OPTIMIZATION OF A LARVAL MIGRATION INHIBITION ASSAY (LMIA) FOR DETERMINING *IN VITRO* SUSCEPTIBILITY OF EQUINE CYATHOSTOMINS TO MACROCYCLIC LACTONE ANTHELMINTICS, AND EVALUATION OF VARIATION IN SPECIES-SPECIFIC DRUG TOLERANCE LEVELS USING A 454 LARGE-SCALE PARALLEL PYROSEQUENCING SYSTEM

by

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(Under the Direction of Ray M. Kaplan)

ABSTRACT

Cyathostomins are the most important parasites of horses worldwide. The macrocyclic lactones (ML) constitute the most effective drug class available. However, increasing numbers of suspected ML-resistance cases currently exist. No validated bioassay for ML-resistance in cyathostomins is currently available and the presence of multiple species is a complicating factor. The objective of this study was to optimize and validate a larval migration inhibition assay (LMIA) protocol to detect and measure levels of ML resistance/susceptibility in cyathostomins and determine the impact of species composition. We developed and optimized a LMIA protocol and validated a 454 sequencing protocol for species identification based on ITS-2 sequences. The dose response curves generated using four different analogs in two different parasite populations showed good consistency. The 454 protocol exhibited high sensitivity but did not represent the species abundance accurately. This limitation did not allow a valid evaluation of the levels of innate tolerance among species.

INDEX WORDS: 454 sequencing, Anthelmintic resistance, Cyathostomins, *Cyathostominae*, ITS-2, Larval migration inhibition assay, Pyrosequencing, Macrocyclic lactones

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DEDICATION

Above all I dedicate this work to God, the reason behind everything, to my beloved wife Amalia, and my dear son Alvaro; the main inspirations of my life. I also dedicate this achievement to my parents, Carmen and Raul, without their guidance and support I would not have accomplished anything in my life.

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CHAPTER 1

LITERATURE REVIEW

Cyathostomin parasites

The Family *Strongylidae* (strongyles) includes the most common and pathogenic parasitic nematodes of horses. Two subfamilies are included: *Strongylinae* (large strongyles) and *Cyathostominae* (small strongyles) (Lichtenfels et al., 2008). Members of the *Cyathostominae* are also called cyathostomins or cyathostomes and the disease produced by them is called cyathostominosis (Lichtenfels et al., 2002). The adults of the *Cyathostominae* are usually (but not always) smaller than the adults of the *Strongylinae* (Gasser, 2004). *Cyathostominae* were formerly grouped in the family *Trichonematidae* and most of the species are classified in the genus *Trichonema* (Ogbourne, 1975).

Even though a morphology-based criteria of classification of these nematodes is currently the most accepted, molecular approaches designed for this purpose have failed to fully support the actual division of subfamilies and genera within the *Cyathostominae* based on morphological characteristics (Hung, 2000). According to a phylogenetic classification of the Phylum Nematoda based on small subunit ribosomal DNA (rDNA) analysis, strongyles are included in the clade number five, separated from other orders of important parasitic nematodes which are included in other clades (Dorris et al., 1999).

The subfamily *Cyathostominae* comprises 13 genera with 52 valid species. 10 species have been reported only in zebras or donkeys; some species are very rare, being sporadically reported in horses (Hodgkinson et al., 2001). These parasites were underestimated as pathogenic parasites for a long time, being largely eclipsed by the large strongyles. However, this has changed dramatically in recent years as cyathostomins have surpassed the large strongyles in importance (Pilo et al., 2012; Steinbach et al., 2006).

The terminology used to name genera and species in the *Cyathostominae* has been problematic. Consensus obtained through scientific meetings and the use of molecular biology studies have defined the most accepted taxonomic classification (Lichtenfels et al., 2002). The most updated list of cyathostomin species recognizes 50 valid species included in 14 genera as shown in Fig. 1.1 (Lichtenfels et al., 2008).

Cyathostomum:

Cyathostomum tetracanthum
Cy. alveatum
Cy. catinatum
Cy. montgomeryi
Cy. pateratum

Cylicostephanus:

Cylicostephanus calicatus
Cs. asymetricus
Cs. bidentatus
Cs. goldi
Cs. hybridus
Cs. longibursatus
Cs. minutus

Coronocyclus:

Coronocyclus coronatus
Co. labiatus
Co. labratus
Co. sagittatus
Co. ulambajari

Cylicocyclus:

Cylicocyclus brevicapsulatus
Cc. nassatus
Cc. asini
Cc. ashworthi
Cc. triramosus
Cc. auriculatus
Cc. ultrajectinus
Cc. insigne
Cc. gyalocephaloides
Cc. elongatus
Cc. adersi
Cc. leptostomum
Cc. radiatus

Skrjabinodentus:

Skrjabinodentus longiconus
S. tshoijoi
S. caragandicus

Parapoteriostomum:

Parapoteriostomum mettami
P. euproctus
P. schuermanni
P. mongolica

Cylicodontophorus:

Cylicodontophorus bicoronatus
C. reineckei

Cylindropharynx:

Cylindropharynx longicauda
C. brevicauda
C. intermedia

Petrovinema:

Petrovinema poculatum
P. skrjabini

Poteriostomum:

Poteriostomum imparidentatum
P. ratzii

Tridentoinfundibulum:

Tridentoinfundibulum gobi

Gyalocephalus:

Gyalocephalus capitatus

Hsiungia:

Hsiungia pekingensis

Caballonema:

Caballonema longicapsulatum

Fig. 1.1: Taxonomic classification of cyathostomins. Based on Lichtenfels et al., 2008

Life cycle

Cyathostomin parasites have a direct life cycle. Adult worms live in the large intestine and produce eggs that are passed in the feces of the host into the environment. Specific details of individual species life cycle have not been determined, and only a generalized life cycle model has been established (Hodgkinson, 2006).

The egg development in the environment is optimal in the range of 25° to 33°C, with an upper limit of 38°C and a lower limit of about 7.5°C (Nielsen et al., 2007). An embryo within the egg develops to the first stage larva (L_1) which then hatches. In the environment, the L_1 moults twice and reach the infective third stage (L_3) (Lyons et al., 1999). Although egg hatching does not occur below a threshold of 7.5 to 10°C, egg embryonation can take place even at lower temperatures, with a lowest limit of 4°C (Nielsen et al., 2007). Only the first and second stage larvae are feeding stages. The L_3 is surrounded by a double-layered cuticle that keeps it from feeding but at the same time protects it from environmental conditions, enabling it to survive extremes conditions (Nielsen et al., 2007). Third stage larvae develop mostly in pastures and in peak seasons during the year as many as 200 000 L_3 per kg of grass may be present (Uhlinger, 1993).

The infection of horses takes place through the ingestion of infective L_3 on the grass. After ingestion, the L_3 exsheaths in the intestinal lumen and actively penetrates the mucosa of the caecum and/or colon. Normally, the larvae encyst in mucosal tissues where they continue their development to the fourth stage (L_4). Four stage larvae then emerge from the submucosa into the intestinal lumen, where they moult into adults and complete their life cycle (Brianti et al., 2009).

The different developmental stages that take place in the mucosal tissues are usually denominated: early third stage larva (EL_3), which can enter hypobiosis inside the intestinal mucosa depending upon various environmental and biological factors; the late developing third stage (LL_3), which has a characteristic tubular buccal capsule; and the developing fourth stage (L_4), that has a distinguishable cup-shaped buccal capsule (Chapman et al., 1999). The development of the late L_3 to L_4 occurs within the

fibroblastic cyst that forms when the larva enters the mucosa. In a non-hypobiotic cycle this process can take 6 to 12 days. The L_4 usually stays encysted for 30 to 60 days before emerging into the intestinal lumen. This period can be much longer, up to 2 years, in the case of developmental arrest (Collobert-Laugier et al., 2002a).

One of the main reasons for the lack of information on species-specific life cycle features is the serious difficulty involved in the establishment of successful single-species infection models. *In vitro* culture systems to produce fourth stage larvae have shown limited success. Some genera of cyathostomins, such as *Cylicostephanus*, have shown the greatest tendency to develop to fourth stage *in vitro*, whilst other genera show a minor tendency. Some species, such as *Cyathostomum catinatum*, have shown no evidence of development under *in vitro* culture conditions (Brianti et al., 2009). Evidence generated through studies on recovery and identification of adult specimens has suggested that some cyathostomin species such as *Cylicocyclus nassatus* have a short life cycle of about 2 months, while species such as *Cy. catinatum*, *Cylicostephanus longibursatus* and *Cylicostephanus goldi* have longer life cycles of about 3 to 4 months, producing a single generation per year (Ogbourne, 1975).

Perhaps one of the most remarkable characteristics of the life cycle is the existence of an alternative developmental cycle, involving the encystment of L_3 and their developmental arrest for long periods until specific stimulation; whereupon larvae re-start their life cycle and moult to L_4. The encysted stages have been identified as early L_3, developing L_3 and late L_3 and they have been morphologically described in detail (Brianti et al., 2009). Arrested hypobiotic larvae can halt their development for up to 2 years (Dargatz et al., 2000). The most common feature of this inhibited mucosal life cycle is the trickle emergence of encysted larvae to the lumen. However, sometimes because of unknown factors, massive numbers of larvae can be released in a short period of time, producing severe tissue damage and clinical disease, a syndrome known as larval cyathostominosis (Lyons et al., 1999). This syndrome has important implications in equine health and it will be discussed in more detail in the pathogenesis section of this review.

The phenomenon of inhibited larval development is a common feature of the life cycle of several other parasitic nematodes and even though it is well known to occur in cyathostomins, the specific factors that trigger it are still unknown (Davidson et al., 2005). Based on the seasonal pattern observed, it seems to be an adaptation to survive periods of environmental adversity (Ogbourne, 1975) in a similar way that has been reported for ruminant nematodes. Observations made in Europe suggest that, indeed, cooler environmental conditions may be a trigger for mucosal arrestment. A large proportion of L_3 ingested at the end of the summer and in the fall enter hypobiosis and then emerge to start their luminal life cycle from winter to the next summer (Eysker et al., 1990). The natural trend to enter hypobiosis in *Cyathostominae* larvae has been confirmed experimentally by *in vitro* exposure to low temperatures, as in other parasitic nematodes (Brianti et al., 2009). However, scientific evidence failed to fully support cold temperatures as the major trigger for hypobiosis in cyathostomins (Davidson et al., 2005). Certain associations between larval hypobiosis and particular species has been suggested but there is still little scientific evidence to support this hypothesis (Ogbourne, 1975). Some experiments have shown that the acquired immune response of the host against parasite infection could promote hypobiosis (Davidson et al., 2005) but this has not been totally confirmed. In conclusion, the major factors underlying the development of hypobiosis in cyathostomins are still poorly understood and the lack of single species *in vitro* models makes it difficult to explore this field.

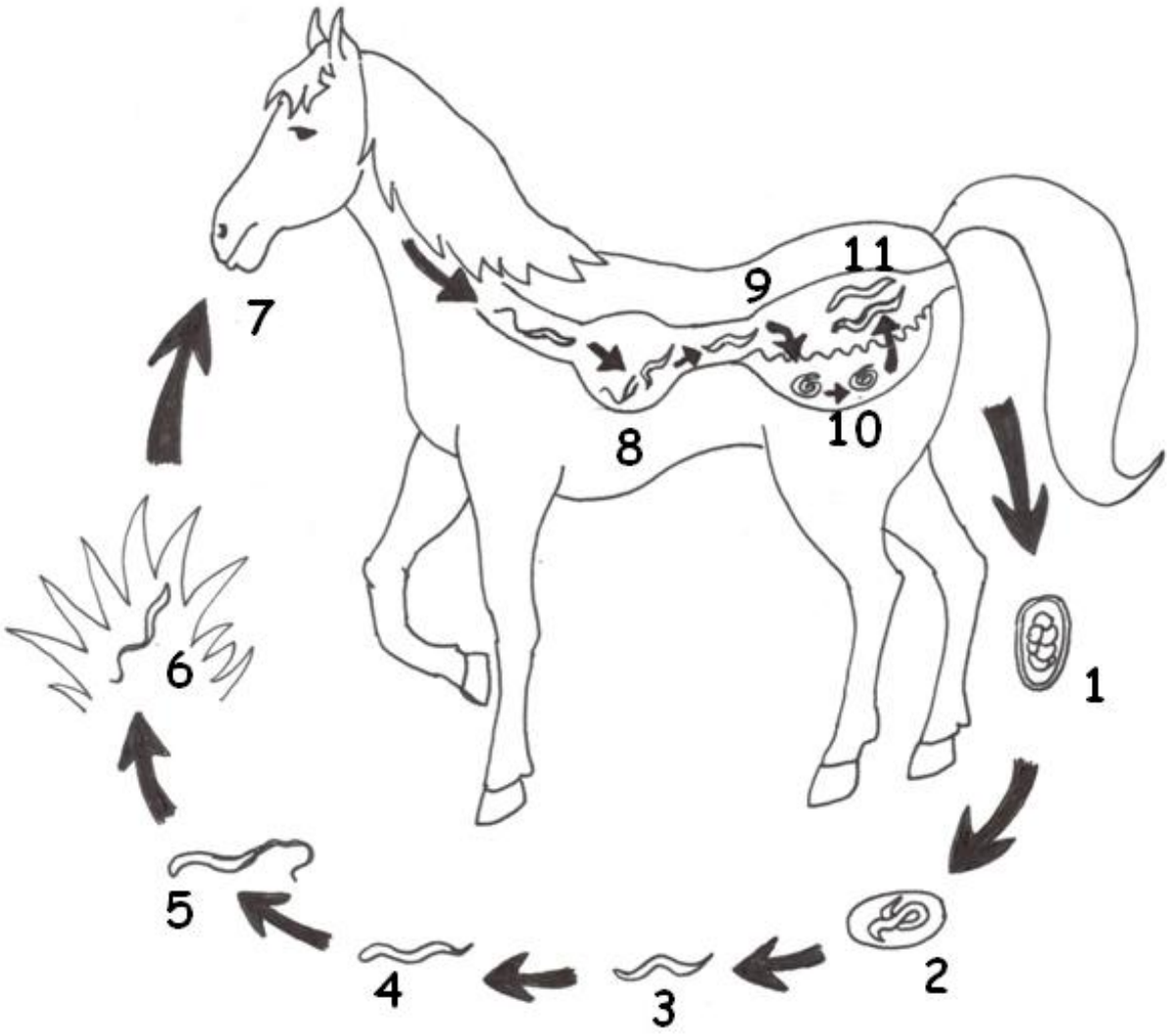


Fig. 1.2: Life cycle of cyathostomins. **1**, eggs are shed in feces. **2**, in good conditions a first stage larva (L_1) develops inside the egg. **3**, L_1 hatches and live in the environment feeding on bacteria. **4**, L_1 moults to second stage larva (L_2). **5**, L_2 moults to third stage larvae (L_3) which keeps the L_2 sheath and is the infective stage. **6**, L_3 survive long periods in the environment and stay at the top of pastures. **7**, L_3 is ingested by the horse with pasture. **8**, L_3 exsheaths in the small intestine. **9**, exsheathed L_3 penetrates the large intestine mucosa to continue its development. **10**, L_3 develops to fourth stage larva (L_4) within the intestinal mucosa. **11**, L_4 leaves the mucosa and enter the intestinal lumen where they develop to adults, males and females helminths mate, and females lay eggs which are passed in the feces.

Prevalence

Cyathostomins are the most common parasites in horses (Kornas et al., 2010). Prevalence of cyathostomin infection in horses can be as high as 100%, as confirmed by several studies worldwide (Abbott et al., 2007; Güiris et al., 2010; Kornaś, 2011; Kyvsgaard et al., 2011; Lyons et al., 1999; Reinemeyer et al., 1984). Cyathostomins constitute the most abundant group of parasitic nematode in the large intestine of horses and occur in very large numbers, frequently of hundreds of thousands and even millions (da Silva Anjos and de Lurdes, 2006; Kyvsgaard et al., 2011). For example it is estimated that 85 to 100 % of the total helminth intestinal fauna in horses correspond to cyathostomins (Uhlinger, 1993) and more than 95% of L_3 obtained from coproculture in horses belong to the *Cyathostominae* species (Reinemeyer et al., 1984).

Cyathostomins not only infect horses, there are also widespread in other equids such as working donkeys, with prevalences close to 100% being reported (Getachew et al., 2010). Wild equids, such as zebras, are also susceptible hosts for cyathostomin infections. There are published studies on naturally acquired cyathostomin infections in captive zebras (Lia et al., 2010).

Environment

Even though it has been suggested that other factors, such as different varieties of grass, may enhance the survival and migration of cyathostomin L_3 (Quinelato et al., 2008), climatic conditions have by far the greatest impact on the development and survival of the free-living environmental stages and consequently the worm burdens (dos Santos et al., 2011; Nielsen et al., 2007). The seasonal dynamics of infection have been described. Cool summers and cold winters favor high rates of larvae survival and warm summers are highly unfavorable to L_3 survival (Kornas et al., 2010; Ogbourne, 1975). However, worm burdens may be affected by management factors, such as the time kept in stables vs. the time on pasture and anthelmintic treatments, rather than weather factors (Kornas et al., 2010).

Studies have confirmed the existence of a seasonal variation in the egg development to infective larva on pasture in response to rainfall and temperature. Low temperatures cause little larval development while frequent rainfall and higher temperatures increase development dramatically (Ramsey et al., 2004). Feces are the most important reservoir for infective larvae. Third stage larvae migrate to the grass mainly in response to rainfall since moisture is a major factor for stimulation of larval migration (Couto, 2011; dos Santos et al., 2011). The importance of this phenomenon is confirmed by the fact that larvae tend to remain in the feces during dry periods (dos Santos et al., 2011). Environmental conditions favoring larval development may not favor larval survival. In climates where the larval development is accelerated, larval survival is limited, while on the other hand, dry seasons favor longer larval survival (Quinelato et al., 2008). In subtropical and tropical climates the infection with *Cyathostominae* infective larvae occurs throughout the year but is especially high during autumn and winter (Couto, 2011) and it is related to the permanent occurrence of infective larvae on horse pastures (Quinelato et al., 2008). In tropical areas summer is the critical season for larval survival, with reduced numbers of infective larvae in pastures. In colder climates, with long periods of snow cover, a greater proportion of larvae may survive on pasture (Nielsen et al., 2007).

Host

The equine host plays an important role in the development of naturally acquired cyathostome infections, especially regarding parasite burden and parasite development. Age is the most important variable in this phenomenon (Love and Duncan, 1992). Scientific evidence supports a relationship between age and cyathostomin egg shedding with younger animals shedding more eggs than older ones (Becher et al., 2010). Another factor that may be influenced by age is the species diversity. In a surveillance study on the prevalence of cyathostomin species on a horse farm, yearling animals carried more species of cyathostomins than foals. The reason behind this finding could be the greater time of exposure to infection in the older animals than the young horses (Ionita et al., 2010).

The immune response of the horse in cyathostomin infections is a major component of the host reaction and influences the outcome of disease. Exposure to cyathostomin infection induces an adaptive immune response against subsequent challenges and can lead to a self-cure phenomenon which is not species specific (Monahan et al., 1997). Acquisition of resistance occurs over time as evidenced by the fact that older animals show a significant reduction of the parasite burdens when compared to young animals, although this response is not effective against incoming infective larvae (Monahan et al., 1998). The development of protective immunity under pasturing conditions is a relatively slow process that depends mainly on the level of infection (Chapman et al., 2002). However, despite the correlation between increase in age and decrease in levels of infection, and the confirmed development of immunity, a lifelong susceptibility to cyathostomin infections has been recognized (Kornas et al., 2010; Lind et al., 1999).

Even though no significant influence of sex or breed on the cyathostomin infection prevalence has been reported by some authors (Collobert-Laugier et al., 2002a; Mughini Gras et al., 2011), other studies have shown a higher level of infection in geldings (Kornas et al., 2010). The real influence of sex in parasite burden is still unclear.

Species distribution

Even though the number of recognized cyathostomin species is more than 50, commonly as few as 4 to 5 species tend to dominate the parasite population (Gawor, 1995; Lyons et al., 1999; Ogbourne, 1975) and the prevalence of these major species has not dramatically changed over time, not even with the introduction of the modern anthelmintic drugs. Studies worldwide have mostly agreed regarding the 10 most prevalent species: *Cs. longibursatus, Cy. catinatum, Cc. nassatus, Cs. goldi, Cylicostephanus (Cylicocyclus) calicatus, Coronocyclus (Cyathostomum) coronatum, Cylicostephanus minutus, Cylicocyclus insigne, Cylicocyclus leptostomum* and *Cyathostomum pateratum.* In most of the cases the first five species comprise more than 80% of the total adult count (Cutolo, 2011; Eysker et al., 1990;

Hodgkinson et al., 2008; Kuzmina and Kharchenko, 2008; Lind et al., 2003; Ogbourne, 1975; Ogbourne, 1976; Reinemeyer et al., 1984; Stancampiano et al., 2010). Other reports also include *Coronocyclus labiatum, Cylicocyclus ultrajectinus, Poteriostomum imparidentatum* and *Poteriostomum ratzii* in the list of the ten most common species (Collobert-Laugier et al., 2002a; Collobert-Laugier et al., 2002b; Gawor, 1995; Kinsella et al., 2002; Kornaś, 2011; Kuzmina and Kharchenko, 2008; Mughini Gras et al., 2011; Traversa et al., 2010).

Several publications have explored the species distribution in cyathostomin infections in detail and they have produced valuable information concerning the dynamics of naturally acquired infections in horses. One of the earliest studies on this topic in the US was done by Reinemeyer et al. (1984). They carried out a necropsy-based study during a year to recover and identify cyathostomin species in the US. They found 21 species, with a species distribution per horse that ranged from 2 to 11 species. The total burden of adult worms ranged from 680 to 663,100. The colon contained 94.2% of the total lumen population of parasites. The 5 most prevalent species were *Cy. catinatum, Cc. nassatus, Cs. longibursatus, Co. coronatum* and *Cs. goldi*, comprising 84% of the total cyathostomin population. Together with 5 other species, *Cs. calicatus, Cs. minutus, Cc. leptostomum, Cc. insigne* and *Cy. pateratum*, they accounted for the 98.9% of the total burden recovered. The other 11 species found represented only 1.15% of the total population. In a similar study done in Europe, almost 20 years later, six of these same species, *Cc. nassatus, Cy. catinatum, Cs. longibursatus, Cc. lepostomum, Cs. minutus* and *Cs. calicatus* comprised more than 90% of the total burden in naturally infected horses and the range of species diversity was from 6 to 13 species (Lind et al., 2003). In Brazil, more recently, Anjos and Rodriguez (2006) found that four of the same species were highly prevalent: *Cc. nassatus, Cs. minutus, Cs. calicatus* and *Cs. goldi*. With the inclusion of *Cyathostomum tetracantum*, these were the five most prevalent species. Together with *Cs. longibursatus* and *Cc. leptostomum*, these species comprised almost 90% of the cyathostomin population. In contrast with this study, other large scale work on cyathostomin prevalence in a different region of Brazil did not find any specimens of *Cy. tetracantum*, even though the other species were found to be dominant (Silva et al., 1999).

This comparison of studies provides evidence for the concept that, despite a few exceptions and a certain level of variability, the same major species have been described in the different climatic regions of the world and that this scenario has not changed dramatically over the years. The introduction of the broad spectrum modern anthelmintic drugs has not significantly altered this scenario. A study aimed to molecularly identify the cyathostomin species composition on a commercial horse farm in central Kentucky (US) found a very similar distribution of cyathostomin species before and after drug treatment (ivermectin), with the same major species dominating the parasite population (*Cc. nassatus, Cs. longibursatus, Cs. minutus, Cc. insigne* and *Cs. calicatus*) (Ionita et al., 2010).

In general, a great variability in adult cyathostomin burdens in individual horses has been reported. The caecum, when compared with the colon, is a minor source of adult *Cyathostominae* populations and most of the worms are located in the ventral colon (Collobert-Laugier et al., 2002b; Reinemeyer et al., 1984; Stancampiano et al., 2010). Even though cyathostomin species show a specific preference of location in the large intestine (ventral, dorsal colon and caecum), one species can have a high prevalence in more than one compartment which could suggest a greater adaptive capacity of certain species and/or competition with other species in a single compartment (da Silva Anjos and de Lurdes, 2006). A certain level of specificity in anatomic location in the large intestine has also been reported for encysted larval stages (Reinemeyer and Herd, 1986; Reinemeyer and Powell, 1986).

Even though there are no species found only in donkeys (Lichtenfels et al., 2008), some species can be more frequently detected in donkeys than in horses, such as *Cyathostomum montgomeryi*. However, in general, the same major species described in horses dominate the adult worm population in donkeys (Getachew et al., 2010). The case is somewhat different in zebras where there are 6 species reported to infect exclusively these wild equids although two of the common species found in horses have been also identified in captive zebras (Lia et al., 2010).

Pathogenesis

Cyathostomins are the most important parasitic pathogens of horses (Love and Duncan, 1992; Love et al., 1999). Adult helminths feed on the intestinal mucosa and they are normally considered to produce a low to moderate pathogenicity (Love et al., 1999). The cardinal pathological feature of cyathostominosis is an inflammatory enteropathy caused by the penetration and/or emergence of larval stages from the intestinal tissues; this may lead to a protein-losing enteropathy and alteration of intestinal motility. It has been suggested that the age of the horse, previous infections and the species composition may be major factors that determine the pathogenicity of a cyathostomin infection (Love and Duncan, 1992; Love et al., 1999). Experimental and naturally acquired infections with cyathostomins can produce several detectable clinical signs, the most important being a reduction in weight gain, and the alteration of peripheral blood values, with hypoalbuminemia and hyperglobulinemia (Love and Duncan, 1992; Murphy and Love, 1997). However, because of inherent difficulties in working with individual cyathostomin species, there are no reports of species-specific pathogenicity (von Samson-Himmelstjerna, 2012).

Larval cyathostominosis, or so called winter cyathostominosis, can be a severe clinical syndrome, with up to 50% mortality (Dargatz et al., 2000), and it is associated with the sudden development of vast numbers of encysted cyathostomin larvae, often ranging from thousands to more than a million, in the mucosa of the large intestine and caecum (Peregrine, 2006). Since most of the anthelmintic drugs do not affect encysted mucosal stages, the lack of specific treatment can lead to dangerous increase in the number of mucosal larvae and this, in turn, may increase the risk for the development of larval cyathostominosis (Dowdall et al., 2004). Clinical signs of larval cyathostominosis include diarrhea, acute weight loss and colic. Gross lesions at necropsy may consist of colonic mucosal congestion, nodules, colonic erosion and ulcers and colonic edema. Histopathological lesions in the intestinal mucosa may comprise cellular infiltrates with different combinations of lymphocytes, eosinophils, plasma cells and macrophages (Peregrine, 2006). Fever, depression and hypoalbuminemia are also common features (Dowdall et al., 2004).

The specific molecular mechanism that governs the synchronous emergence of large numbers of immature stages in cases of larval cyathostominosis is still unknown (Matthews et al., 2008). A possible role for proteins containing LIM domains has been suggested. This domain is a novel class with a cysteine-rich zinc-finger motif that has been associated with regulation of gene transcription and physiological synchronization (Matthews et al., 2008). The existence of cysteine-class proteinases in the excretory-secretory products (ESP) of cyathostomins and their role in the pathogenesis of larval cyathostominosis has been described (Kinsella et al., 2002).

Clinical diagnosis of this syndrome may be complicated because of some non-specific clinical signs, such as fever and colic (Peregrine, 2006). The monitoring of the serum levels of lactate dehydrogenase (LDH) has been suggested as a clinical test for measuring mucosal damage caused by larval cyathostominosis (Francisco et al., 2011). Conventional coprological methods can give an estimate of the adult cyathostomin burden but do not provide any indication of the larval mucosal burden, which can constitute up to 90% of the total parasite population, with several hundreds of thousands of encysted larvae (Dowdall et al., 2002).

The search for a specific molecular marker for larval cyathostominosis diagnosis is one of the challenges faced by ongoing research on this area. The identification of Cy-GALA-1 protein as a potential candidate for the diagnosis of cyathostomin larval mucosal burdens has been documented. This novel protein has been shown to be specific for mucosal stages only; it is detected by specific antibodies produced in cases of heavy mucosal larval burdens with no cross reactivity with other equine helminths. Anti-Cy-GALA-1 antibodies are present in cases of larval cyathostominosis and this protein has been detected in major cyathostomin species from different geographical origins such as the UK and US (McWilliam et al., 2010).

Diagnosis

As in the case of parasitic nematodes in ruminants and other domestic species, fecal egg count (FEC) techniques are the test of choice for *in vivo* diagnosis of strongyles in general (reviewed by Nielsen et al, 2010 b). These tests have been used for clinical diagnosis and for estimation of parasite burden levels for quite a long time. In general, a value between 200 and 500 eggs per gram (epg) has been used as a cut off for anthelmintic treatment in strongyle infections and FEC of over 1000 epg has been suggested as indicative of high rates of infection (Uhlinger, 1993).

Despite their wide use, FEC are generally considered a poor predictor of parasite infection level because of the poor correlation between test results and the actual number of worms in the host (Lyons et al., 2001; Uhlinger, 1993). More recently, a good qualitative capacity of FEC but with a limited value for the actual egg count numbers have been recognized. An optimal cut off of 500 epg in young horses for estimating a critical parasite burden has been reported, but, in general, a non linear correlation between FEC and number of worms has also been confirmed (Nielsen et al., 2010a). Still further research in this area is needed before definitive conclusions on the real value of FEC to estimate a cyathostomin burden can be drawn (Nielsen, 2012). Moreover, some factors can influence the accuracy of a test, such as storage conditions of the fecal samples and environmental conditions at sampling, which have been shown to be crucial in determining the outcome of a fecal analysis for strongyle eggs (Nielsen et al., 2010c).

The modified McMaster technique is the most commonly used FEC procedure. There are several protocols published but, in general, the basis for this technique lies in the suspension of a weighed amount of feces in a pre-determined volume of a saturated flotation solution. The fecal suspension is mixed and strained, and then a small aliquot of the total volume is transferred to a specially designed slide. The number of eggs is counted and mathematically corrected for weight and dilution. Depending on the protocol used the minimum level of detection is between 1 and 200 epg. Like the other FEC protocols, this technique can show low reliability and lack of repeatability. A negative value rarely indicates a worm

free horse, even though repeated negative results may indicate a very low parasite burden (Uhlinger, 1993).

The identification of cyathostomin species is another complicated area. Identification of species or genus based on eggs is impossible (Lichtenfels et al., 2002). The identification of cultured L_3 for the same purpose is not satisfying (Kornas et al., 2009; Reinemeyer et al., 1984) and it only identifies *Strongylus* spp. and differentiates them from cyathostomins (Hodgkinson, 2006). A novel system based on morphological measurements and the arrangement of the intestinal cells to classify cyathostomin L_3 into 4 types has been reported. However, this system is cumbersome, impractical and its routine application for identification of cyathostomins to the species level is questionable (Kornas et al., 2009).

The identification of adult worms is the gold standard for determination of species but it is laborious, since it can be done only by comparative morphology. Another drawback for this approach is that it is done mostly at necropsy, or sometimes with specimens expelled from live animals after treatment (Kornas et al., 2009; Lichtenfels et al., 2002), which makes it impractical for most epidemiological field studies. It is based on a thorough morphological examination of the anterior end structures, such as buccal capsule, mouth collar, external leaf-crowns, internal leaf-crowns and esophageal funnel (Kuzmina et al., 2011). The use of morphological features of the fourth stage larvae is still considered important for taxonomic studies, mainly because this developmental stage has specific and useful simple-siomorphic characteristics and key anatomical structures which are simpler than those of the adults. However, the difficulty in getting suitable specimens for identification and the lack of information on morphology make this enterprise too time-consuming and complicated to be used routinely for most species (Kharchenko and Kuzmina, 2010). Moreover, since the alternative molecular methods commonly used in research have only been elaborated for detecting the most prevalent and common species, classical morphological identification methods on adult specimens obtained at necropsy are still needed (Kuzmina et al., 2011).

Because of the importance of larval cyathostominosis and its diagnosis, methods to assess the burden of encysted mucosal stages at necropsy have been developed. Two basic techniques have been developed

to enumerate encysted mucosal stages of cyathostomin larvae: The transmural illumination (TMI) method and the digestion of the mucosa with pepsin (DIG) or so called mucosal digestion method. The TMI can only detect larger developing larvae (LL_3 and L_4), so in order to detect and quantify EL_3 and developing L_3, DIG should be used (Chapman et al., 1999). Mucosal digestion is the technique most recommended for the quantification of mucosal stages in drug efficacy trials against encysted stages (Reinemeyer, 2011, personal communication). A detailed description of the TMI and DIG protocols has been provided by Chapman et al. (1999) and by Monahan et al. (1996). Dowdall et al. (2004) have studied the humoral immune response of horses presenting clinical cases of larval cyathostominosis and they have identified and purified two antigen complexes with promising application as immunodiagnostics. The use of serum protein electrophoresis to measure changes in serum proteins levels has proved not to be a reliable tool for assessing cyathostomin burden and anthelmintic treatment efficacy (Abbott et al., 2007).

Molecular methods for diagnosis: DNA based approaches

Among the molecular approaches currently available, DNA based assays constitute the most suitable for the identification of parasites and for genetic studies of parasites in general (McManus, 1996). Developing semi-quantitative DNA based assays to assess the approximate number of cyathostomin larvae, eggs or adult worms is a feasible option and they would be useful tools for research and clinical diagnosis (Gasser, 2004; Hodgkinson, 2006).

