance that these cyathostomins will get into contact with anthelmintics and this might increase resistance development within certain species. For determining the PPP of more individual species, parasite naïve foals could be infected and with the RLB technique available would not need to be necropsied to identify the species. For the purpose of establishing species specific PPP feces would need to be collected every day probably from day 21 onwards and eggs or cultured larvae could be differentiated.

Hodgkinson et al. (2003) showed that *Cya. catinatum*, *Cys. goldi*, *Cys. longibursatus*, *Cyc. ashworthi* and *Cyc. nassatus* 4th stage larvae were expelled in horses with larval cyathostominosis. These species belong to the most prevalent species in many countries worldwide (Reinemeyer et al., 1984; Krecek et al., 1989; Bucknell et al., 1995; Collobert et al., 2002). Whether these species are more pathogenic than others remains to be elucidated. Larvae breaking loose from their capsules in the (sub) mucosa, resulting in inflammation of the gut wall, can cause disease especially after massive re-activation. The pathology of the infection, after larvae break loose, could for example also be related to species specific inflammation based on their size as L4, their location in the gut wall or secretory-excretory material all leading to more severe inflammation by certain species. Given the fact that some species appear to become predominant in horses with shortened ERP early after treatment and the possibility that some species may be more pathogenic than others, warrant further work on the role of different species.

Macrocyclic lactone resistance detection

To interpret parasitological data on a worldwide level, without having to discuss methods first, consensus on guidelines are needed. Unfortunately, no recent adjustments were made to WAAVP anthelmintic resistance detection guidelines (Coles et al., 1992) which are not host specific. Therefore, recent guidelines for resistance detection in horses are not available while research methods and statistical approach, due to variables between farms and between horses, should be established per host species (Kaplan, 2002; Pook et al., 2002; Vidyashankar et al., 2007; 2012). The WAAVP guidelines, although still useful, do not suffice for ML resistance detection in horses (Coles et al., 2006). Fortunately, many people have published on the interpretation of equine parasitological diagnostic results since 1992 and more recent guidelines for veterinarians performing equine parasitological research have been adopted in the US (AAEP, 2012).

Despite the lack of consensus among equine parasitologists, for *in vivo* anthelmintic resistance detection measuring the reduction in egg counts is considered the gold standard. As shortened ERP has developed on certain premises the counting of strongyle eggs for efficacy testing should therefore not be restricted to days 0 and 14, but also be performed at day 42 (IVM) or day 56 (MOX) after treatment (**chapter 3 and 4**). It might also be useful to include larval cultures in the next WAAVP protocol to improve ML resistance detection. Larval cultures are far more sensitive than egg counts (Bello and Allen, 2009). In our study a detection limit of 0.04 Larvae Per Gram (LPG) was achieved which is far more sensitive than the sensitivity of 25 or 50 EPG with the McMaster method. Furthermore, differentiation of the cultured larvae can reveal which species produce eggs during shortened ERP.

As stated the FECRT is the most widely adopted technique to *in vivo* determine reduced efficacy of an anthelmintic. The widely used McMaster method, although easy to perform, is not a very sensitive method for fecal egg counts. Furthermore, at least 2 farm visits and individual collection of feces from as many horses as possible are required for a proper FECRT. *In vivo* determination of ML efficacy is therefore labor intensive and insensitive and consequently an *in vitro* diagnostic system for detection of ML resistance in cyathostomins would be very welcome.

No *in vivo* resistant worm population was found by FECRT in the early stages of the work described in this thesis. This made it difficult to study the relationship between *in vivo* and *in vitro* efficacy of the MLs. Although MLs could have a different activity against L3 than against adults, because other genes or products are expressed in L3s than in adults, it is far easier to study ML resistance in larvae *in vitro* than in adult cyathostomins *in vivo*. The combination of an *in vitro* assay and differentiation of cyathostomin species with RLB allowed selecting *in vitro* susceptible and unsusceptible L3 and studying ML resistance. Although LDA appeared to be suitable as an *in vitro* technique for sheep nematodes, this technique did not produce reliable results in studies with cyathostomins (Tandon and Kaplan, 2004; Osterman Lind et al., 2005). Also since obtaining fresh eggs for an *in vitro* assay is more time consuming than working with larvae (that can be stored) we chose to study susceptibility of L3 to ML with a larval migration assay. In the related genera *Cooperia, Ostertagia* and *Haemonchus* a good correlation between *in vivo* resistance and larval migration was observed (Wagland et al., 1992; Gill and Lacey, 1998; Kotze et al. 2006; Demeler et al., 2010a, 2010b; El-Abdelatti et al., 2010) suggesting that this might be similar in cyathostomins. Robinson et al. (2008) showed a relation between *in vivo* and *in vitro* ML susceptibility in donkey cyathostomins. However, studies performed by Matthews et al. (2012) failed to show that LMIA could reliably distinguish ML-susceptible from ML-resistant populations. It should be mentioned that they did not differentiate L3 to species. Since it is not likely that resistance will develop simultaneously in all species and especially because the migration in the presence of IVM differs between species, differentiation of migrated larvae is necessary in cyathostomins.

Our work also indicated a role of GluCls in cyathostomin resistance development because the presence of L-glutamate combined with IVM resulted in the rLMIA in a decrease of migration percentage of L3 (**chapter 6**). The same synergistic effect of L-glutamate and IVM was found on the activation of GluCls by allosteric binding (Forrester et al., 2002). Further research on the involvement of GluCls and ML resistance development in cyathostomins therefore is justified. If the resistance mechanism in cyathostomins would be clarified assays to identify markers for resistance detection can be developed.

Cya. catinatum was the least ML susceptible species in our *in vitro* studies (**chapters 6 and 7**). However, this species is not prominent in horses with shortened ERP suggesting that perhaps no relation between resistant adults and larvae exists. The combined rLMIA RLB is also labor-intensive because larvae have to migrate through two sieves and should then be processed individually in RLB. Therefore, our rLMIA RLB is currently not suitable for routine large-scale studies. Probably we are still a long way from a useful *in vitro* diagnostic technique to evaluate ML-resistance in cyathostomins in the field. There are high throughput techniques that could be developed for differentiation of cyathostomin species. Variation in copy numbers of the rDNA genes within and between the different species is probably the main difficulty in differentiating and quantifying of cyathostomins.

