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# *In vitro* selection and differentiation of ivermectin resistant cyathostomin larvae

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ABSTRACT

Cyathostomins are considered to be the primary helminth pathogen of horses and macrocyclic lactones (ML) are the most frequently used anthelmintics. Therefore, ML resistance is a serious threat for the control of these parasites. In the present study ivermectin resistant cyathostomin L3 were *in vitro* selected, using a reiterative larval migration inhibition assay (rLMIA) and differentiated by reverse line blot (RLB). Larvae were obtained from two populations, one from a never treated, free-roaming horse population in the nature reserve Oostvaardersplassen (OVP) and the other from regularly ivermectin-treated ponies of Utrecht University (UU). In the rLMIA the proportion of larvae that migrated increased with each passage, demonstrating that the applied procedure indeed selects for larvae the least susceptible for ivermectin. This was further supported by the fact that glutamate addition to this procedure reversed the selection effect, which also suggests that glutamate-gated chloride channels (GluCls) play a role in the ivermectin resistance of the selected L3. In both populations the predominant species were *Cyathostomum catinatum*, *Cylicostephanus longibursatus* and *Cylicocyclus nassatus*. After *in vitro* selection in the rLMIA in the presence of ivermectin the predominant species became *C. catinatum* in both larval populations, while *C. nassatus* disappeared in the never treated OVP larval population but not in the regularly ivermectin-exposed UU population. It is concluded that the rLMIA and RLB can be used to study anthelmintic resistance in cyathostomin populations and to study differences and changes in species composition between populations with different anthelmintic exposure histories.



## 1. Introduction

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

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

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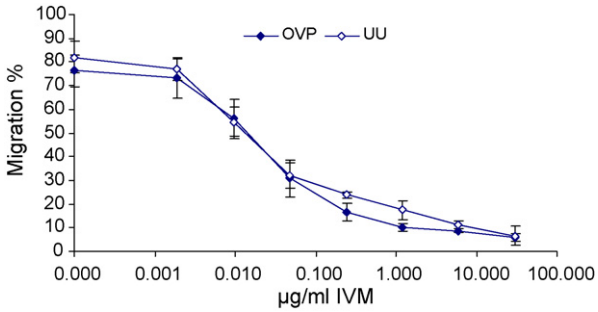


**Fig. 1.** Effect of different ivermectin concentrations on migration of xL3 in the LMIA. For each larval population (OVP and UU) two migration assays were done, both in duplicates. Results are given as the mean (±SD).

This resistance is a threat for controlling cyathostomins in horses.

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

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

Molecular detection of resistance is also hampered by the existence of 50 species of cyathostomins (Lichtenfels et al., 2008) of which eggs and larvae cannot be differentiated morphologically. Therefore, the availability of techniques that enable detection of resistance and the differentiation of the infective larvae (L3) would be useful. The larval migration inhibition assay (LMIA), where L3s migrate in the presence of different concentrations of ML is such a technique. Robinson et al. (2008) used this method and showed a dose response curve for exsheathed L3 migrating in different concentrations of ivermectin and moxidectin. They also found slightly lower LD50's for susceptible than for resistant populations. With respect to species differentiation, the reverse line blot assay (RLB) has proven to be a reliable molecular technique (Kaufhold et al., 1994) which has also been applied for differentiating 13 cyathostomin species (Traversa et al., 2007). The RLB is based on hybridization of a species specific probe with an amplified fragment of the inter genic spacer (IGS) region.

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

## 2. Materials and methods

### 2.1. Parasites

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

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

### 2.2. Larval migration inhibition assay (LMIA)

In the LMIA, ML paralyses the exsheathed larvae (xL3) and inhibit migration through a sieve. The method is based on Robinson et al. (2008). L3 were exsheathed with 0.1% hypochlorite the day before LMIA and viable xL3 were collected in a Baermann glass overnight. A known number of larvae were pre-incubated (2 h, 37 °C in the dark) in PBS containing different concentrations of ivermectin (Sigma–Aldrich) from a stock solution in dimethylsulfoxide (DMSO) with an end concentration of 1% DMSO. After pre-incubation the larvae were allowed to migrate in the same solution through a sieve (cell strainer 40 µm, Becton Dickinson) for 1 h at 37 °C in the dark. For reasons of accuracy numbers of xL3s applied to the sieve were such that each fraction after migration contained at least 50 larvae. xL3 that migrated through the sieve were fixed and stained in iodine, counted using a dissection microscope and, when