Among the possible genomic targets for this purpose, the 26s – 18s rDNA intergenic spacer (IGS) has been used as a genetic marker for 16 major cyathostomin species, with promising results (Kaye, 1998). Four oligoprobes designed by alignment of reported IGS sequences have been used to identify 4 cyathostomin species with optimal results; a fifth oligoprobe was used to identify all the 16 species tested, including the most common species (Hodgkinson et al., 2001). Other species-specific oligonucleotide probes based on IGS alignment have been used in a reverse line blot (RLB) hybridization assay for molecular identification of 13 major cyathostomin species reported worldwide: *Cy. catinatum, Cc.*

nassatus, Cc. insigne, Cylicocyclus ashworthi, Cs. calicatus, Cs. minutus, Cs. goldi, Cs. longibursatus, Coronocyclus labratus, Co. labiatus, Co. coronatum, Cy. pateratum, Cc. leptostomum, and *Strongylus* spp. This molecular test can simultaneously diagnose the presence of the most common species in a sample (Traversa, 2007). Recent application of this assay has confirmed its potential as a diagnostic tool for characterization to the species level of field cyathostomin populations (Ionita et al., 2010). The application of the RLB for species identification during *in vitro* bioassays has proved the validity of the molecular techniques to assist in the correct interpretation of results by assessing the species composition of the larvae pools used in these assays (van Doorn et al., 2010).

The use of another rDNA targets, the first and second internal transcribed spacer (ITS-1 and ITS-2) regions has shown to be a reliable options for the molecular diagnosis of equine strongyles, mainly because of their abundance and reduced sequence variation within species (Campbell et al., 1995; Hodgkinson, 2006). The three major species of the genus *Strongylus* can be differentiated based on ITS-2 sequences and a PCR linked restriction fraction length polymorphism (RFLP) can be used to identify them even at the single egg or larva level (Campbell et al., 1995). Using published ITS-2 sequences, Nielsen et al. (2008) have developed a qPCR assay specific for *Strongylus vulgaris*. This molecular test is capable of detecting semi-quantitatively DNA extracted from *S. vulgaris* eggs present in fecal samples. This work confirmed the usefulness of the ITS-2 as a genetic marker for the diagnosis of equine strongyles.

The ITS region sequences have great potential as genetic markers for species identification of cyathostomin species and they can provide data for determining the taxonomic status of several species within the sub-family *Cyathostominae* as well as their evolutionary relationships (Chilton, 1997; Gasser, 2004; Hung, 1997, 2000). Based on the magnitude of interspecies differences observed in the ITS-1 region (0.6 – 23.7%) and even higher variation in the ITS-2 region (1.3 – 56.3%) found during the analysis of rDNA sequences from 22 cyathostomin species and 6 *Strongylinae* species, it was concluded that they are suitable as species-specific markers (Gasser, 2004). Moreover, specific PCR primers have

been designed for specific amplification of *S. vulgaris, Cy. catinatum, Cc. nassatus, Cs. longibursatus* and *Cs. goldi* ITS-1 and ITS-2 regions for species detection (Hung et al., 1999). The ITS-1 and ITS-2 sequences analysis have also identified the existence of cryptic species within *C. minutus* (Hung, 1999a).

Pioneering work done almost 20 years ago, targeting the ITS-2 region proved the validity of the DNA sequencing approach as a highly sensitive diagnostic tool in parasitic nematodes (Gasser, 1993). Considering that the development of molecular tests to identify not only cyathostomins at species level but also other strongyles is a major area of current research, the use of DNA sequencing approaches is a potential field to explore for this purpose. The next generation high throughput DNA sequencing assays may constitute a tool for this purpose (Nielsen, 2012).

Introduction to the next generation sequencing: 454 pyrosequencing

Next-generation sequencing (second and third generation) technologies have been developed as a more convenient alternative to the traditional Sanger capillary DNA sequencing method (Mardis, 2008). The second generation methods required the amplification of the template and the detection of newly incorporated bases by different methods for sequencing (Travis, 2011). One of these methods of detection is the pyrosequencing assay.

Developed in late 1980's and first described as the enzymatic luminometric inorganic pyrophosphate detection assay (ELIDA), the pyrosequencing protocol was greatly improved in the mid 1990's, constituting the first real time method by synthesis used for DNA sequencing (Ronaghi et al., 1996). The pyrosequencing method uses the pyrophosphate molecule (PPi) released during the enzymatic synthesis of complementary DNA to synthesize ATP, which, in turn, fuels the luciferase-mediated conversion of luciferin into visible light. In this way light is produced every time a nucleotide is incorporated and detected in real time (Mardis, 2008). Because of its high accuracy, flexibility, ease-of-use and practicality in a relative short period of time, pyrosequencing was widely adopted as a popular platform to perform single nucleotide polymorphism (SNP) analysis and other genotyping studies. This, in turn, led to the

development of commercially available multi-channel versions for multiple SNP (Fakhrai-Rad et al., 2002). One of these automated systems, the PSQ^{TM} 96 well (Biotage AB, Uppsala, Sweden), has been shown to be suitable for the analysis of genetic variations in large scale studies in human populations (Nordfors et al., 2002). This same instrument has been successfully used for the detection of SNP in the codon 200 of the β-tubulin gene, the molecular basis of resistance to BZ drugs, with a higher sensitivity than the standard *in vitro* assays therefore proving the validity of this protocol (von Samson-Himmelstjerna et al., 2009). The same platform has been applied to the study of the role of these same SNP and other genetic polymorphisms in cases of BZ resistance in horse cyathostomins, and to the further characterization of this codon in individuals from the major species. These studies have confirmed the optimal performance of this approach for this type of genetic analysis in cyathostomins (Hodgkinson et al., 2008; Lake et al., 2009).

In 2005, Margulies et al., working for the 454 Life Sciences Company, reported the incorporation of the multi-channel pyrosequencing principle in a massive high throughput sequencing platform. This was the official birth of the 454 technology (Margulies et al., 2005). In the 454 sequencing protocol, large genomic DNA samples are fractioned into small 300-800 bp fragments by mechanical nebulization, or smaller DNA samples, such as PCR amplicons, are directly attached to short adaptor sequences. Then single stranded DNA (ssDNA) fragments with linked adaptors are included in a sample library. The next step is called emulsion PCR (emPCR), where the ssDNA are bound to sepharose beads that carry an oligonucleotide, complementary for the adaptor sequence. The beads carrying the ssDNA samples are emulsified in a mixture, in which each bead is "wrapped" into a PCR micro-reactor micelle. The PCR reaction that takes place afterwards produces an enormous clonal amplification with a final product of about 10 million identical DNA copies per bead. Later the beads are combined with reagents such as DNA polymerase, sulfurylase and luciferase and are layered onto a specially designed microtitre plate. The plate contains 1.6 million wells, with a diameter of 44 μm, allowing only one bead (about 30 μm) per well. The plate is taken to the genome sequencer FLX (GS FLX) machine where the pyrosequencing

process takes place and hundreds of thousands of beads each carrying millions of a DNA sample are sequenced in parallel (Droege and Hill, 2008).

In general, the GS FLX 454 technology offers high throughput sequencing with single read accuracies equivalent to or better than the traditional capillary Sanger technique, exhibiting optimal performance with microbial genomes with high plasticity and producing much longer high quality readings than any other second generation sequencing platforms (Loman et al., 2012; Luo, 2012). A large run in the GS FLX produces about 1 million reads in 10 hours with an average length of 400 bp. The smaller version, the GS Junior, produces only about 100 000 reads. The upgraded version (GS FLX +) released in 2011 is expected to reach an average of 700 bp per read (Travis, 2011). A sample-specific multiplex identifier (MID), which is added to the ends of the DNA strand, allows pooling of several samples in one run, in this way, from 1 to 2304 samples can be sequenced in parallel in a large run (Droege and Hill, 2008). In general, the 454 platform is considered to be the most mature of the next-generation DNA sequencing options available (Travis, 2011).

The 454 technology has been used for multiple applications such as *de novo* sequencing, whole genome re-sequencing, full length/shotgun sequencing (Loman et al., 2012), transcriptome analysis (Fujie et al., 2010), among others. Regarding parasitic nematodes of veterinary importance, the 454 sequencing technology has been used to fully sequence the mitochondrial genome of the McMaster isolate of *Haemonchus contortus* (Jex et al., 2008).

One another important application of the 454 platform is the study of complex microbial communities, to evaluate their species composition and ecology. This novel area of genetics is called metagenomics (Wommack, 2008). In metagenomic studies, the highly accurate and relatively long reads of the 454 sequencing can identify an individual organism or gene in an unknown complex environmental sample (Gomez-Alvarez et al., 2009). The 454 technology offers unique possibilities to study microbial diversity and it has been successfully used in several publications in this area. This platform constitutes, together with Illumina, the only second generation sequencing protocols suitable for metagenomic

studies. However, the longer readings and much shorter running period makes 454 the best option for most of the cases (Travis, 2011). For example, the GS FLX platform has been successfully used to investigate the species diversity and distribution of unicellular eukaryotic organisms in freshwater ecosystems, based on sequencing of a 450 bp variable region within the 18S ribosomal DNA gene. This confirms the great potential of this technology in the determination of overall eukaryote species diversity with a sensitivity high enough to detect minute cell numbers (Bråte et al., 2010).

The use of 454 is also a powerful tool in metagenomic studies in viruses, bacteria and parasites. It has been used for assessing virus biodiversity in plants (Roossinck et al., 2010). A modified software has been incorporated in the 454 pipeline technology and it has been validated in the detection of mutations levels in human viruses (Astrovskaya et al., 2011). This high throughput technology has been extensively used in studies of the human oral and intestinal microbiota (Andersson et al., 2008; Luo, 2012). The potential of the assay in metagenomics research in parasites has been also explored. In the protozoan parasite, *Plasmodium falciparum*, the 454 platform proved to be superior to the conventional Sanger sequencing method for AT rich genomes. This technique can therefore be successfully applied to genetic studies on this major human pathogen (Samarakoon et al., 2011).

The applicability of 454 sequencing in metagenomics studies of nematode communities has also been explored. An experiment using different artificial metagenomics pools with different combinations of handpicked adult free-living nematodes from 41 species and 22 genera has demonstrated the suitability of the 454 massive parallel sequencing in the characterization of species composition in nematode populations. This experiment sequenced two genetic targets, both located in the rDNA genomic region, from genomic DNA extracted from the artificial worm pools. In general, the results showed an optimal accuracy to assess species richness, with most of the species identified positively, but there was a distortion of the real species abundance, with no correlation between the real proportions of individuals placed in the pools and the percentages per species produced by the sequencing (Porazinska et al., 2009).

Chemotherapy against cyathostomins

From an historical perspective, the use of chemical compounds (anthelmintics) has always been the main, and most of the time the only, component of any treatment or control program against cyathostomin infections in horses. Nowadays, three major classes of anthelmintics, the benzimidazoles (BZ), tetrahydropyrimidines and macrocyclic lactones (ML), are the only available options for this purpose (Allison, 2011; Kaplan and Nielsen, 2010).

Phenothiazine was the first anthelmintic effective against cyathostomins to be introduced in the 1940's. In the 1950's piperazine proved to be the first drug that was efficacious against more than one group of equine nematodes (Lyons et al., 1999). The modern era of broad-spectrum anthelmintics began in the 1960's with the introduction of thiabendazole, the first drug within the BZ class to be developed (Dargatz et al., 2000). BZ drugs changed the anthelmintic industry because they were the most broad-spectrum nematocidal drugs available as single products, and they were extremely safe. In the 1970's dichlorvos, a organophosphate drug, was developed and was highly active against *Cyathostominae* parasites. Probenzimidazoles, such as febantel, and the tetradyropyrimidine, pyrantel, were also introduced in the 1970's. (Lyons et al., 1999).

The ML drug class was introduced to the veterinary market in the early 1980's with ivermectin (Gokbulut et al., 2010), which proved to be the first drug to be efficacious against immature cyathostomin stages (Campbell, 1985). Then, in 1989, the first milbemycin drug, moxidectin, was commercially introduced, and an oral formulation was then approved for use in horses (Cobb and Boeckh, 2009).

Before moxidectin, the last drug to be introduced, the list of drugs approved for horses in the US comprised: butonate, tioxidazole, febantel, dichlorvos, trichlorfon, thiabendazole, cambendazole, fenbendazole, mebendazole, oxfendazole, oxifendazole, levamisole, phenothiazine, piperazine, pyrantel pamoate and tartrate and ivermectin. It should be mentioned that there are drugs still approved, but no longer commercially available, for use in horses (Conder, 1995).

Encysted EL_3 stages are refractory to most of the anthelmintic drug classes, making their removal complicated (Abbott et al., 2007; Dowdall et al., 2004; Love and McKeand, 1997). Therapeutic schemes to reduce encysted mucosal larval stages are the use of fenbendazole at a dose of 10 mg/kg for 5 consecutive days or a single dose of moxidectin at a single dose of 0.4 mg/kg (Rossano et al., 2010). Steinbach et al (2006) performed a comparison of the outcomes of the larvicidal effects of both drugs to try to detect adverse effects to the host. Fenbendazole showed a faster killing of encysted larvae (4-6 days after the last treatment) than moxidectin (6-14 days after the last treatment). However, the effects of both treatments were quite different. Fenbendazole induced a severe inflammation and tissue damage while moxidectin showed a weaker tissue reaction and much reduced tissue damage. The authors concluded that both drugs were highly effective against luminal, encysted and hypobiotic stages, but moxidectin would be better tolerated by the host.

Macrocyclic lactones

Macrocyclic lactones (ML) are derived from fermentation products of the soil actinomycete, *Streptomyces* sp., originally discovered in a soil sample sent to the Kitasato Institute in Japan as part of a new microbial compounds screening program (Campbell, 1985). All members of the ML group posses a lactone macrocyclic ring and they all have hydrophobic characteristics. The group comprises two families: the avermectins and the milbemycins. Avermectin B1 (abamectin) is the natural original avermectin and mother molecule from which ivermectin was later derived. Other members of the family such as eprinomectin, doramectin and selamectin are synthetically produced. The family of the milbemycins differs from the avermectins mainly in the lack of the sugar moiety on carbon 13 (C-13) of the macrocyclic ring. The main compound in the milbemycin group is moxidectin (Lespine et al., 2007).

Despite their similar basic core chemical structure, one remarkable feature of the ML analogs is their narrow structure-activity relationship, as revealed by the dramatic effects on the pharmacology of different molecules produced by minor changes in the structural components (Banks et al., 2000; Michael

et al., 2001). Changes in peripheral substituents at C-5, C-13, C-22, C-23, and C-25 affect the pharmacology and biological activity of the different ML drugs. This has been confirmed through *in vitro* studies on the structure/activity in avermectin drugs. The hydrophobic/lipophilic nature of the substituent, the presence or absence of the oleandrose mono or disaccharide substituent, among other chemical features, proved to be the most important changes related to ML drug potency. It should be mentioned that the analogs with the highest potency *in vitro* are not necessarily the commercially available drugs. Most of the analogs with optimal performance *in vitro* do not exhibit good pharmacokinetics *in vivo* (Michael et al., 2001).

The avermectin analogs have two variants: A and B. About 60% of the avermectins have a hydroxyl group in the position C-5 and are termed B components, while the other 40% have a methoxyl group in this position and are termed A components. The chemical conversion of avermectin B components to avermectin A components consists in the transferring of the methyl group of S-adenosylmethionine (SAM) to the C-5 hydroxyl of avermectin B. This reaction is catalyzed by the enzyme avermectin B O-methyltransferase (Schulman et al., 1986). The analogs denominated 1, such as B1, have a double bond between C-22 and C-23 and a hydrogen atom at C-23. The analogs of type 2, such as B2, have a single bond and an hydroxyl group instead (Campbell, 1985). The avermectin $B1_a$, in a mixture with avermectin $B1_{b,}$ is called abamectin, and it was the first fermentation-derived natural ML to be tested as an effective anthelmintic agent against parasitic nematodes in ruminants, canids and poultry in tests *in vivo* (Egerton, 1979).

Ivermectin

Ivermectin is the chemical derivative of the natural avermectin B1; it is an off-white powder, insoluble in water but very soluble in organic solvents. Sold as Ivomec® by Merck and Co; nowadays by Merial, it was the first anthelmintic of the ML family to be commercially released (Conder, 1995). The synthesis of ivermectin results from the hydrogenation of the *cis* carbon-carbon double bond at carbons 22 and 23 of

the homologous avermectins $B1_a$ and $B1_b$ (Mariano, 2011). The chemical name of ivermectin is 22, 23-dihydroavermectin and, like abamectin, it occurs in the form of two homologues: ivermectin $B1_a$, with a sec-butyl group at C-25 and ivermectin $B1_b$, with an isopropyl group instead. The $B1_a$ component comprises at least 80% and the $B1_b$ not more than 20% of the formulation. However, both components show the same pharmacological effect (Campbell, 1985). Ivermectin is used to treat nematode, insect and acarine parasites, showing high potency in ruminants, swine and companion animals including horses (Campbell, 1985). Ivermectin is also used to treat human parasitic diseases; it has been used in mass drug administration programs to control human filariasis since the 1980's (Moreno et al., 2010).

Eprinomectin

Eprinomectin was synthesized by Merck Laboratories with the chemical name of 4"-epi-acetylamino-4"-deoxy-avermectin B1 (Shoop et al., 1996). Eprinomectin has the core structure of abamectin with the only difference being the substitution of the hydroxyl group in the 4" position of the distal sugar with an epimethylano group ($NHCOOH_3$). Similar to most of the avermectins, it also has $B1_a$ and $B1_b$ forms (Lespine et al., 2007). The modification in the sugar moiety increases dramatically the insecticide activity of the molecule, as confirmed by efficacy against crop pests (Rugg et al., 2005).

Eprinomectin is a good example of the structure-activity relationships in ML since only one major structural change in the abamectin molecule causes dramatic changes in solubility, distribution, stability and diversity of spectrum while maintaining a high potency. However, the principal difference is in the pharmacokinetics of the drug, with minimal excretion in milk (Holste et al., 1997). Based on this characteristic and its high efficacy against immature and adult helminth stages, and major ectoparasites, eprinomectin constituted the first successful topical endectocide approved for use in lactating dairy cattle (Shoop et al., 1996). Although not available for use in equids, eprinomectin has been shown to be highly effective against *Dyctiocaulus arnfieldi*, the equine lung worm, when used as a pour-on in donkeys, with no adverse effects (Veneziano et al., 2011).

Other avermectins

Doramectin was the first commercially available avermectin analog prepared by directed mutational biosynthesis using mutated strains of *S. avermitilis*. This novel approach allowed the introduction of changes in the C-25 position of the avermectin ring (Goudie et al., 1993). Doramectin is structurally more similar to abamectin than to ivermectin, and was introduced in the market in 1993, available only for cattle and swine (Michael et al., 2001; Rugg et al., 2005).

Other members of the ML group are: emamectin and selamectin. Emamectin is a member of the second generation of avermectins, denominated aminoavermectins. This drug is closely related to eprinomectin and it is produced by a multistep semi-synthetic chemical process, with a 1500-fold increase in potency against major insect crop plagues when compared with other avermectins (Cvetovich, 1994). Selamectin is one of the most recent avermectins to be introduced in animal health, being a semi-synthetic derivative of doramectin (Michael et al., 2001), with only one sugar in C-13, therefore exhibiting intermediate properties between the disaccharide avermectins and the milbemycins. This highlights the importance of the distal sugar in the pharmacology of the avermectin analogs (Lespine et al., 2007; Prichard et al., 2012). Another avermectin, selamectin, was identified in a targeted analog program, searching for novel endectocides with increased potency against fleas and a better safety index in dogs (Banks et al., 2000).

Milbemycins: moxidectin

The milbemycin sub-group was isolated almost 10 years before the discovery of the avermectin subgroup. Milbemycin, the type molecule of the class, was discovered to be produced by another member of the genus *Streptomyces*, *S. hygroscopicus* (Prichard et al., 2012). Milbemycins are structurally equivalent to 13-deoxy-ivermectin, lacking the sugar side chain at C-13 (Conder, 1995). However, they do not share the same properties of the avermectin aglycones, since they are protonated and not hydroxylated at the C-13 position and they have other substituents at the level of C-25. Similar to the avermectins, milbemycins,

have a narrow structure-activity relationship, with differences among analogs mainly at C-5 and C-25 (Prichard et al., 2012). Milbemycin oxime, the commercially available derivative of milbemycin, is a mixture of A_4 and A_3 oximes of milbemycin D (Conder, 1995) and it is used exclusively in dogs and cats to treat gastrointestinal nematodes, ear and mange mites, and for heartworm prevention (Prichard et al., 2012).

Paradoxically, although the milbemycin class was discovered about a decade before the avermectins, the most important member of the class, moxidectin, was commercially first released almost a decade after ivermectin, as Cydectin® for cattle (Cobb and Boeckh, 2009). Moxidectin is a semi-synthetic derivative of the naturally occurring milbemycin nemadectin (F-alpha), with an olefinic side chain at C-25 and a methoxime moiety at C-23 (Prichard et al., 2012). Similar to abamectin, naturally occurring ancestor of ivermectin, nemadectin is produced by other soil bacteria from the genus *Streptomyces*, *S. cyanogriseus* subspecies *noncyanogenus*, originally isolated from a sand sample taken from an Australian beach in 1983 (Cobb and Boeckh, 2009).

Moxidectin shares the 16-membered ML ring core with the avermectins and it also shares some of the same chemical properties and pharmacodynamics, but some important differences in pharmacology and activity *in vitro* have been described between moxidectin and the avermectins (Ardelli et al., 2009). Moxidectin is widely used against nematodes and insects in animal health (Rugg et al., 2005). It shows no adverse reactions at a dose of 0.4 mg/kg and it is environmentally safer than ivermectin as confirmed by effects on dung beetles (Conder, 1995). The use of moxidectin for the treatment of human onchocerciasis is presently under evaluation (Prichard et al., 2012).

Mode of action of macrocyclic lactones

Early attempts to elucidate the biochemical mechanisms involved in the paralytic effects of ML on arthropods and nematodes suggested the involvement of gamma aminobutyric acid (GABA) mediated opening of chloride channels with hyper polarization of neurons and blockage of neuro-muscular signal

transmission (Campbell, 1985). Later, the pioneering electrophysiology data published by Arena et al. (1991, 1992), working with *Xenopus laevis* oocytes expressing *C. elegans* RNA, were the first descriptions of an interaction of ivermectin with glutamate-gated chloride channels (GluCl) (reviewed by Conder et al, 1995). Later work demonstrated that ML drugs acted mainly on the GluCl, opening them and increasing the influx of chloride ions into muscle cells, such as the pharyngeal muscles. This pharmacological action leads to an inhibition of muscle contraction and flaccid paralysis of the parasite (Cobb and Boeckh, 2009; Sheriff et al., 2005; Wolstenholme and Rogers, 2005).

Present specifically in invertebrates only, GluCl are members of the family of cys-loop ligand gated ion channels, forming pentameric structures composed of one or more type of subunits across the cell membrane. Among these sub-units, AVR-14 isoforms A and B are reported to form ML-sensitive channels (Wolstenholme and Rogers, 2005). The sub-units GLC-3 (Horoszok et al., 2001) and GLC-5 (called GluCla by Forrester et al., 2003) forms ML-sensitive channels as well. ML drugs pharmacological effects are thought to be mediated by direct opening of these channels and by potentiating the action of their natural ligand, glutamate (Yamaguchi et al., 2012). Research on the molecular mechanism of action of ivermectin suggested that its effect on the GluCl channel is that of a non-conventional agonist and that it may interact with the transmembrane domains of the channel. Amino acid residues near the extracellular end and membrane spanning region have showed to be important for the ivermectin effects on the channels (reviewed by Wolstenholme, 2010).

Hibbs and Gouaux (2011) have recently published a paper describing the three-dimensional structure of the *Caenorhabditis elegans* GluClα glutamate-gated chloride channel and its interactions with ivermectin. The architecture of the assembled ion channel has a pinwheel-like scaffold, with a cylindrical homopentamer of five either identical or homologous subunits. The alpha helical transmembrane segments of each subunit line the channel pore. In this work, the role of ivermectin as a partial allosteric agonist has been confirmed. Ivermectin molecules bind the channel through extensive hydrophobic interactions and hydrogen bonding at subunit interfaces on the periphery of the transmembrane domains,

proximal to the extracellular side of the membrane bilayer. These interactions induce local changes in the membrane domain and global conformational changes in the entire receptor, opening the ion channel, and pre-activating and stabilizing the binding site for the natural ligand, glutamate. The glutamate binding site is located in the extracellular domain also, between the subunits interface. The chloride channel blocker, picrotoxin, binds the subunits interface in a different site, near the cytosolic side of the transmembrane pore. A more recent study has explored the nature of the binding of the milbemycin drugs to the GluCl channels in the parasitic nematode, *H. contortus,* by constructing a software-based homology model. Using docking simulation based on *in vitro* responses to the drug in receptors with point mutations, the model showed certain similarities with the ivermectin binding but with the presence of a second site of interaction located in the extracellular domain of the subunits (Yamaguchi et al., 2012). Differences in the core structure between the avermectins and milbemycins may explain the differences predicted by this study (Prichard et al., 2012), but further research in this area is still needed before definitive conclusions can be drawn.

Even though it is widely assumed that the molecular basis for ML pharmacology is due to its interactions with ligand gated ion channels, participation of additional targets has been suggested (Wolstenholme, 2012). These may account for the differences in response observed in *in vitro* studies (Laing et al., 2012), and differences in the pharmacodynamics of ML between the invertebrate and vertebrate host. One of these molecules are the P-glycoprotein pumps (PgP) (Yan et al., 2012). There are abundant data that support a major role of these membrane transporter proteins in the modulation of the pharmacokinetics of several ML drugs, including ivermectin. The higher affinity for the PgP showed by the avermectins has suggested their use as inhibitors of these pumps in cases of multi drug resistance (MDR) in cancer therapy in humans (Lespine et al., 2007).

Although there is evidence to support a common mechanism of action for the avermectins and milbemycins, differences in pharmacokinetics, pharmacodynamics and toxicology have also been reported (reviewed by Prichard et al, 2012). A study comparing the effects of ivermectin and moxidectin

in the *C. elegans* model has suggested some differences in both, phenotypic responses (pharyngeal pumping and locomotory behavior) and transcription profiles. However, similar studies involving parasitic nematodes are required to further support these preliminary findings (Ardelli et al., 2009).

Experiments done on *C. elegans* have shown that the oral route may not be the only one involved in the penetration of avermectins in nematodes but penetration through the cuticle or through cuticular openings may also be important. This possibility is also supported by the *in vitro* effect of ivermectin on the non-feeding L_3 of parasitic nematodes (O'Lone and Campbell, 2001). Additional work done with ivermectin showed that the GluCl sensitive to ML in nematodes is located mainly in neurons and not in muscles (Portillo et al., 2003). Wild type *C. elegans* exposed to low concentrations of ivermectin (0.1 μM) for one hour show dramatic paralysis. However, certain mutations in GluCl subunits render resistance to this paralytic effect on the body muscles but do not confer protection against the effects on the pharyngeal activity. This confirms the different effects exerted by the drug in specific areas of this model organism (Laing et al., 2012). It has been suggested that feeding inhibition through pharyngeal pumping paralysis and not paralysis of the body could be the major effect involved in the *in vivo* effect against *Teladorsagia circumcincta*. In *Trichostrongylus colubriformis* and *H. contortus* it is the paralytic effect which may be the most important, based on the different times needed for worms to be expelled (Gill and Lacey, 1998). Further *in vivo* studies in sheep, measuring the uptake of radioactive labeled inulin, suggest that a very reduced effect of ivermectin on feeding inhibition of *H. contortus* exists, however, this result does not discard the possibility of feeding inhibition as a component in the mechanism of action *in vivo* (Sheriff et al., 2005).

Most of the knowledge of the pharmacology of ML drugs has been obtained through *in vitro* works with the non-target organism *C. elegans* but *in vitro*, ML are also very potent inhibitors of parasitic nematode development and motility (Gill and Lacey, 1998). Studies done with the major sheep parasite, *H. contortus*, have described two different effects of avermectins *in vitro*. At a very low concentration, of < 1 nM, no effect on motility is observed but there is a reduction in larval development, produced by

starvation, as a consequence of pharyngeal paralysis. At much higher concentrations (50 000 nM) a marked and fast effect on larval motility can be observed. This confirms different sensitivities for the pharyngeal muscle, responsible for feeding, and for somatic muscles (Gill et al., 1995). Nevertheless, the similar patterns observed in the pharmacology of different ML drugs *in vitro* may suggest that these putative sites of action could be different, although somewhat related (Gill and Lacey, 1998).

One important feature observed in *in vitro* bioassays with ML which is directly related to the structure-activity relationship of the class is the wide range of *in vitro* potencies obtained when different drugs are examined in specific parasite, using a determined phenotypic test (Gill and Lacey, 1998; Michael et al., 2001; Sheriff et al., 2005). In some cases, an intermediate analog may be the most potent compound for a particular bioassay in a determined nematode species (Michael et al., 2001). Moreover, minimum structural changes in substituents at C-5, C-13, C-25 and C-23, or disruption of the planarity of the lactone ring in both the avermectins and the milbemycins, produce changes in the drug potency *in vitro* of several orders of magnitude (Gill and Lacey, 1998). For example, avermectin B1 is a more potent *in vitro* inhibitor of larval development and motility in *H. contortus* when compared to avermectin B2 or ivermectin. Avermectin monosaccharide and avermectin aglycone are much less potent inhibitors of larval development, and they do not inhibit larval motility, even at very high concentrations (50 μM) (Gill and Lacey, 1998; Gill et al., 1995). Regarding other avermectin analogs, work carried out by Sheriff et al (2002) showed that eprinomectin was about a 5-fold more potent as inhibitor of feeding activity in *T. colubrifornis* larvae than ivermectin and, similarly to previous findings, 200-fold more potent than avermectin aglycone; furthermore, doramectin has been reported to be as potent as ivermectin and these two drugs about 100 fold more potent than selamectin at inhibiting larval development in *H. contortus*. Remarkably, in the same study, the most potent analog observed was a non commercially available novel compound derived from selamectin (Michael et al., 2001).

Toxicity of macrocyclic lactones

ML drugs show low toxicity to vertebrates and relatively short environmental persistence, which reduces the toxic effects in non target organisms in the environment (Rugg et al., 2005). The relative high therapeutic index these drugs exhibit is not because of their extraordinary safety in mammals but rather because of their extraordinary potency against parasites (Conder, 1995), thus, the doses given are extremely low.

Toxicity of ML in animals is related to the transporter activity of the PgP across cell membranes. Low expression of PgP leads to passage of the drug across the blood-brain barrier, leading to over-accumulation of the drug in the nervous system, producing symptoms of toxicity (Cobb and Boeckh, 2009). Several herding breeds of dog, but especially collies, show an increased susceptibility to ivermectin toxicity. Clinical symptoms of toxicity include depression and hypersalivation. A genetic mutation in the gene encoding a member of the Pgp family has been shown to be responsible for this phenotype (Han et al., 2010).

Pharmacology of macrocyclic lactones in horses

Among the ML analogs available for livestock, only ivermectin and moxidectin are sold and used in horses. Abamectin, doramectin and eprinomectin are not licensed for use in horses (Conder, 1995; Molento et al., 2012). Both ivermectin and moxidectin are available for oral administrations as a gel (moxidectin), paste (ivermectin) or liquid (ivermectin). As discussed before, despite sharing common chemical features, ivermectin and moxidectin exhibit some major differences in their pharmacology in horses.

The first reported use of a ML in horses was in 1980, with the use of ivermectin subcutaneously at a dose of 0.1 to 0.8 mg/kg (Slocombe, 1980), Egerton et al. (1981) later confirmed its remarkable efficacy against major endoparasites using the same route. The intramuscular route was used later with similar good results (Slocombe and McCraw, 1981). Although first commercially introduced as an intramuscular

injection for horses, rare reports of adverse reactions were responsible for withdrawal of this parenteral form and the later introduction of the oral paste (Campbell, 1985) as well as the liquid formulation for nasogastric intubation (Gokbulut et al., 2010), which are the only commercially available forms for use in horses at present.

When used orally in horses, ivermectin reaches plasma peak levels after 2 to 5 hours and is better absorbed as a paste formulation. Most of the drug is excreted in feces as intact drug (Conder, 1995). A complete comparative analysis of the pharmacokinetics of ivermectin in horses following oral or topical , (a pour on formulation licensed for cattle) administration has been published by Gokbulut et al. (2010). In this study, a higher efficacy and almost seven times greater bioavailability was detected for the oral route than the topical route, with a 14-fold higher peak plasma concentration and about three-fold larger area under the curve (related to the amount of drug available in circulation over time), and despite the lower dose used in the oral route (0.2 mg/kg vs. 0.5 mg kg for the pour on). More recent work has further explored the use of a higher dose (1 mg/kg) via pour on with relatively good results against intestinal nematodes (Francisco et al., 2011), however, the oral route is still the only approved route for use in horses.