Advisory role for veterinarians

Clearly, as shown above, cyathostomin species respond to the use of anthelmintic drugs, including MLs, rendering those drugs less effective. Therefore, cyathostomin control strategies should not only aim to prevent disease but also aim to preserve the efficacy of the available anthelmintics as much as possible. One of the measures taken was to make anthelmintics POM in 2008. This strengthened the advisory role of veterinarians concerning parasite control. However, this advisory role should imply more than only providing the anthelmintics. It should be based on a solid understanding of the epidemiology and control of helminth infections in horses. Following the new legislation and (obligatory) post academic education it was expected that veterinarians would conduct more fecal examinations for worm eggs (monitoring) and that decisions to treat horses would become more dependent on the result of such fecal examinations. Indeed, this was found to be true (**chapter 5**). However, the results also showed that further improvement on the understanding of worm control of veterinarians should be aimed for.

Much could be gained if sensible farm management would be advocated and explained to horse owners on a larger scale. This task could be performed by veterinarians. For example, it is well known that a small minority of adult horses are 'wormy', while most of them will shed very few worm eggs in the feces and will not require anthelmintic treatments (Döpfer et al, 2004; Nielsen et al. 2006; Becher et al., 2010). When these low shedders have been sorted out on a particular farm, fecal monitoring and anthelmintic treatments can be concentrated on the minority of shedders. To minimize the amount of work involved in fecal examination pooled fecal samples could be performed with a maximum of 10 animals and these could be used to determine if the group needs treatment (Eysker et al., 2008). Individual or pooled EPGs should be performed and proper thresholds for strongyle egg counts in feces should be used (www.parasietenwijzer.nl). In other countries a similar situation is present. The first country to tackle the problem of increasing anthelmintic resistance by legislation

was Denmark. They made anthelmintics POM and at the same time diagnostics prior to (possible) treatment were made obligatory by law. This may work as well in the Netherlands, but requires a regulatory change made by the government.

Veterinarians indicated they still felt a need for more knowledge (**chapter 5**). The implementation of monitoring through strongyle egg counts, based on the epidemiology of the parasites, needs further guidance from veterinarians and should be paid for by horse-owners. At post-academic courses the majority of veterinarians indicated not to register egg counts of animals. Since the EPGs of horses are related to the ones that were performed weeks or months before it would be very useful to record these data in the veterinary clinic and use these for parasite management schemes. A firm relation between veterinary practice and horse owners is needed so the veterinarian knows the circumstances in which the horses live (Sloet van Oldruitenborgh-Oosterbaan et al., 2009). Although in the past few years the perception of anthelmintics as a medicine has changed for the better, still a lot of blind treatments are given. If a new anthelmintic drug for horses would come to the market it is necessary to use this product wisely, allowing for parasite refugia for example, this way the drug would have the longest life span possible.

Main conclusions of this thesis

It was concluded that a high efficacy of IVM and MOX was still achieved 14 days after treatment yet a shortened ERP after these treatments on many farms was detected. Most often *Cylicocyclus* species were found in horses shortly after ML treatment. On the farm with the most pronounced ERP shortening, species from other genera were also found early after treatment indicating that other genera can be selected as well.

From our *in vitro* studies it was learned that *Cya. catinatum* is a candidate for future studies, because it is an abundant species with a relatively high percentage of resistant L3 in many of the studied larval populations. Because of the demonstrated effect of glutamate on larval migration in the presence of ivermectin, it is justified to focus on the role of GluCls in ML resistance of the cyathostomins. Larvae were successfully separated in IVM *in vitro* susceptible and less susceptible larvae. The *in vitro* unsusceptibility of specifically *Cya. catinatum* appears to be related to anthelmintic treatment history on the horse premises suggesting at least *in vitro* resistance of *Cya. catinatum*.

After the introduction of the legislation that made anthelmintics prescription only medicines supported by post academic courses some significant improvements in the prudent use of anthelmintics by veterinarians were accomplished because treatments appeared to be increasingly based on fecal diagnosis. However, continuing efforts need to be focused on improving the number of quantitatively processed fecal samples and the interpretation of fecal diagnosis results.

References

AAEP Parasite Control Guidelines.Developed by the AAEP Parasite Control Subcommittee of the AAEP Infectious Disease Committee.

Becher A.M., Mahling M., Nielsen M.K., Pfister K. 2010. Selective anthelmintic therapy of horses in the Federal states of Bavaria (Germany) and Salzburg (Austria): an investigation into strongyle egg shedding consistency. Vet. Parasitol. 15, 116-122.

Bello T.R., Allen T.M., 2009. Comparison of two fecal egg recovery techniques and larval culture for cyathostomins in horses. Am. J. Vet. Res., 70, 571-573.

Boersema J.H., Borgsteede F.H.M., Eysker M., Elema T.E., Gaasenbeek C.P.H., van der Burg W.P.J., 1991. The prevalence of anthelmintic resistance of horse strongyles in The Netherlands. Vet. Q. 13: 209–217.

Boersema JH, Eysker M, van der Aar WM., 1998.The reappearance of strongyle eggs in the faeces of horses after treatment with moxidectin. Vet Q. 20, 15-17.

Bucknell D.G., Gasser R.B., Beveridge I., 1995. The prevalence and epidemiology of gastrointestinal parasites of horses in Victoria, Australia Int. J. for Parasitol. 25, 711-724.

Canever R.J., Braga P.R.C., Boeckh A., Grycajuck M., Bier D., Molento M.B., 2013.Lack of Cyathostomin sp. reduction after anthelmintic treatment in horses in Brazil. Vet. Parasitol.194, 35-39.

Chapman M.R., French D.D., Monahan C.M., Klei T.R., 1996. Identification and characterization of a pyrantel pamoate resistant cyathostome population. Vet. Parasitol. 66, 205-212.

Chapman M.R., French D.D., Klei T.R., 2002, Gastrointestinal helminths of ponies in Louisiana: a comparison of species currently prevalent with those present 20 years ago. J. Parasitol. 6, 1130-1134.

Coles G.C., Bauer C., Borgsteede F.H., Geerts S., Klei T.R., Taylor M.A., Waller P.J., 1992. World Association for the Advancement of Veterinary Parasitology (W.A.A.V.P.) methods for the detection of anthelmintic resistance in nematodes of veterinary importance. Vet. Parasitol. 44, 35-44.

Coles G.C., Rhodes A.C., Wolstenholme A.J., 2005. Rapid selection for ivermectin resistance in *Haemonchus contortus*. Vet. Parasitol. 129, 345-347.

Coles G.C., Jackson F., Pomroy W.E., Prichard R.K., von Samson-Himmelstjerna G., Silvestre A., Taylor M.A., Vercruysse J., 2006. The detection of anthelmintic resistance in nematodes of veterinary importance. Vet. Parasitol. 136, 167–185.