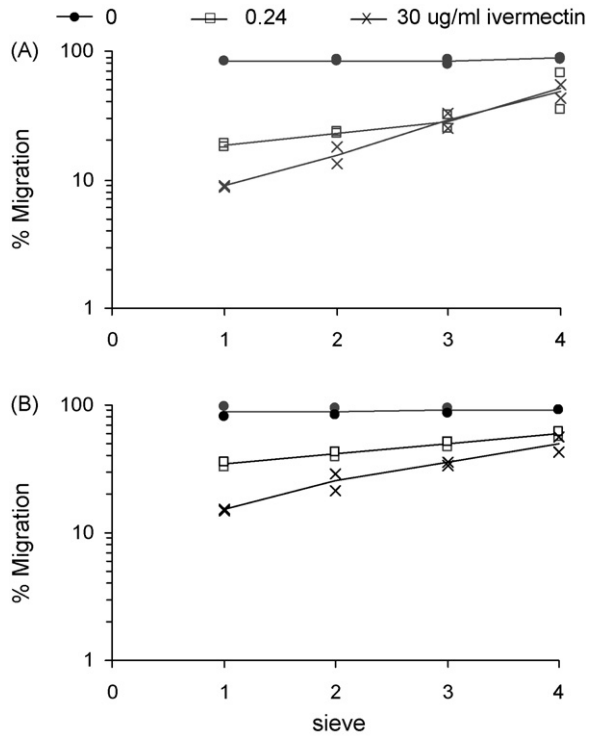


**Fig. 2.** Migration percentage through four consecutive sieves in the rLMIA. xL3s from OVP, culture II (A) and UU, culture I (B) were allowed to migrate in the presence or absence of ivermectin. The percentage migration through each sieve was determined by duplicate measurements. The mean (line) and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

appropriate, isolated individually and stored at −80 °C until analysis by reverse line blot (RLB).

### 2.3. Reiterative LMIA (rLMIA)

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

### 2.4. Lysis of individual xL3

Each individually isolated xL3 was lysed in 25 μl proteinase K /Worm Lysis Buffer (WLB) (4.5 U/ml proteinase K, 50 mM KCl, 10 mM tris pH 8.3, 2.5 mM $MgCl_2$, 0.45% NP-40, 0.45% tween-20 and 0.01% gelatin). The mixture was frozen for 15 min at −80 °C and the xL3s were lysed overnight at 56–60 °C. Proteinase K was heat inactivated the next day at 95 °C for 15 min.

### 2.5. Amplification of inter genic spacer (IGS) fragment

With RLB we were able to detect the amplified IGS from a single individual xL3. Conditions and primers for amplification of the IGS fragment were as described for adult worms by Traversa et al. (2007), except that we used 2.5 μl lysate of individual xL3 per 25 μl PCR reaction. Primers were 5′ biotinylated as described by Traversa et al. (2007).