As a member of the milbemycin group, moxidectin has a different pharmacology when compared with ivermectin. In general, moxidectin exhibits exceptionally higher potency against nematodes, longer half life and better distribution than ivermectin (Prichard et al., 2012). Some differences in the pharmacokinetics of the compound when compared with ivermectin also exist. When orally administrated in horses as a gel, moxidectin is not extensively transformed to less active metabolites, remaining mostly as active parent compound. Similar to ivermectin, the main route of elimination is the feces (Cobb and Boeckh, 2009). In their reference work on this topic, Perez et al. (1999) compared the pharmacokinetics of moxidectin vs. ivermectin following oral administration in adult horses. Despite showing similar patterns of absorption, and no significant difference in time corresponding to peak plasma level or absorption, moxidectin showed a two-fold higher mean plasma concentration and a maximum

concentration of about 1.5-fold greater. Remarkably, moxidectin had about a six times longer elimination half-life (23 days vs. 4 days), and almost a three-fold higher overall amount of drug in the bloodstream (area under the curve). These results confirmed the better bioavailability and longer persistence of moxidectin in horses. The two-fold higher oral dose recommended for horses (Pérez et al., 1999), low susceptibility to ABC transporters (Pgp family) (Prichard et al., 2012) and higher lipophilicity of moxidectin when compared to ivermectin, which creates a natural body fat reservoir of drug with a slow release (Cobb and Boeckh, 2009), have been suggested as contributing factors to explain partially some of these differences. The remarkable pharmacokinetic features of moxidectin have a positive impact on its efficacy against parasites, especially against stages not readily controlled by other ML (Cobb and Boeckh, 2009).

Spectrum of activity of macrocyclic lactones in horses

Egerton et al. (1981), in his pioneering paper using an experimental preparation of ivermectin, established that a dose of 0.1 mg/kg applied subcutaneously had an efficacy of >99% against *Strongylus* spp., *Gasterophilus* spp., *Oxyuris equi*, *Parascaris equorum*, *Onchocerca cervicalis* and cyathostomins. One notable finding of this study was that in order to reach a >90% of efficacy against one cyathostomin species, *Cs. goldi*, a higher dose (0.5 mg/kg) was required. This study determined that 0.2 mg/kg should be the standard dose in horses and also gave the first indication of different ML-susceptibility among cyathostomin species. Later, the oral route using the same dosage, proved to have a similar spectrum of efficacy and even extended it to almost all the major internal equine parasites, including lungworm (*D. arnfieldi*) and stomach worms (*Habronema muscae* and *Draschia megastoma*) (Campbell, 1985).

Although highly effective against adult luminal stages of cyathostomins, ivermectin does not show significant efficacy against encysted mucosal stages at recommended doses and even at five times higher doses than those recommended (1.0 mg/kg) (Klei et al., 1993). This lack of activity against hypobiotic larvae has been related to pharmacological characteristics of the drug and the location or metabolic state

of the parasite (Klei et al., 1993; Monahan et al., 1996). In contrast with ivermectin, moxidectin demonstrates high efficacy against encysted luminal stages of cyathostomins (Steinbach et al., 2006). Cobb and Boeckh (2009) have published a detailed review of the pharmacokinetics and efficacy of moxidectin in horse endoparasites. At a dose of 0.2 mg/kg and administrated as oral gel this drug is nearly 100% effective against luminal cyathostomin stages, larval and adult stages of *Strongylus* spp., *Triodontophorus* spp., *Oesophagodontus robustus, P. equorum, O. equi* and *H. muscae* (Monahan et al., 1996). The high efficacy of moxidectin against the immature migrating stages of *Strongylus* spp. and its better performance than other ML against encysted cyathostomin larvae are probably related to the differences in the pharmacology of the molecule. One additional singular characteristic of ivermectin is the paralytic effects seen in cyathostomins that induces the expulsion of both adult and luminal fourth stage specimens in a period of approximately 2 days after treatment, allowing the recovery of intact specimens (Lind et al., 2003).

The egg reappearance period (ERP) is an important parameter for assessing the efficacy of anthelmintic drugs against tissue dwelling larval stages in horses, with special reference to cyathostomins (Duncan et al., 2002). The ERP is defined as the duration of the total suppression of detection of eggs in feces (Lyons et al., 2008) or the period that it takes for the number of eggs in the feces to reach a certain detectable threshold (von Samson-Himmelstjerna et al., 2007). Because of their different levels of efficacy against encysted mucosal cyathostomin larvae, ivermectin and moxidectin exhibit also significantly different ERP. According to earlier publications, the original ERP for ivermectin ranged from 8 to 13 weeks (Demeulenaere, 1997; Jacobs, 1995; Piché, 1990) and about 12 to 24 weeks for moxidectin (Demeulenaere, 1997; DiPietro et al., 1997; Jacobs, 1995).

As mentioned before, since their introduction, ML anthelmintics have showed an outstanding efficacy against large strongyles, including migratory immature stages (Slocombe, 1980), and they have been the main factor in the dramatic reduction in the prevalence of large strongyle on horse farms. According to more recent studies ivermectin still has a high efficacy against *Strongylus* spp. with no cases

of suspected resistance reported (Ionita et al., 2010). Regarding non-strongyle nematodes, *Parascaris equorum* is the dose limiting parasite for ML in horses (Nielsen et al., 2010b). However, ivermectin is highly effective against this horse ascarid at recommended doses (Larsen et al., 2011).

The therapeutic use of ML in wild and other domestic equine species has been also documented. Oral administration of ivermectin has been shown to have 100% efficacy against cyathostomins in naturally infected zebras in a safari park in Italy (Lia et al., 2010). Administered as pour on preparation, eprinomectin can be used to treat *D. arnfieldi* (equine lungworm) in naturally infected donkeys (Veneziano et al., 2011).

Anthelmintic resistance

Anthelmintic resistance is defined as the inherited ability of individual worms in a population to survive the effects of a drug that is generally effective against the same species and stage of infection (Sangster, 1999). This change in susceptibility in the population results from the decrease in the frequency of susceptible individuals when compared to the same population upon initial exposure to the drug (Conder, 1995). Resistance should not be confused with cases of inherent tolerance, which is defined as the innate unresponsiveness of a determined species or specific developmental stage to a determined drug, independent of prior exposure (Conder, 1995; Sangster, 1999).

The genetic basis for the development of resistance to anthelmintic drugs is the increase in frequency of alleles for resistance which is determined by the selection pressure exerted by the drug treatment (Sangster, 1999). Alleles for resistance (R) may be present in a drug naïve worm population, though they should initially be rare. After anthelmintic treatment is started, the frequency of the R alleles increase as the treatment survivors reproduce (Dargatz et al., 2000). The frequency of anthelmintic treatment and the efficacy of treatment are also important in the development of resistance (Sangster, 1999). The frequency of treatment is directly related to the maintenance of refugia, the parasite population not exposed to the drug and therefore not selected for resistance. Parasite refugia is a major consideration when wanting to

delay the development of resistance, since it is a potential source for susceptible genes, to dilute out the resistant ones in the parasite population (Soulsby, 2007).

The emergence of anthelmintic resistance is a major limitation in animal production (Conder, 1995). This phenomenon has introduced into common use certain specialized terms used to describe different types of resistance. Side resistance occurs when parasites resistant to one drug in a specific class develop resistance to other member of the same class; for example, development of side-resistance against moxidectin in ivermectin resistant strains has been reported in sheep, goat and cattle nematodes. Multiple resistance occurs when a parasite develops resistance to more than one class of anthelmintics, and cross resistance is when the development of resistance to one drug class leads to resistance to members of a different class (Dargatz et al., 2000).

The emergence of early stages of resistance to a specific anthelmintic compound in a parasite population can be very difficult to detect, especially in mixed populations of worms (Sangster, 1999). The most common situation that occurs is the report of treatment failure that leads to the detection of clinical resistance (Dargatz et al., 2000). However, this is the final consequence of the accumulation of resistant alleles in the parasite population until they reach very high levels (Sangster, 1999). According to abundant data, reversion of resistance to the susceptible original state is unlikely. Even the interruption of the use of the drug for many years will not eliminate resistance and restore efficacy (Lyons et al., 2001; von Samson-Himmelstjerna, 2012).

Currently, increasing numbers of cases of multiple resistance and numerous cases of multigeneric resistance have indicated a worsening scenario. These situations highlight the importance of anthelmintic resistance in the current epidemiology of parasitic diseases in animals (Kaplan and Vidyashankar, 2012). Anthelmintic resistance is a currently a serious threat to equine health and cases involving major parasite pathogens have been extensively documented (von Samson-Himmelstjerna, 2012).

Anthelmintic resistance in horses

Anthelmintic resistance is a major concern in the equine industry worldwide and is mostly limited to the cyathostomins and *P. equorum* (Dargatz et al., 2000; Nielsen, 2012). Emergence of anthelmintic resistance in these horse nematodes against the three major drug classes used: BZ, tetrahydropyrimidines and ML has been documented (Kaplan et al., 2004; Kaplan and Nielsen, 2010; Kaplan and Vidyashankar, 2012).

Benzimidazoles

Resistance against BZ is reported mainly in cyathostomins, since the drug is still highly effective against other equine nematodes, including *P. equorum* (Kaplan and Vidyashankar, 2012). Thiabendazole was the first member of the BZ class to be associated with resistance in cyathostomins, just a few years after its introduction on the market. Resistance against this drug was reported in cyathostomins in 1965 in a horse farm in Kentucky, and this parasite population was later named Population B. It was reported to be resistant to other members of the BZ class such as cambendazole, mebendazole, fenbendazole and oxfendazole (Drudge et al., 1988). Resistance against fenbendazole and oxibendazole have been extensively reported worldwide, with prevalences ranging from 10 to 98% among horse farms (Eysker et al., 1989; Kaplan et al., 2004; Kuzmina and Kharchenko, 2008; Traversa et al., 2007).

Multiple cyathostomin species are reported to be resistant to BZ drugs. Kuzmina and Kharchencko (2008) reported that BZ resistance cases in Ukrainian horse farms concerned ten dominant species: *Cc. nassatus, Cy. catinatum, Cs. calicatus, Cy. ashworthi, Cs. longibursatus, Cs. goldi, Cy. pateratum, Cs. minutus, Co. coronatus, Cc. leptostomum*; with only one species from the group of the non-dominant or so called satellite species, *Cc. labiatus*. These results suggest that the emergence of BZ resistance may be associated with the high genetic diversity reached by the major cyathostomin species because of their high abundance in parasite populations (Kuzmina and Kharchenko, 2008).

Tetrahydropyrimidines (pyrantel)

Moderate levels of resistance to pyrantel in cyathostomins has been detected in horses in Italy, the UK and Germany (Traversa et al., 2009b). Greater and more widespread levels of resistance have been reported in the US, and it is thought to be related to the daily administration of the drug (Kaplan et al., 2004). The horse ascarid, *P. equorum* has been also involved in cases of anthelmintic resistance against pyrantel in horse farms. Craig et al (2007) have documented a case of pyrantel-resistant *P. equorum* on a large horse farm in Texas.

Other anthelmintic classes

The first report of anthelmintic resistance in cyathostomins against phenothiazine appeared in the early 1960's in the UK and US (Lyons et al., 1999). Later, resistance to piperazine was also documented in a horse farm in Kentucky, after a period of 18 years of periodic treatment with this drug in combination with different drugs. This was the first case of piperazine resistance in cyathostomins or any other major parasitic nematode (Drudge et al., 1988). Resistance against closantel and the organophosphate, trichlorfon, have been most recently reported in equine cyathostomins in Brazil, based on fecal egg count reduction tests (FECRT) performed 7 days after treatment (Borges et al., 2010).

Anthelmintic resistance against macrocyclic lactones in horses

The problem of anthelmintic resistance against ivermectin and moxidectin in small ruminant nematodes (sheep, goats, llamas and alpacas) has been extensively documented throughout the world and the cases are currently increasing in cattle (Kaplan and Vidyashankar, 2012). The phenomenon of resistance against ML in parasitic nematodes has been shown to have complex characteristics which depend on the parasite species. This is exemplified by different resistant isolates of sheep nematodes, which have demonstrated different "resistant phenotypes" when tested *in vitro* (Gill and Lacey, 1998).

Since the introduction of the ML in the early 1980's, no new major drug class has been introduced in the horse market and the release of a new one in the short term seems to be very unlikely. Moreover, the ever increasing documentation of resistance against BZ and pyrantel in *P. equorum* and cyathostomins leaves the ML as the most effective anthelmintic class in horses (Matthews et al., 2012; Molento et al., 2012). However, ML resistance in horse parasites is no longer an uncommon finding but rather an increasing concern. ML resistance is common in *P. equorum* and although is not yet a widespread problem in cyathostomins, the first reports of ML resistant have been confirmed (Molento et al., 2012). Although there are some reports of possible cases of failure of ML to fully control severe clinical cases of *O. equi* (Durham and Coles, 2010), the emergence of anthelmintic resistance to ML has not been officially reported for this parasite (Reinemeyer et al., 2010b). In the case of stomach worms, *Draschia/Habronema*, some anecdotal suspected cases of ML resistance have occurred, but have not been officially reported (Kaplan and Vidyashankar, 2012).

Parascaris equorum

Resistance against both ivermectin and moxidectin has been reported extensively in *P. equorum* in foals on horse farms in Europe and the Americas (Craig, 2007; Molento et al., 2008; Nareaho et al., 2011; Reinemeyer et al., 2010a; Slocombe et al., 2007; von Samson-Himmelstjerna et al., 2007) and is mostly related to overtreatment of foals (reviewed by von Samson-Himmelstjerna, 2011).

Cyathostomins

Sangster (1999), making a comparison between sheep trichostrongyloids and horse cyathostomins, and considering the widespread resistance against BZ and pyrantel in cyathostomins, predicted the imminent emergence of resistance against ML in cyathostomins, but at a significantly slower rate. Considering the current scenario, this event would constitute a serious threat to the horse industry (Dargatz et al., 2000). However, despite almost three decades of use in horses, and contrary to the expected scenario, resistance

to ML in cyathostomins is not yet widespread, as shown by several reports of high efficacy (>99%) of ivermectin and moxidectin in horse herds around the world (Borges et al., 2010; Cutolo, 2011; Ionita et al., 2010; Kuzmina and Kharchenko, 2008; Kyvsgaard et al., 2011; Larsen et al., 2011; Lind et al., 2003; Traversa et al., 2007; van Doorn et al., 2012). However, there are also an increasing numbers of reports of ML failure to fully control cyathostomin infections and a reduction in efficacy against the immature stages, which are indicative of the early stages of drug resistance (Lyons et al., 2011; Rossano et al., 2010; von Samson-Himmelstjerna et al., 2007). Moreover, resistance against moxidectin in cyathostomins has been confirmed already (Molento et al., 2012).

Generally, a small reduction in the FECRT and a shortened ERP have been considered as early signals of ML resistance in cyathostomins (Molento et al., 2012), but this reduction in efficacy can be a product of other variables different from the reduced efficacy of the drug (Conder, 1995). Another confounding factor to consider when evaluating ERP between studies, is the lack of uniformity in the experimental design and in cut off values between studies, making a fair comparison complicated (Molento et al., 2012). Based on this information, most of the published cases of reduced efficacy of ML in cyathostomin populations should be considered as suspected cases of ML field resistance until they are confirmed by compelling scientific evidence.

The first official documented case of apparent ML resistance in equine cyathostomins was reported in a herd of donkeys in the UK. After moxidectin treatment, a shortened ERP of only 8 weeks was demonstrated by FECRT. Even though treatment failure is normally the first signal for clinical resistance, it should be considered that a commercial product was used extra-label in this case and the different pharmacokinetic profiles may occur between donkeys and horses. These factors could also explain the reduced efficacy observed (Trawford et al., 2005 cited by Molento et al., 2011).

A failure to fully control cyathostomins using three ML drugs, abamectin, moxidectin and ivermectin, was later reported in Brazilian horses (Molento et al., 2008), and in the same year, a case was published of clinical failure of ivermectin to fully control strongylid infections, most likely

cyathostomins, on a horse farm in Australia (Edward and Hoffmann, 2008). In the latter a reduced ERP for moxidectin was also documented. A more recent case of suspected resistance against ivermectin in cyathostomins has been reported in Finland (Näreaho et al., 2011) but the authors of this study assumed that the type of strongyle eggs seen on FEC belonged to the cyathostomins, and did not perform species identification. Another major drawback was the lack of a control group. Even with this considerations, and based on the high efficacy (>99%) demonstrated by MLs in other similar studies, these results could be considered warning signals of the potential emergence of ML resistance.

As mentioned before, the ERP is one important parameter for evaluation of efficacy of ML drugs against cyathostomins in horses. However, ERP in cyathostomins are sometimes difficult to evaluate because of the nature of field experiments, the presence of inhibited larval stages and the complex species composition of parasite populations (Sangster, 1999). Comparison among studies is complicated by the use of different criteria in determining ERP (Molento et al., 2012), however, shortening of the ERP could indicate selection of a more resistant phenotype and some degree of resistance present in cyathostomin populations (Lyons et al., 2008).

One of the first reports of reduced ERP after ivermectin treatment in cyathostomins was a large scale study done between 2003 and 2004 comprising 63 horse farms in western Germany (von Samson-Himmelstjerna et al., 2007). These authors tested the efficacy of ivermectin in cyathostomins and *P. equorum.* Regarding the cyathostomins, despite the high efficacy found on almost all of the farms, one farm presented a suspected case of resistance based on the FECRT, but the most important finding was that the ERP had been reduced to less than 5 weeks. Later Lyons et al. (2008 and 2009) in Kentucky, published the results of one of the most complete studies on the shortening of the ivermectin ERP in cyathostomin populations. Based on FEC, the ERP was only 3 to 4 weeks, almost half of the length it was supposed to be. In a follow-up study on the same parasite population, a reduced efficacy (55%) of ivermectin against the luminal immature stages (fourth stage and possibly fifth larvae stage) was reported. This was thought to be the reason for the reduced ERP observed. A reduced efficacy of ivermectin against

the luminal fourth stage larvae has been previously reported (Klei et al., 1993), but considering that the efficacy of the drug the against adult stages was still high, this was not considered as true case of resistance (Monahan et al., 1996). In the Kentucky study, ivermectin was also still highly effective against adult worms, but the significant decrease in the ERP related to the reduction in susceptibility of the larval stages suggested early stages of anthelmintic resistance.

A similar situation of reduction in the ERP has been reported for moxidectin. Recently, a study in Kentucky has found a ERP after moxidectin treatment of only 42 days (6 weeks) in naturally infected horses (Rossano et al., 2010).The expected ERP for moxidectin is from 12 to 24 weeks. In order to test the efficacy of moxidectin and ivermectin in cyathostomins, two independent trials were conducted on a horse farm in Kentucky using the FECRT. Two oral products were tested. Results obtained showed a shortening in the ERP for both drugs, with 7 to 8 weeks for moxidectin and 4 to 5 weeks for ivermectin in the first study, and 4 to 6 weeks for both drugs in the second experiment. The high variability among the results obtained from the different experimental groups in the first experiment could not be explained by the authors (Lyons et al., 2011).

Research into the phenomenon of anthelmintic resistance in cyathostomins is limited because of the high costs and difficulties in working with these parasites both *in vivo* and *in vitro*. Knowledge generated on major sheep nematodes should be cautiously extrapolated to cyathostomins, since the extent of parallelism between both groups is unknown (Dargatz et al., 2000; Sangster, 1999). One important conclusion that can be drawn from this section is that anthelmintic resistance in cyathostomins against all the drug classes, including ML, is emerging. Additionally, a singular feature of these parasites is the complexity of the species composition of parasite populations. This unique scenario implies the need for studies on species-specific associations with level of resistance (Hodgkinson et al., 2008). Experimental studies on the development of anthelmintic resistance against BZ and pyrantel has demonstrated that different species exhibit different levels of susceptibility to the drugs and the emergence of resistance is related to some specific species in the population (Lyons et al., 2001; Lyons et al., 1996a). The emergence

of anthelmintic resistance may influence the species distribution and some specific species may be more involved than others in the development and spread of resistance (Traversa et al., 2010). It has even been suggested that the pressure of selection exerted by frequent anthelmintic treatments may produce a reduction in the species diversity in cyathostomin communities (Kuzmina and Kharchenko, 2008), however, there is little scientific evidence to support this. Nevertheless, these hypotheses stress the importance of epidemiological studies in resistant cyathostomin populations to understand better the influence of species composition in the emergence of resistance.

Mechanism of anthelmintic resistance

Two basic mechanisms for anthelmintic resistance have been documented: reduced drug level in the nematode because of decreased uptake, increased removal or drug metabolism, and changes in the drug target. Resistance to BZ in nematodes is a clear example of the second mechanism. A mutation in the β-tubulin gene, the molecular target of the drug, induces a lower binding affinity (Dargatz et al., 2000). The molecular mechanism of resistance against BZ has been extensively studied and even molecular assays for detection have been developed (Conder, 1995). It is well known that a single SNP in the β-tubulin isotype 1 gene is responsible for a BZ-resistant phenotype in trichostrongyloid parasites of sheep and cattle, such as *H. contortus* and *T. circumcincta*. Three SNP have been identified, at codons 200, 198 and 167 of the gene (von Samson-Himmelstjerna et al., 2009). In cyathostomins, BZ resistance has been associated with the polymorphism TTC to TAC, producing a tyrosine instead of a phenylalanine, in two of these codons, 200 and 167 (Drogemuller et al., 2004a; Hodgkinson et al., 2008; von Samson-Himmelstjerna et al., 2003).

The BZ-resistant associated mutation in the codon 200 of the β-tubulin isotype 1 has been used to develop a PCR assay to specifically genotype the resistant allele in cyathostomins. A field study done on Chilean horse farms showed a good correlation between this novel DNA-based assay, FECRT and the *in vitro* test, egg hatch assay. These findings support a major role of the β-tubulin codon 200 polymorphism

in the mechanism of BZ resistance in horse cyathostomins (von Samson-Himmelstjerna et al., 2002). However, the lack of molecular characterization in cyathostomin species to determine the levels of intra- and interspecies variability in the group makes it difficult to determine the real significance of these polymorphisms (Drogemuller et al., 2004b; Lake et al., 2009). An additional factor that complicates the situation even more is the already discussed abundant number of species grouped in the taxon *Cyathostominae* (von Samson-Himmelstjerna, 2012).

Mechanisms of resistance against macrocyclic lactones

Preliminary studies on abamectin-resistance in insects indicated at least two mechanisms for resistance: a decrease in cuticular penetration and increased drug metabolism. These studies together, with others in different organisms, also suggested a polygenic basis for ML resistance (Conder, 1995). Genetic studies on resistance against ivermectin in laboratory strains of the free-living model nematode *C. elegans* confirmed this polygenic nature, requiring simultaneous mutation of three genes. However, all of these three genes encode GluCl sub-units, which suggested the central role of these channels in the development of resistance to ML in this nematode (Dent et al., 2000).

More recently, a naturally occurring four amino acid deletion in the ligand-binding domain of a GluCl alpha sub-unit has been shown to confer resistance to avermectins in *C. elegans* (Ghosh et al., 2012). This confirms that polymorphisms in the GluCl sub-unit genes can act as the molecular mechanism for ML resistance in *C. elegans*, and this may have some implications in understanding the nature of this phenomenon in parasitic nematodes. However, the scenario in parasitic nematodes is much more complex and despite considerable effort invested, the genetic basis for ML resistance is still poorly understood (Wolstenholme, 2012).

Much of the effort to elucidate the genetics involved in the generation of ML resistance has been focused on parasitic nematodes of ruminants, and *H. contortus* is the best example of this. Using a genetic system of multiple backcrossing in this species, a specific locus has been linked to the resistant

phenotype, but the reduced knowledge of the full genome of this parasite hampers any possibility for further identification of specific genes (Redman et al., 2012). Nevertheless, considering their role in the pharmacology of the ML, special attention has been focused on the GluCl and the PgP (Wolstenholme and Rogers, 2005).

Mutations in the GluCl subunits have been suggested as a possible mechanism of reduced susceptibility to avermectins. Point mutations in one residue abolished ivermectin sensitivity in *H. contortus*, while mutations in the arachnid *Tetranychus urticae* induced resistance to abamectin (reviewed by Wolstenholme, 2010). Further characterization of specific sequence polymorphisms in GluCl alpha sub-unit genes in ML resistant phenotypes in parasitic nematodes have been confirmed using *C. elegans* as a genetic system (Glendinning et al., 2011). Specific variants of one GluCl alpha sub-unit, AVR-14, have been related to ivermectin resistant field isolates in the cattle nematodes, *Cooperia oncophora* and *Ostertagia ostertagi*, even though specific markers for resistance have not been determined and further research is needed before establishing the relationship of these findings with specific mechanisms of resistance (El-Abdellati et al., 2011). Molecular studies on the GluCl in cyathostomins are much more infrequent. Tandon et al. (2006) have characterized GluCl subunits in *Cc. nassatus*, but similar studies in other cyathostomin species are lacking and the role of GluCl in the development of ML resistance in cyathostomins is still poorly understood.

Despite the compelling evidence of a relationship between the GluCl and ML resistance, the participation of the PgP in conferring resistance should not be discarded. This has been suggested by molecular studies in multi-resistant strains of *H. contortus* (Williamson et al., 2011). The drug efflux capacity of these ATP-binding transporters has been associated with the development of anthelmintic resistance against BZ, closantel and ML in this major ruminant parasite (reviewed by Drogemuller et al., 2004c). Moreover, this is the only possible mechanism for ML resistance that has been explored in cyathostomins. The characteristic of the PgP sequences present in the cyathostomins have been related to

the scarcity of confirmed ML resistance reports, although still further research is needed to confirm this hypothesis (Drogemuller et al., 2004c).

Detection of anthelmintic resistance

Resistance is generally not recognized until it becomes a clinical problem (treatment failure), and when clinical drug failure happens, it is too late to implement any corrective measure (Conder, 1995). The concepts of clinical resistance and treatment failure should not be confused. A case of clinical resistance can lead to treatment failure, but treatment failure can also be caused by other reasons different from resistance (Dargatz et al., 2000). Detection of anthelmintic resistance at initially low levels is, therefore, a key concept for optimal parasite control, because it makes it possible to design and implement appropriate measures to extend drug efficacy (Matthews et al., 2012; Molento et al., 2012; von Samson-Himmelstjerna, 2012). However, assessment of anthelmintic resistance is currently poorly developed (Dargatz et al., 2000). Detection of anthelmintic resistance can be performed by four major methods: controlled test, fecal egg count reduction test (FECRT), *in vitro* bioassays or so called phenotypic tests, and molecular tests.

The controlled test requires the recovery at necropsy of the total number of worms present in after drug treatment, and comparison of the worm data of a treated group with a non treated (control) group. This is considered the gold standard for assessing anthelmintic efficacy (Duncan et al., 2002), but it has the main disadvantages that it is expensive; it is impractical for routine monitoring resistance since it requires the necropsy of the horses. Additionally, this test mainly detects only the late stages of resistance (Conder, 1995).

Because of its simplicity and practicality the FECRT is the most commonly used method to detect anthelmintic resistance to all drug classes in horse nematodes (Nielsen et al., 2010b). However, this method does not detect early stages of resistance, but only high levels of resistance and it is greatly influenced by the biological characteristics of the parasite, such as fecundity (Conder, 1995; Matthews et

al., 2012). One of the limitations of the data generated by the FECRT is the difficulty in addressing the high variability produced by several factors including individual variability between different herds and uneven distribution in parasite burdens, so that Vidyashankar et al. (2007) have suggested that improvements in the design of statistical models should be made to generate more valuable data. Despite these problems, and because of the lack of *in vitro* or molecular assays available for routine use, the FECRT is still the gold standard for detection of anthelmintic resistance in horse nematodes, including cyathostomins (Vidyashankar et al., 2007).

Molecular tests are based on the detection and analysis of molecular markers specific for resistance. They have been shown to be highly sensitive, more practical, and faster than other conventional methods (von Samson-Himmelstjerna et al., 2003; von Samson-Himmelstjerna et al., 2002; von Samson-Himmelstjerna et al., 2009), and they are considered to be the ultimate goal for detection of anthelmintic resistance. However, their implementation requires a deep knowledge and characterization of the molecular bases for resistance in field isolates, and this field is still poorly understood in almost all the major drug classes, with the only exception being the BZ (Matthews et al., 2012; von Samson-Himmelstjerna, 2012).

The *in vitro* tests, or phenotypic bioassays, for the detection of anthelmintic resistance measure the *in vitro* effects of drugs on free-living parasite stages (reviewed by Kotze at al., 2009) . The application of phenotypic tests is a valid option to monitor drug susceptibility in parasites (Kotze et al., 2009). A number of the most common assays for *in vitro* detection of resistance are based on the anthelmintic effects on development, growth and/or movement of nematode species. These assays include the egg hatch assay (EHA), the larval development assay (LDA) and the larval migration inhibition assay (LMIA) (Conder, 1995). Other *in vitro* bioassays based on different larval activities reported in the literature include the larval artificial exsheathment assay (Alonso-Diaz et al., 2011), and the larval feeding inhibition assay (Álvarez-Sánchez et al., 2005), among others.

Currently, no *in vitro* assay has been correctly validated for use in horse parasites. Among the available options, the EHA for detection of BZ resistance may be a potential option since it has been extensively validated and standardized for routine use in the major nematodes of ruminants (von Samson-Himmelstjerna et al., 2002). However, this assay has not been standardized for its use in horse cyathostomins (von Samson-Himmelstjerna, 2012), and because of the high levels of BZ resistance worldwide, the usefulness of a bioassay restricted to the BZ class in these parasite can be arguable (Matthews et al., 2012).

The LDA measures the effects on development of early L_1 stages to L_3 when exposed to different drug concentrations during a period of days. In the case of the major nematodes of small ruminants (*H. contortus, T. colubriformis* and *T. circumcincta*), the LDA is a good detector of resistance and this has been confirmed by testing known susceptible and resistant parasite isolates (Gill et al., 1995). In the case of the ML, the LDA measures the paralytic effects on pharyngeal pumping that leads to the starvation and lack of larval development (Kotze et al., 2006). A modified LDA protocol for ivermectin resistance detection has been successfully evaluated in the cattle nematodes, *O. ostertagi* and *C. oncophora* (Demeler et al., 2010b). The applicability of the LDA has also been extended to moxidectin for *H. contortus* in goats (Kaplan et al., 2007).

Tandon and Kaplan (2003) evaluated a commercially available protocol of LDA (Drenchrite®) to detect anthelmintic resistance to BZ, levamisole (which was used as a surrogate for pyrantel resistance levels) and ML in cyathostomins. For this purpose, several parasite populations from horse farms with different levels of susceptibility, as determined by FECRT, were used. A ML naïve population, Population S, which was isolated from an experimental herd at the University of Kentucky, was used as a ML sensitive control and to examine the repeatability of the test using the same population. In contrast to results obtained in two parasitic nematodes of ruminants, *H. contortus* and *Trichostrongylus* sp., the results obtained in this case demonstrated the inaccuracy of the LDA when used with cyathostomins. The test showed poor repeatability, a large variation in the IC_{50} (the concentration that inhibits the

development of 50% of the parasites) for ivermectin, and a poor correlation between the IC_{50} values and the sensitivity *in vivo* for the three classes. Different behavior of cyathostomin larvae *in vitro* to that exhibited by ruminant nematodes, and especially the complex species composition of the group may be responsible for the failure of the LDA described in this paper. Another study to validate the same commercial LDA was done in Sweden using equine strongyle larvae (>95% cyathostomins) obtained from several horse farms (Lind et al., 2005). Despite lower IC_{50} values obtained in comparison to Tandon and Kaplan (2003), the major conclusion of the authors was the same; the commercial LDA does not seem to be a useful tool for the detection of anthelmintic resistance in cyathostomins. The presence of mixed infections with multiple different cyathostomin species was again mentioned as a factor that complicates the interpretation of LDA results in horse cyathostomins.

A further group of bioassays for the assessment of anthelmintic resistance are based on larval motility. In this group we find the larval motility assay (LMA), which consists of the subjective categorization of larval movement. One protocol for the LMA has been successfully used in human hookworms (Kotze et al., 2009). One obvious flaw in this test is the subjectivity that makes standardization very difficult. The objective measurement of larval motility using a micromotility meter to detect changes in motility patterns has also been used in ruminant nematodes with relative success (Demeler et al., 2010b), however, the equipment required for this assay can be prohibitively expensive. Finally, another test in this group and the most commonly used in many studies is the larval migration inhibition assay (LMIA).

Larval migration inhibition assay

The larval migration inhibition assay (LMIA) is based on the measurement of larval migration *in vitro*. The principle of the test is the ability of larvae to migrate through a sieve after exposure to different concentrations of drugs (Conder, 1995). The use of a sieve creates a physical separation of motile larvae from non-motile larvae after exposure to drugs (Kotze et al., 2006). Since it is based on measurement of

the paralytic effects of drugs on somatic muscles, the LMIA is a suitable *in vitro* test for ML-resistance detection. This is not the case for BZ, for which this assay has been shown to be inappropriate (Demeler et al., 2010b). This bioassay has also been used for assessing anthelmintic properties in plant extracts, with variable results (Alonso-Diaz et al., 2011; Alonso-Díaz et al., 2008a; Alonso-Díaz et al., 2008b; Howell, 2009).

One of the pioneering works on larval migration inhibition assays was published by Douch et al. (1983) . In this work, a test based on the migration of ruminant nematode larvae from agar gel blocks was used to determine the paralytic effects *in vitro* of several host intestinal factors extracted from mucus. As a positive control for inhibition of migration these authors used levamisole, and it produced about 99% of inhibition of migration. Different species of parasites were used and each showed a different percentage of migration in the negative control. The main objective of this study was to determine which substances were responsible for resistance against nematodes in sheep. One major observation was that the protocol used proved to be highly sensitive to the action of paralytic drugs such as levamisole. Based on this observation, Wagland et al. (1992) developed a simplified protocol for the measurement of levamisole susceptibility in *T. colubriformis*, confirming the usefulness of this approach for detection of resistance to anthelmintic drugs with paralytic effects, such as ML. Using exsheathed L_3 of different isolates of *H. contortus,* d'Assonville et al. (1996) devised a protocol to determine the susceptibility to ivermectin using a LMIA. In this protocol, a 38 μm sieve with a layer of agar was used. Significant differences in the mean percentage of migration inhibition between isolates were detected, with a good correlation with the resistance/susceptibility status of the isolate.