Collobert-Laugier C., Hoste H., Sevin C., Dorchies P., 2002. Prevalence, abundance and site distribution of equine small strongyles in Normandy, France. Vet Parasitol 110,77-83

Demeler J., Küttler U., von Samson-Himmelstjerna G., 2010a. Adaptation and evaluation of three different *in vitro* tests for the detection of resistance to anthelmintics in gastro intestinal nematodes of cattle. Vet Parasitol. 170, 61-70.

Demeler J., Küttler U., El-Abdellati A., Stafford K., Rydzik A., Varady M., Kenyon F., Coles G., Höglund J., Jackson F., Vercruysse J., von Samson-Himmelstjerna G., 2010b. Standardization of the larval migration inhibition test for the detection of resistance to ivermectin in gastro intestinal nematodes of ruminants. Vet Parasitol. 174, 58-64.

Dent J.A., Smith M.M., Vassilatis D.K., Avery L., 2000. The genetics of ivermectin resistance in Caenorhabditis elegans. PNAS 97, 2674–2679.

Döpfer D., Kerssens C.M., Meijer Y.G.M., Boersema J.H., Eysker M., 2004. Shedding consistency of strongyle-type eggs in Dutch boarding horses. Vet. Parasitol. 124, 249-258.

Drudge J.H., Lyons E.T., Tolliver S.C., Fallon E.H., 1990. Phenothiazine in the origin of benzimidazole resistance in population-B equine strongyles. Vet Parasitol. 35, 117-130.

El-Abdellati A, Geldhof P, Claerebout E, Vercruysse J, Charlier J., 2010. Monitoring macrocyclic lactone resistance in *Cooperia oncophora* on a Belgian cattle farm during four consecutive years. Vet Parasitol. 171, 167-171.

Eysker M., Jansen J., Boersema J.H., Lewing-van der Wiel P.J., 1983. Development of benzimidazole resistance in a *Haemonchus contortus* strain in the Netherlands following fenbendazole treatment of ewes at parturition. Res. Vet. Sci. 34, 46-49.

Eysker M., Bakker J., van de Berg M., van Doorn D.C.K., Ploeger H.W., 2008. The use of age-clustered pooled faecal samples for monitoring worm control in horses. Vet. Parasitol. 151, 249-255.

Forrester S.G., Prichard R.K., Beech R.N., 2002. A glutamate-gated chloride channel subunit from *Haemonchus contortus*: expression in a mammalian cell line, ligand binding, and modulation of anthelmintic binding by glutamate. Biochem Pharmacol. 63, 1061-1068.

Ghosh R., Andersen E.C., Shapiro J.A., Gerke J.P., Kruglyak L., 2012. Natural variation in a chloride channel subunit confers avermectin resistance in *C. elegans*. Science 335, 574-578.

Gill J.H., Lacey E., 1998. Avermectin/milbemycin resistance in trichostrongyloid nematodes, Int.J. Parasitol. 28, 863-877.

Hodgkinson J.E., Love S.,Lichtenfels J.R., Palfreman S., Ramsey Y.H., Matthews J.B., 2001. Evaluation of the specificity of five oligoprobes for identification of cyathostomin species from horses. Int. J.Parasitol. 31, 197-204.

Hodgkinson J. E., Lichtenfels J.R., Mair T. S., Cripps P., Freeman K. L., Ramsey Y. H., Love S., Matthews J. B., 2003. A PCR-ELISA for the identification of cyathostomin fourth-stage larvae from clinical cases of larval cyathostominosis. Int. J. Parasitol. 33, 1427-1435.

Hodgkinson J. E., Freeman K. L., Lichtenfels J. R., Palfreman S., Love S., Matthews J. B., 2005. Identification of strongyle eggs from anthelmintic-treated horses using a PCR-ELISA based on intergenic DNA sequences. Parasitol. Res. 95, 287-292.

Hodgkinson J.E., Clark H.J., Kaplan R.M., Lake S.L., Matthews J.B., 2008. The role of polymorphisms at b-tubulin isotype 1 codons 167 and 200 in benzimidazole resistance in cyathostomins. Int. J. Parasitol. 38, 1149-1160.

Hung G.C., Chilton N.B., Beveridge I., Gasser R.B., 2000. A molecular systematic framework for equine strongyles based on ribosomal DNA sequence data. Int. J. Parasitol. 30, 95–103.

Kaplan R.M., 2002. Anthelmintic resistance in nematodes of horses. Vet. Res. 33, 491–507.

Kaye J.N., Love S., Lichtenfels J.R., McKeand J.B., 1998. Comparative sequence analysis of the intergenic spacer region of cyathostome species. Int. J. Parasitol. 28, 831-836.

Kotze A.C., Le Jambre L.F., O'Grady J.,2006. A modified larval migration assay for detection of resistance to macrocyclic lactones in *Haemonchus contortus*, and drug screening with Trichostrongylidae parasites. Vet. Parasitol. 137, 294–305.

Kopp S.R., Coleman G.T., Traub R.J., McCarthy J.S., Kotze A.C., 2009. Acetylcholine receptor subunit genes from *Ancylostoma caninum*: altered transcription patterns associated with pyrantel resistance. Int. J. Parasitol. 39, 435-441.

Krecek R.C., Reinecke R.K., Horak I.G., 1989. Internal parasites of horses on mixed grassveld and bushveld in transvaal, Republic of South Africa. Vet. Parasitol. 34, 135–143.

Kwa M.S.G., Veenstra J. G., Roos, M.H., 1994. Benzimidazole resistance in *Haemonchus contortus* is correlated with a conserved mutation at amino acid 200 in b-tubulin isotype 1. Mol. Biochem. Parasitol. 63, 299–303.

Lichtenfels J. R, Gibbons L. M., Krecek R. C., 2002."Recommended Terminology and Advances in the Systematics of the Cyathostominea (Nematoda: Strongyloidea) of Horses". Vet. Parasitol. 107, 337–342.

Lichtenfels J.R., Kharchenko V.A., Dvojnos G.M., 2008. Illustrated identification keys to strongylid parasites (strongylidae: Nematoda) of horses, zebras and asses (Equidae). Vet. Parasitol. 156, 4-161.

Lyons E.T., Tolliver S.C., Collins S.S., 2009. Probable reason why small strongyle EPG counts are returning "early" after ivermectin treatment of horses on a farm in Central Kentucky.Parasitol Res.104, 569-574.

Lyons E.T., Kuzmina T.A., Tolliver S.C., Collins S.S., 2011. Observations on development of natural infection and species composition of small strongyles in young equids in Kentucky. Parasitol. Res. 109, 1529-1535.

Lyons E.T., Tolliver S.C., 2013.Further indication of lowered activity of ivermectin on immature small strongyles in the intestinal lumen of horses on a farm in Central Kentucky.Parasitol Res. 112, 889-891.