### 2.6. Reverse line blot (RLB) hybridization

The RLB for species specific hybridization of IGS amplicons from cyathostomins is a modification of the method of Traversa et al. (2007). Probes specific for *Coronocyclus coronatus*, *C. labiatus*, *C. labratus*, *Cylicostephanus goldi*, *C. longibursatus*, *C. minutus*, *Cylicocyclus ashworthi*, *C. insigne*, *C. leptostomum* and *C. nassatus* were based on IGS sequences (Kaye et al., 1998) and are given by Traversa et al. (2007). We confirmed the specificity of the probes for the common species (*C. goldi*, *C. insigne*, *C. labratus*, *C. longibursatus* and *C. nassatus*) by hybridisation with PCR products from morphologically differentiated adults (Lichtenfels et al., 2008). Probes specific for *Cyathostomun catinatum* (CATD5, CGACTAGGCGTACATCATA), *C. pateratum* (PAT5, CATACAGTTGTAACATTCTCG) and *Cylicostephanus calicatus* (CAL2, ACATGCAACACCCTGTTCAA) were designed by us, because we could not achieve specific hybridization for these species with the probes described by Traversa et al. (2007). For that, we aligned the contemplated probes (see Supplementary data) with recently corrected IGS sequences for *C. catinatum* (accession no. HM142927) and *C. calicatus* (accession no. HM142928), and a new *C. pateratum* consensus sequence. Specificity of the three newly designed probes was confirmed by hybridisation with PCR products from morphologically differentiated adults (Lichtenfels et al., 2008) of these three species. Specificity of the probes for *C. ashworthi*, *C. leptostomum* and *C. nassatus* was confirmed by alignment

with new consensus sequences showing a greater level of intraspecific variation (Hodgkinson unpublished data). Alignments of all specific probes and IGS sequences are given as Supplementary data with the exception of the IGS sequences from *C. labratus* (not present in any database) and *C. labiatus* (too different from the others for a meaningful alignment). Ten microlitres of the IGS amplicon per lane was used for hybridization. A mixture of PCR products of xL3s were used as positive control for each hybridization. Hybridized products were detected with streptavidin-POD conjugate (Roche), followed by incubation in ECL detection reagent and exposure to Amersham Hyperfilm ECL (both GE Healthcare).

### 2.7. Statistics

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

## 3. Results

### 3.1. Dose dependent inhibition of migration

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

### 3.2. Selection of ivermectin resistant xL3

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

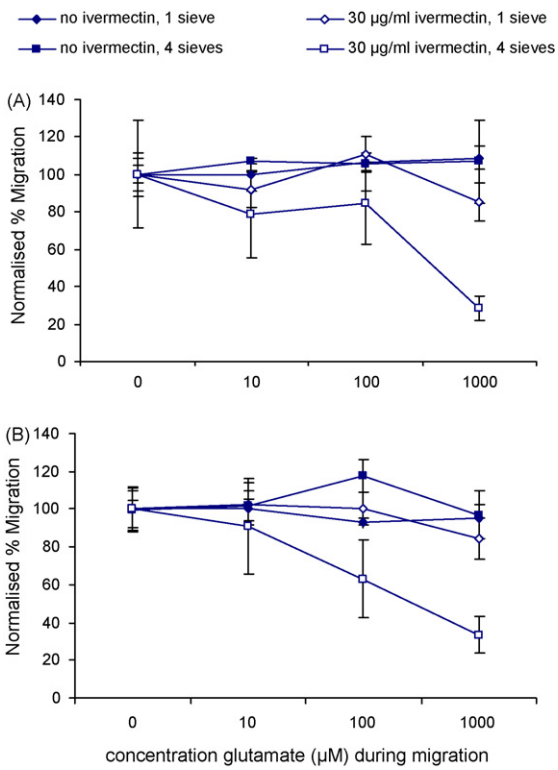


**Fig. 3.** The effect of different glutamate concentrations on larval migration in the absence or presence of ivermectin. xL3's from population OVP, culture III (A) and population UU, culture I (B) were allowed to migrate through one (control) or four consecutive sieves in the presence of glutamate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

### 3.3. Selection of ivermectin resistant xL3 is reduced in the presence of glutamate

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

### 3.4. Differentiation of ivermectin resistant xL3

Three cultures from the OVP population and one from UU population were analysed with RLB before and after