Kotze at al. (2006) developed a 96-well microtitre plate based LMIA using a 20 μm sieve to detect resistance to three ML drugs: ivermectin, eprinomectin and abamectin. Isolates of *H. contortus, T. colubriformis* and *T. circumcincta* with different degrees of resistance/susceptibility were tested. The use of a layer of commercial agar on the top of the mesh was shown to increase the sensitivity of the assay and to decrease the EC_{50}. Detection of resistance was good in *H. contortus* with a high sensitivity, but

results with for *T. colubriformis* and *T. circumcincta* were not promising. These results may suggest that the LMIA can be used to measure the inhibitory effects of ML *in vitro*, different from those effects detected by the LDA, but only for some specific species.

More recently, a modified protocol of the LMIA has been developed and evaluated for the detection of ivermectin resistance in bovine nematodes (Demeler et al., 2010b). This novel version of the test included the recovery and counting of the non-migrating larvae. Consistent dose response curves were seen and a resistance ratio, product of dividing the IC_{50} of the resistant isolate by the IC_{50} of the susceptible one, of 5.5 between a resistant isolate of *C. oncophora* vs. a susceptible one was obtained. In general, the protocol reported clearly showed the potential of the LMIA to assess and measure ivermectin resistance in this cattle parasite. The same protocol was taken to the field to monitor development of ivermectin resistance in a Belgian cattle herd. The conclusions of this work reinforce the usefulness of this phenotypic test (El-Abdellati et al., 2010).

Because of the lack of standardization and uniformity among the published protocols, analysis of repeatability and reproducibility of LMIA results from different laboratories using the same assay on the same parasite have been missing. Additional standardization in different research laboratories (ring testing) would be required before the LMIA could be adopted as a routine test for resistance detection worldwide. To address this issue, the protocol for the LMIA described by Demeler et al. (2010) for detection of ML-resistance in *C. oncophora* and ML-susceptibility in *O. ostertagi* was used in a ring test in parasitology laboratories from several European countries. The data generated by this study indicated a high reproducibility of the protocol and the capacity for detection of resistance in *C. oncophora* even at low resistance ratios (Demeler et al., 2010a).

The LMIA has been tested for *in vitro* assessment of emerging ML resistance in cyathostomin populations with different protocols and different preliminary results. McArthur (2007) developed a protocol of LMIA for detection of moxidectin resistance in cyathostomins. The protocol consisted of incubating exsheathed larvae in different moxidectin concentrations for 2 hours and then allowing them to

migrate through a 20 μm mesh for a period of two hours at 30°C. Pools of cyathostomin larvae from two groups of donkeys, one group suspected to be resistant to moxidectin, and the other known to be susceptible to the same drug, plus larvae from a group of horses with a susceptible cyathostomin population were included. Because there was no known resistant strain of cyathostomins available, an ivermectin resistant isolate of *H. contortus* was included as a positive control for resistance. The analysis of the dose response curves suggested that the assay yielded fairly consistent results. Statistically significant differences between the susceptible and the suspected resistant cyathostomin populations were found, and, together with previous reports of shortened ERP in another closed donkey population, strongly suggested that the LMIA had promising potential as a detector of ML resistant in horse cyathostomins.

At the same time, but independently, a similar format of the LMIA using a 30 μm mesh and a 48 well-plate was standardized, testing three avermectin analogs, eprinomectin, ivermectin and doramectin, in three different horse cyathostomin populations. A modification in the exsheathment procedure was introduced when compared to McArthur's protocol (McArthur, 2007), with washing of the exsheathed larvae on filter paper to try to reduce losses produced by repeated centrifugation. The major conclusions of this work were: a better performance of DMSO as a drug solvent, up to a maximum concentration of 0.5% when compared with Tween 20; and no effect of temperature in the performance of the test when 30°C was compared to 24°C. Regarding the incubation solution, 0.85% saline and Earle's balanced salt solution both showed a better performance than distilled water, especially at higher drug concentration. This was because the solutions more closely resemble physiological ionic concentrations. The analysis of the dose response curves obtained showed a significantly better performance of eprinomectin and doramectin than ivermectin, with good consistency when used in a ML-naïve population. Only eprinomectin showed a significant difference between two populations, which strongly suggested that it should be evaluated further. However, high levels of variability between results from different ML-susceptible populations and inconsistency in the migration in control wells were the major drawbacks observed. A lack of larval motility leading to low migration rates in the negative controls also demanded

further investigation. In general, these results, although encouraging, needed further validation and research before a working protocol could be fully established (Monopoli, 2007).

More recently, van Doorn et al. (2010) have used a modified version of the LMIA protocol developed by McArthur (2007). Exsheathed L_3 were incubated in different concentrations of ivermectin for two hours. After the incubation period, larvae were allowed to migrate through a 40 μm nylon mesh. No statistically significant differences were reported between two different cyathostomin populations with different ML exposure history, one ML-naïve population vs. one with a history of frequent exposure to ML treatments. Most notably, the ivermectin selected population presented a significantly lower IC_{50}. Similar to previous reports, this study also found very low migration percentages in the absence of drug, which ranged from only 46 to 64%. Despite ignoring any correlation between reduced migration and long incubation periods, this finding suggested that natural inhibition of migration in negative controls was one of the major limitations of the LMIA protocols in cyathostomins.

However, even more informative data were generated in this study when a modification of the protocol was used to perform a reiterative migration at two defined concentrations of ivermectin: 0.24 and 30 μg/ml. The protocol was combined with the molecular identification of larvae by the RLB protocol developed by Traversa et al. (2007) and was used to perform *in vitro* selection with ivermectin in both parasite populations. In relation to species composition, the reiterative LMIA did not select for any particular species in absence of drug and the L_3 species included in the RLB oligoprobes were found to be 3 species, *Cy. catinatum, Cs. longibursatus* and *Cc. nassatus*, which clearly dominated both parasite populations. The *in vitro* selection with ivermectin induced significant changes in the species composition of both populations and large differences in susceptibility between the species were detected. *Cy. catinatum* was by far the most prevalent species after the *in vitro* selection, even in the naïve population, while *Cc. nassatus* declined significantly only in one population. This last finding confirmed that differences in drug tolerance between both populations exist, even among members of the same species. Even though the authors have suggested what the implications might be with regard to selection for

resistance *in vivo*, and concerning the pre-existence of resistant phenotypes in cyathostomin populations, the most important conclusions that can be drawn from this study are the existence of different levels of inherent tolerance to ML *in vitro* among particular species of cyathostomin larval stages that can alter the results of *in vitro* tests, such as the LMIA.

On the data discussed here, it can be concluded that at the present time, no bioassays for detection of ML resistance in horse nematodes have been fully validated. In the case of cyathostomins, and in contrast to the case in ruminant nematodes, one major complication in the optimization and validation of *in vitro* tests is the presence of multiple species that can be identified at larval stages only with the use of molecular tools. Therefore, the influence of species composition on bioassays should be considered and further investigated due to the species different level of natural tolerance to drugs (Matthews et al., 2012).

Cyathostomin populations used in this study (Population S and Population Mox)

A closed experimental herd of Shetland type ponies was established in 1974 at the University of Kentucky (Lyons et al., 1996a). The structure of the herd has varied since then from 25 to 35 females and one stud. Male foals and some replacement females born in the herd have been used to monitor the parasite fauna using critical tests over the years (Lyons et al., 2001). The population of cyathostomins infecting this herd has been given the designation Population S (Pop S) (Lyons et al., 1996a). Beginning in 1974, all ponies in the herd were treated every 8 weeks with a BZ drug for 22 years; first with cambendazole for 4 years and then oxibendazole for 18 years. The primary objective of this original long-term experiment was to study the effects of BZ treatment on the cyathostomin species prevalence in this population (Lyons et al., 1996b).

Differences in the rate of resistance development between drugs were observed, with resistance against cambendazole developing faster than resistance to oxibendazole. Critical tests identified six species to be resistant to BZ: *Cy. catinatum, Co. coronatum, Cc. nassatus, Cs. longibursatus, Cs. goldi* and *Cs. calicatus*. The evolution of levels of resistance in specific species had different patterns over time

and some differences in susceptibility against different BZ drugs among species was also reported (Lyons et al., 1996a), further supporting the notion that different species may exhibit different levels of susceptibility to specific drugs. The dynamics of species composition in the cyathostomins of Pop S during this period included 28 species grouped into 8 genera. This was similar to that reported for cyathostomin infections in the same geographical area. *Cy. catinatum* was the most prevalent species (96%), and four other species: *Cs. longibursatus, Cs. goldi* , *Cc. nassatus* and *Cs. calicatus* were present in more than 80% of the animals. Another four species, *Co. coronatum, Cc. leptostomum, Cs. minutus* and *Cc. insigne*, had prevalences of between 40 and 60%. Sixteen additional species had less than 40% prevalence and three species were rarely found (<1%). The relative intensity of infection among the various species revealed the same typical pattern that is observed worldwide, with *Cs. longibursatus, Cy. catinatum* and *Cc. nassatus* accounting for more than 80% of the total worm population. Six species accounted for most of the remaining worms (19%), and 19 other species accounted for only about 1% of the total. The six previously reported as BZ resistant species were among the 9 dominant species. In general, no dramatic effect of the long term BZ treatment on the dynamics of Pop S was detected (Lyons et al., 1996b).

Starting in 1992 and for a period of 7 years, a bimonthly treatment with pyrantel was applied to the herd. This new anthelmintic treatment scheme did produce some changes in the species composition of cyathostomins, with *Cc. nassatus, Cs. calicatus* and *Cs. minutus* as the new dominant species. Four species developed pyrantel resistance, and from this group only *Cs. minutus* had not been previously reported as BZ resistant. This study confirmed that different levels of susceptibility/resistance may be exhibited by individual cyathostomin species to different anthelmintics (Lyons et al., 2001). Even prior to long-term exposure to the drug, only an average of 72% efficacy for pyrantel was detected in two animals at the end of the 18 years of BZ selection (Lyons et al., 1996a). This may be indicative of a natural tolerance phenomenon or of resistance that had developed previously in the population (Lyons et al., 2001). A similar case was reported by the same authors in a small strongyle population in the same geographical area (Drudge et al., 1988). In this case, pyrantel showed a lower percentage for efficacy than

reported previously in other areas and resistance had developed at a relatively faster rate. One possible reason in this particular case was the development of a multiple resistance matrix in the parasite population, created by a long standing anthelmintic resistance, to piperazine, in this case (Drudge et al., 1988). Other possible explanations may include a previous non recorded exposure to the drug on pasture; pyrantel salts were introduced in the 1970's and they have been used in a daily, low-level dosage, in-feed formulation for a long time (Lyons et al., 1999). Despite having a different mechanism of action from the BZ drugs, involving nicotinic receptors as the pharmacological target, pyrantel shares this mechanism of action with levamisole (Kopp, 2009).

In 2000, a small group of 9 ponies, 7 males and 2 intact males, were separated from the Pop S herd and placed on a small pasture in order to begin an experiment on moxidectin selection. A sub-therapeutic dosage of 0.05 mg/kg was administrated orally. The Pop-S cyathostomin population infecting these ponies, now under ML selection, was designated as Population Mox (Pop Mox). The frequency of moxidectin treatments and the number of animals treated have varied during the experiment. From the beginning of the drug selection experiment, from February 2000 to May 2001, a total of 15 treatments, mostly at monthly intervals, were applied to all 9 animals in the herd. After a period of 5 months without any drug treatment, and from October 2001 to August 2005, 13 bimonthly drug treatments were applied to some specific individuals, selected on the basis of a positive FEC. In this way, mostly 5 out of the 9 animals were regularly treated during this period. From September 2005 to November 2006, a monthly interval was used again (with a break of 3 months between November 2005 and February 2006), but again selecting only ponies with positive FEC. Thus, only 3 out of the 13 treatments administered covered the 9 animals of the group, and mostly 1 to 6 animals were treated regularly. In January 2007, the drug selection was restarted at an interval of 2 to 3 months until November of the same year. Most of these treatments covered all the ponies in the herd. In 2008, and after a pause of 6 months, from July through November 2008, just 3 bimonthly treatments were applied to most of the ponies. In May 2009, the dosage of moxidectin was increased to 0.1 mg/kg. From the period between May 2009 and July 27, 2010, date of the last recorded treatment in the group, only 7 treatments were applied. Since a positive FEC criteria

were also used for treatment, only one treatment, done in March 2010, comprised all the animals in the herd (n=10). The number of ponies in the herd has been kept mostly constant at 9 animals, with new animals added to the herd as pony mares have foaled and some of the original members have been taken out of the experiment for different reasons. From June 2007 to September 2008, the number dropped to 8, and in March 2010, the number increased to 10 ponies.

Regarding FEC, the average has always been low to moderate since the beginning of the selection experiment with only a few isolated cases having high values. Moreover, true clinical resistance has never been detected in the herd since the percentages of reduction rarely showed suspected resistance. For example, during the years 2009 and 2010, the mean reduction in FEC reduction showed no resistance, with 100% reduction in FEC for most of the animals. Only in one case with less than 90%, however, its pretreatment FEC was too low to draw strong conclusions. Additionally, the sub-therapeutical dose, two-fold lower than that recommended for use in horses, used in this herd discards any possible case of resistance. The effect of the moxidectin selection on the genetic diversity of the parasite population was evaluated by amplified fragment length polymorphism (AFLP) in individual *Cc. nassatus* specimens collected from Pop Mox. When compared to Pop S (Tandon et al., 2005). A reduced level of genetic polymorphism caused by the drug pressure was detected, but it was not possible to relate these genetic changes to the emergence of resistance. This study also confirmed the high level of inherent genetic polymorphism in a cyathostomin population No details on the species composition changes induced by the moxidectin selection were described in this work or in any other related publication.

Conclusions

Cyathostominae nematodes are the most important parasite of horses worldwide. More than 50 species have been described in this group and the presence of multiple species complicates the knowledge on epidemiology and diagnosis of infection. Because of difficulties in the morphological identification of free living stages, molecular approaches are considered the most efficient tool for species identification.

Use of anthelmintic drugs is the only available practical control option and only three major drug classes are used: benzimidazoles (BZ), tetrahydropyrimidines and macrocyclic lactones (ML). Resistance to the BZ and tetrahydropyrimidines has been extensively reported worldwide while the ML currently remain still highly effective. However, the increasing number of suspected ML-resistance cases and the first reports of confirmed cases of ML resistance in cyathostomins constitute a serious threat to the horse industry.

Early detection and measurement of ML-resistance is the best way to monitor the effectiveness of control strategies and prevent the spread of resistance. Because of the inherent drawbacks in the standard *in vivo* assays and the lack of molecular tools available, *in vitro* bioassays are the most attractive option for detecting early stages of resistance development. Among the *in vitro* assays previously developed, the larval migration inhibition assay (LMIA) is the most promising option, but more research is needed to fully validate this approach. One major complicating factor in the validation of *in vitro* tests for cyathostomins is the presence of multiple species, which may have different levels of innate *in vitro* tolerance to ML drugs. Thus, determining the influence of species composition on the results of bioassays should be considered and further investigated. The objective of the present study was to optimize and validate an LMIA protocol to detect and measure levels of resistance/susceptibility to ML in cyathostomin populations, and determine the impact that multiple cyathostomin species have on the interpretation of the results of the LMIA.

CHAPTER 2

INTRODUCTION

Infection with cyathostomins is major cause of parasitic disease in horses with high prevalence worldwide and producing clinical and subclinical problems (Cutolo, 2011; Kornas et al., 2010). These parasites have a direct life cycle and horses get infected by ingestion of infective third stage larvae (L_3) on pastures (Lind et al., 2003). The use of anthelmintic drugs is the only available option for control. In horses, only three classes of drugs are routinely used, benzimidazoles (BZ), tetrahydropyrimidines (pyrantel) and macrocyclic lactones (ML) (Conder, 1995). There is abundant evidence of anthelmintic resistance in cyathostomins against BZ and tetrahydropyrimidines (Kaplan and Vidyashankar, 2012). The ML, such as ivermectin and moxidectin, are still considered to be highly effective against cyathostomins. However, there are increasing reports of a reduction in the egg reappearance period (ERP) after treatment with ML, indicating a progressive loss of effectiveness (von Samson-Himmelstjerna, 2012). Moreover, resistance against moxidectin has already been reported (Molento et al., 2012). Therefore, there is a need for routine monitoring of the drug efficacy on farms where ML based products are used with high frequency (Nielsen, 2012). The early detection and measurement of drug resistance is the only way to evaluate the effectiveness of control strategies for the prevention and control of resistance in horse populations (Kaplan and Nielsen, 2010).

The traditional method for detection of anthelmintic resistance under field conditions is the fecal egg count reduction test (FECRT). This *in vivo* method has been used widely in studies to characterize resistance in cyathostomin populations (Kaplan et al., 2004; Kuzmina and Kharchenko, 2008; von Samson-Himmelstjerna et al., 2007). However, this test does not detect early stages of resistance and

evidence suggests that it only detects cases where high numbers of resistant individuals occur in the population. It is greatly influenced by the biological characteristics of the parasite (Matthews et al., 2012) and the accurate analysis and comparison of the data generated are difficult because several variables cannot be effectively controlled (Vidyashankar et al., 2007). Use of molecular techniques for the detection of resistance purpose is not yet a viable option because of a lack of knowledge of the molecular mechanisms of anthelmintic resistance in the main drug classes, with the exception of the BZ (von Samson-Himmelstjerna, 2012). Based on this, we can conclude that phenotypic bioassays are for now the best option for monitoring the emergence of ML resistance in equine cyathostomins. Several *in vitro* methods, such as egg hatch assays (EHA), larval development assays (LDA) and larval motility assays, have been successfully validated to detect anthelmintic resistance in parasitic nematodes of ruminants (Conder, 1995). However, among these options, only the larval migration inhibition assay (LMIA) has been shown to have potential as an alternative test for ML resistance in cyathostomins (McArthur, 2007; Monopoli, 2007), but further research and validation for the LMIA is needed before its routine use can be recommended in cyathostomins.

The epidemiology of infection with cyathostomins is still poorly understood. Cyathostomins consist of a complex group of 51 species, although the large majority of infections are made up of about 10 of the most common species (Traversa et al., 2009b). Studies done on this issue have showed that mostly 4 to 10 species predominate in infected animals (Gasser, 2004) although, using molecular approaches, up to 13 species have been identified in horses in Europe (Traversa et al., 2010). Adult cyathostomins can be identified to the species level only by a few authorities using comparative morphology. Larval stages are exceptionally difficult to identify and eggs are impossible to identify even to the subfamily level (Lichtenfels et al., 2008). DNA-based molecular approaches have been developed for this purpose, targeting specific regions of the parasite genome (Gasser, 2004). A reverse line blot (RLB) hybridization test has been successfully developed based on specific probes within the intergenic spacer (IGS) region. This assay proved the validity of molecular tests based on genomic ribosomal DNA (rDNA) to identify simultaneously most of the common equine cyathostomin species (Ionita et al., 2010). The second

internal transcribed spacer region (ITS-2) genomic sequences have shown potential as genetic markers for species identification of cyathostomin species (Gasser, 1997; Hung, 1999b) and they have been successfully used in the molecular identification of cyathostomin larval (L_3 and L_4) stages (Brianti et al., 2009). The accurate identification of cyathostomin species involved in drug resistant cases is very important to increase the understanding of the biology and epidemiology and the pathogenesis associated with these parasites (von Samson-Himmelstjerna, 2012). Moreover, determining the influence of species composition on the performance of bioassays because of the nematodes different level of natural tolerance to drugs, should be considered and further investigated (Matthews et al., 2012). The development of further molecular tools for future research on cyathostomins needs to focus only on the major species that have proved to be important in terms of prevalence and/or anthelmintic resistance.

The main goal of this study was to optimize and validate an LMIA protocol to detect and measure levels of ML susceptibility/resistance in cyathostomin populations. A second aim was to evaluate the impact of cyathostomin species composition on the LMIA performance, by using a novel molecular approach for species identification, based on sequencing of the ITS-2 region using a 454 massive parallel pyrosequencing platform.

CHAPTER 3

MATERIALS AND METHODS

Parasite isolates

Population S

Six adult male ponies from the Population S (Pop S) experimental herd (Lyons et al., 1996a) at the University of Kentucky (Lexington, Kentucky) were moved to the University of Georgia campus (Athens, Georgia) in November, 2010. These animals have never been treated with any ML analog. Due to the ML-naïve status of the cyathostomin population infecting them, Pop S parasites were considered to be as a ML-susceptible population in the performance of the bioassays. The ponies were located in a pasture belonging to a former sheep production unit. This pasture had never been used for pasturing horses before and was therefore considered to be cyathostomin-free. The experimental animals were identified with letters (A to F) based on coat color and particular markings. Periodically, fecal samples were collected in a non-invasive way. This method was used because of the untamed nature of these animals, which did not allow proper handling. Recently shed fresh feces were collected from the ground, and only the top part of the fecal pile was sampled to avoid any possible contamination with free-living nematodes on the ground. Animals were sampled from November 2010 to June 2011. Samples were taken to the laboratory in labeled polyethylene bags inside a plastic cooler with ice packs. Samples were kept at 4°C until subjected to fecal egg count (FEC) and coproculture.

Population Mox

Fecal samples were rectally collected from ponies numbered 11, 33 and 45 in the Population Mox (Pop Mox) (Tandon et al., 2005) located in an experimental pasture of the University of Kentucky (Lexington, Kentucky) from June 2010 to July 2011. These animals were selected based on their consistently positive FEC results. The ponies in Pop Mox were originally derived from Pop S. However, they had been periodically treated with moxidectin at sub-therapeutical levels from 2000 to 2010. Because of this repeated exposure to moxidectin, these parasites were considered to be a ML-selected population in the experiments. The last moxidectin treatment had been given on July 27, 2010. Samples collected during a period of 4 to 5 consecutive days were mailed overnight to the laboratory in airtight bags placed in boxes with ice packs. Upon arrival, the samples were stored at 4°C until they were used for FEC and coproculture.

Population Snyder Barn

Standardization experiments were performed with third stage cyathostomin larvae (L_3) derived from coprocultures of fecal samples collected rectally from naturally infected mares of the horse farm, Snyder Barn, of the University of Georgia (Athens, Georgia). This group of animals had a previous history of periodic treatments with ivermectin and other anthelmintic drugs. The cyathostomin population present on this farm has shown high susceptibility to ML-drugs. However, since the parasites obtained from this population were used for standardization experiments and not for drug dose-response assays, the naïveté/exposure of the isolate was irrelevant. Samples were taken to the laboratory in a plastic cooler with ice packs in labeled polyethylene bags. Samples were kept at 4°C until use for FEC and coproculture.

Fecal egg count, coproculture and recovery of third stage larvae (L_3)

A FEC using a modified McMaster technique was performed to have an estimate of the eggs per gram (epg) of fecal material. This modified protocol has been developed in our laboratory. Four grams of feces were homogenized in 26 ml of sodium nitrate and strained through 2 layers of cotton woven cheesecloth with a pattern of 44x36 (Electron Microcopy Sciences, Hatfield, Pennsylvania, US). The solution was loaded into a 3-chamber modified McMaster counting slide (Focal Point, South Africa). Since the final concentration of feces in the 30 ml suspension was 0.133 g/ml and each chamber holds a volume of 0.15 ml, the amount of feces contained in each chamber was 0.05999 g. Since 1 g divided by 0.05999 is equal to 16.67, the total eggs counted in the 3 chambers were multiplied by a conversion factor of 16.67 to obtain the epg value. The FEC data were used to determine the numbers of larvae expected to be recovered and, based on the actual number recovered, to obtain the rate of recovery (Table 3.1). For the coprocultures, the feces were placed into a 1-gallon metal bowl and mixed with an equal amount of commercially available high quality aggregate gardening vermiculite (Ray Brown Enterprises, Woodruff, South Carolina, US). Distilled water was added to obtain a homogeneous, moistened mixture. The coprocultures were covered tightly with foil. Small holes were made with a pair of clean scissors for ventilation purposes. The coprocultures were labeled and incubated at room temperature (~ 21°C) for 10 days. The coprocultures were stirred and sprayed with distilled water every two days in order to keep them moistened. After the incubation period, the coprocultures were placed in a modified large-scale Baermann apparatus for larval recovery. The fecal culture was wrapped in one layer of cheese cloth and one layer of Kimwipes® (Kimberly-Clark Corp., Irving, Texas, US) and placed in a large metal sieve inside a big metal Baermann funnel. Lukewarm tap water was poured into the funnel until the coproculture was entirely covered. The coproculture was left to sit overnight. Larvae were collected from the bottom of the rubber hose of the Baermann apparatus by opening the valve at the end of the funnel and collecting about a liter of fluid in a pharmacological, graduated 1-L polypropylen beaker. The beaker was kept at 10°C for 6 to 12 hours to allow the larvae to sediment to the bottom. After siphoning off the supernatant, the sediment was transferred to a 50-ml polypropylene centrifugation tube. The larval

suspension was transferred to a small Baermann apparatus assembled with one layer of cheese cloth and one layer of Kimwipes®. The purpose of this second baermannisation procedure was to get rid of small contaminating particles. Three aliquots of 10 µl were observed microscopically for confirming that contaminants were absent, for positive identification of the L_3 as cyathostomins, based on their morphological characteristics (total length and number and shape of intestinal cells; Monopoli, 2007), and for determining the approximate concentration of L_3 in the suspension. Larvae were stored at 10°C in a labeled 50-ml polypropylene centrifugation tube until use. Only larvae less than one month old were used for the assays.

Pre-baermannisation of L_3

The night before an assay was performed, a Baermann apparatus was assembled using a layer of Sefar Nitex® nylon mesh with a 25 µm pore of the same material used for making the migration wells (see below) and lukewarm distilled water. The larval suspension was warmed to 37°C and vortexed vigorously for 15 seconds before it was poured into the Baermann apparatus. The larvae were allowed to migrate through the mesh for at least 15 hours. Before use, three aliquots of 10 µl were counted and checked to establish the larval concentration and to confirm larval motility. If possible, the final volume of the larval suspension was reduced to the desired concentration of larvae, e.g., for one migration assay, a concentration of about 4000 to 6000 larvae per ml was used.

Exsheathment of L_3

Considering the losses that occur after exsheathment, at least 3000 to 4000 larvae were required to be exsheathed to guarantee sufficient larvae for one assay. The larval suspension was incubated at room temperature (~ 21°C) for 10 to 20 minutes. One to three ml of the larval suspension (in distilled water) were poured into a 15-ml polypropylene centrifuge tube. Seventy-five microliters of a 2% sodium

hypochlorite solution per each ml of larval suspension was added. To make the 2% sodium hypoclorite solution, a 6% solution (commercial bleach; Clorox, Pleasanton, California, US) was diluted 1:3 in distilled water. Immediately after adding the 2% sodium hypochlorite solution, the suspension was vortexed vigorously for 15 seconds and incubated in a heated dry bath block (VWR, Scientific Products, Suwanee, Georgia, US) at 40°C for 3 minutes. During the incubation, the solution was vortexed twice. After the incubation, the suspension was transferred to a new 3-ml (or 5-ml, if a greater volume was required) disposable plastic syringe. Using the syringe, the suspension was forced to pass through a 5 µm Whatman® cellulose nitrate membrane filter disc (Whatman, Miami, Florida, US) held in a Millipore Swinnex-25® heartworm filter holder. The membrane filter, with the exsheathed larvae on it, was rinsed with phosphate buffered saline (PBS; pH 7.4; Sigma, St. Louis, Missouri, US), using a 1000-µl manual pipette (Pipetman®, Gilson, Middleton, Wisconsin, US) and the suspension poured into a 15-ml polypropylene centrifugation tube, using a small plastic funnel. The filter disc was then placed in a 55 mm x 15 mm small Petri dish using a pair of tweezers and washed in 2 ml of PBS. The washing thus obtained was then poured into a 15-ml centrifugation tube. The concentration of larvae and their exsheathed status was confirmed, examining three aliquots of 10 µl microscopically on a glass slide. The optimal concentration for the migration assays was approximately 60 to 100 larvae per 100 µl. The concentration of larvae was corrected as needed by diluting the larval suspension or by changing the volumes of media used during the exsheathment process.

Table 3.1: Data from corpocultures from the parasite populations used in this study. Date of collection of pooled fecal samples, average FEC (in epg), amount of feces cultured (in g), number of L_3 expected to be recovered, actual number of L_3 recovered and rate of recovery from each of the three parasite populations.

Pop Snyder					
Date collected	**FEC**	**Amount (g)**	**Expected**	**Recovered**	**Rate of Recovery**
1/20/2010	1255	895	1123224	101090	9.00%
1/20/2010	367	860	315907	1580	0.50%
2/16/2010	185	1450	267784	40837	15.25%
2/16/2010	185	210	38784	10006	25.80%
2/17/2010	1050	574	602700	230834	38.30%
2/24/2010	467	1077	503019	67405	13.40%
3/8/2010	1330	1200	1596610	47100	2.95%
6/3/2010	580	634	368202	4418	1.20%
6/3/2010	1039	1156	1200650	45625	3.80%
7/16/2010	538	1200	645600	12912	2.00%
9/13/2010	440	500	220000	26840	12.20%
10/10/2010	1989	807	1605123	43338	2.70%

Pop S					
Date collected	**FEC**	**Amount (g)**	**Expected**	**Recovered**	**Rate of Recovery**
11/3/2010	517	580	299860	9596	3.20%
11/13/2010	167	476	79492	19635	24.70%
11/13/2010	200	675	135000	50625	37.50%
12/30/2010	468	792	370725	143100	38.60%
1/21/2011	507	887	449709	162795	36.20%
3/3/2011	494	793	391742	43092	11.00%
3/15/2011	607	869	527483	163520	31.00%
3/21/2011	110	648	71280	8981	12.6%
3/23/2011	380	813	308940	191542	62.00%
4/10/2011	586	528	309353	86000	27.80%
5/16/2011	1125	786	884250	70740	8.00%
5/23/2011	311	640	199040	86582	43.50%
5/23/2011	257	84	21588	2526	11.70%
6/10/2011	878	512	449612	58000	12.90%

Pop Mox					
Date collected	FEC	Amount (g)	Expected	Recovered	Rate of Recovery
6/30/2010	125	332	41600	18720	45.00%
6/30/2010	117	266	31200	6989	22.40%
6/30/2010	55	453	25080	4489	17.90%
6/30/2010	46	409	18696	841	4.50%
9/16/2010	83	440	36520	3652	10.00%
9/16/2010	183	380	69540	13004	18.70%
10/15/2010	1432	427	611464	67017	10.96%
10/16/2010	1600	458	732880	32980	4.50%
10/17/2010	12313	448	5516129	342000	6.20%
10/18/2010	1551	295	457627	54000	11.80%
1/2-3/2011	1184	450	532822	42626	8.00%
1/4-6/2011	824	566	466446	57840	12.40%
3/21-25/2011	135	563	76166.7	45929	60.30%
4/4-6/2011	1129	628	708772	202000	28.50%
05/28-06/01/2011	1161	1046	1214406	173660	14.30%
06/06-10/2011	300	2934	880200	22005	2.50%
06/06-10/2011	1833	2014	3691662	310100	8.40%

Categorization of cyathostomin L_3 motility

A subjective scale to categorize larval motility was developed as described in Table 3.2.

Table 3.2: Categories for evaluation of motility of exsheathed cyathostomin L_3.

Category	Description
1	Highly active larvae with constant and fast movements.
2	Larvae with jerky, slow movements, or twitching. Also coiled but moving larvae
3	Larvae straight or coiled. Not moving at all.

The larvae were observed individually under an Eclipse TS 100 inverted microscope (Nikon, Shinjuku, Japan), at a magnification of 40X for a period of approximately 5 seconds and classified into one of the three categories.

Evaluation of the effect of different media on cyathostomin L_3 motility

After being exsheathed, cyathostomin L_3 were suspended in any of three different media: phosphate buffered saline (PBS; pH 7.4; Sigma); phosphate based buffer solution (pH 6.2; Harleco, EMD Millipore) with potassium phosphate monobasic, sodium phosphate dibasic and sodium azide (Merck KgaA, Darmstadt, Germany); Earle's Balanced Salt Solution (Sigma); or Bio Whittaker® RPMI-1640 (with L-glutamate; Lonza BioWhittaker, Basel, Switzerland). The concentration of larvae was adjusted to approximately 20 larvae per 100 µl of suspension. One hundred microliters of larval suspension were plated in 6 replicates, in a 24-well, cluster, flat bottom, treated, non-pyrogenic polystyrene, sterile tissue culture plate (Costar®, Corning Inc., Corning, New York, US). One hundred microliters of media were added. Once plated, samples were shaken gently for 15 seconds and then kept in darkness (in a drawer) at room temperature (~ 21°C) for 2 or 22 hours. After the incubation period, the plates were examined under an Eclipse TS 100 inverted microscope (Nikon) where the larvae were categorized at a magnification of 40X. Approximately 100 larvae per treatment were categorized in each experiment.

Evaluation of the effect of different time of incubation on cyathostomin L_3 motility

Exsheathed L_3 were suspended in PBS (pH 7.4; Sigma). The concentration of larvae was adjusted to approximately 20 larvae per 100 µl of suspension. A hundred microliters of larval suspension were plated in 6 replicates in a 24-well tissue culture plate of the same type as described above. One hundred microliters of PBS were added. Samples were shaken gently for 15 seconds and then kept in darkness (in a drawer) at room temperature (~21°C) for an incubation period of 2, 4 or 22 hours. After the incubation period, the plates were examined under an Eclipse TS 100 inverted microscope (Nikon) where they were categorized at a magnification of 40X for larval motility. Approximately 100 larvae per treatment were categorized in this way.