Matthews J.B., McArthur C., Robinson A., Jackson F., 2012. The *in vitro* diagnosis of anthelmintic resistance in cyathostomins. Vet. Parasitol. 185, 25-31.

McCavera S., Walsh T.K., Wolstenholme A.J., 2007. Nematode ligand-gated chloride channels: an appraisal of their involvement in macrocyclic lactone resistance and prospects for developing molecular markers. Parasitol. 134, 1111-1121.

Molento M.B., Antunes J., Bentes R.N., Coles, G.C., 2008. Anthelmintic resistant nematodes in Brazilian horses. Vet. Rec. 162, 384-385.

Monahan C.M., Chapman M.R., French D.D., Taylor H.W., Klei T.R. 1995. Dose titration of moxidectin oral gel against gastrointestinal parasites of ponies. Vet. Parasitol. 59, 241-248.

Nielsen M.K., Haaning N., Olsen S.N., 2006. Strongyle egg shedding consistency in horses on farms using selective therapy in Denmark. Vet. Parasitol. 135, 333-335.

Ogbourne C.P.,1978. Pathogenesis of cyathostome (Trichonema) infections of the horse. A review. CAB abstracts. CIH miscellaneous publication No 5.

Osterman Lind E., Uggla A., Waller P. Hoglund J., 2005. Larval development assay for detection of anthelmintic resistance in cyathostomins of Swedish horses. Vet. Parasitol. 128, 261-269.

Pook J.F., Power M.L., Sangster N.C., Hodgson J.L., Hodgson D.R., 2002. Evaluation of tests for anthelmintic resistance in cyathostomes. Vet. Par. 106, 331–343.

Prichard R.K, 2007. Ivermectin resistance and overview of the Consortium for Anthelmintic Resistance SNPs. Expert Opin. Drug Discov. 2, 41-52.

Reinecke, R.K. 1980, Chemotherapy in the control of helminthiasis. Vet. Parasitol. 6, 255-292.

Reinemeyer C.R., Smith S.A., Gabel A.A., Herd R.P., 1984. The prevalence and intensity of internal parasites of horses in the U.S.A. Vet. Parasitol. 15, 75–83.

Robertson A.P., Bjørn H.E., Martin R.J., 2000. Pyrantel resistance alters nematode nicotinic acetylcholine receptor single-channel properties. Eur J Pharmacol. 394, 1-8.

Robinson A., McArthur C., Burden F.A., Goss L., Trawford A.F., Jackson F., Matthews J.B., 2008. Use of the larval migration inhibition assay to investigate suspected macrocyclic lactone resistant cyathostomin populations. Proc. of the Int. Equine Parasite Drug Resistance Workshop. Copenhagen Denmark.

Sloet van Oldruitenborgh-Oosterbaan M.M., van Doorn D.C.K., Holland W., van Herten J., Ploeger H.W., Fink-Gremmels J., 2009. Antiparasitaire middelen en de receptplicht voor paarden. Tijdschr. Diergeneesk. 134, 288-295.

Tandon R., Kaplan R.M., 2004. Evaluation of a larval development assay (DrenchRite®) for the detection of anthelmintic resistance in cyathostomin nematodes of horses. Vet. Parasitol. 121, 125–142.

van Wyk J.A., 2001. Refugia-overlooked as perhaps the most potent factor concerning the development of anthelmintic resistance. Onderstepoort J. Vet. Res. 68, 55–67.

Vercruysse J., Eysker M., Demeulenaere D., Smets K., Dorny P., 1998. Persistent efficacy of a 2% equine moxidectin gel on establishment of Cyathostominae in horses. Vet Rec.143, 307-309.

von Samson-Himmelstjerna G., Fritzen B., Demeler J., Schurman S., Rohn K., Schnieder T., Epe C., 2007. Cases of reduced cyathostomin egg-reappearance period and failure of *Parascaris equorum* egg count reduction following ivermectin treatment as well as survey on pyrantel efficacy on German horse farms. Vet. Parasitol. 144, 74-80.

Von Samson-Himmelstjerna G., Walsh T.K., Donnan A.A., Carriere S., Jackson F., Skuce P.J., Rohn K., Wolstenholme A.J., 2009. Molecular detection of benzimidazole resistance in *Haemonchus contortus* using real-time PCR and pyrosequencing. Parasitol. 136, 349–358.

Vidyashankar A.N., Kaplan R.M., Chan S., 2007. Statistical approach to measure the efficacy of anthelmintic treatment on horse farms. Parasitol. 134, 2027-2039.

Vidyashankar A.N., Hanlon B.M., Kaplan R.M. 2012. Statistical and biological considerations in evaluating drug efficacy in equine strongyle parasites using fecal egg count data. Vet. Parasitol. 185, 45-56.

Wagland B.M., Jones W.O., Hribar L., Bendixsen T., Emery D.L., 1992. A new simplified assay for larval migration inhibition. Int. J. Parasitol. 22, 1183–1185.

Waller P.J., 2006. From discovery to development: Current industry perspectives for the development of novel methods of helminths control in livestock. Vet. Parasitol. 139, 1-14.

Wolstenholme A.J., 2011. Ion channels and receptor as targets for the control of parasitic nematodes. Int. J. Parasitol.: Drug and drug resistance 1, 2-13.

Xiao L., Herd R.P., Majewski G.A., 1994. Comparative efficacy of moxidectin and ivermectin against hypobiotic and encysted cyathostomes and other equine parasites. Vet. Parasitol. 53, 83-90.

Young K.E., Garza V., Snowden K., Dobson R.J., Powell D., Craig T.M., 1999. Parasite diversity and anthelmintic resistance in two herds of horses. Vet. Parasitol. 85, 205–214.

Summary

Samenvatting

Dankwoord / Acknowledgements

Curriculum Vitae

Summary

Horses on pasture always acquire helminth infections as parasitic larvae or eggs will be ingested. Cyathostomins are the primary helminths pathogens of young (1-3 years) horses.

The most pathogenic form of the disease (larval cyathostominosis) mainly develops in this age class (**chapter 1**). Worldwide anthelmintic resistance development of cyathostomins against several anthelmintic drug classes has been shown. Since macrocyclic lactones (MLs) is the last group of anthelmintics that was introduced it was considered necessary by us to determine if resistance against these drugs had developed and if risk factors and/or the resistance mechanism could be determined.