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

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

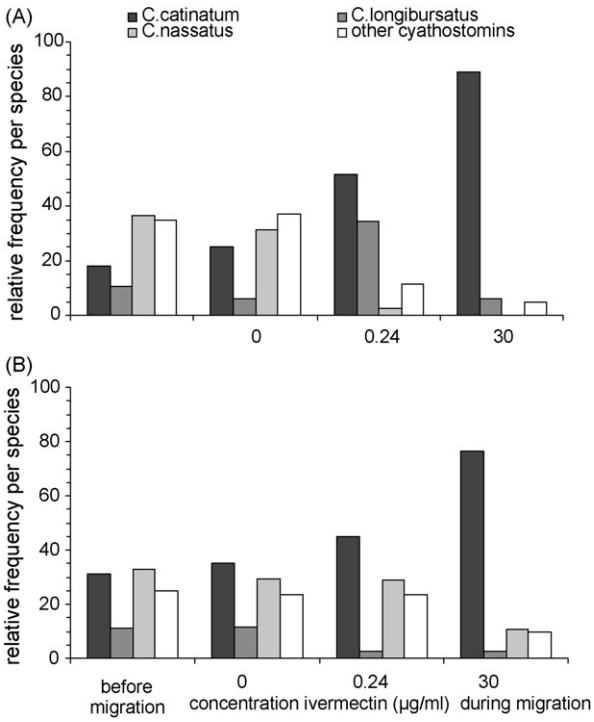


**Fig. 4.** Species composition of cyathostomins before and after selection by reiterative LMIA and differentiated by RLB. xL3s from population OVP, culture II (A) and UU, culture I (B) were allowed to migrate in rLMIA with ivermectin concentrations as indicated. Randomly picked xL3 were differentiated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

rLMIA (Table 1). In the absence of ivermectin no significant differences were found between the fraction not subjected to LMIA and the fraction obtained after the rLMIA, indicating that migration itself was not selective. All 13 species for which a specific probe was present were found. The three cultures of OVP showed comparable species composition and the three most abundant species in both the OVP and UU population were *C. catinatum, C. longibursatus* and *C. nassatus.* We analysed from both populations the cultures collected at the same period (April 2009). The species other than *C. catinatum*, *C. longibursatus* and *C. nassatus* were grouped together as other cyathostomins (Fig. 4), because prevalence of these species was generally too low to be analysed separately. *In vitro* selection for resistance resulted in a changed species composition in both the OVP and UU population ($P < 0.001$).

Before the rLMIA 18% of the OVP population was *C. catinatum* and after selection it increased to 51% and 89% in the presence of 0.24 or 30 μg/ml ivermectin, respectively. Within this population, *C. nassatus* appeared to be very susceptible to ivermectin. Before the rLMIA, the relative frequency was 36% and this decreased to 3 and 0% after selection with 0.24 or 30 μg/ml ivermectin, respectively. The species composition of the UU population also changed after *in vitro* selection. The relative frequency of *C. catinatum* increased from 35% before to 45% and 76%

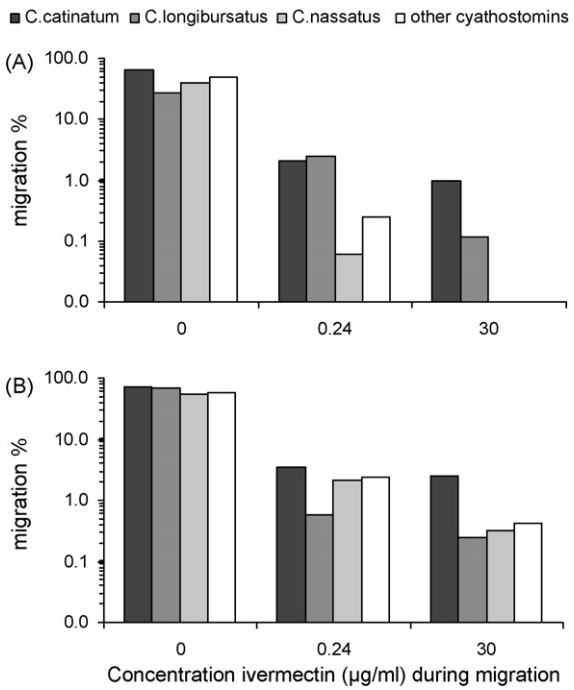


**Fig. 5.** Percentage migration per cyathostomin species through four sieves of the OVP (A) and UU (B) population in 0, 0.24 or 30 μg/ml ivermectin. Data were calculated from the rLMIA results as shown in Table 1 and based on actual numbers of larvae as used in the assays.

after selection with 0.24 and 30 μg/ml ivermectin, respectively, with changes not being significantly different from those observed in the OVP population. However, the relative frequency of *C. nassatus* in the UU population did not change after selection with 0.24 μg/ml ivermectin, while after selection with 30 μg/ml *C. nassatus* still made up 11% of the population. These changes for *C .nassatus* did differ significantly from those seen in the OVP population ($P < 0.001$).