Drugs

Stock solutions of ML analogs

Drugs in a powdered form [eprinomectin, ivermectin and abamectin (Sigma) and moxidectin (Fort Dodge Animal Health, Princeton, New Jersey, US)] were dissolved in 100% dimethyl sulfoxide (DMSO; Sigma) to make stock solutions of 10 mM, as described in Table 3.3.

Table 3.3: Composition of 10 mM drug stock solutions.

Drug	Molecular weight	Powder dissolved in 1 ml of DMSO
Moxidectin	639.82 g/mol	6.4 mg
Eprinomectin	914.14 g/mol	9.1 mg
Ivermectin	875.10 g/mol	8.8 mg.
Abamectin	873.11 g/mol	8.7 mg.

After dissolving the powder in DMSO, the suspension was vortexed vigorously for 10 minutes. The final solution was stored at -80°C until use.

Working solutions (100x) and plate (2x and 1x) drug solutions

The protocol to make 7 two-fold dilution concentrations of each of the ML analogs in DMSO at 100 times the final concentration in the plate, the working solutions, is described in Table 3.4.

Table 3.4: Composition of drug working solutions.100 times the final concentration.

In plate	100x	Procedure
20 µM	2 mM	400 µl of the 10 mM stock to 1600 µl of DMSO
10 µM	1 mM	1 ml of the 2 mM solution to 1 ml of DMSO
5 µM	0.5 mM	1 ml of the 1 mM solution to 1 ml of DMSO
2.5 µM	0.25 mM	1 ml of the 0.5 mM solution to 1 ml of DMSO
1.25 µM	0.125 mM	1 ml of the 0.25 mM solution to 1 ml of DMSO
0.625 µM	0.0625 mM	1 ml of the 0.125 mM solution to 1 ml of DMSO
0.3125 µM	0.03125 mM	1 ml of the 0.0625 mM solution to 1 ml of DMSO

These working solutions were stored at 4°C in 2-ml vials covered in foil, to protect them from light, up to 15 days before being used. The working solutions were diluted in PBS to obtain a concentration of 2x the final plate concentration for the incubation drug solutions, and 1x for the migration drug solutions. An example of the composition of the incubation and migration solutions for one LMIA assay is described in detail in Table 3.5.

Table 3.5: Composition of incubation and migration drug solutions.

Incubation solution (2x)	**Migration solution (1x)**
392 µl PBS	5940 µl PBS
8 µl working solution	60 µl working solution
400 µl final volume	6000 µl final volume

For the control solution (final plate concentration of 1% DMSO in PBS), the working drug solution was replaced by pure DMSO. These incubation and migration solutions were stored at 4°C, but they were used within hours of being made. These solutions were shaken frequently before being used.

Larval migration inhibition assay

The 24-well plate design for the LMIA with 7 drug concentrations and one control, and three replicates per treatment is shown in Figure 3.1.

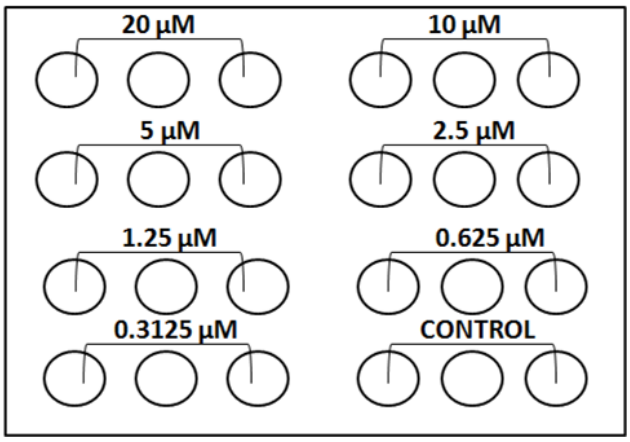


Fig 3.1: Experimental design for the 24-well plate larval migration inhibition assay

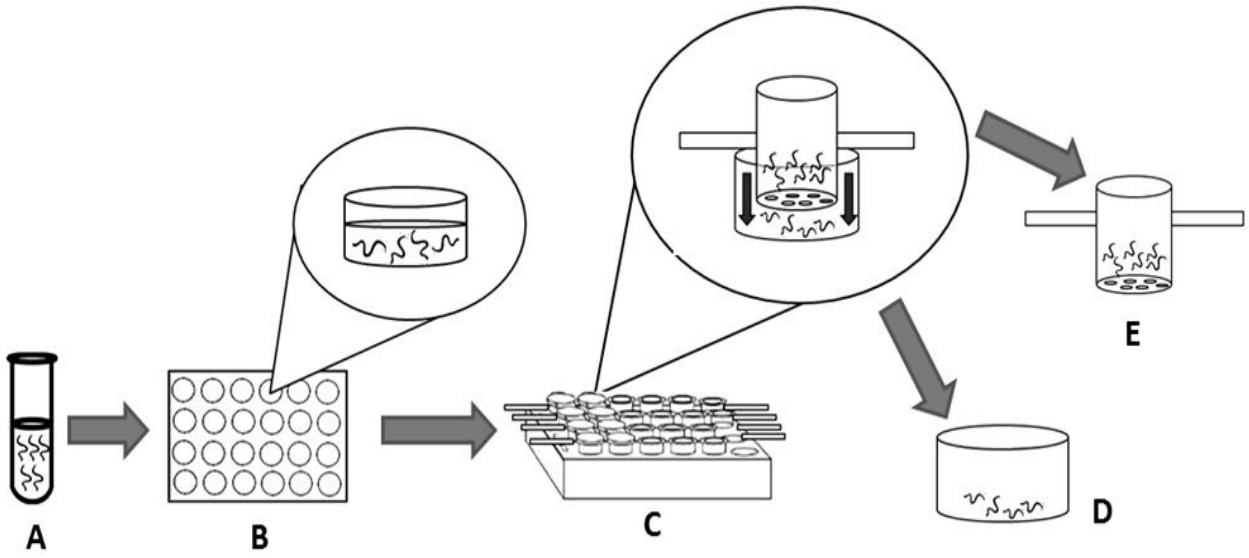


Fig. 3.2: Diagram of the larval migration inhibition assay protocol. **A**, Cyathostomin L3 were exsheathed. **B**, exsheathed L3 were incubated in a 24-well plate for two hours at ~21°C, in different drug concentrations. **C**, L_3 were transferred to migration sets, assembled on a 24-well plate, and they allowed to migrate for 45 minutes at 37°C. D, migrating L3, in the bottom of the migrating plate were counted. E, non migrating L_3 were collected from the migration sets and counted. For details, refer to the text.

Incubation

Using a 200-µl manual pipette (Pipetman®, Gilson), 100 µl of exsheathed L_3 suspension was pipetted into each of the corresponding wells of a 24-well culture plate. The tube containing the larval suspension was vortexed vigorously for 15 seconds after 3 wells had been pipetted to ensure the homogeneous distribution of larvae in the wells. Using a 1000-µl electronic digital pipette (Rainin, Oakland, California, US), 100 µl of the incubation solution was added to each well following the plate layout described in the experimental design (Fig. 3.1). The drug solution was also vortexed vigorously after a series of 3 replicates had been pipetted to ensure the homogeneity of the solution. After pipetting the incubation solution, the plate contents were gently mixed by moving the plates from side to side. The plate was then shaken in a TSZ-S-04 mini microplate shaker (TSZ Scientific LLC, Framingham, Massachusetts, US) for 5 minutes at maximum speed (2800 rpm). The plates were placed in darkness (in a drawer) at room temperature (~ 21°C) for 2 hours. At the end of the first hour of incubation, the plates were shaken for 5 minutes in the microplate shaker at maximum speed again.

Migration

Definition of terms:

- Migration well: A modified segment of a Polymethylmethacrylate tube (Plexiglas®) acrylic with a ½" outer diameter, ¼" inner diameter and a 1" length, with a 25 µm pore nylon mesh Sefar Nitex® glued to the bottom of the tube with a water-resistant epoxy.
- Migration rack**:** A group of 6 individual migration wells glued to a plastic stick in such way that they correspond to a row in a 24-well plate and fit as a group when placed over the 24-well plate.
- Migration set**:** A group of four individual migration racks arranged so that they fit a 24-well plate. The sets were identified by different colored tape.

At least 40 minutes before the incubation period was over, the migration solutions were warmed to 37°C for at least 10 minutes. The migration sets were placed in a 24-well plate. Once the migration wells were in position, on the top of each well of the migration plate, the wells were loaded with 1800 µl of migration solution by using a 5000-µl manual pipette (Research®, Eppendorf, Hamburg, Germany). This process was done gently and slowly to guarantee the passage of the solution through the mesh to the bottom of the well, avoiding spills or air bubbles. The loading of the migration wells was done following the same 24-well plate design used for the incubation (Fig. 3.1). Right before transferring, the incubation plate was shaken in a TSZ-S-04 mini microplate shaker (TSZ Scientific) for 1 minute at maximum speed (2800 rpm) and then vortexed vigorously for 15 seconds. Transfer of contents (200 µl) was done by using a 1000-µl manual pipette (Pipetman®, Gilson). After finishing a row (6 wells and two drug concentrations), the incubation plate was vortexed for 15 seconds again before starting with the next row. After transferring larvae, the migration plate was placed in a culture incubator (Fisher Scientific, Hampton, New Hampshire, US) at 37°C for 45 minutes in darkness. During the transfer process and the migration period, all kinds of movements and vibrations were avoided to reduce the possibility for non-specific migration. After finishing the migration period, the migration plate was taken from the incubator and the racks were gently removed. The non-migrating larvae (still on the mesh) were washed with distilled water and poured into another new 24-well plate by using a washing bottle. For the washing procedure, the racks were inverted above the corresponding well and a stream of water was gently applied, using the washing bottle, from the bottom of the well and through the mesh. The 24-well migration plate and the non-migration plate were carefully labeled so that the corresponding wells could be matched for counting purposes. Ten microliters of a 50% Lugol's solution was added to each well before counting. This last step was omitted when larvae were selected to be recovered and stored for genomic DNA extraction (see below). Plates were examined under an Eclipse TS 100 inverted microscope (Nikon), where they were observed at a magnification of 40X. The number of larvae in each well of the migration plate and the corresponding non-migration plate were counted.

Migrating L_3 recovery and storage

Based on the consistency of the dose response curves and number of replications performed, only larvae from LMIA' assays using the analogs, moxidectin (milbemycin) and eprinomectin (avermectin), were used for genomic DNA extraction and molecular procedures. For this purpose, 8 independent assays were performed with each population (Pop S and Pop Mox) using each ML analog. After the larvae were counted, the 24-well plates were kept at 4°C until used for larval recovery. Prior to L_3 recovery from the plate, 1 ml of solution was drawn from each well so that the volume of the well was reduced to approximately 1 ml. The contents of all the replicate wells for each drug concentration (or control) from each independent assay were transferred to a 50-ml polypropylene centrifuge tube. Thus, one 50-ml tube per drug concentration was used for each drug in each of the two populations. The tubes were centrifuged at 2000 x g for 10 minutes at 4°C. The supernatant was discarded and the sediment (~2 ml) was transferred to a 15-ml polypropylene centrifugation tube. Distilled water was added up to make up the volume to 15 ml. The tubes were centrifuged at 2000 x g for 10 minutes at 4°C. The supernatant was discarded and the sediment (~2 ml) was resuspended in distilled water made up to 15 ml. The centrifugation step was repeated once and the sediment (~2 ml) was transferred to a 2-ml archiving cryovial. The 8 archiving vials obtained per population were labeled and stored in a cardboard freezer box at -80°C until use for pooling and DNA extraction.

Pooling of migrating L_3

In order to reduce the number of L_3 pools to be used for genomic DNA extraction and molecular identification, migrating L_3 from different drug concentrations were pooled in three pools according to the global dose response curve obtained in the analysis of all the replicates performed (see below and results section). Only migrating L_3 present in drug concentrations above the inhibitory concentration 50 (IC_{50}) were considered. Three pools of migrating larvae were created and termed: high pool (highest drug concentrations), median pool (intermediate drug concentrations above the IC_{50}) and control pool (all the control wells in each population). The detailed compositions of these pools were as follows:

- High pool: All larvae migrating at the two highest concentrations tested (20 and 10 μM) were pooled for each population and for each drug.
- Median pool: If the 95% confidence interval (CI) for the IC_{50} included an assay concentration tested in the assay, migrating larvae from wells of that concentration were included in the median pool. If the 95% CI for the IC_{50} fell between 2 drug concentrations, then the migrating larvae for both concentrations, immediately above and immediately below the IC_{50}, were included.
- Control pool: All migrating larvae in the control wells were pooled and used for molecular identification.

A fourth pool was created and included both parasite populations. This pool was termed "runners" and consisted of very active L_3 after a period of incubation of 12 hours. The objective of this pool was to identify highly active larvae despite long periods of incubation, and correlate these findings with the species composition of larvae migrating above the IC_{50}. For making the runners pools, L_3 were exsheathed and plated in a 24-well plate as previously described. These larvae were treated like controls, therefore, 100 μl of PBS with 1% DMSO were added to each well. The plate was incubated in darkness for 12 hours. After incubation the plate was examined under an Eclipse TS 100 inverted microscope (Nikon) at 40X. Using a 200-μl manual pipette (Pipetman®, Gilson), only the highly active larvae

(category 1) were sucked up using a 100-µl tip and put into an archiving cryovial. Three independent assays per population were carried out and up to 100 larvae were recovered. The detailed composition of each L_3 pool is described in Table 3.6.

Table 3.6: Composition of each pool of migrating cyathostomin L_3.

Population	Drug/group	Drug concentrations	Number of larvae
Pop S	Moxidectin high	20 µM + 10 µM	444
	Moxidectin median	1.25 µM	781
	Eprinomectin high	20 µM + 10 µM	920
	Eprinomectin median	1.25 µM + 2.5 µM	1951
	Control	None	3132
	Runners	None	~100
Pop Mox	Moxidectin high	20 µM + 10 µM	493
	Moxidectin median	1.25 µM + 0.625 µM	1637
	Eprinomectin high	20 µM + 10 µM	630
	Eprinomectin median	1.25 µM + 2.5 µM	1289
	Control	None	2361
	Runners	None	~100

Primer design

The second internal transcribed spacer (ITS-2) genomic regions nucleotide sequences from 22 *Cyathostominae* species were downloaded from the Nucleotide GeneBank® (NCBI). Using the software Geneious® Basic 5.0.3 (Biomatters Ltd., Auckland, New Zealand) the sequences were aligned and a set of degenerate primers was visually designed to amplify a specific internal region of about 250 base pairs (bp) in all the species analyzed. This region was selected based on the highly conserved priming region and the species-specific non-consensus sequences. Degenerate primers were designed and termed: CyatITS-2reg forward (fw): ACATTGTTTGTCGAATGGCGC; and CyatITS-2reg reverse (rv): ACAMRTCARCAGTGACAACG.

Genomic DNA extraction from adult cyathostomins

Genomic DNA was extracted from adult cyathostomin specimens collected at necropsy from the Pop S ponies (University of Kentucky) from November 1998 to April 2000 by researchers at the University of Kentucky and the University of Georgia. These specimens had been identified to the species level using morphological criteria and they were stored at -80°C in different media including, liquid nitrogen, 70% ethanol, homogenized in TRIS, or frozen without media. In the case of whole specimens preserved in liquid nitrogen, or 70% ethanol, or frozen without media, 3 to 10 adult worms (depending on the availability of specimens) were washed in PBS, air dried and placed in a clean 1.5 ml microcentrifuge tube. Using a pair of small metallic tweezers, the specimens were crushed against the tube walls until they were broken up into very small pieces or an amorphous mass. The tweezers had been previously thoroughly washed with soap, rinsed with nuclease free distilled water and autoclaved. Samples with specimens already homogenized in TRIS were centrifuged at 13000 x g for 1 minute. The supernatant was discarded and the pellet was resuspended in 500 µl of DNA extraction buffer. DNA extraction buffer was prepared to contain: 100 mM NaCl, 50 mM Tris pH 8.05, 50 mM EDTA pH 8.0 and 1% SDS. The tubes were centrifuged again at 13000 x g for 1 minute and the supernatant was discarded. Five hundred microliters of DNA extraction buffer were added to the tube, rinsing all the remnants of the worm. Three microliters of Proteinase K (Sigma) from *Tritiratium album*, at a concentration of > 800 units/ml were added. The tube was vortexed vigorously for 15 seconds. The sample was placed in an orbital incubator at 37°C for at least 10 hours of digestion. The sample was then subjected to a modified protocol of phenol-chloroform genomic DNA extraction in a fume hood. In brief, 500 µl (equal volume) of a phenol-chloroform-isoamyl alcohol mixture (25:24:1; Sigma) was added to the tube and vortexed vigorously for 15 seconds. The tube was then centrifuged at 13 000 x g for 5 minutes. The top liquid layer was transferred to a clean centrifuge tube and the previous step was repeated once. Then the top liquid layer was transferred to a clean centrifuge tube and approximately 400 µl (equal volume) of chloroform > 99% containing amylenes as a stabilizer (Sigma) were added. The tube was vortexed vigorously for 15 seconds. The tube was then again centrifuged at 13 000 x g for 5 minutes. The top liquid layer was

transferred to a new centrifuge tube and 1.5 volumes of ice cold 100% ethanol was added to the tube and the tube mixed gently 10 times by inversion. Ten microliters of sodium acetate (~3M in water; Sigma) and 5 µl of Glycoblue® (blue co-precipitant; Ambion, Austin, Texas) were added to the sample. The tube was incubated at -80°C for 2 hours or at -20° C for at least 10 hours. The tube was then incubated at room temperature (~ 21°C) for 5 minutes and centrifuged at 13000 x g for 10 minutes. All the supernatant was very carefully discarded, leaving only the DNA pellet. The pellet was air dried for 10 to 15 minutes and then resuspended with 25 µl of high quality nuclease-free distilled water (Promega, Fitchburg, Wisconsin, US). The concentration of DNA was determined with a Nanodrop® 2000 spectrophotometer (Thermo Scientific, Waltham, Massachusetts, US). Samples were stored at -20°C until use.

Validation of primers

For the validation of the degenerate set of primers, 1 µl of extracted genomic DNA was used as a template. Concentrations of extracted genomic DNA ranged from 2 to 170 ng/µl. A final volume of 50 µl of a conventional PCR reaction mix was prepared with 1 µl of each, the forward and reverse primers, at a concentration of 10 µM (final primer concentration of 0.2 µM), 25 µl of 2X GoTaq® Green Master Mix (400µM dATP, 400µM dGTP, 400µM dCTP, 400µM dTTP and 3mM $MgCl_2$; Promega) and 22 µl of nuclease-free distilled water (Promega). The PCR was carried out in a Peltier Thermal Cycler (PTC 200, MJ Research Inc., Waltham, Massachusetts, US). The cycle used was: 94°C for 2 minutes followed by 35 cycles of 94°C for 30 seconds (denaturing), 57°C for 30 seconds (annealing), 72°C for 40 seconds (extension) and 72°C for 5 minutes (final extension). PCR amplicons were subjected to electrophoresis in a 1% agarose gel (Bio-Rad, Hercules, California, US) at 100 volts for 60 minutes. Gels were visualized in a Gel Imager G: BOX HR® (Syngene, Frederick, Maryland, US) using the software GeneSnap® 7.05 (SynGene, Synoptics) at 150 to 200 ms exposure using ethylene bromide (EtBr)/ultraviolet (UV) filter. Once a single PCR product was confirmed, the DNA amplicon was purified from the PCR reaction mixture using a DNA purification kit, either the Illustra® GFX PCR DNA and Gel Band Purification Kit

(GE Healthcare, Waukesha, Wisconsin, US) or the QIAquick® Gel Extraction Kit (Qiagen, Venlo, Netherlands). The procedure was done following the specific directions for the kit and the DNA was eluted in 20 µl of nuclease-free distilled water (Promega). The DNA concentration was determined in a Nanodrop 2000 spectrophotometer (Thermo Scientific). Aliquots of the DNA samples were sent on ice to the Georgia Genomics Facility (GGF) (University of Georgia) for single tube capillary Sanger sequencing using the CyatITS-2reg primers at a concentration of 3.3 µM as sequencing primers. The sequences obtained were checked and trimmed by reading the chromatograms produced in the sequencing reaction with the software Chromas Lite® 2.01 (Technelysium, Pty. Ltd., Eden Prairie, Minnesota, US). A nucleotide BLAST® (National Library of Medicine) search was used to confirm their specific cyathostomin species identity. The specificity of the primers for *Cyathostominae* species was tested using genomic DNA from *Trichostrongylus colubriformis* and *Strongylus vulgaris* as negative controls. A negative control, with no DNA, was also included to check that non-specific products did not form.

454 titanium fusion primers design and validation

Based on the CyatITS-2reg primers, titanium fusion primers were designed following the directions included in the 454 Sequencing Technical Bulletin No. 013-2009 "Amplicon Fusion Primer Design Guidelines for GS FLX Titanium Series Lib-A Chemistry" (Roche, Basel, Switzerland). Fusion primers were labeled as Pyro-CyatITS-2reg, forward and reverse. These primers were basically the product of adding adaptors to the original CyatITS-2reg primer sequences. The adaptors were A (CGTATCGCCTCCCTCGCGCCATCAG) for the forward primers and B (CTATGCGCCTTGCCAGCCCGCTCAG) for the reverse primers, plus the specific 10-nucleotide midtag identifier. The titanium fusion primers were diluted in nuclease-free distilled water (Promega) at a concentration of 20 µM. To test the fusion primers, a PCR amplification cycle was carried out in a Peltier Thermal Cycler (PTC 200, MJ Research Inc.) using the CyatITS-2reg primers. The PCR amplicons obtained were purified as described previously. One microliter of amplicon extraction was used in a 50 µl

PCR reaction following the same design and using the same cycles described for the validation of the CyatITS-2reg primers, but using the titanium fusion primers at a concentration of 20 μM (to give a final concentration of 0.4 μM instead of 0.2 μM). In the same reaction, a PCR amplification reaction using the CyatITS-2reg primers (conventional primers) was included. The PCR amplicons were subjected to electrophoresis in a 2% agarose gel (Bio-Rad), in matching pairs (conventional and fusion primer set), in adjacent lanes, at 100 volts for 60 minutes. Gels were visualized in a Gel Imager G: BOX HR® (Syngene) as previously described.

Lysis of migrating exsheathed cyathostomin L_3 and genomic DNA extraction

A lysis buffer was prepared to have the following composition: 50 mM KCl, 10 mM Tris (pH 7.5), 2.5 mM $MgCl_2$, 0.45% Triton X-100 and 0.01% Gelatin. Once prepared, the buffer was autoclaved and stored at room temperature (~21°C). After thawing, suspensions containing migrating L_3 were transferred to clean new 1.5 ml microcentrifuge tubes and centrifuged at 13000 x g for 5 minutes. The supernatant was discarded, leaving about 0.2 ml of sediment. In this step, the tube contents were pooled according to the pool design established previously (Table 3.5). After pooling the samples the lysis buffer was added to the tubes to make up the volume to 1.5 ml. The tubes were centrifuged again for 13000 x g for 5 minutes; the supernatant was discarded, leaving about 0.2 ml of sediment. Additional lysis buffer was added to make up the volume to 1.5 ml and the tubes were centrifuged again at 13000 x g for 5 minutes. The supernatant was discarded, leaving ~50 μl of sediment. Lysis buffer was added again to make up the volume to 100 μl. One microliter of Proteinase K (Sigma) from *Tritiratium album*, at a concentration of > 800 units/ml was added. The tubes were shakes vigorously for 15 seconds and then centrifuged at 1000 x g for 10 seconds. The tubes were then placed in a Peltier Thermal Cycler (PTC 200, MJ Research Inc.) for a lysis cycle of 60°C for 10 hours, for Proteinase K activation and 94°C for 15 minutes, for inactivation. The DNA concentration was determined in a Nanodrop 2000® spectrophotometer (Thermo Scientific) and the tubes were stored at -80°C until use.

Validation of the 454 high throughput DNA sequencing protocol

A validation step was included in the 454 sequencing experiment to evaluate the accuracy of the 454 sequencing platform in assessing species richness and abundance in cyathostomin L_3 pools. For this purpose, artificial DNA pools were made by combining DNA from different species in known proportions. To establish the composition of the artificial pools, the following criteria were considered:

- The prevalence of the cyathostomin species based on the information reported by Lyons et al. (1996).
- The availability of genomic DNA extracted from cyathostomin species.
- Because 454 optimal run conditions require a homogeneous amplicon size of $\geq$ 250 bp, species with a smaller ITS-2 region size (< 200 bp), i.e. *Co. coronatum* and *Cs. minutus*, were excluded from the sequencing run to avoid interferences in the readings and bias in the PCR and 454 sequencing. Therefore, these two species were not considered for the validation experiment either.

DNA from cyathostomin species was diluted to approximately the same standard concentration of ~3.2 ng/µl (based on the DNA sample with the lowest concentration) as determined by a Nanodrop 2000® spectrophotometer (Thermo Scientific). DNA samples were combined according to the artificial pool design, and submitted for 454 sequencing. The results obtained were unexpected, so the DNA samples used for the making of the pools were further analyzed using a Qubit® 2.0 Fluorometer (Life Technologies, Carlsbad, California, US). Based on large differences between the Nanodrop readings and the Qubit® ones and given the considerably higher sensitivity of the Qubit® (Beaudet et al., 2007), the readings produced by it were considered to be the correct ones. In this way, the real percentages of the total DNA for each species were deduced for each pool. Details on the corrected proportions of each species in the artificial pools are presented in the results section, Tables 4.14 through 4.18.

Preparation of DNA amplicons for 454 sequencing

CyatITS-2reg amplification of genomic DNA from L_3 pools

For the PCR amplification of the ITS-2 region, 2 µl of genomic DNA extraction lysate from a larval pool was run in triplicate (3 technical replicates) in a Peltier Thermal Cycler (PTC 200, MJ Research Inc.) in a 50 µl PCR reaction prepared by adding 1 µl of each CyatITS-2reg primer (final primer concentration of 0.2 µM), 25 µl of 2X GoTaq® Green Master Mix (400µM dATP, 400µM dGTP, 400µM dCTP, 400µM dTTP and 3mM $MgCl_2$; Promega) and 21 µl of nuclease-free distilled water (Promega). The PCR cycle performed was the same as described previously. The PCR amplicons were subjected to electrophoresis in a 1% agarose gel (Bio-Rad) at 100 volts for 60 minutes. The gels were visualized in a Gel Imager G: BOX HR® (Syngene) using the software GeneSnap® 7.05 (SynGene, Synoptics) at 150 to 200 ms exposure using an EtBr/UV filter.

Band excision and DNA gel extraction

After visualization, gels were visualized with the ultraviolet screen of a Dual Transiluminator Foto Spectrum® (Fotodyne, Hartland, Wisconsin, US). A PCR amplicon of about 250 bp and a smaller one of about 120 bp, if two bands were present, or the unique PCR amplicon, if only one band was present, were carefully excised using a clean new single edge razor blade N° 9. The extracted bands were kept in a clean 1.5 ml microcentrifuge tube and stored at 4°C until DNA gel extraction was performed. DNA gel extraction was done using a QIAquick® Gel extraction kit (catalog number 28704; Qiagen) following the directions described in the QIAquick® Spin Handbook. The DNA was eluted in 20 µl of nuclease-free distilled water (Promega). DNA concentrations were determined using the Nanodrop 2000® spectrophotometer (Thermo Scientific). Samples were labeled and kept at -20°C until use. DNA amplicons from larger sized bands were used for titanium fusion primer amplification. DNA amplicons from smaller sized bands (< 200 bp) were sent directly to the GGF (University of Georgia) for sequencing

by the Sanger capillary technique, using the CyatITS-2reg primers at a concentration of 3.3 µM as the sequencing primers. The sequences obtained were checked and trimmed by reading the chromatograms with the software Chromas Lite® 2.01 (Technelysium, Pty. Ltd.). Nucleotide BLAST® (National Library of Medicine) searches in the Nucleotide GeneBank® (NCBI) were used to obtain the specific cyathostomin species identities.

Fusion primers amplification of DNA amplicons

One microliter of cyatITS-2reg PCR amplicon extraction was used as a template in a 50 µl PCR reaction in a Peltier Thermal Cycler (PTC 200, MJ Research Inc.). The reaction was prepared by adding 1 µl of each primer (fw and rv) from a determined set of titanium fusion primers (tagged with a specific 10-nucleotide midtag identifier) according to the experimental design (final primer concentration of 0.4 µM), 25 µl of 2X GoTaq® Green Master Mix (400µM dATP, 400µM dGTP, 400µM dCTP, 400µM dTTP and 3mM $MgCl_2$; Promega) and 21 µl of nuclease-free distilled water (Promega). The PCR cycle was the same as previously described. The PCR amplicons were subjected to electrophoresis in a 1% agarose gel (Bio-Rad) at 100 volts for 60 minutes. The gels were visualized in a Gel Imager G: BOX HR® (Syngene) using the software GeneSnap® 7.05 (SynGene, Synoptics) at 150 to 200 ms exposure using an EtBr/UV filter.

Band excision, DNA gel extraction and submission of samples

After visualization, the gels were visualized with the ultraviolet screen of a Dual Transiluminator Foto Spectrum® (Fotodyne). Single bands were carefully excised using a clean new single edge razor blade N° 9. The extracted bands were kept in a clean 1.5 ml microcentrifuge tube and stored at 4°C until DNA gel extraction was performed. For the DNA gel extraction, a QIAquick® Gel Extraction Kit (catalog number 28704; Qiagen) was used, following the directions described in the QIAquick® Spin Handbook. The DNA was eluted in 20 µl of nuclease-free distilled water (Promega). DNA concentrations were

determined using the Nanodrop 2000® spectrophotometer (Thermo scientific). The samples were labeled and kept at -80°C until sent to the GGF for sample preparation and the 454 pyrosequencing experiment.

454 sequencing experiment

Once in the GGF, the samples were analyzed in both a 2100 Bioanalyzer (Agilent Technologies, Santa Clara, California, US) using a DNA 7500 series II kit (Agilent Technologies), and in a qPCR reaction to determine the size, quality and quantity of the products. All the technical replicates were pooled together in approximately equal amounts of DNA and then included in a DNA library. This DNA library was processed following the following steps: DNA library capture in capture beads A and B, emulsification, amplification, bead recovery, DNA library enrichment, and sequence primer annealing. Beads loaded with the DNA samples were shipped to the Roche 454 Core Facility at Branford, Connecticut, US where they were loaded in ¼ of a 454 titanium Picrotiter sequencing plate and run in a GS FLX plus titanium. The data generated was processed and filtered by the 454 software pipeline and the quality filtered reads and associated quality scores in FASTA format along with the files in standard flowgram format (SSF) were electronically delivered to GGF.

Quantitative PCR (qPCR) for determination of differences for ITS-2 number of repeats in *Cyathostominae* genera

Primers

For the amplification of the ITS-2 region, CyatITS-2reg primers were used at a concentration of 10 μM. β-tubulin isotype-1 gene was used as a single-copy reference gene, and for its amplification, Btubmgb1 fw (AATGCTACCCTATCCGTTCATC) and Btubmgb1 rev (CAAATATCATAGAGAG CTTCATTGTCAAT) were used (von Samson-Himmelstjerna et al., 2003) at a concentration of 10 μM.

DNA

The genomic DNA extracted for the validation of the CyatITS-2reg primers was used. These samples included 5 cyathostomin species, representing the 5 major genera reported for Pop S (excluding *Coronocyclus*): *Cyathostomum (Cy. pateratum); Cylicostephanus (Cs. goldi); Cylicocyclus (Cc. nassatus); Petrovinema (Pe. poculatum)* and *Poteriostomum (Po. imparidentatum).* These species were selected based on the availability of DNA. Because of their previous use for making artificial DNA pools for 454 sequencing validation experiment, the genomic DNA concentrations were assumed to be similar.

qPCR experiment

Samples were run in triplicate in a 20 µl qPCR reaction with 10 µl of Maxima® SYBR Green/Rox qPCR Master Mix 2x (Fermentas, Vilnius, Lithuania), 7 µl of nuclease free distilled water, 1 µl of fw and rv primer (final primer concentration of 0.5 µM) and 1 µl of genomic DNA suspension. A sample without DNA (2 µl of distilled water instead) was included as a control to check for the formation of primer dimers. To reduce pipetting error, a stock mixture of Maxima® SYBR green Master Mix, water and primers was made previously and distributed in all of the wells used in the experiment, so that only DNA was added to the corresponding individual well. The experiments were run in an Mx 3000 P® Real Time PCR system (Stratagene, La Jolla, California, US) and using the software Mx Pro-Mx 3000 P v 4.0.1 (Stratagene). For both sets of primers, a SYBR Green amplification cycle with a dissociation curve was applied. Both set of primers were run separately in different cycles. The amplification cycle for β-tubulin was: at 94°C 10 minutes the first cycle (Segment 1), followed by 40 cycles each consisting of 94°C for 30 seconds, 50°C for 30 seconds (annealing step) and 72°C for 40 seconds (extension step) (Segment 2). For amplification of the β-tubulin, one experiment was performed. The amplification cycle for ITS-2 was similar, but the annealing step was carried out at 57°C for 30 seconds. For the ITS-2 amplification, three independent experiments were done. The melting curves were visually compared to test the homogeneity of the PCR products. To confirm this, 20 µl of reaction product of one replicate per treatment was mixed

with 4 µl of Blue/Orange 6X Loading Dye (Promega) and subjected to electrophoresis in a 2% agarose gel (Bio-Rad) following the same protocol previously described for the validation steps.