The aim of the first study described in this thesis (**chapter 2**) was to look for ivermectin (IVM) resistance on Dutch horse premises and to determine possible risk factors for resistance development. This study was performed according to the guidelines of the World Association of the Advancement of Veterinary Parasitology (WAAVP) using the fecal egg count reduction test (FECRT). Seventy premises of which half were raising farms were investigated through pooled fecal samples and larval cultures. The FECRT was only performed (by the investigators) on horses that showed positive egg counts or larval cultures after IVM treatment by their owners. Questionnaire data showed that the frequency of treatment was high (on average 5 times per year) on most farms. Thirty-four percent of these 70 owners dewormed frequently within the egg reappearance period (ERP). None of the farmers tried to use pasture rotation as worm control measure. Only 9 out of the 15 farms claiming to remove feces from pasture did this at least once every 2 weeks. Fecal examination before deworming (monitoring) was only done on 2 (3%) farms. Only in one horse an efficacy against cyathostomins of IVM less than 95% was found. This implies that resistance of cyathostomins against IVM was not detected in this study and risk factors for ML resistance development could not be established.

To further study ML resistance development with FECRT and to study shortening of the ERP, premises in Italy, Belgium and the Netherlands were selected based on a history of (frequent) deworming with MLs (**chapter 3**). At least 10 study

sites in each country were selected and animals were allocated to one of two treatment groups based on the pre-treatment fecal egg counts (FEC). Animals were treated with a ML and this was either IVM or moxidectin (MOX). In total, 320 horses on 32 premises were examined. The FECRT 14 days after treatment indicated a 100% efficacy in 59 of the 64 treatment groups and >92% efficacy in the remaining 5 groups. In the Netherlands an efficacy for IVM of 92% was found on one farm and shortening of ERP on 6 premises. In total the ERP for IVM was decreased on 17 out of 32 study sites and for MOX this was 15 out of 32 study sites. On 9 sites the ERP was decreased for both IVM and MOX.
To investigate the species that contributed to a shortened ERP, 4 Dutch horse premises were selected of which 2 from the study described in chapter 3 and 2 premises without previous knowledge on ERP. Fecal samples from 21 horses, before and after ML treatment, were cultured and 40 larvae obtained from each culture were individually differentiated with reverse line blot (RLB) (**chapter 4**). The RLB showed that early egg shedding was dominated by species of the genus *Cylicocyclus* (Cyc.). Before treatment species composition was generally more diverse with species from several other genera also present and no differences in composition of the larval cultures of horses with a shortened ERP and those who had regular ERPs were found. It is concluded that particularly *Cylicocyclus* spp. are responsible for a shortened ERP.

In the Netherlands in 2008, as a result of an EU directive, regulatory changes were made which made anthelmintics prescription only medicines (POM). Questionnaire surveys at the beginning of POM and again 4 years later were performed (**chapter 5**). The aim was to study whether the regulatory changes, post-academic education, the website www.parasietenwijzer.nl and a paper on Good Veterinary Practice regarding helminths and deworming (Sloet van Oldruitenborgh-Oosterbaan et al., 2009) resulted in changes in horse practitioners' practices. Results showed that in 2012 significantly more fecal samples were examined than in 2008, more fecal samples were taken for monitoring purposes, more fecal samples were examined quantitatively and more use was made of pooled fecal samples. The number of veterinarians that routinely examined a fecal sample before deciding whether to treat or not increased by three-fold from 2008 to 2012. Results also showed that continuing efforts need to be focused on improving the number of quantitatively processed fecal samples and the interpretation of fecal diagnostics.

In the 1st *in vitro* study of this thesis (**chapter 6**) cyathostomin eggs were obtained from 2 populations of horses, one from never treated and the other from regularly IVM treated ponies. The larvae, cultured from eggs, were *in vitro* selected using a reiterative Larval Migration Inhibition Assay (rLMIA) and were differentiated by RLB. In the rLMIA the proportion of larvae that migrated through 4 sieves increased with each passage, demonstrating that the applied procedure selects for the least IVM susceptible larvae. Adding L-glutamate to the procedure reversed the selection effect, which suggested that glutamate gated chloride channels (GluCls) play a role in IVM susceptibility of the selected

larvae. In both larval cultures the predominant species were *Cyathostomum* (*Cya.*) *catinatum*, *Cyc. nassatus* and *Cylicostephanus* (Cys) *longibursatus*. After *in vitro* selection in the rLMIA in the presence of IVM the predominant species became *Cya. catinatum* in both cultures, while *Cyc. nassatus* disappeared in the never treated but not in the regularly IVM exposed larval population. It was concluded that the rLMIA RLB is useful in studying *in vitro* ML susceptibility of larvae.

In the 2nd *in vitro* study a two-sieve rLMIA was performed (**chapter 7**) to study the intra-specific variation in IVM susceptibility and to examine the variation in outcome in the assays using 8 worm populations with different histories of ML exposure. In all 8 populations the pre-dominant species were *Cya. catinatum*, *Cyc. nassatus*, *Cys. longibursatus* and *Cya. pateratum*. After *in vitro* separation of larvae in the presence of IVM by rLMIA the most prevalent species were *Cya. catinatum* and *Cys. longibursatus*. The combined rLMIA RLB demonstrated a higher migration percentage for the 2nd sieve compared with the 1st sieve, indicating selection of larvae with low IVM susceptibility. The highest migration percentage for the 2nd sieve was found for *Cya. catinatum*. Overall, a significantly lower migration percentage for the 2nd sieve was observed for *Cya. catinatum* in the never treated horses compared with the treated horses. It was found that within cyathostomin species there is heterogeneity with respect to *in vitro* IVM susceptibility and this was also found in larvae obtained from never treated horses. It was concluded that rLMIA using IVM and 2 sieves separated susceptible from less susceptible *Cya. catinatum* L3s and that their heterogeneity was related to treatment frequency.

In the discussion section (**chapter 8**) the relationship between the use of anthelmintics and resistance development against several drug classes and how to detect (ML) resistance in cyathostomins was discussed. The three main conclusions of this thesis mentioned in chapter 8 are:
although a high efficacy of IVM and MOX was still achieved 14 days after treatment, a shortened ERP after these treatments on a considerable proportion of the investigated farms was detected. Most often *Cylicocyclus* species were found in horses shortly after ML treatment. On the farm with the most pronounced ERP shortening, species from other genera were also found early after treatment indicating that other genera can be selected as well.
It was learned that reduced susceptibility for IVM of specifically *Cya. catinatum* appears to be related to anthelmintic treatment history on the horse premises suggesting *in vitro* resistance at least.

After the introduction of the legislation that made anthelmintics prescription only medicines some significant improvements in the prudent use of anthelmintics by veterinarians were accomplished.