The relative frequency of one species is influenced by the abundance of other species. Therefore, the migration percentage per species before and after selection was calculated (Fig. 5). The migration percentage in the absence of ivermectin was approximately 60% for all species. The percentage migrated *C. catinatum* was for both populations 1–2% in both the 0.24 and the 30 μg/ml ivermectin concentration, respectively. Hardly any selection for *C. catinatum* occurred between 0.24 and 30 μg/ml ivermectin. For the other species the migration percentages after selection with 30 μg/ml ivermectin were lower and ranged from 0% to 0.12% and from 0.24% to 0.42% for the OVP and UU population, respectively.

Including glutamate in the reiterative LMIA decreased the migration (Fig. 3), but no influence on the species composition was observed (Table 1).

## 4. Discussion

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

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

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

Larvae are not adults and even when GluCls are involved in ivermectin resistance in both stages, the effect may differ. For example, xL3 do not feed and therefore ivermectin that binds to GluCls located in the pharynx (if they are present) will probably not strongly affect the motility of those larvae, but may kill an adult worm. Whatever the mechanism, the LMIA for ML susceptible cyathostomin populations gave different results than for resistant ones (Robinson et al., 2008), indicating that at least some effect involving motility is similar in xL3's and adults. In the more thoroughly studied *H. contortus* it was found that a correlation between *in vitro* LMIA results and *in vivo* resistance depended on the way the strains were selected (Gill and Lacey, 1998). When sub-therapeutic doses were used for selection, there was no correlation. However, when strains were isolated in the field with therapeutic doses there was a good correlation between motility as measured in the LMIA and observed *in vivo* resistance. Although it remains to be demonstrated that different behavior in larval migration reflects *in vivo* ML resistance in cyathostomins, the LMIA has been identified as a promising diagnostic tool (McArthur et al., 2009; Kaplan, 2009). This is supported by the present results. Modifying the LMIA to an rLMIA increases the detection of resistant larvae while at the same time the resistance of the population as a whole can be quantified as increase in migration percentage. The rLMIA also offers the possibility to study pre-existing resistant variants, because there is no new generation necessary for selection, meaning no recombination due to sexual reproduction. rLMIA also offers the possibility to isolate

individual xL3 for differentiation, allowing examination of possible cyathostomin species differences.

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

Differentiation of xL3 from both the OVP and UU populations demonstrated that *C. catinatum, C. nassatus* and *C. longibursatus* are the most abundant species, as has been found elsewhere (Gasser et al., 2004). After *in vitro* selection *C. catinatum* is by far the most prevalent species in both populations. This suggests that, also in a never treated population resistant larvae are present in low numbers. This was earlier suggested by Young et al. (1999) for cyathostomins, without differentiating them. So despite *C. catinatum* being the least susceptible species in the rLMIA the majority of the larvae were still susceptible. *C. nassatus* declined in numbers after selection and it even disappeared completely from the OVP population. Interestingly, the life cycle of *C. nassatus* is much shorter than that of *C. catinatum*, because *C. nassatus* seems to lack the possibility to inhibit its development unlike *C. catinatum* (Ogbourne, 1975). It might be that a longer cycle increases the selection pressure upon the parasite when exposed to anthelmintics, resulting in higher numbers of *C. catinatum* resistant larvae compared with *C. nassatus*, although this can not explain the high numbers of resistant *C. catinatum* in the never treated OVP population. For a proper evaluation why some species appear to be more prone for developing anthelmintic resistance than others, more information is required about the entire life-history traits of each species influencing their refugia status in time as well as their inherent susceptibility to certain drugs.

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

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

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## Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.vetpar.2010.08.021.

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