Data analysis

For determining the effect of different media on cyathostomin L_3 motility, data were entered in a Microsoft Excel® sheet (Microsoft, Redmond, Washington, US) and the number of larvae in each of the three categories was calculated as a percentage of the total larvae categorized. A Chi-square test was performed using the software Minitab® 15.1.30.0 (Minitab, Inc., State College, Pennsylvania, US). Analysis of differences in L_3 migration percentages between different treatments during optimization trials were determined by one way analysis of variance (ANOVA) and Tukey's Studentized Range (HSD) using the software SAS® 9.2 (SAS Institute, Cary, North Carolina, US).

For the LMIA data analysis, to evaluate the consistency and repeatability of the independent replicates, the data were entered in Microsoft Excel® to determine the percentage of migration and migration inhibition. The model "one population" logistic regression of the Fit Logit Program (Dobson et al., 1987) was used to determine the dose response curve, corrected for natural inhibition of migration. Data generated were entered in GraphPad Prism® version 5 (GraphPad Software, Inc., San Diego, CA) and a mixed model approach was used to examine variability between assays, and the variability between individual assays for each drug and for each population. For determining the dose response curve and the inhibitory concentration 50 (IC_{50}, also called EC_{50} or LD_{50}) the data were previously corrected for natural inhibition using Microsoft Excel®. The average migration for the three control replicates per assay was determined and used to correct the migration in the drug concentration replicate. In this way, the percentage inhibition corrected for the natural inhibition was determined as a function of the percentage of migration (100 - migration %). The average of the three replicates for each drug concentration was obtained and plotted against the natural logarithm of the concentration. Migration inhibition in the control group was set to 0. Using the corrected average of inhibition of migration, the sigmoidal dose-response curves were determined with GraphPad Prism® version 5 using a variable slope nonlinear regression

model. A four-parameter logistic equation was applied, with the bottom set to zero and the percent migration inhibition plotted against the log-transformed concentration for each drug treatment. Controls were plotted against a value of -1.0. For each treatment, the drug concentration producing inhibition halfway between zero and the maximum y-value, defined as the IC_{50}, was calculated using the same software. Significant differences in the IC_{50} between both parasite populations were calculated using GraphPad Prism® using the extra sum-of-squares F test and these were reported as a *p*-value. The resistance ratio for each analog was calculated by dividing the IC_{50} of Pop Mox by the IC_{50} of Pop S.

For qPCR determination of differences for the ITS-2 number of repeats in *Cyathostominae* genera, amplification plots were analyzed individually for testing the consistency of the replicates and collectively to test the amplification curves. In order to test significant differences between ITS-2 amplification levels among different genera, the CT values obtained for ITS-2 in each experiment were divided by the CT values generated in the β-tubulin experiment (the single copy reference gene). The values obtained were analyzed using one way ANOVA and Tukey's Studentized Range (HSD) using the software SAS® 9.2.

The numbers of sequences per species per pool, after the 454 sequencing, were determined using the software Megablast® (NCBI) to assign the species identity, matching the sequences with the database available at GeneBank® (NCBI). The standard M9 format and a minimum bit score of 200 bp were used. The most highly similar accession number sequence was selected for each individual sequence. The prevalence of specific species per pool was calculated in Microsoft Excel®. Differences in the prevalence of species between the control pools and the drug treatment, or between the control pools and the runners pool in each population, were determined by the Chi-square test of independence using SAS® 9.2 to determine whether species distribution was independent of control and drug exposure. Differences in the proportions of individual species were tested using Fisher's exact test.

CHAPTER 4

RESULTS

Media

During the preliminary standardization experiments, a positive correlation between larval migration and larval motility was observed, and in general, a low migration percentage was obtained (data not shown). Therefore, it was decided to assess the effects of different media (with different composition and different pH) on larval motility over time. Four different media were used for this purpose and a subjective scale of categorization of larval motility was developed and applied. This scale consisted in three categories: 1, highly active larvae; 2, larvae with slow and/or jerky movements, and 3 larvae not moving at all (Table 3.2) ,In a first series of four independent experiments with six replicates per treatment, Earle's balanced salt solution (NaCl, 6.8 g/L; KCl, 0.4 g/L; $CaCl_2$, 0.2 g/L; $MgSO_4$, 0.1 g/L; NaH_2PO_4, 0.1 g/L; $NaHCO_3$, 2.20 g/L; D-glucose, 1 g/L), phosphate buffered saline (PBS; NaCl, 0.138 M; KCl, 0.0027 M) and a phosphate based buffer solution (KH_2PO_4 , 6.63 g/L; Na_2HPO_4, 2.56 g/L; NaN_3, 1.0 g/L)were compared. These media were selected for two main reasons: their common widespread use for *in vitro* assays with nematode larvae (PBS and Earle's solution) and the fact that they exhibit different pH values raging from acid to alkaline (6.2, 7.4 and 9.0 respectively). Using two different incubation periods (2 and 22 hours), no significant differences (p=0.082 and p=0.123, respectively) were observed (Table 4.1 and Table 4.2). When PBS was compared to RPMI in a series of similar experiments, no significant differences were detected (p=0.054) (Table 4.3). Based on these results, PBS was chosen for future assays because of its lower cost, ease of preparation and simple storage.

Table 4.1: Effect of 3 different media on cyathostomin L_3 motility after 2 hours of incubation. Percentage for each motility score is indicated, with the number of larvae in parenthesis. Incubation was done at 21°C. Buffer, phosphate based buffer solution: pH 6.2; PBS, phosphate buffered saline: pH 7.4; Earle's, Earle's salt balanced solution: pH 9*

Motility score	Buffer	PBS	Earle's
1	69.4% (68)	67.3% (66)	67.2% (43)
2	18.4% (18)	29.6% (29)	21.9% (14)
3	12.2% (12)	3.1% (3)	10.9% (7)
TOTAL	**100% (98)**	**100% (98)**	**100% (64)**

***Chi-Sq = 8.261, DF = 4, *P*-Value = 0.082

Table 4.2: Effect of 3 different media on cyathostomin L_3 motility after 22 hours of incubation. Percentage for each motility score is indicated, with the number of larvae in parenthesis. Incubation was done at 21°C. Buffer, phosphate based buffer solution: pH 6.2; PBS, phosphate buffered saline: pH 7.4; Earle's, Earle's salt balanced solution: pH 9*

Motility score	Buffer	PBS	Earle's
1	31.9% (30)	43.8% (49)	31.4% (27)
2	52.1% (49)	39.3% (44)	57% (49)
3	16% (15)	17% (19)	11.6% (10)
TOTAL	**100% (94)**	**100% (112)**	**100% (86)**

*Chi-Sq = 7.252, DF = 4, *P*-Value = 0.123

Table 4.3: Effect of PBS and RPMI on cyathostomin L_3 motility after 2 hours of incubation. Percentage for each motility score is indicated, with the number of larvae in parenthesis. Incubation was done at 21°C*

Motility score	PBS	RPMI
1	50.6% (45)	61.6% (69)
2	30.3% (27)	16.1% (18)
3	19.1% (17)	22.3% (25)
TOTAL	**100% (89)**	**100% (112)**

*Chi-Sq = 5.821, DF = 2, *P*-Value = 0.054

Incubation period

An optimal incubation period should allow sufficient time for the drug to exert an effect on larval motility, but it should not be too long to induce a reduction in the normal activity of larvae not exposed to the drug (negative control group). Previous studies on the LMIA with cyathostomins have showed that a period of two hours was suitable (Monopoli, 2007). In order to rule out the effects of extended incubation periods as a cause of reduction in larval motility that we observed, the effect of the length of the incubation period on larval motility was also evaluated. Using PBS as the media during three different incubation periods (2, 4 and 22 hours), no significant differences (p=0.702) were observed (Table 4.4). Based on these results, an incubation period of two hours was set as the standard.

Table 4.4: Effect of 3 different incubation times on cyathostomin L_3 motility. Percentage for each motility score is indicated, with the number of larvae in parenthesis. Three different periods of incubation, 2, 4 and 22 hrs, at 21°C were used. PBS was used as the media*

Motility score	2 hr	4 hrs	22 hrs
1	33.3% (51)	32.5% (49)	31.6% (54)
2	6.5% (10)	5.3% (8)	9.4% (16)
3	60.1% (92)	62.3% (94)	59.1% (101)
TOTAL	**100% (153)**	**100% (151)**	**100% (171)**

*Chi-Sq = 2.182, DF = 4, *P*-Value = 0.702

Standardization of the protocol

DMSO concentration

In order to obtain a homogeneous solution with ML analogs in water based media, such as PBS, drugs first need to be dissolved in organic solvents, such as DMSO. High levels of DMSO are needed to obtain higher concentration of ML drugs (10 to 30 μM) in solution. However, a too high a concentration of DMSO can have a deleterious effect on larval viability *in vitro*. In order to test for potential negative

effects of 1% DMSO in PBS, an experiment was performed to compare the migration rates of larvae exposed to 0.5% and 1% DMSO, the former concentration having successfully been used in preliminary studies (Monopoli, 2007). No significant differences (p=0.720) were detected on ANOVA analysis (Table 4.5). A concentration of 1% DMSO was set as standard

Table 4.5: Migration of cyathostomin L_3 at 0.5 and 1% DMSO. Average percentage is indicated, with the 95% CI in parenthesis. DMSO was dissolved in PBS. A 25 μm pore sized mesh was used during the larval migration. †

DMSO	Migration (C.I.)
0.5%	68.46 (61.01-75.91)
1%	60.47 (54.81-66.13)

† Two independent experiments with 6 repetitions for each treatment were made; p-value=0.720

Mesh pore size

In the LMIA protocol, the mesh pore size should allow only actively moving larvae to migrate through it, but it should prevent the migration of paralyzed larvae due to non specific movements (non-active migration). Based on the range of the width of exsheathed cyathostomin L_3 measured in a sample of 47 individual larvae from Pop S (16.18-24.19 μm), pore sizes of 25 μm and 30 μm were selected to perform a comparison experiment. In this experiment, the percentages of migration in the negative control (larvae in 1% DMSO in PBS) and in the positive control for the inhibition of migration (heat-killed larvae) were determined. Significant differences (p<0.05) were detected in the case of the migration of the positive controls, with the 30 μm pore size mesh allowing more heat-killed larvae to pass through. No significant differences were observed for the negative controls (Table 4.6). Based on these results, a pore size of 25 μm was used in the LMIA protocol.

Table 4.6: Migration of living and heat-killed cyathostomin L_3 using 25 µm and 30 µm mesh. Average percentage is indicated, with the 95% CI in parenthesis. Two different pore size mesh were used during the migration†

Mesh diameter	Living (CI)*	Heat-killed (CI)*
25 µm	50.51 (45.31-55.71) [a]	4.31 (3-5.62) [a]
30 µm	51.05 (45.55-56.55) [a]	18.75 (12.38-25.12) [b]

† Three independent experiments with 6 repetitions for each treatment were made
* Rows within columns with the same letter are not significantly different

Use of vortexing in the LMIA protocol

Preliminary results obtained in the previous experiments showed low migration rates in the negative controls. This was directly correlated with a significant reduction in larval motility over time during the incubation period. However, it was observed that mechanical stimulation of the incubation plate by shaking it and/or vortexing it, or even just the mere fact of transferring larvae from the well plate to a glass slide, triggered a significant reactivation of larval motility, even after longer incubation periods. To exclude a possible increase in larval migration in the negative control by vortexing the larvae, we carried out an experiment where the incubation plate was vortexed immediately before transferring it to the migration plate and at 30 minute intervals during a 2 hour migration period. Positive controls for inhibition of migration (heat killed larvae) were used and the results were compared with those obtained in a similar protocol but without vortexing. Significant differences ($p<0.05$) were observed only for the negative controls between both protocols, with a higher rate of migration seen in the protocol using vortexing. A similar rate of migration for the positive control was detected (Table 4.7). Based on these results, vortexing of larvae was incorporated into the LMIA protocol.

Table 4.7: Migration of living and heat-killed cyathostomin L_3 using 2 different protocols. Average percentage is indicated, with the 95% CI in parenthesis. A 25 μm mesh was used in two different protocols of the LMIA†

Protocol	Living (CI)*	Heat-killed (CI)*
No vortexing	54.89 (51.33-58.45)[a]	6.07 (2.95-9.19) [a]
Vortexing	84.17 (81.33-87.01)[b]	4.03 (-1.77-9.83)[a]

† Two independent experiments with 6 repetitions for each treatment were made
* Rows within columns with the same letter are not significantly different

During a time course experiment to assess the duration of larval motility after reactivation by vortexing/shaking of the plate, it was observed that after 30 to 40 minutes, the larval motility significantly decreased until reaching very low levels again (data not shown). Previous studies on the LMIA using ML in cyathostomins done in our laboratory had shown that most of the migration happened during the first hour of migration (Monopoli, 2007). Based on these data, we decided to modify the protocol by shaking the incubation plate in an electronic plate shaker only at the end of the incubation period and immediately before transferring to the migration plate. Moreover, the migration period was reduced to 45 minutes. In order to obtain a truly positive control for inhibition of migration and to avoid the non-specific migration of killed larvae due to the straight shape induced by the heat-killing process, larvae exposed to 25 μM moxidectin (the highest concentration obtained without precipitation under assay conditions) for two hours were then fixed in 10% formalin. The results obtained showed a rate of migration of more than 95% in the negative control and less than 1% in the positive control (Table 4.8).

Table 4.8: Migration of living and moxidectin-killed-formalin-fixed cyathostomin $L_{3.}$ Average percentage of migration is indicated, with the 95% CI in parenthesis. A 25 μm pore sized mesh was used in an improved protocol †

MESH DIAMETER	Living	MOX-formalin fixed
25 μm	95.3 (94.29 – 96.37)	0.50 (0.18-0.82)

† Three independent experiments with 6 repetitions for each treatment were made

When the positive control was changed to include larvae exposed to 25 μM moxidectin without exposure to formalin, the rate of migration in the positive control increased slightly up to around 4%. The migration in the negative control was similar to the one obtained in the previous experiment. These results confirmed the validity of the new optimized and standardized LMIA protocol that had been developed and suggested that even at the highest ML concentration obtained under these conditions, a certain degree of larval motility was still expected.

Table 4.9: Migration of living and moxidectin-killed cyathostomin $L_{3.}$ Average percentage of migration is indicated, with the 95% CI in parenthesis A 25 μm pore sized mesh was used in an improved protocol †

MESH DIAMETER	Living	MOX-killed
25 μm	93.5 (91.85 – 95.19)	3.9 (2.62- 5.26)

† Three independent experiments with 6 repetitions for each treatment were made

Larval migration inhibition assay

Four different ML analogs, abamectin, ivermectin, eprinomectin and moxidectin, were used for performing LMIA experiments in exsheathed cyathostomin L_3 pools from both Pop S and Pop Mox. These ML analogs were selected based on availability and previously reported results (McArthur, 2007; Monopoli, 2007). The number of individual replicates per drug in each population ranged from 6 to 22. Despite the optimization of the protocol (as previously described) the percentage migration in the control wells was still variable, ranging from as low as 55% to as high as 93% (or 45% to 7% inhibition of migration). The inhibition of migration in the highest drug concentration (20 μM) was rarely 100%, with an average of 86.81% and a 95% CI from 85.18 to 88.44. In a preliminary analysis of repeatability between replicates, dose response curves corrected for the natural inhibition of migration were generated in FitLogit® Excel sheets as described in the materials and methods. The data generated by the software were plotted in Graphpad Prism® to generate dose response curves using a mixed model approach. A good consistency in the dose response curves generated was observed, with all the replicates plotting tightly together (data not shown).

For a more rigorous analysis of the dose response curves and the IC_{50}, only assays with an average migration in the control wells equal to or higher than 70% were considered for the analysis, since assays with lower migration in the control group would yield reduced information after the correction for natural inhibition, as observed in the preliminary analysis. A total of 65 assays out of the 88 done (73.86%) fulfilled this threshold and, therefore, were considered for the analysis. The dose response curves for each analog in each population are shown in Figures 4.1 to 4.4; the number of individual replicates included in the analysis is indicated in parentheses (n) and ranged from 3 to 13. Eprinomectin and moxidectin had the highest number of replicates. Dose response curves were very similar for both populations for each drug, with no obvious deviation to the right in the ML-selected isolate (Pop Mox). Table 4.10 shows the IC_{50} (and the 95% C.I.), the resistance factor (RR) for the Pop Mox (Pop Mox IC_{50}/Pop S IC_{50}) and the coefficient of determination (r^2) of the dose response curve for each ML analog used. Based on the r^2

values, all the dose response curves fitted the nonlinear regression model very closely (0.88-0.96). Only with abamectin and moxidectin were statistically significant differences between both populations detected (p=0.0127 and p=0.0002 respectively), but only moxidectin showed differences in the 95% CI for the IC_{50} for the two populations. The IC_{50} values for abamectin, ivermectin, and moxidectin were similar, ranging from ~0.9 to ~1.5 μM. Only eprinomectin showed higher IC_{50} values for both populations (~2.5 μM). None of the resistance ratios were significantly greater than 1, therefore, any statistical difference was considered of no biological relevance.

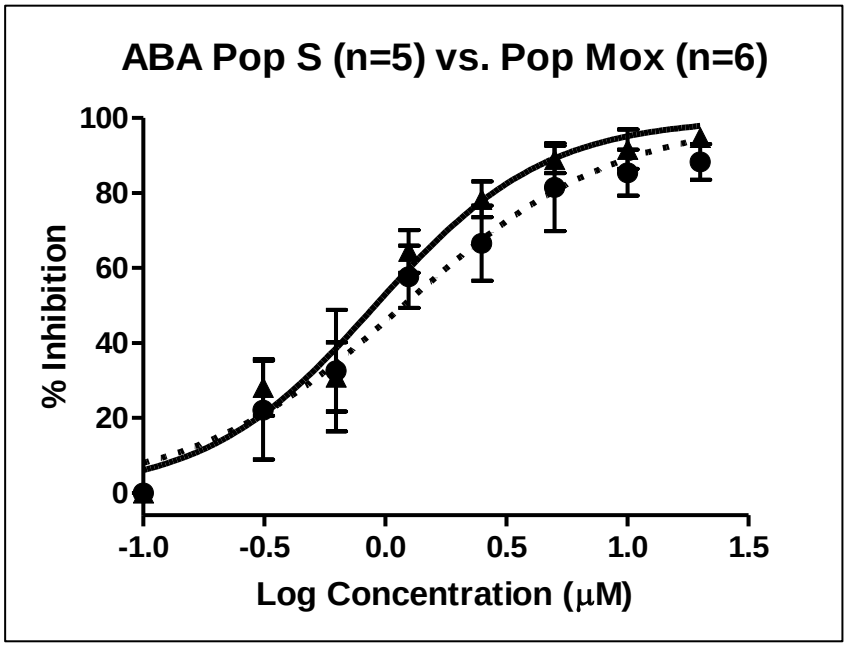


Fig 4.1: Larval migration inhibition assay dose-response curves for abamectin. Dose-response curves using abamectin (ABA) in Pop S (dotted line) and Pop Mox (continuous line) are plotted together. Each data point represents the mean ± SD from a different number of independent assays done in each population and indicated in parenthesis (n).

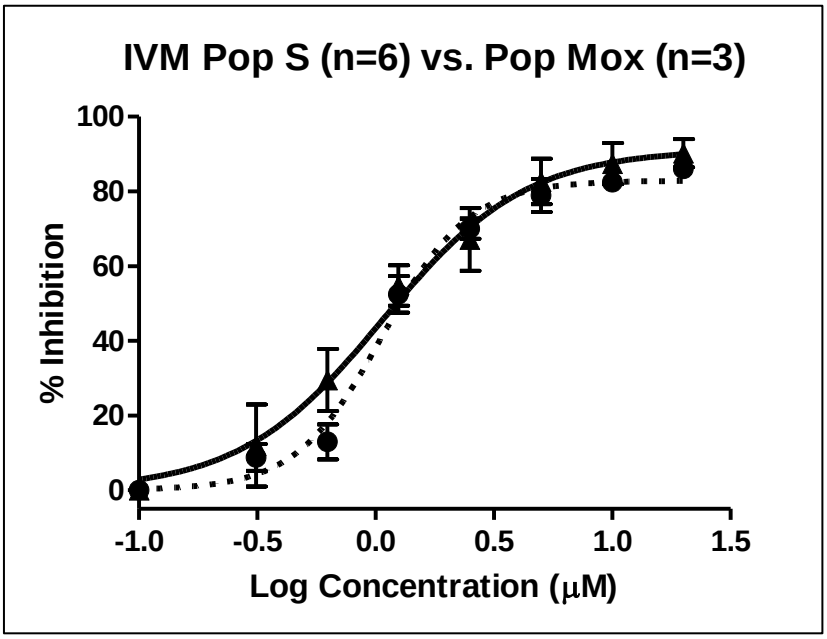


Fig 4.2: Larval migration inhibition assay dose-response curve for ivermectin. Dose-response curves using ivermectin (IVM) in Pop S (dotted line) and Pop Mox (continuous line) are plotted together. Each data point represents the mean ± SD from a different number of independent assays done in each population and indicated in parenthesis (n).

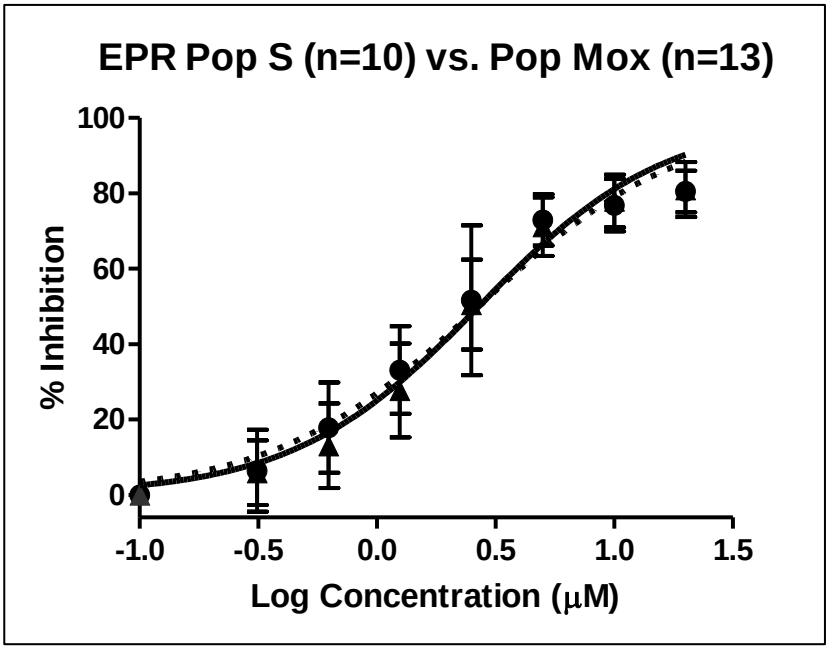


Fig 4.3: Larval migration inhibition assay dose-response curve for eprinomectin. Dose-response curves using eprinomectin (EPR) in Pop S (dotted line) and Pop Mox (continuous line) are plotted together. Each data point represents the mean ± SD from a different number of independent assays done in each population and indicated in parenthesis (n).

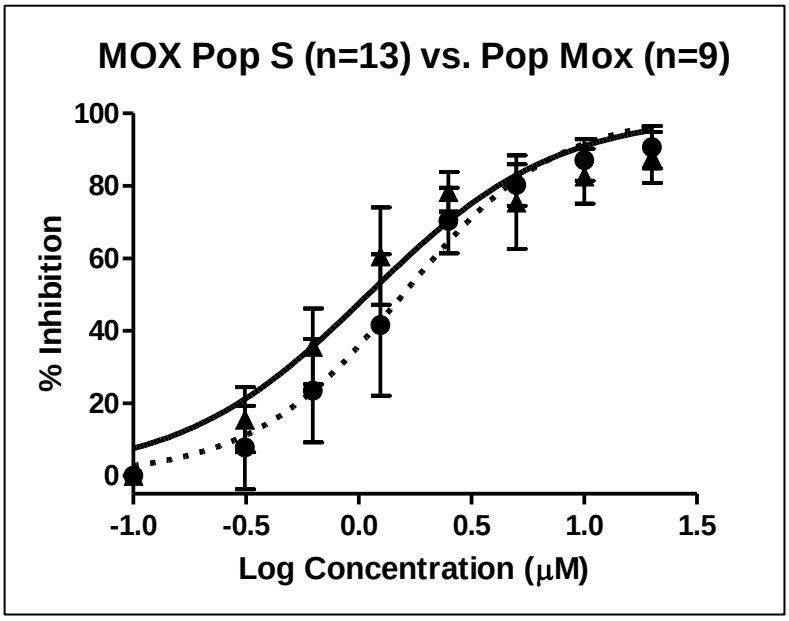


Fig 4.4: Larval migration inhibition assay dose-response curve for moxidectin. Dose-response curves using moxidectin (MOX) in Pop S (dotted line) and Pop Mox (continuous line) are plotted together. Each data point represents the mean ± SD from a different number of independent assays done in each population and indicated in parenthesis (n).

Table 4.10: Comparison of dose-response data from larval migration inhibition assays. Data using ABA, abamectin; IVM, ivermectin; EPR, eprinomectin and MOX, moxidectin in Pop S and Pop Mox. The IC_{50} (μM) of Pop S was compared to that of Pop Mox using the extra sum-of-squares F test with *p*-values reported. Resistance ratio (RR) = Pop Mox IC_{50} / Pop S IC_{50}. R^2, coefficient of determination.

	ABA		**IVM**	
	Pop S	**Pop Mox**	**Pop S**	**Pop Mox**
IC_{50} (μM)	1.19	0.910	1.08	1.03
CI (μM)	0.98-1.45	0.82-1.02	0.95-1.24	0.84-1.26
RR	n/a	0.76	n/a	0.95
R^2	0.9011	0.9568	0.9425	0.9681
p-value	n/a	0.0127	n/a	0.7244
	EPR		**MOX**	
	Pop S	**Pop Mox**	**Pop S**	**Pop Mox**
IC_{50} (μM)	2.55	2.79	1.57	1.10
CI (μM)	2.21-2.95	2.51-3.10	1.41-1.76	0.94-1.28
RR	n/a	1.09	n/a	0.70
R^2	0.8805	0.9104	0.9085	0.8823
p-value	n/a	0.3185	n/a	0.0002

Validation of primers

The CyatITS-2reg primer set was successfully used to amplify the specific ITS-2 region of interest from genomic DNA extracted from adult specimens of 11 cyathostomin species: *Co. coronatum*; *Cy. catinatum; Cy. pateratum*; *Cc. nassatus; Cc. lepostomum*; *Cs. longibursatus; Cs. goldi; Cs. calicatus; Cs. minutus*; *Po. imparidentatum* and *Pe. poculatum.* Additionally, to our knowledge, this is the first report of an ITS-2 incomplete sequence from *Cs. asymetricus*. Fig 4.5 shows examples of PCR amplicons from a validation reaction subjected to electrophoresis in a 1% agarose gel.

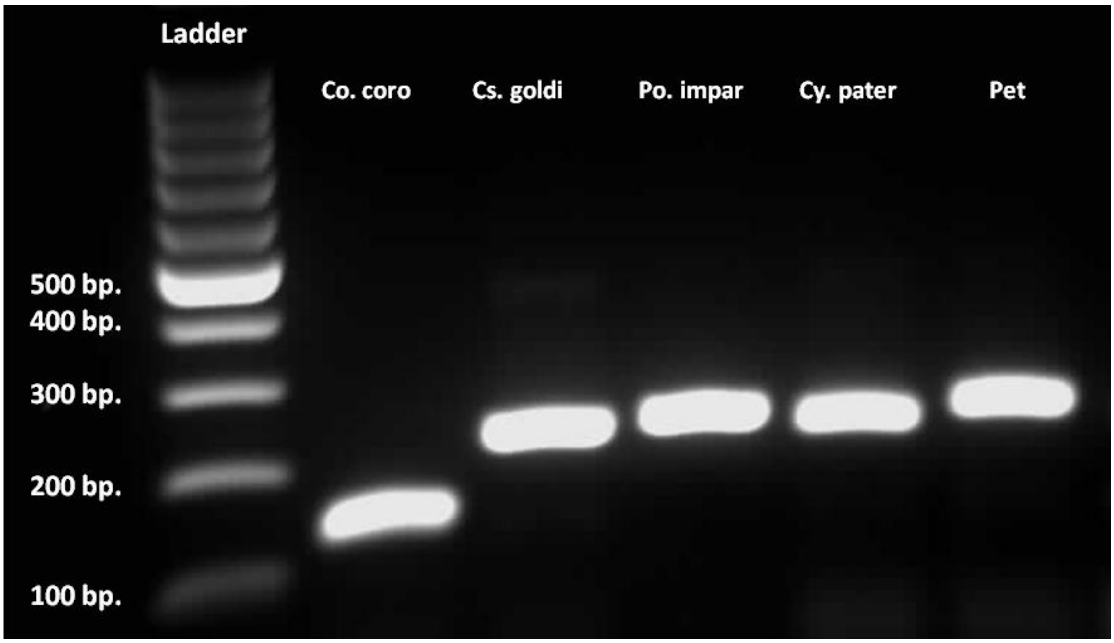


Fig 4.5: Validation of the CyatITS-2reg primers. Digital image of a 1% agarose gel with PCR bands obtained from amplification of the ITS-2 specific region during the validation of the CyatITS-2reg primers. The sizes of the fragments of the 100 bp ladder are indicated on the left. Letters on the top of each lane indicate the cyathostomin species: Co. coro, *Coronocyclus coronatum*; Cs. goldi, *Cylicostephanus goldi*; Po. impar, *Poteriostomum imparidentatum*; Cy. pater, *Cyathotomum pateratum*; Pet, *Petrovinema poculatum*.

Validation of 454 titanium fusion primers

454 titanium fusion primers (Pyro-cyatITS-2reg) successfully produced CyatITS-2reg amplicons from the 11 cyathostomin species used in the validation of the primers. When analyzed in a 2% agarose gel next to a CyatITS-2reg (conventional primer) amplicons, a difference in size of ~70 bp between both could be seen in each species validated. In Fig 4.6, a gel with PCR products from the amplification of cyatITS-2reg of *Co. coronatum* using different combinations of conventional and fusion primers, the extra ~70 bp difference can be seen when using the fusion primer set. This difference was reduced to ~35 bp when just one fusion primer was used. This was done to confirm the expected differences in size between amplicons due to the presence of the extra nucleotides in the fusion primers and the validity of both fusion primers.

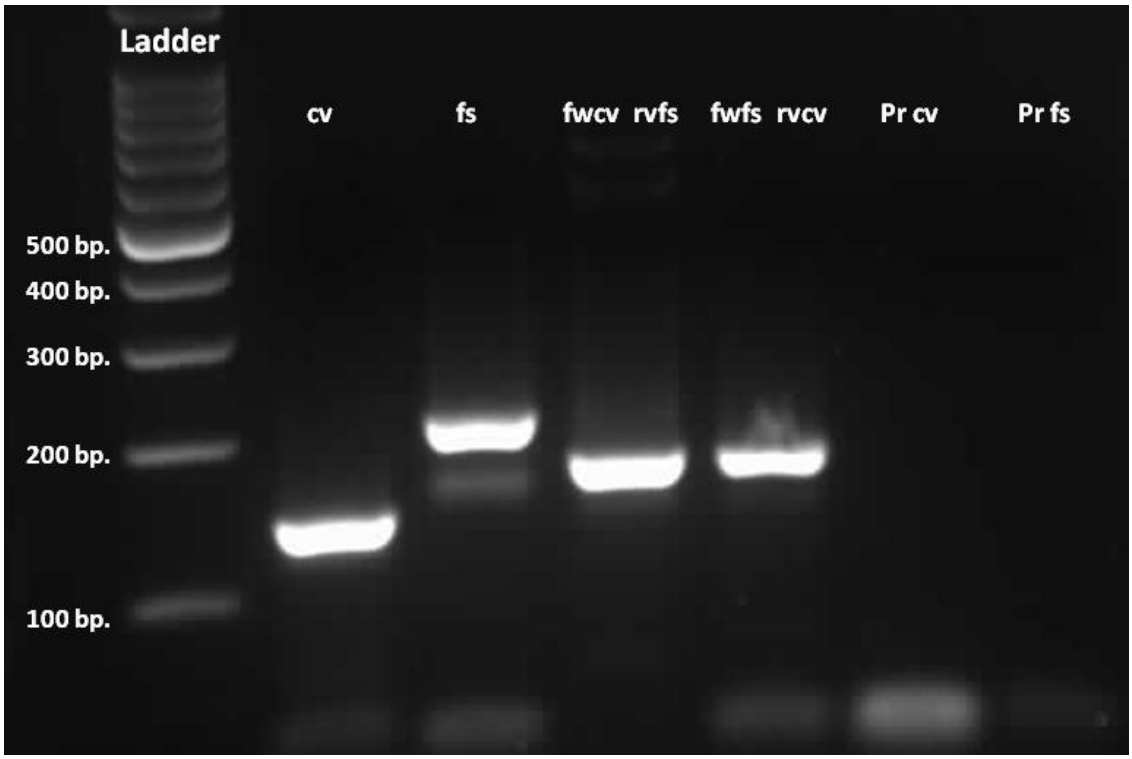


Fig 4.6: Validation of 454 titanium fusion primers. Digital image of a 2% agarose gel with PCR bands obtained from amplification of the ITS-2 specific region of *Coronocyclus coronatum* during the validation of the fusion primers. The sizes of the fragments of the 100 bp. ladder are indicated on the left. Letters on the top of each lane indicate the primers used: cv, conventional CyatITS-2 reg; fs, fusion Pyro-CyatITS-2 reg; fwcv rvfs, forward conventional and reverse fusion; fwfs rvcv, forward fusion and reverse conventional; Pr cv; conventional primers and no DNA; Pr fs, fusion primer only and no DNA.