Samenvatting

Paarden die weiden zullen altijd worminfecties hebben door opname van parasitaire larven of eieren. Cyathostominae zijn de meest pathogene wormen bij jonge (1-3 jaar leeftijd) paarden. De ernstigste vorm van de ziekte (larvale cyathostominosis) ontwikkelt zich voornamelijk in deze leeftijdscategorie (**hoofdstuk 1**). Ontwormingsmiddelen (anthelmintica) worden gebruikt om deze parasieten te beheersen. Wereldwijd is anthelminticum resistentie van cyathostominae tegen verschillende groepen van anthelmintica inmiddels aangetoond. Sinds de introductie van de macrocyclische lactonen (MLs), de laatst geïntroduceerde groep van middelen, werd het door ons noodzakelijk geacht om te bepalen of resistentie tegen deze groep van anthelmintica al was ontwikkeld en of risicofactoren voor het resistentiemechanisme konden worden bepaald.

Het doel van de eerste studie in dit proefschrift (**hoofdstuk 2**) was om ivermectine (IVM) resistentie op Nederlands paardenbedrijven vast te stellen en om eventuele risicofactoren voor resistentie-ontwikkeling te bepalen. Deze studie werd uitgevoerd volgens de richtlijnen van de World Association for the Advancement of Veterinary Parasitology (WAAVP) met de fecal egg count reduction test (FECRT). Dit is een test om bij een levend dier de reductie (%) van het aantal eieren na een behandeling te meten. Zeventig bedrijven, waarvan de helft opfokbedrijven, werden onderzocht met behulp van gepoolde feces monsters en larvenkweken. De FECRT werd vervolgens alleen uitgevoerd (door de onderzoekers) bij paarden die na IVM behandeling door de eigenaren nog positieve ei-tellingen of larvenkweken hadden. De risicofactoren van resistentie ontwikkeling werden geprobeerd vast te stellen met behulp van een enquête. Uit de enquêtes bleek dat de frequentie van de behandeling hoog was (gemiddeld 5 keer per jaar) op de meeste bedrijven. Vierendertig procent van de 70 deelnemers ontwormden hun paarden binnen de egg reappearance periode (ERP). Dit is de periode na een anthelmintische behandeling tot de eieren opnieuw in de feces verschijnen. Geen van de managers/eigenaren probeerden weiderotatie te gebruiken als controlemaatregel. Maar 9 van de 15 bedrijven die aangaven feces uit de weide te verwijderen deden dit minstens eenmaal per 2 weken. Fecesonderzoek voorafgaand aan een eventuele behandeling (monitoring) werd maar gedaan op 2 (3%) bedrijven. Slechts in 1 paard werd

een werkzaamheid van IVM tegen cyathostominae van minder dan 95% gevonden. Dit houdt in dat resistentie ontwikkeling van cyathostominae tegen IVM en mogelijke risicofactoren niet konden worden vastgesteld in deze studie.

Om ML resistentie ontwikkeling met de FECRT en verkorting van de ERP verder te bestuderen, werden bedrijven in Italië, België en Nederland geselecteerd op basis van hun historie van (frequente) ontwormingen met MLs (**hoofdstuk 3**). Tenminste 10 paardenbedrijven in ieder land werden geselecteerd en dieren werden op basis van hun fecal egg count (FEC) in een van twee behandelingsgroepen ingedeeld. Dieren werden behandeld met MLs en dit was ofwel IVM of moxidectine (MOX). In totaal werden 320 paarden op 32 bedrijven onderzocht. Uit de FECRT 14 dagen na ontwormen bleek de behandeling 100% effectief in 59 van de 64 behandelingsgroepen en meer dan 92% effectief in de resterende 5 behandelingsgroepen. In Nederland werd op een bedrijf een effectiviteit van IVM van 92% gevonden en een verkorting van de ERP op 6 bedrijven. In totaal was de ERP na IVM verkort op 17 van de 32 bedrijven en voor MOX was dit 15 van de 32 bedrijven. Op 9 bedrijven was de ERP voor zowel IVM als MOX verkort.
De cyathostominae zijn een groep van wormen die bestaan uit 50 soorten die paardachtigen infecteren. Om te onderzoeken welke soorten bijdragen aan een verkorte ERP, werden 4 Nederlandse paardenbedrijven geselecteerd waarvan 2 met verkorte ERP uit de ERP-studies (**hoofdstuk 3**) en 2 bedrijven met een onbekende ERP. Feces van 21 paarden, van voor en na de ML behandeling, werd gekweekt en 40 larven uit elke cultuur werden individueel gedifferentieerd met de reverse line blot (RLB) (**hoofdstuk 4**). Na differentiatie bleek dat vroege ei uitscheiding werd gedomineerd door soorten uit het genus *Cylicocyclus* (*Cyc.*). De soortensamenstelling voor behandeling was over het algemeen meer divers dan na behandeling, met ook soorten uit andere geslachten. Er werden daarnaast vooraf geen verschillen in soortensamenstelling gevonden tussen paarden met een verkorte ERP en paarden met een reguliere ERP. Geconcludeerd wordt dat vooral *Cylicocyclus* soorten verantwoordelijk zijn voor een verkorte ERP.

In Nederland heeft in 2008, als gevolg van een EU-richtlijn, een wijziging in de regelgeving plaats gevonden die van anthelmintica recept plichtige geneesmiddelen (Prescription Only Medicines, POM) maakte. Vragenlijsten werden bij de invoering van POM en 4 jaar later afgenomen (**hoofdstuk 5**). Het doel was om te onderzoeken of de wijzigingen in deze regelgeving, maar ook post-academisch onderwijs, de website www.parasietenwijzer.nl en bijvoorbeeld een paper over goede diergeneeskundige praktijk (Good Veterinary Practices) met betrekking tot wormen en ontwormingsmiddelen (Sloet van Oldruitenborgh - Oosterbaan et al., 2009) leiden tot veranderingen in het handelen van Nederlandse paardendierenartsen. De studie toonde aan dat in 2012 aanzienlijk meer feces monsters werden onderzocht dan in 2008, meer monsters werden onderzocht voor monitorings-doeleinden, meer monsters werden kwantitatief onderzocht en er werd voor de diagnostiek meer gebruik gemaakt van gepoolde

feces monsters. Het aantal dierenartsen dat routinematig feces monsters onderzocht alvorens te besluiten te behandelen (of niet) verdrievoudigde tussen 2008 en 2012. De uitkomsten toonden ook aan dat verdere acties gericht moeten worden op het verbeteren van het aantal kwantitatief onderzochte feces monsters en de interpretatie van feces diagnostiek.

In de 1e *in vitro* studie van dit proefschrift **(hoofdstuk 6**) werden strongylus type eieren uit 2 populaties paarden verkregen, uit een nooit behandelde populatie en uit regelmatig IVM behandelde pony's. De larven, gekweekt uit eieren, werden *in vitro* geselecteerd met behulp van een reiterative larval migration inhibiton assay (rLMIA, of herhaalde larven migratie remmingstest) en deze larven werden gedifferentieerd met de RLB. In de rLMIA in de aanwezigheid van IVM nam het percentage van de larven dat migreerde door 4 zeven met elke passage toe, waaruit blijkt dat de procedure selecteert voor IVM ongevoelige larven. Het vervolgens toevoegen van L-glutamaat aan de rLMIA procedure deed het eerder gevonden selectie-effect van IVM te niet. Dit suggereerde dat de glutamaat gereguleerde chloride kanalen (GluCls) een rol spelen in IVM gevoeligheid van de geselecteerde larven. In beide populaties waren de dominante soorten *Cyathostomum* (*Cya.*) *catinatum*, *Cylicocyclus* (*Cyc.*) *nassatus* en *Cylicostephanus* (*Cys.*) *longibursatus*. Na *in vitro* selectie met behulp van de rLMIA in aanwezigheid van IVM waren de dominante soorten *Cya. catinatum* in beide culturen, terwijl *Cyc. nassatus* verdween in de nooit behandelde maar niet in de regelmatig aan IVM blootgestelde populatie. Geconcludeerd word dat de rLMIA RLB nuttig is bij het bestuderen van *in vitro* IVM gevoeligheid van larven.

De 2e *in vitro* studie, met een rLMIA met 2 zeven, werd uitgevoerd (**hoofdstuk 7**) om de intra-specifieke variatie in IVM gevoeligheid en de variatie tussen 8 populaties met verschillende ontwormingsfrequenties te bestuderen. De 8 populaties bestonden uit 2 populaties die nooit waren behandeld en 6 populaties die frequent waren behandeld. In alle 8 populaties waren de veel voorkomende soorten *Cya. catinatum*, *Cyc. nassatus*, *Cys. longibursatus* en *Cya. pateratum*. Na *in vitro* selectie van ongevoelige larven met behulp van de rLMIA in aanwezigheid van IVM bleken de meest voorkomende soorten *Cya. catinatum* en *Cys. longibursatus*. De gecombineerde rLMIA RLB liet een hoger migratie percentage voor de 2e zeef in vergelijking met de 1e zeef zien, wat wees op selectie van larven met een lage gevoeligheid voor IVM. Het hoogste migratie percentage voor de 2e zeef werd gevonden bij *Cya. catinatum*. Daarnaast werd in de gebundelde groep van niet behandelde paarden ten opzichte van de gebundelde groep van frequent behandelde paarden een significant lager migratie percentage voor de 2e zeef voor *Cya. catinatum* waargenomen. Binnen een soort werd heterogeniteit met betrekking tot in vitro IVM gevoeligheid gevonden bij larven verkregen uit niet behandelde paarden. Geconcludeerd word dat de rLMIA in de aanwezigheid van IVM de gevoelige van de ongevoelige

Cya. catinatum L3s kunnen scheiden en dat hun heterogeniteit kan worden gerelateerd aan de ontwormingsfrequentie van de paarden.
In de discussie (**hoofdstuk 8**) werd de relatie tussen het gebruik van anthelmintica en resistentie ontwikkeling en de detectie van ML resistentie in cyathostominae besproken.

De drie belangrijkste conclusies van dit proefschrift zoals in **hoofdstuk 8** vermeld zijn:

1) Hoewel een hoge effectiviteit van IVM en MOX op 14 dagen na de behandeling kon worden aangetoond, werd er wel een verkorte ERP na ML behandelingen op een aanzienlijk deel van de onderzochte bedrijven vastgesteld. Meestal werden kort na een ML behandeling *Cylicocyclus* soorten gevonden. Op het bedrijf met de kortste ERP werden ook soorten van andere genera gevonden, wat aangeeft dat deze ook door ML behandelingen kunnen worden geselecteerd.

2) De *in vitro* ongevoeligheid van *Cya. catinatum* L3s voor IVM is hoger dan voor de ander cyathostominae L3s en lijkt gerelateerd te zijn aan de ontwormingsgeschiedenis van een bedrijf.

3) Na de introductie van de nieuwe wetgeving, die van anthelmintica uitsluitend op recept (POM of in het Nederlands URA) verkrijgbare geneesmiddelen maakte, werd een aantal belangrijke verbeteringen in het rationeel voorschrijven van ontwormingsmiddelen door dierenartsen gerealiseerd.

Dankwoord
Acknowledgements

Op de allereerste plaats wil ik mijn promotor, co-promotoren en begeleider heel hartelijk bedanken voor de geboden kans en voor hun inspanningen ten aanzien van mijn promotieonderzoek. Bedankt Jaap Wagenaar, Harm Ploeger, Frans Kooyman en Maarten Eysker. Maarten kwam in zijn welverdiende vrije tijd zelfs uit Oegstgeest naar het maandelijkse overleg in Utrecht.

Aan mijn collega's (en voormalige "hoekpunten") Jos van Putten, Herman Egberink, Maarten Eysker en Victor Rutten dank ik mijn fijne baan binnen het Departement Infectieziekten en Immunologie (I&I). Binnen de afdeling Klinische Infectiologie (KLIF-I&I) van Jaap Wagenaar zijn alle parasitologen ondergebracht en daar mocht ik ook mijn promotieonderzoek doen.

Mijn directe collega's van de afdeling KLIF wil ik bedanken voor hun interesse en ondersteuning. Hartelijk dank voor jullie hulp: Lea, Mirjam, Rolf en Stephanie. Bedankt ook Mirjam dat je mijn paranimf wilt zijn. Maar natuurlijk ook (op alfabetische volgorde van voornamen) dank aan Anky, Birgitta, Carolien, Coen, David, Dik, Els, Haitske, Joost, Linda, Lisa, Maarten, Marian, Marjolijn, Marloes, Masha, Nicole, Nicole, Niels, Sylvia, Wendy, Wim en al onze stagiaires alsmede onze "gast-kliffers" van LH.

Wim Hendrikx wil ik bedanken voor de introductie in de parasitologie en voor de prachtige ets die hij voor mij heeft gemaakt en die nu op de voorkant prijkt. Fijn dat Lotte en Emiel van Sitestorm deze vervolgens weer prachtig hebben vormgegeven.

Hartelijk dank ook aan mijn stagiaires (en inmiddels allemaal dierenarts) Eva, Ilja, Jasper, Manuela, Marieke, Marije, Muriël en Sepha voor hun werkzaamheden. Maar ook de stagiaires van de HAS Den Bosch en HAS Dronten en hun respectievelijke begeleiders wil ik bedanken voor hun werk.