Quantitative PCR (qPCR) for determination of differences for ITS-2 number of repeats in *Cyathostominae* genera

Equal concentrations of DNA were used in the amplification plots for the β-tubulin isotype 1 in *Cylicostephanus, Petrovinema* and *Poteriostomum*, *Cylicocyclus* and *Cyathostomum.* The amplification plots showed differences with slightly lower and slightly higher cycle threshold (CT) values, respectively (Fig 4.7). The analysis of the melting curve showed a single product different from primer dimers. This was also confirmed by subjecting the products to electrophoresis in a 2% agarose gel. Regarding the experiments on the ITS-2, in general, a good consistency in the amplification plots among the three replicates was seen. Again, the melting curves analysis and the products analyzed in a 2% agarose gel showed single products in each sample. Even though *Cylicocyclus* showed, on average, the lowest CT ITS-2/CT β-tubulin value (indicating the most efficient amplification) and *Petrovinema* the highest (indicating the least efficient amplification), the ANOVA revealed statistically significant differences (p<0.0001) for *Petrovinema* when compared to *Poteriostomum, Cylicostephanus, Cylicocyclus* and *Cyathostomum*. *Poteriostomum* was similar to *Cyathostomum* but different from the others and there were no significant differences among *Cylicostephanus, Cylicocyclus* and *Cyathostomum* (Table 4.12).

Smaller size ITS-2 species: *Cs. minutus* and *Co. coronatum*

As explained in the materials and methods section, *Cs. minutus* and *Co. coronatum* were excluded from the 454 experiment to avoid any bias or interference in the sequencing run, since they showed the smallest sized ITS-2 regions. However, based on the presence or absence of the typical small band around 120 bp in the gels, we could measure their presence in a qualitative way. As can be seen in the example in Fig 4.8, differences in the presence and intensity of this small band were observed. In general, the presence of small bands was more evident and stronger in Pop Mox pools than in Pop S pools. Differences between the different pools within each population were not observed, with the exception of the pool "runners" in Pop Mox where the small bands were less clear when compared to other pools in that population. The

small bands were always less bright than the larger sized band (~250 bp) in all the replicates. Based on these observations, it was concluded that *Cs. minutus* and/or *Co. coronatum* were never the dominant species and the populations comprised mostly cyathostomin species with ITS-2 region of ~250 bp. The results of the Sanger capillary sequencing of DNA extracted from the small band indicated that most of the samples of Pop S had very low DNA concentrations, too low to produce a sequencing reading (which was corroborated by means of chromatogram readings). These samples were considered as "not determined". The pool of runners was the only one in Pop S that could be properly sequenced, and the BLAST search showed a sequence compatible with *Cs. minutus*. For Pop Mox, almost all the sequences from the pools, with the Pool of runners being the only exception, showed clearly that *Co. coronatum* was the species indicated by the small band. In the case of the runners, the sequence reaction failed, but the chromatogram indicated the presence of more than one species in the samples. The assumption in this case was the that both species, *Cs. minutus* and *Co. coronatum*, were present in the sample, based on previous reports of species prevalence in Pop S (Lyons et al., 1996b). However, since the presence of other species cannot be discarded, the results were considered as "not determined".

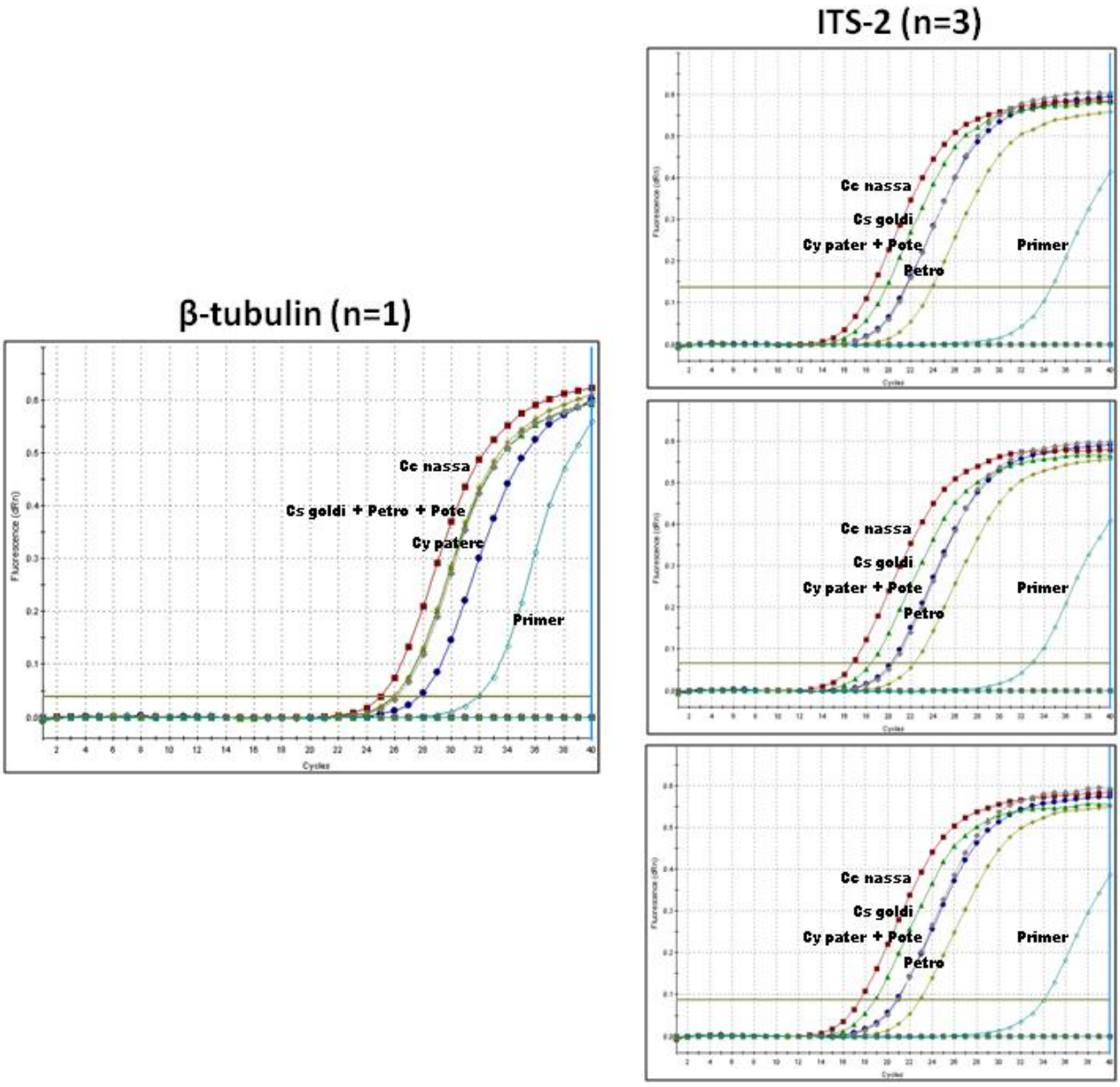


Fig 4.7: qPCR amplification plots of β-tubulin and ITS-2 region experiments. The number of independent experiments is indicated in parenthesis (n). Cyathostomin species are indicated by labels on the left of the plot line: Cc nassa, *Cylicocyclus nassatus*; Cs goldi, *Cylicostephanus goldi*; Petro, *Petrovinema poculatum*; Pote, *Poteriostomum imparidentatum*; Cy pater, *Cyathostomun pateratum*; Primer, primers.

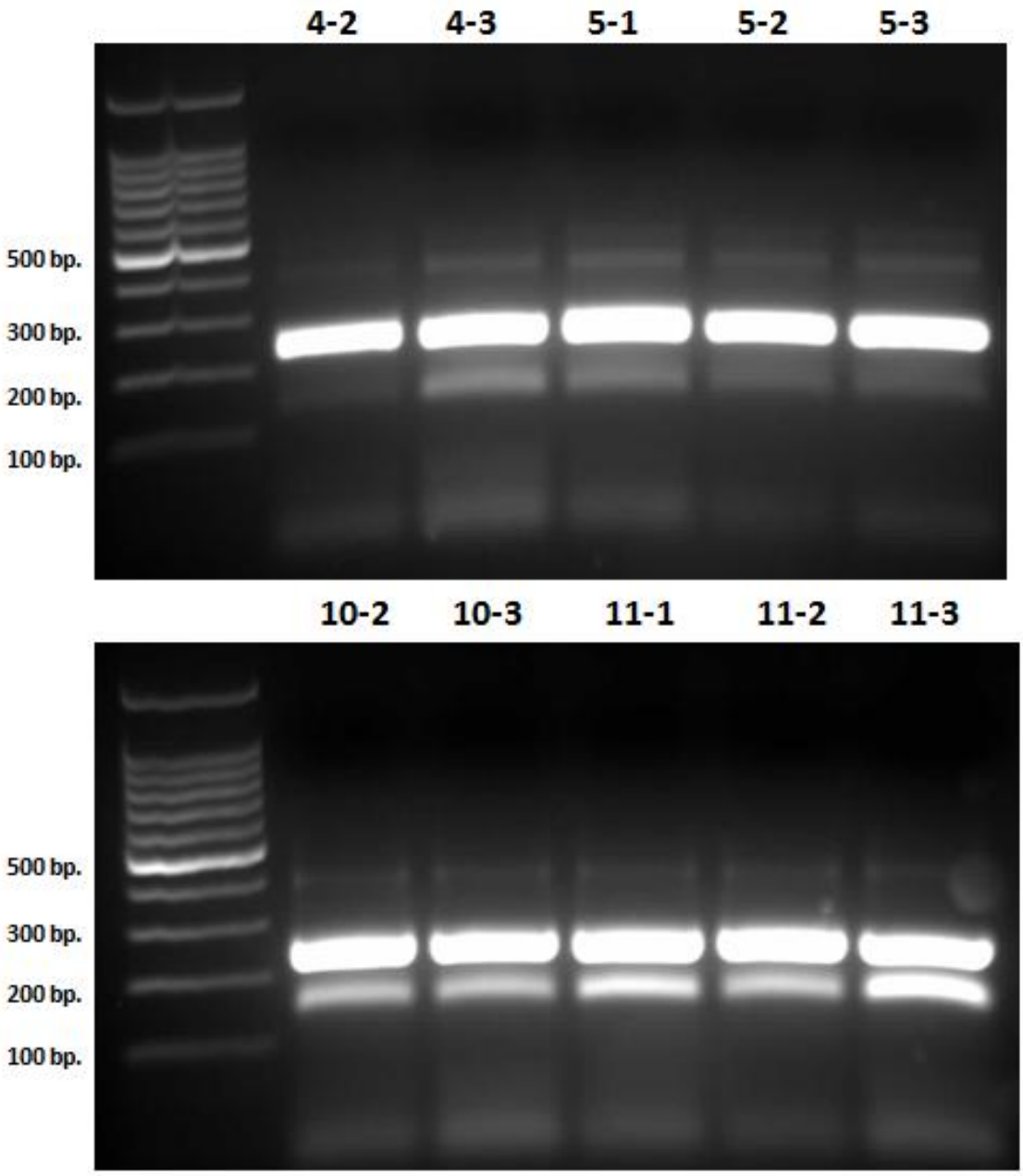


Fig 4.8: Differences in the presence of the smallest sized ITS-2 region. Differences between different migrating L_3 pools after PCR amplification using the conventional primers CyatITS-2reg. PCR products were subjected to electrophoresis in a 1% agarose gel and visualized as described in the material and methods. Two different gels are shown. Pools from Pop S are in the top panel and from Pop Mox in the bottom panel. Numbers in the top of each gel indicate pool number (left) and replicate number (right). Top panel: Pop S EPR Median (4-2 and 4-3); Pop S Control (5-1, 5-2 and 5-3). Bottom panel: Pop Mox EPR Median (10-2 and 10-3); Pop Mox Control (11-1; 11-2 and 11-3). The sizes of the fragments of 100 bp DNA ladder are indicated on the left of the images.

Table 4.11: Real time qPCR data plot from the β-tubulin and ITS-2 region experiments. The first three columns indicate the cycle threshold (CT) value as obtained for each independent experiment. Columns labeled with R1 through R3 show the values as a result of the division of each ITS-2 replicate CT by the β-tubulin CT value.

Genus†	**CT β tubulin**	**CT ITS-2 01**	**CT ITS-2 02**	**CT ITS-2 03**	**R1**	**R2**	**R3**	**Average**
Cyathostomum **b,c**	27.73	21.66	20.62	20.86	0.7811	0.7436	0.7523	0.7590
Cylicocyclus **c**	24.95	18.62	17.17	17.58	0.7463	0.6882	0.7046	0.7130
Cylicostephanus **c**	25.97	19.88	18.83	18.88	0.7655	0.7251	0.7270	0.7392
Poteriostomum **b**	26.20	21.75	20.82	20.95	0.8302	0.7947	0.7996	0.8082
Petrovinema **a**	25.93	24.01	22.72	22.9	0.9260	0.8762	0.8831	0.8951

†Rows within columns with the same letter are not significantly different

454 sequencing experiment

The 454 sequencing experiment successfully produced a total of 158,876 high quality readings (sequences) with an average read length of 250 bp and a mode read length of 254 bp. Less than 3% of the sequences produced were filtered. The read length distribution showed a very homogeneous distribution of the sequences generated. After the preliminary analysis approximately 10% of the sequences were shown to be < 120 bp, too short for the average expected reading, as such, all sequences shorter than 120 bp were discarded from the bioinformatics analysis. After this trimming, a total of 140,856 remained to be assigned to species. The parameter set for the Megablast query was established with a bit score minimum of 200 bp. After the Megablast® search, all the sequences generated matched a cyathostomin species ITS-2 accession number available in the GeneBank®.

Validation of the 454 sequencing protocol

Five artificial pools containing different concentrations of genomic DNA extracted from cyathostomin species were made. The concentration of genomic DNA was established originally by a Nanodrop 2000® spectrophotometer and the design of the pools was explained in detail in the materials and methods

section. The corrected original concentration of DNA was subsequently determined by a Qubit® 2.0 Fluorometer. Tables 4-12 through 4.16 show the results of the validation experiment using the artificial pools of genomic DNA and their comparison with the actual proportions of DNA from each species contained in each pool as established by the Qubit® 2.0 Fluorometer. In order to identify the relationship between the true proportions of species per pool and the proportion of sequences that resulted for each species, produced in the 454 experiment, the lower and upper 95% confidence limits (CL) were calculated for the 454 sequence proportions and compared with the known proportion in each pool. The results of this comparative analysis were classified as too high, if the lower CL of the 454 proportion was greater than the true proportion; too low, if the upper CL of the 454 proportion was lower than the true proportion; good, if the CL contained the true proportion; and as a odd reading, if the species detected by the 454 experiment was not included in the artificial pool. In general, our results did not reflect accurately the true species percentages used in the artificial pools, but they did represent almost the entire species diversity, with 90% (9/10 and 9/10) of the species detected in the two pools that contained 10 species, and an average of about 90% (4/4, 4/4 and 3/4) in the three pools which contained four species. The 454 platform was not able to discern between *Cy. catinatum* and *Cy. pateratum*. This outcome was expected because of the high level of similarity (99-100%) between the ITS-2 sequences for these two species. Moreover, the region selected for sequencing showed no difference between those two species. For that reason, *Cy. pateratum* and *Cy. catinatum* were considered as a complex in the interpretation of the 454 results. The validity of this two-species complex was confirmed by the fact that sequences identified as *Cy. pateratum* were detected in all the pools comprising only four species and none of those pools contained DNA from *Cy. pateratum* but *from Cy. catinatum*. Another finding that supports this two-species complex is the fact that *Cy. pateratum* was detected in the pools that contained 10 species in proportions that increased as the percentage of inclusion for *Cy. catinatum* increased in the pool, although these increments were not in the same proportions in the 454 data. However, the 454 sequencing did identify *Cs. goldi*, which also shows a very high level of similarity (98%) with *Cy. pateratum* and *Cy. catinatum*. *Cs. goldi* was detected consistently in all the artificial pools. A somewhat similar situation

occurred with *Cs. asymetricus*. In this case, no accessible sequence was available in the GeneBank but we successfully produced a sequence form adult worms of this species. The Megablast search, therefore, assigned its sequences to the closest species in the group, *Cs. bidentatus*, as was confirmed by the results of pools containing *Cs. asymetricus*. One important finding was the obvious over estimation of *Cc. nassatus* in all the artificial pools. This species was by far the dominant species in all the pools, with prevalences of 72 – 99% of all the reads. A certain level of correlation with the real percentage included in the sample could not be determined but, in general, there was an over estimation ranging from 1.7 to 57-times the known proportions included in the pools. With the only exception of *Petrovinema poculatum* (never detected) every single species included in each pool was identified by the assay, including species with prevalences of 1%. In one of the three pools with four species, *Cs. longibursatus* was not detected at a prevalence of 10%, but it was detected in the other three pools, with prevalences of 25% and 75%, and it was even detected at a lower prevalence (3%) in both pools with 10 species.

Table 4.12: Detail of DNA composition of artificial pool 01. The total amounts of DNA (ng) and their comparison with the results of the 454 sequencing experiment are shown. The original proportion of genomic DNA is compared with the percentage of sequences determined by the number of sequence reads from specific species produced by the 454 experiment. The column of analysis characterizes the relationship of these confidence limits of the measured proportions with the real proportions placed in the pools, as explained above.

Artificial Pool 01				
Species	**DNA(ng)**	**Original**	**Determined**	**Analysis of CL**
Cc. nassatus	0.254	4.68%	92.33%	CL too high
Cs. goldi	1.07	19.70%	5.51%	CL too low
Cy. catinatum/ pateratum	2.03	37.34%	1.27%	CL too low
Cs. longibursatus	0.525	9.67%	0.30%	CL too low
Pe. poculatum	0.72	13.25%	0%	CL too low
Cs. calicatus	0.354	6.52%	0.06%	CL too low
Cs. asymetricus/bidentatus	0.278	5.12%	0.49%	CL too low
Po. imparidentatum	0.199	3.66%	0.01%	CL too low
Cc. leptostomum	0.003	0.06%	0.01%	CL too low
Cc. ashworthi	0	0%	0.01%	Odd reading
TOTAL	5.433	100.00%	100.0%	

Table 4.13: Detail of DNA composition of artificial pool 02. The total amounts of DNA (ng) and their comparison with the results of the 454 sequencing experiment are shown. The original proportion of genomic DNA is compared with the percentage of sequences determined by the number of sequence reads from specific species produced by the 454 experiment. The column of analysis characterizes the relationship of these confidence limits of the measured proportions with the real proportions placed in the pools, as explained above.

Artificial Pool 02				
Species	**ng**	**Original**	**Determined**	**Analysis of CL**
Cc. nassatus	0.032	1.26%	72.38%	CL too high
Cs. goldi	0.321	12.73%	19.69%	CL too high
Cy. catinatum/ pateratum	1.622	64.32%	5.39%	CL too low
Cs. longibursatus	0.0213	0.84%	0.10%	CL too low
Pe. poculatum	0.18	7.14%	0%	CL too low
Cs. calicatus	0.106	4.21%	0.20%	CL too low
Cs. asymetricus/bidentatus	0.139	5.51%	2.10%	CL too low
Po. imparidentatum	0.0995	3.95%	0.10%	CL too low
Cc. leptostomum	0.001	0.04%	0.04%	Good
TOTAL	2.522	100.00%	100.0%	

Table 4.14: Detail of DNA composition of artificial pool 03. The total amounts of DNA (ng) and their comparison with the results of the 454 sequencing experiment are shown. The original proportion of genomic DNA is compared with the percentage of sequences determined by the number of sequence reads from specific species produced by the 454 experiment. The column of analysis characterizes the relationship of these confidence limits of the measured proportions with the real proportions placed in the pools, as explained above.

Artificial Pool 03				
Species	**ng**	**Original**	**Determined**	**Analysis of CL**
Cc. nassatus	0.212	8%	81.25%	CL too high
Cs. goldi	2.14	78%	17.76%	CL too low
Cy. catinatum/pateratum	0.248	9%	0.82%	CL too low
Cs. longibursatus	0.142	5%	0.17%	CL too low
TOTAL	2.742	100%	100.00%	

Table 4.15: Detail of DNA composition of artificial pool 04. The total amounts of DNA (ng) and their comparison with the results of the 454 sequencing experiment are shown. The original proportion of genomic DNA is compared with the percentage of sequences determined by the number of sequence reads from specific species produced by the 454 experiment. The column of analysis characterizes the relationship of these confidence limits of the measured proportions with the real proportions placed in the pools, as explained above.

Artificial Pool 04				
Species	**ng**	**Original**	**Determined**	**Analysis of CL**
Cc. nassatus	0.064	8%	81.31%	CL too high
Cs. goldi	0.642	41%	16.21%	CL too low
Cy. catinatum/pateratum	0.223	14%	0.71%	CL too low
Cs. longibursatus	0.639	41%	1.78%	CL too low
TOTAL	1.568	100%	100.00%	

Table 4.16: Detail of DNA composition of artificial pool 05. The total amounts of DNA (ng) and their comparison with the results of the 454 sequencing experiment are shown. The original proportion of genomic DNA is compared with the percentage of sequences determined by the number of sequence reads from specific species produced by the 454 experiment. The column of analysis characterizes the relationship of these confidence limits of the measured proportions with the real proportions placed in the pools, as explained above.

Artificial Pool 05				
Species	**ng**	**Original**	**Determined**	**Analysis of CL**
Cc. nassatus	0.339	56%	99.11%	CL too high
Cs. goldi	0.214	35%	0.81%	CL too low
Cy. catinatum/ pateratum	0.025	4%	0.09%	CL too low
Cs. longibursatus	0.028	5%	0.00%	CL too low
TOTAL	0.606	100%	100.00%	

454 sequencing identification of species composition of migrating L_3 pools

The results of the identification of the cyathostomin species by Megablast® search of the 454-generated sequence reads produced for each pool are shown in Figure 4.9 for Pop S and Figure 4.10 for Pop Mox. Two bar graphs per population were produced, one showing the percentages for each species detected in

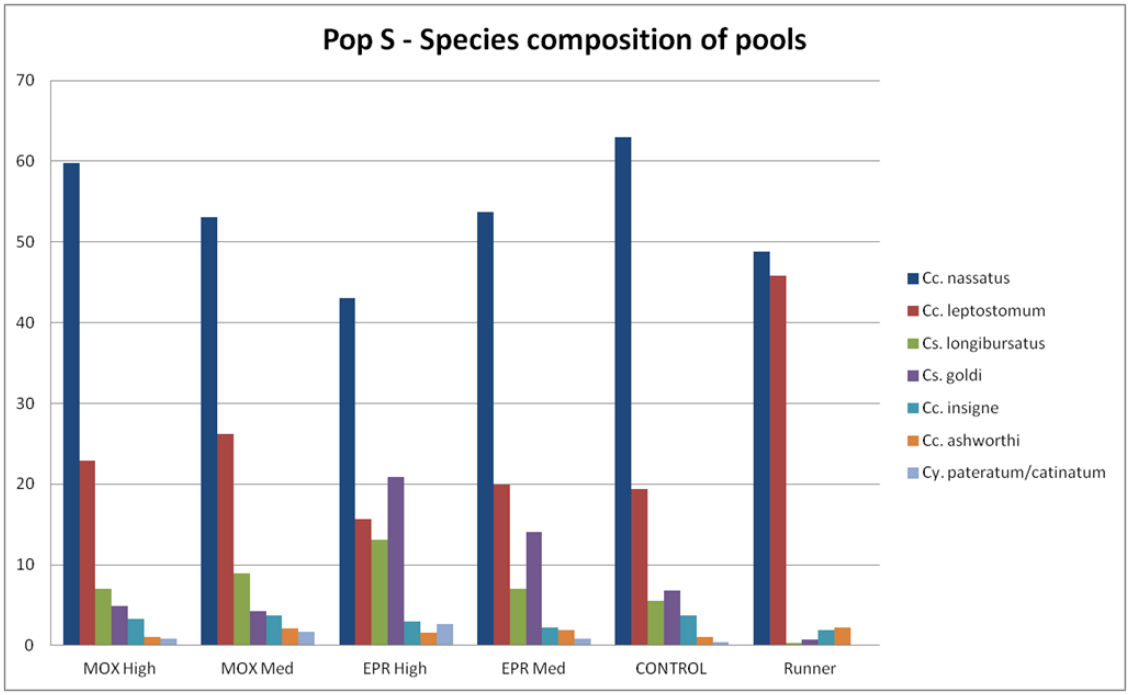
each larval pool while the other compares the distribution of each specific species across the 6 different pools. More importantly, and similar to the results of the validation experiment, *Cc. nassatus* was by far the most prevalent species found in each pool. This coincidental finding confirms that the presence of this species was overestimated by the 454 protocol used. Therefore, the interpretation of these results is impossible.

Regarding detection of species present in both populations, no major differences in the species composition of both populations were observed, with the only exception being *Cs. bidentatus/asymetricus*, which was detected only in Pop Mox. However, there were remarkable differences in the apparent prevalences of species in the controls of both populations. Up to 6 species, *Cc. nassatus, Cc. leptostomum, Cs. goldi, Cs. longibursatus, Cc. insigne* and *Cc. ashworthi*, comprised ~99% of the control pool in Pop S, leaving three minor species making up less than 1%. In the control pool of Pop Mox, only 3 species, *Cc. nassatus, Cs. longibursatus* and *Cs. goldi* accounted for ~99% of the population, while the other 7 species identified made up less than 1% of the population. Remarkably, however, the percentages of species per pool across the different pools of each population remained fairly consistent.

Regarding changes in the prevalence of species in the drug-exposed pools when compared to the control pool, and despite the absence of dramatic switches in species composition, the Chi-square test and the Fisher exact test analysis indicated significant differences for certain species (Table 4.17). For Pop S, the proportions of *Cs. goldi, Cs. longibursatus* and the *Cy. catinatum/pateratum* complex increased significantly, while *Cc. leptostomum* decreased significantly in the eprinomectin high pool and *Cc. nassatus* decreased significantly in both eprinomectin pools ($p<0.0001$). These changes were more dramatic, percentage-wise, in the high pool. The results were different in this population for both moxidectin treated pools, with *Cc. leptostomum, Cs. longibursatus* and the *Cy. catinatum/pateratum* complex showing an increase, while *Cs. goldi* and *Cs. nassatus* exhibited a decrease. *Cc. ashworthi*

increased significantly only in the moxidectin median pool (p<0.0001). These changes, percentage-wise, were more dramatic in the median concentration (Table 4.17).

In the case of the results for Pop Mox, based on the distribution of species, the interpretation of the results is more complicated. All the 7 species with less than 1% of prevalence across the pools were grouped as "others" to allow a better statistical analysis. There was a significant increase in *Cs. longibursatus* in both eprinomectin treated pools, while *Cs. goldi* and the *Cy. catinatum/pateratum* complex significantly increased only in the highest concentration pool. The prevalences of *Cs. goldi* were reduced in the eprinomectin median pool (p<0.0001). In the moxidectin treated pools, *Cs. longibursatus* increased in both pools, while *Cs. goldi* increased only in the high pool (p<0.0001). *Cc. nassatus* showed a significant decrease in all the drug treated pools (p<0.0001). One remarkable finding was that the presence of *Cs. longibursatus* increased significantly in all the drug treated pools in both populations. No dramatic changes in the species composition of the group of highly active larvae subjected to long incubation periods ("runners") were detected when compared with the control pool, but statistically significant changes in the prevalences of some species were found, with a significant increase in the prevalence of *Cc. ashworthi* and *Cc. leptostomum* in Pop S, and only *Cc. nassatus* increasing in Pop Mox (p<0.0001) (Table 4.17).



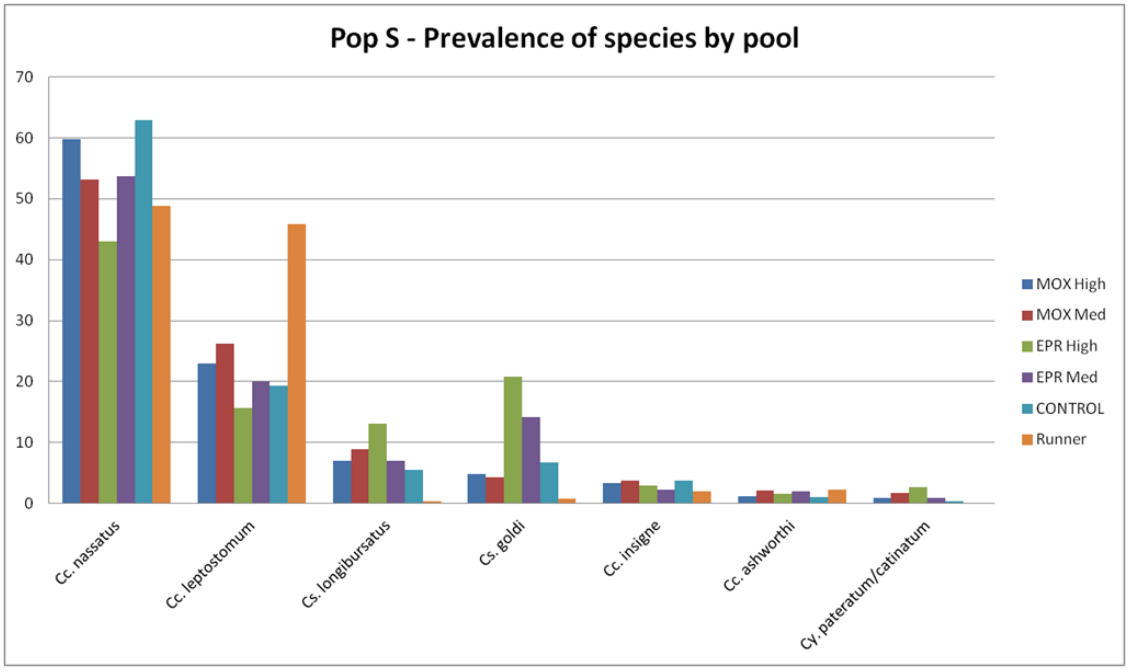
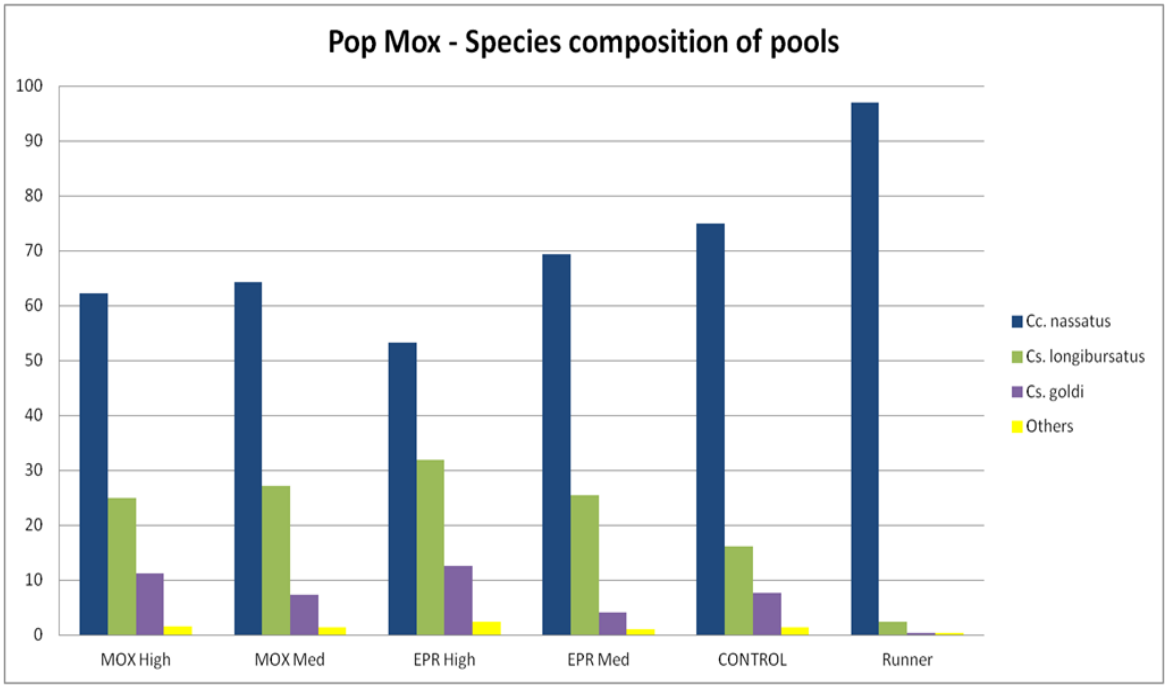


Fig. 4.9: Species composition of Pop S pools. Species composition of each pool and the prevalence of species by pool in Population S (Pop S) pools are based on bioinformatics analysis of the 454 sequencing data. MOX High, pool moxidectin high; MOX Med, pool moxidectin median; EPR High, pool eprinomectin high; EPR Med, pool eprinomectin median, CONTROL, pool control; Runner, pool runners. The results of the Megablast designation of sequence reads produced in each pool by the 454 run were plotted in a Microsoft Excel® sheet to generate a 2-D column graph. Because of their low prevalence, *Cs. calicatus* and *Co. labiatum* are not shown