Mijn leuke collega's bij GD Deventer, en dat zijn er velen, wil ik voor hun getoonde interesse bedanken. Met name ook bedankt aan de collega's van de afdeling R&D waar Theo Lam leiding aan geeft. Zo ook mijn EPI collega's en die van de diverse andere afdelingen. Omdat dit boekje toch over paarden en wormen gaat noem ik hier Emiel, Linda en oud collega Bram in het bijzonder. En omdat parasieten ook een onderdeel van hun werkzame leven is (of is geweest) noem ik nog speciaal Anika, Anouk, Barbara, Coen, Daphne, Edwin, Erik, Guillaume, Harold, Jet, Kees, Lammert, Marian, Menno, Remco, René en Thea. Bedankt voor de (parasitologische) samenwerking.

Oud collega's zoals Angela, Barend (in memoriam) Berit, Esther en Pawel wil ik hier ook bedanken. Maar ook Herman Cremers voor het delen van zijn morfologische parasitologische kennis. Dank aan Fred Borgsteede en Cor Gaasenbeek. Mijn dank gaat ook uit naar Frens Westenbrink, van hem heb ik over kwaliteitsborging in een laboratorium geleerd.

Hartelijk dank voor de voortdurende samenwerking aan Marianne Sloet van Oldruitenborgh Oosterbaan van het Departement Gezondheidszorg Paard. En ook aan de (toenmalige) medewerkers van de ambulante kliniek (Astrid, Floor en Jessica) voor hun medewerking.

Mijn dank gaat ook uit naar Thomas Geurden en medewerkers van Zoetis voor de goede samenwerking. Herman Kruijsen dank ik voor de gelegenheden mijn kennis over paardenparasieten, bijvoorbeeld tijdens de Klinische Avonden Paard, met dierenartsen te mogen delen. Mijn dank gaat ook uit naar David van Doorn als secretaris van de "6th EEHNC". Jan van de Veen dank ik hierbij voor de wormen.

Mijn mede bestuursleden bij de Nederlandse Vereniging voor Parasitologie (Jaap, Jaco, Joke en Rob) hartelijk dank voor het delen van jullie kennis en netwerken. Zo leer ik ook nog het een en ander over humane parasieten. De leden van de werkgroep veterinaire parasitologie van de NVP wil ik allemaal danken voor het in vertrouwen delen van allerhande parasitologische informatie. Ik noem daarbij speciaal nog even Frans van Knapen omdat hij recent met emeritaat is gegaan. Alle mede specialisten in opleiding veterinaire microbiologie bedankt voor jullie interessante lezingen en discussies.

Hartelijk dank aan de leden van de leescommissie (Theo Lam, Kurt Pfister, Marianne Sloet van Oldruitenborgh Oosterbaan, Arjan Stegeman, Lodewijk Tielens). Fijn dat ik van jullie mijn proefschrift mag verdedigen.
My special thanks to Kurt Pfister, from Ludwig-Maximilians-Universität Munchen, for our interesting discussions on equine parasitology.

Many thanks to Krystyna Cwiklinski, Jane Hodgkinson and Jacqui Matthews for our collaboration on cyathostomin species differentiation. Thanks also go to Anne Becher, Ray Kaplan and Martin Nielsen for the collaboration on the questionnaire surveys. Thanks go to Georg von Samson-Himmelstjerna for our collaboration in the past. Thank you Eva Osterman Lind for having us in Uppsala. Thanks, for sharing their knowledge, go to all the parasitologists from Ghent University. My thanks go to the people from Moredun Institute, who do research on parasites and who shared their knowledge. Thanks also to all the fantastic (equine) parasitologists that I encountered at conferences for sharing their knowledge. Some of them have such a fine sense of humor!

Mijn lieve (schoon) familie wil ik bedanken voor hun ondersteuning. Mijn ouders en schoonouders dank ik in het bijzonder, bedankt Robert en Tineke, Lieske en Herman. Fijn Chantal, lieve zus, dat je mijn paranimf wilt zijn. Aan mijn vrienden wil ik laten weten dat ze de allerleukste, slimste en liefste mensen zijn die ik ken. Hartelijk dank voor jullie gezelschap. Graag kampeer, borrel, Vrijmibo, oud en nieuw of kerst, kopjes thee of koffie ik binnenkort weer met jullie in Alphen ad Rijn, Amsterdam, Borne, Bunnik, Cairo, Delft, Den Haag, Den Hoorn, Deventer, Doetinchem, Elsloo, Enschede, Florianópolis, Gorssel, Gouda, Hengelo, London (see you Gareth), Ommen, Reeuwijk, Renkum, Rotterdam, Stein, Someren-Heide, Twello, Utrecht, Wageningen. En (oud) buren en familie jullie zijn natuurlijk ook mijn vrienden.

En aan het einde van dit dankwoord gaat mijn liefde en dank uit naar de twee leukste mannen, Jean-Pierre en Tei van Mulken. Wat zijn jullie toch geduldig geweest. Onze zoon Tei is alweer negen jaar en van hem heb ik de afgelopen jaren waarschijnlijk het meest geleerd. Op een goede tweede plaats dan toch het promotieonderzoek.

In het Twents zeggen we "*Aait vedan*".

D

Curriculum Vitea

Deborah van Doorn werd geboren op 4 april 1969 in Hengelo (O). In 1987 haalde ze haar VWO diploma aan het Twickel College in Hengelo.
Na eerst uitgeloot te zijn kon begonnen worden aan de studie Diergeneeskunde aan de Universiteit van Utrecht. Binnen de opleiding werd gekozen voor de differentiatie Landbouwhuisdieren met speciale interesse voor herkauwers en in 1998 werd de opleiding afgerond. Er werd vervolgens een kleine 2 jaar als dierenarts in diverse landbouwhuisdierenpraktijken gewerkt.
Van januari 2000 tot januari 2003 werkte Deborah als docent diergezondheid aan het Groenhorstcollege in Barneveld.

Als docent Veterinaire Parasitologie is ze vanaf januari 2003 werkzaam bij het departement Infectieziekten en Immunologie van de faculteit Diergeneeskunde. In september 2008 is ze binnen de afdeling Klinische Infectiologie begonnen aan haar promotieonderzoek naar anthelminticum resistentie ontwikkeling bij cyathostominae van paarden.

Vanaf oktober 2009 tot op heden is ze voor een halve dag per week gedetacheerd bij de afdeling R&D van GD Deventer. Daarnaast is Deborah sinds 2010 bestuurslid van de Nederlandse Vereniging van Parasitologie.