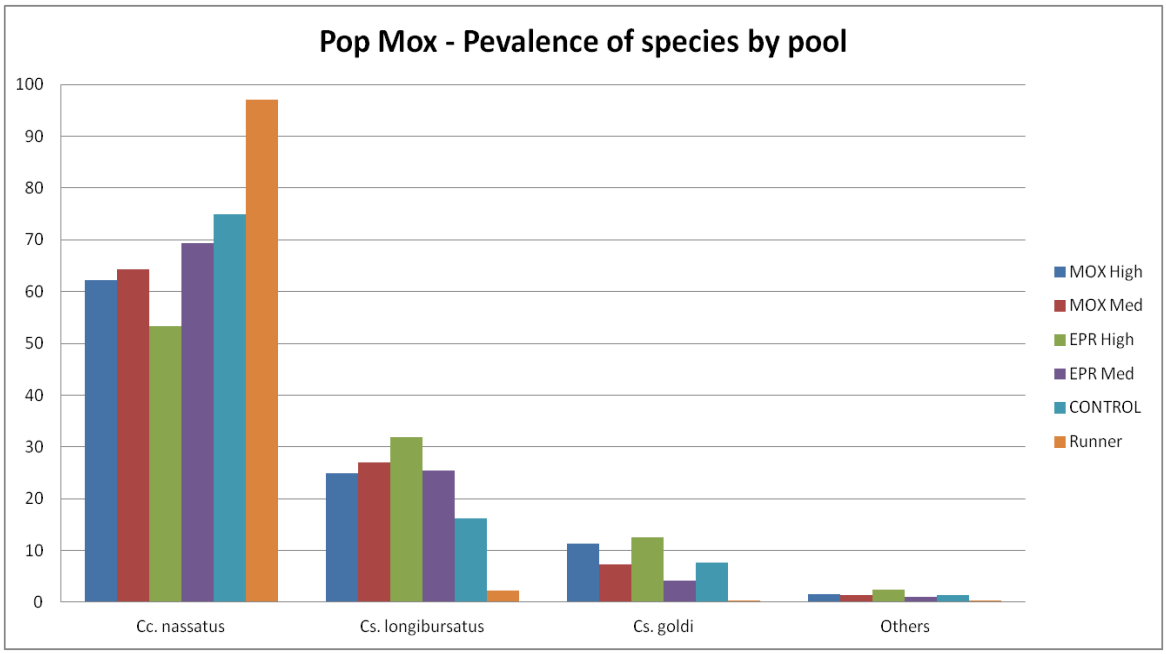


Fig. 4.10: Species composition of Pop Mox pools. Species composition of each pool and the prevalence of species by pool in Population Mox (Pop Mox) pools are based on bioinformatics analysis of the 454 sequencing data. MOX High, pool moxidectin high; MOX Med, pool moxidectin median; EPR High, pool eprinomectin high; EPR Med, pool eprinomectin median, CONTROL, pool control; Runner, pool runners. The results of the Megablast designation of sequence reads produced in each pool by the 454 run were plotted in a Microsoft Excel® sheet to generate a 2-D column graph. Others comprises: *Cc. leptosotomum, Cc. insigne, Cc. ashworthi, Cy pateratum/catinatum, Cs. calicatus, Co. labiatum* and *Cs. bidentatus*

Table 4.17: Species with significant differences in prevalence across the migrating L_3 pools. Cyathostomin species with a significant increase or decrease in the drug treated pools and runners when compared to the control pool in each population are shown. EPR High, pool eprinomectin high; EPR Median, pool eprinomectin median; MOX High, pool moxidectin high; MOX Median, pool moxidectin median; Runners, pool runners

Population	Pool	Increased	Decreased
Pop S	EPR High	*Cs. goldi; Cs. longibursatus* *Cy pateratum/catinatum*	*Cc. nassatus* *Cc. leptostomum*
	EPR Median	*Cs. goldi; Cs. longibursatus* *Cy. pateratum/catinatum*	*Cc. nassatus*
	MOX High	*Cc. leptostomum* *Cs. longibursatus* *Cy. pateratum/catinatum*	*Cc. nassatus* *Cs. goldi*
	MOX Median	*Cc. ashworthi* *Cc. leptostomum* *Cs. longibursatus* *Cy. pateratum/catinatum*	*Cs. goldi* *Cc. nassatus*
	Runners	*Cc. ashworthi* *Cc. leptostomum*	*Cs. calicatus; Cs. goldi* *Cc. insigne* *Cs. longibursatus* *Cc. nassatus* *Cy. pateratum/catinatum*
Pop Mox	EPR High	*Cs. goldi; Cs. longibursatus* *Cy. pateratum/catinatum*	*Cc. nassatus* others
	EPR Median	*Cs. longibursatus*	*Cs. goldi; Cc. nassatus*
	MOX High	*Cs. longibursatus; Cs. goldi*	*Cc. nassatus*
	MOX Median	*Cs. longibursatus*	*Cc. nassatus*
	Runners	*Cc. nassatus*	All the rest

CHAPTER 5

DISCUSSION

In this study, a modified protocol for a larval migration inhibition assay (LMIA) was developed to determine the dose responses of equine cyathostomin third stage larvae (L_3) to ML drugs. Moreover, a novel molecular approach for cyathostomin species identification based on a 454 high throughput DNA sequencing platform was tested. Our hypotheses were (1) that the LMIA is a suitable method for measuring levels of resistance/susceptibility to ML in cyathostomin populations; (2) that the species composition of cyathostomin populations has an impact on the resulting dose response measured in the bioassay and (3) that the 454 massive parallel pyrosequencing system is a valid option to quantitatively characterize the species composition of cyathostomin populations. Our main goals were to measure the levels of *in vitro* susceptibility to ML in different cyathostomin populations, and to evaluate the effect of cyathostomin species composition on the interpretation of the LMIA results. To accomplish this objective, we tested two parasite populations with different levels of exposure to ML anthelmintics; a ML-naïve population (Pop S) and an experimentally moxidectin-selected population (Pop Mox). Using four different ML analogs; ivermectin, abamectin, eprinomectin and moxidectin, the LMIA was repeated in multiple independent replicates at different drug concentrations. The species composition in the original untreated L_3 pools and the migrating L_3 pools at different drug concentrations (at the IC_{50} level and at the highest 2 concentrations tested) were characterized using a 454 massive parallel pyrosequencing platform, to evaluate differences in inherent tolerance among the cyathostomin species, and differences in species composition between the two cyathostomin populations.

The LMIA showed good repeatability, as indicated by the dose-response data generated. Significant differences between both populations were found at the analysis of the IC_{50} only for abamectin and moxidectin; however, these differences were small and without any clinical relevance. In both cases, the ML-selected population remarkably exhibited a lower IC_{50} than the susceptible one. Moreover, the resistance ratios (IC_{50} of resistant population/IC_{50} of susceptible population) obtained for the ML-selected population did not indicate any relevant differences. Based on these findings, it can be concluded that the LMIA detected small differences in ML susceptibility in two different cyathostomin populations in 2 of the 4 ML analogs tested, although it failed to demonstrate a lower susceptibility in a drug-selected population. However, resistance has not been demonstrated clinically for Pop Mox. Therefore, the validity of the LMIA to detect resistance to ML in cyathostomins remains to be tested, but our results are highly encouraging based on the consistency of our results using up to four different ML-analogs.

The 454 massive parallel pyrosequencing strategy did provide a good accounting of the species composition of the DNA pools, but it did not represent the relative species abundance accurately, as demonstrated by the validation experiment performed. Thus, a valid analysis of the differences in the prevalence of species across the migrating L_3 pools was not possible, and we could not determine differences in the *in vitro* tolerance among the different cyathostomin species to ML. However, based on the consistency of the results in both cyathostomin populations, this novel application of this molecular approach did indicate differences in the species composition in both populations. To our knowledge, this is the first report of the use of a 454 high throughput sequencing protocol for species identification in parasitic nematodes and our results indicate that, even though highly sensitive at detection, this approach has several drawbacks that makes it poorly suitable for this purpose when compared to other molecular techniques.

Among the *in vitro* tests available for detecting and measuring levels of emerging ML resistance in equine cyathostomin populations, the LMIA is the most promising option because it is suitable for anthelmintic drugs with paralyzing effects such as ML analogs. However, no consistent and validated

working protocol has yet been established (Matthews et al., 2012). Other bioassays based on L_3 motility use direct observation and evaluation of motility by an index or using electronic devices (e.g. a micromotility meter). The former technique is prone to subjectivity and poor repeatability, the latter has shown poor correlation with *in vivo* results (Matthews et al., 2012). In our experience, working with a simplified scale of categorization of L_3 motility, we found that the use of this system is cumbersome and time consuming. The LMIA eliminates the subjectivity and difficulty of motility evaluation by using a sieve as a physical means to separate motile from non motile worms (Kotze et al., 2006). Originally, the LMIA was developed to measure the paralyzing effects of abomasal factors in *Trichostrongylus colubriformis*, using levamisole as a control for paralysis, with a ~95% migration inhibition (Douch et al., 1983). Later, Wagland et al. (1992) modified the protocol of LMIA to test different anthelmintic drugs. Then d'Asonville et al. (1996) developed a protocol using ML drugs (ivermectin) exclusively. The modified protocol developed in the present study is based on the protocols developed by Kotze at al. (2006), Monopoli (2007) and McArthur (2007), and is similar to the protocol used by Demeler et al. (2010).

During the process of optimization of this protocol we observed that one of the major parameters affecting larval migration in cyathostomin L_3 was the innate level of larval motility. Compared to other nematode species, cyathostomin L_3 tend to be much less active. Previous studies on LMIA in cyathostomins have encountered similar difficulties. For example, van Doorn et al. (2010), using a 40 μm mesh, which was almost twice the pore size used in our study, obtained similar migration rates in the controls (~75 to 80%). During a protocol for successive LMIA's, these authors also found that the migration in the absence of drug was as low as 60%. These previous reports indicate that low levels of migration in the no-drug controls are a major challenge in developing a LMIA working protocol in cyathostomins. For this reason, several variables that could affect larval motility were tested during our optimization experiments. During this testing it was noticed that mechanical stimulation of the larvae by vortexing or shaking increased the activity of the larvae, thus increasing the migration in the no-drug controls.. To our knowledge, this constitutes the first report on the use of this procedure in a LMIA

protocol. Some authors have reported the addition of warm water to well plates in order to stimulate larval motility in ruminant nematodes (Kotze et al., 2009) and the use of vibrations generated by tapping the plate to stimulate increased larval movements (Howell, 1999). Using this modification, we were able to achieve an average rate of migration in the control wells of approximately 88%. Though our goal was to achieve 90% or better, the levels we achieved are deemed sufficient to yield good quality dose response data in most all of the assays performed.

The dose response curves obtained with each of the 4 analogs in both populations showed good consistency and they fitted the generated nonlinear regression model very closely, with coefficient of determination (r^2) values ranging from 0.88 to 0.97. In the major species of ruminant nematodes, for which the LMIA has been successfully and extensively validated, the r^2 values reported are greater than 0.90 (Demeler et al., 2010a; Demeler et al., 2010b). Therefore, our results likely indicate a truly optimized LMIA protocol. The IC_{50} values obtained (Tables 4.10) were similar in all the analogs used with the exception of eprinomectin, which had a slightly higher value. However, there were no biologically relevant differences between the two cyathostomin populations. Moreover, the 95% confidence interval for the IC_{50} did not indicate significant differences between the populations for abamectin, ivermectin and eprinomectin. Only in the case of moxidectin, the 95% CI for the IC_{50} did not overlap; however, the moxidectin-selected population (Pop Mox) showed a lower value (more susceptible) than the ML-naïve population (Pop S). On statistical analysis, significant differences between both populations were detected for abamectin (p=0.0172) and moxidectin (p=0.0002), but these differences do not seem to have biological relevance since the highest resistance ratio found was 1.09 (for eprinomectin) and because the Pop Mox showed a lower IC_{50} than the Pop S in both cases. The resistance ratio is an important parameter when comparing the resistance status of a parasite population with a known susceptible one. In the previously mentioned LMIA studies in ruminant nematodes using ML drugs, the RR observed for several known resistant isolates ranged between 1.7 and 28.1 (Demeler et al., 2010a; Demeler et al., 2010b; Kotze et al., 2006). Therefore, the LMIA protocol developed in the present study did not detect important differences in susceptibility/resistance levels to ML in the two populations

studied, and a lower susceptibility to ML, which was expected in the Pop Mox, could not be confirmed by the assay. It must be taken into account that even after more than 10 years of drug selection pressure, the percentage of FEC reduction has never confirmed resistance in the herd (Lyons, 2011, personal communication), though one would expect a lower susceptibility due to years of drug selection. Therefore, considering that Pop Mox still remains susceptible to ML and that this population was originally derived from Pop S, similar dose response characteristics may not be unexpected. Consequently the validation of the LMIA protocol for the detection ML resistance in cyathostomin parasites developed in this study remains somewhat inconclusive and further experiments using cyathostomin populations from confirmed field cases of ML resistance are needed since there are currently no resistant laboratory isolates available.

Even though the IC_{50} values obtained in the present study do not differ significantly from those reported for the major ruminant parasites, *H. contortus, T. circumcincta, O. ostertagi* and *C. oncophora* in previous LMIA studies using ML (Demeler et al., 2010a; Demeler et al., 2010b; Kotze et al., 2006), before drawing major conclusions, our results should be compared to similar studies carried out with cyathostomins. To our knowledge there are only three documented studies on the development of LMIA protocols for the detection of ML-resistance in cyathostomins (McArthur, 2007; Monopoli, 2007; van Doorn et al., 2010). The results reported by these previous studies vary.

In McArthur (2007), a 25 μm mesh was also used for the LMIA. However, this author used a different migration period and temperature, and a different statistical approach for data analysis. Moxidectin was used in the assay to determine ML-resistance in cyathostomins infecting two herds of donkeys. The IC_{50} values found in ML-susceptible populations were between 4.5 and 7.5 μg/ml (7 μM-11.7 μM), while in the known ML-susceptible horse isolate, the values ranged from 3.81 – 11.2 μg/ml (5.7 μM – 17.5 μM). These values are much higher than the values found for the same analog in our study, 1.01 μg/ml (1.57 μM) for Pop S and even lower, 0.65 μg/ml (1.0 μM), for Pop Mox. The much higher IC_{50} values found for McArthur's study when compared to our results, despite the use of a sieve of

the same size may be explained by the differences in the statistical methods used to deduce the IC_{50}, the fewer number of replicates per isolate, the differences in the species composition between the populations used, or the solubility of the drugs in the assay media. This argument could be supported by the fact that in the same ML-susceptible *Haemonchus contortus* isolate, used as a positive control for ML-resistance due to the lack of confirmed ML-resistant isolates in cyathostomins, the IC_{50} reported by McArthur, 5 μg/ml (5.7 μM), was much higher than those reported in other studies using similar protocols (d'Assonville et al., 1996; Demeler et al., 2010a; Kotze et al., 2006). When McArthur tested her LMIA protocol in a suspected moxidectin-resistant parasite population, considerably higher values for the IC_{50}, ranging from 10.6 μg/ml to 13.4 μg/ml (16.6 to 20.9 μM) were found, so that the resistance ratios (RR) for the suspected resistant population in the donkeys ranged from about 2.4 to 1.8 and significant differences were found when compared with the susceptible population. The conclusion from this study was that the LMIA protocol developed was a promising option for the assessment of moxidectin-resistance in cyathostomin populations. In our results, the IC_{50} did not demonstrate a change in susceptibility in the ML-selected population when compared to the ML-susceptible one; the highest RR detected was 1.09 for eprinomectin. However, as previously mentioned, we worked with a parasite isolate that had been subjected to frequent moxidectin treatment, but had not yet developed true clinical resistance while those tested by McArthur had.

The results reported in the present study are similar to those reported by Monopoli (2007) working with three different ML-susceptible cyathostomin populations, including Pop S. This author used a 48-well plate and a 30 μm mesh format of the LMIA and three ML analogs, eprinomectin, ivermectin and doramectin. The IC_{50} values for one of the ML-naïve populations, the Population Assateague, were 0.95 μM, 0.37 μM and 0.71 μM for eprinomectin, ivermectin and doramectin, respectively. For both eprinomectin and ivermectin, our results were higher, 2.32 μM and 0.95 μM respectively. In this case, a different parasite population with a different species composition and some differences in the protocols may account for these differences. Different cyathostomin populations may produce different results and this should be considered when interpreting data from this type of bioassay. This is supported by the

IC_{50}'s obtained by Monopoli using eprinomectin in Pop S. The values obtained were much higher than those obtained in Population Assateague, ~2 μM vs. 0.95 μM, and this value is very similar to the IC_{50} value for the same drug obtained in the present study, 2.55 μM. In spite of the use of a larger pore size mesh (30 μm), the migration percentage obtained by Monopoli in the no-drug controls in a period of two hours was similar to ours (70 – 80%), with up to 80% of migrating larvae having migrated in the first 30 minutes. Using a smaller sized mesh (25 μm), we obtained similar migration rates and even higher rates in the negative controls in a shorter migration period (45 minutes). The optimization the LMIA protocol reported in this study, as described in the previous sections, may explain these differences. The differences in our protocol may also explain the high repeatability observed in our results working with four different ML analogs, in contrast with Monopoli's results that showed only consistent results using eprinomectin.

A more recent report of the application of the LMIA for the detection of ML-resistance in horse cyathostomin has been described by van Doorn et al. (2010). They used a modified version of the protocol developed by McArthur (2007) and our protocol had some of the characteristics of this assay, such as the use of PBS, the format of the migration sets, the migration temperature of 37°C and the use of 1% DMSO. Similar to our experimental design, van Doorn et al. (2010) used a ML-naïve population (OVP) and an ivermectin-selected, but not clinically resistant population, in Shetland ponies. One notable difference with our results is the very low IC_{50} values reported in this study, which were significantly lower than our results and other results reported previously. These divergent results demonstrate the differences in the protocols used. Similar to our findings, the IC_{50} for the ML-selected population was significantly lower than the one obtained for the naïve population: 0.025 μg/ml (0.028 μM) vs. 0.031 μg/ml (0.035 μM). Therefore, the LMIA protocol results also did not correspond with the drug exposure/selection of the population. This finding was common in our results for each analog, with Pop Mox having a similar or significantly lower IC_{50} than Pop S. The reason why a long term drug selection process in a cyathostomin population could produce a phenotype that is equal or slightly more susceptible to the drug *in vitro* as detected by the LMIA is not clear. It can be speculated that the experimental drug

selection protocol applied did not induce a truly resistant status, and that this type of protocol differs from the real scenario emergence of resistance in the field. Another possible explanation is the existence of differences in species composition, with some species having higher levels of inherent *in vitro* drug tolerance than others.

One important issue to consider when using the LMIA in cyathostomin parasites is the difficulty in data interpretation generated by the presence of multiple species in a given population, because apparent shifts in susceptibility/resistance may be produced by differences in the natural *in vitro* drug tolerance exhibited by specific species present in the population (Matthews et al., 2012). This constitutes a unique complication for this group of parasites and creates a completely different scenario when compared to the case of ruminant nematodes, for which the LMIA has been standardized and validated for individual species. However, it should be mentioned that authors studying the application of the LMIA to detect ML-resistance in several species of parasitic nematodes in ruminants have reported differences in susceptibility for different species (Demeler et al., 2010a; Demeler et al., 2010b; Kotze et al., 2006). These findings highlight the need to consider the specific characteristics of the species present in studies for validation of the LMIA. Moreover, early studies on the efficacy of ML drugs in cyathostomins indicate that some cyathostomin species show a higher tolerance *in vivo* (Egerton et al., 1981), while other studies have suggested that some species exhibit a higher innate tolerance to ML *in vitro*, and that this should be a major factor to consider when validating a LMIA protocol in these parasites (van Doorn et al., 2010). In order to determine the differences in levels of natural tolerance to ML *in vitro* in cyathostomin populations, we decided to investigate the species composition of larval pools migrating in the no-drug control wells, to compare it with the species composition of migrating L_3 in the wells near the IC_{50} level and at drug concentrations above the IC_{50}. For this purpose, we obtained pools of L_3 from replicates produced with eprinomectin and moxidectin from the LMIA. Since the molecular approach is the only tool available for the accurate identification of species in cyathostomin L_3, we developed and tested a novel protocol for the DNA-based identification of cyathostomin species by using a 454 high-throughput platform to sequence a region within the second internal transcribed spacer region (ITS-2),

with a highly conserved priming region and sufficient internal divergence levels among the cyathostomin species to be useful for specifies-specific markers.

Previous studies have used different molecular approaches for species identification in these parasites, but all have used genomic ribosomal DNA (rDNA), more specifically, the IGS and ITS-2 as molecular markers (Gasser, 1997; Hodgkinson et al., 2003; Hung et al., 1999; Kaye, 1998). The suitability of the ITS-2 as a specific molecular marker for the cyathostomin species has been previously confirmed in several reports (Brianti et al., 2009; Gasser, 2004; Gasser, 1997; Hung et al., 1999). The selection of the ITS-2 instead of the IGS, with a confirmed suitability as a species-specific marker, was based on the 454 experimental sequencing restrictions to an optimal size of amplicon of 200 to 400 bp. This requirement could be satisfactorily met only for the ITS-2 region. Moreover, available DNA sequences from the ITS-2 have been previously used to successfully identify cyathostomin L_3 and L_4 stages using a sequencing approach (Brianti et al., 2009).

The molecular test used for cyathostomin species identification most described in the literature is the reverse line blot (RLB) hybridization assay developed by Traversa et al. (2007). This assay has been successfully used for cyathostomin species identification using eggs isolated from fecal samples (Ionita et al., 2010; Traversa et al., 2010) and L_3 from coprocultures (Cernanská et al., 2009). This assay has been previously used in identifying cyathostomin species in migrating L_3 in an LMIA, where the authors looked for the presence of specific species in ivermectin-exposed larvae (van Doorn et al., 2010). However, this assay is limited to the qualitative detection of the 13 most common cyathostomin species. Therefore, the underestimation of species not included in this test, even though usually less prevalent, and the lack of quantitative capacity should be considered major disadvantages of this test. It has also been suggested that some species reported as rare or infrequent could have been underestimated in previous epidemiological studies (Lind et al., 2003). Additionally, it should be mentioned that major drawbacks have been reported in the original protocol developed by Traversa et al. (2007), that hampered the optimal detection of some species (Cwiklinski et al., 2012; van Doorn et al., 2010). A PCR-ELISA assay has also

been developed to successfully identify cyathostomin L_4, however, this assay is restricted to the identification of only five species (Hodgkinson et al., 2003). Because of the limitations of these molecular assays, we decided to explore the potential of the 454 platform as a promising alternative for cyathostomin species identification, with the capacity to detect even the least prevalent and rare species, some of which have been reported to occur in Pop S (Lyons et al., 1996b). The use of the 454 technology for the characterization of species diversity of populations has been extensively validated in different prokaryotic and eukaryotic organisms (Bråte et al., 2010; Droege and Hill, 2008; Samarakoon et al., 2011). Poranzinska et al. (2009) successfully validated a 454 high throughput massive sequencing protocol for the identification of free-living nematode species in metagenomic samples. This work showed clearly the feasibility of using a similar approach in metagenomic samples of other nematodes, including parasitic ones. The costs related to the use of 454 sequencing may be prohibitively expensive, because of the equipment and reagents required, and this is one of the main constraints in the use of this approach for research purposes. However, several studies have shown that the improved performance, in terms of efficiency, number of reads and higher quality than conventional sequencing approaches makes this technology a practical alternative for metagenomic studies in nematode communities (Travis, 2011).

One of the objectives of our study was to evaluate this novel DNA-based approach for performing a quantitative detection of species. However, the number of rDNA gene copies is known to vary in other species of pathogenic organisms, and this in turn can affect the outcome of quantitative assays (Herrera, 2009). In order to rule out the possibility of significant differences in the copy numbers in the ITS-2 gene in the cyathostomin species, which could lead to a misrepresentation of species' proportions, we performed a SYBR-Green-based quantitative real time PCR experiment. Our experimental approach was a simplified version of the one reported by Providenti et al. (2006) who worked with bacterial plasmid DNA and used a known single-copy gene as a reference. Similar to our approach, these authors used the cycle threshold (CT) values to derive the copy number; however, we applied a simplified mathematical analysis since our main objective was to detect significant differences and not to determine the actual number of copies. There were differences among the CT values obtained, with *Cylicocyclus* exhibiting the

smallest value (indicating a greater number of ITS-2 copies) and *Petrovinema* the highest (indicating fewer number of ITS-2 copies). However, the ANOVA showed no significant differences among the three major genera: *Cyathostomum, Cylicostephanus* and *Cylicocyclus*. Significant differences were detected for the genera, *Petrovinema* and *Poteriostomum*, which showed a higher CT value and therefore a relatively smaller number of ITS-2 repeats when compared to the other three genera. Based on these results we may conclude that the number of ITS-2 repeats among the *Cyathostominae* genera is relatively similar, but that there are significant differences among certain genera. Considering the reiterative nature of PCR amplifications and the *sui generis* conditions of the emulsion PCR (emPCR) used in the 454 protocol, the effect of even the subtle differences on the performance of the platform is difficult to predict. However, considering the existence of non significant differences among the three major genera reported in Pop S and the fact that *Petrovinema poculatum* is a very rare species in this population, these results may indicate that the number of ITS-2 repeats should not to be a major issue in the performance of the 454 experiment. These results are similar to the ones reported for *Aspergillus fumigatus*, in which different isolates did not exhibit significant differences (Herrera, 2009). Even though in the present study we used a different type of organism, the fact that the sub-family *Cyathostominae* comprises very closely related nematode genera that share several common biological features may explain the similarity of results.

A validation experiment was included in our protocol to determine the accuracy of the test to determine quantitatively the species composition in cyathostomin populations. For this purpose, we tested the performance of the assays in artificial pools of genomic DNA from different species in known proportions. The results obtained with the 454 sequencing did not accurately reflect the true species percentages used in the artificial pools, but they did represent the species diversity very accurately and with high sensitivity. With the exception of *Petrovinema poculatum*, every single species included in each pool was identified by the assay, including the species from which extremely small amounts of DNA (in the order of picograms) were used. One remarkable finding that contributed to the misrepresentation of the species in all the artificial pools was the dramatic over estimation of one specific species, *Cc.*

nassatus. Our results are somewhat similar to those reported by Porazinska et al. (2009) working with artificial pools of adult free-living nematodes from up to 41 different taxa. These authors concluded that their 454 protocol detected most of the species present (~90%) but failed to reflect the true proportion in the sample. These findings would indicate that the 454 approach may be optimal at identifying species in populations (identity) with an optimal sensitivity but may be deficient at determining the species' proportions (abundance). Potential bias in the lysis of organisms, in the DNA extraction and the PCR amplification have been discussed as potential factors responsible for distortion of the representation of species in metagenomic studies using next generation sequencing approaches in human digestive tract microbiota (Andersson et al., 2008; Lazarevic et al., 2009). Considering our experimental conditions, none of these conditions are likely to be responsible for the distortion in our results. The emPCR conditions have significant downsides and failure rates that can contribute to this distortion (Travis, 2011). Based on our results, we cannot rule out the possibility of PCR or emPCR amplification bias. Additionally, PCR bias caused by variation in the number of copies of ribosomal DNA has also been described as an important source for misrepresentation of genera and species in metagenomic studies using the 454 platform (Andersson et al., 2008). This may not be the cause for the misrepresentation of species observed in the present study, as suggested by the qPCR experiments carried out to detect any significant difference in rDNA ITS-2 repeat numbers among cyathostomin genera. However, non significant differences may have been greatly increased by the conditions of our protocol and in the emPCR amplification of the 454, but again this possibility cannot be confirmed. The reasons explaining the overestimation of one specific member of the genus *Cylicocyclus* cannot be determined given the current data. More research would be required to try to determine the causes of this unexpected phenomenon, but the cost of these experiments could be quite high.

Another problem of the 454 approach in metagenomics studies is the presence of chimera sequences that diverge significantly from the reference sequences expected and that can lead to misinterpretation of results (Porazinska et al., 2009). In our results, only a single sequence out of 10921 generated matched a cyathostomin species originally not included in the artificial pool (*Cc. ashworthi*). However, in this case,

the species identified shared a high similarity (97% identity) with *Cc. nassatus* (Hung, 1997); therefore, the presence of a partially incomplete sequence or with a insertion-deletion (indel) may explain this incorrect allocation. Considering the very low percentage of misidentified sequences (0.0092% of one pool) and the high similarity in ITS-2 sequences among the cyathostomins, we may conclude that our protocol did not exhibit the problem of highly divergent chimera sequences, confirming the advantages of the use of this next generation sequencing platform with a reduction in the error of underestimation of species richness. One possible area for further optimization of this protocol would be the determination of the real levels of ITS-2 sequence variation within the cyathostomin species to help in the interpretation of readings. This has proved to be an important variable in molecular protocols using IGS (Cwiklinski et al., 2012; van Doorn et al., 2010).

Regarding the identification of species obtained by the 454 platform in the migrating L_3 pools, no major differences in the species detected in both populations were observed. With the exception of *Cs. bidentatus/asymetricus* complex, which was only detected in Pop Mox, the same species were detected in both populations. The limitations at representing accurately the species proportions detected in the validation experiment, makes any conclusion based on the prevalences reported by the 454 experiment very difficult and questionable. However, the consistency of the species proportions across the different pools in each population may suggest a major difference in the apparent species diversity between both populations. In Pop S, up to 6 species accounted for ~99% of the population, while only 3 species comprised the same proportion in Pop Mox, with the other 7 minor species present made less than 1%. These findings, together with the uneven distribution of *Cs. minutus* and *Co. coronatus*, identified qualitatively by Sanger sequencing because of 454 constrains, may indicate some influence of drug selection in the drug-selected parasite population (Pop Mox), with changes in the prevalence of species. The effects of anthelmintic drug selection in the species composition of cyathostomin populations *in vivo* has been reported for BZ (Traversa et al., 2009a). Using a modified protocol of the RLB assay, van Doorn et al. (2010) investigated differences between a ML-naïve and a ML-selected parasite population, both from different origins, but did not find significant differences either, with the same three major species

dominating both populations. The use of a different molecular test for species identification with a higher performance at determining species richness, and the study of two related populations, one of them with a heavy drug-selection (Pop Mox), are factors that could explain our findings. In van Doorn et al. (2010), two unrelated field populations were used, one which had been subjected to ivermectin selection under field conditions, and the other that was ML-naive. In our case, we worked with two related populations in which the moxidectin-selected one had been derived from the ML-naïve one, and the drug selection had been experimentally attempted under intensive pressure. Even though this drug selection did not induce true clinical resistance, previous molecular studies in Pop Mox did show genetic changes in one specific species (Tandon et al., 2005), and therefore changes in worm susceptibility to ML, and in the species composition may be expected.

More remarkably, the distribution of the dominant species reported in these results somewhat resembled the species distribution in Pop S described in previous long-term epidemiological studies on parasite population dynamics (Lyons et al., 2001; Lyons et al., 1996b), but with a distortion in the species prevalence, which was most dramatic in *Cy catinatum/pateratum*. This may suggest a good accuracy of the 454 protocol at detecting species, and a certain validity of the 454 results in representing the species diversity in both parasite populations. Similar to the results obtained in the artificial pools and remarkably, *Cc. nassatus* was, by far, the dominant species in every single migrating larvae pool, but with much lower percentages than those found in the artificial pools. We do not know to what extent the results of the validation experiment could be extrapolated to the results of the larval pools. The conditions of the artificial pools for validation do not necessarily represent the conditions of the larval pools. In the first case, we have worked with low amounts of genomic DNA extracted from adult worms; in the case of the migrating L_3 pools, a different protocol of genomic DNA extraction was used and the concentrations of DNA obtained per species present were unknown. Further research is, therefore, required to optimize this protocol and determine the relationship between the results for the artificial test and unknown larval pools. A potential area for experimentation would be the use of artificial pools made of individual adult cyathostomins, from known species, in different proportions to try to represent better the real scenario, in

a similar approach to that used by Porazinska et al. (2009). The collection and use of L_3 from specific cyathostomin species have been reported (Kornas et al., 2009) and this would constitute another possible approach for designing artificial metagenomic pools for further research in the validation of the protocol developed in the present study or other similar next generation sequencing approach.

Regarding the species composition of the drug-exposed migrating pools when compared to the control pool, no dramatic changes were observed in either of the populations. The statistical analysis revealed a significant increase in the prevalences of certain species in the drug treated pools, while other species showed a decrease. However, and based on the bias favoring an over estimation of *Cc. nassatus* detected in the validation experiment, any assumptions based on this statistical analysis should be reviewed with caution. In general, the changes in the drug treated populations did not correlate with the species identified in the group of more active larvae ("runners"). There is only one previous report of natural ML tolerance in cyathostomin larvae *in vitro* as measured by a LMIA (van Doorn et al., 2010). In that study, *Cy. catinatum* clearly dominated the larvae population after the ivermectin-selection. Our results did not show such a dramatic change with a single significantly dominant species. However, in our particular case the differences among species prevalences across the pools should be viewed with caution because of the questions generated by the validation experiment.

In conclusion, our results demonstrated that the LMIA can be used successfully to measure *in vitro* dose-response of cyathostomin parasites to four different ML analogs. However, further research testing field cases of ML-resistance is still needed to fully validate the assay. The existence of confirmed field cases of ML-resistant cyathostomins (Molento et al., 2012) opens the possibility for further validation of the modified protocol of LMIA developed. Using a high throughput sequencing platform we were able to identify cyathostomin species in larval pools and from genomic DNA extracted from adult worms with a high sensitivity, however, our protocol did not represent accurately the abundance of species and there was a significant overestimation of one species. These limitations did not allow a valid evaluation of the levels of natural tolerance among cyathostomin species, but the sequencing did provide information

regarding differences in species composition among two parasite populations that had been subjected to different levels of drug selection. Despite the drawbacks reported in our approach, we have demonstrated the validity of a novel molecular tool for cyathostomin species identification in parasite populations based on high throughput DNA sequencing. However, and more important, this approach showed not to be accurate at assessing the species abundance. Considering the high costs related to the 454 platform, and the development of the new alternatives, including the platforms of third generation, future studies on refining this molecular approach may include the use of these new technologies of next generation sequencing.

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