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Keywords

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ABSTRACT

Cyathostomins are the most prevalent parasitic pathogens of equids worldwide. These nematodes have been controlled using broad-spectrum anthelmintics; however, cyathostomin resistance to each anthelmintic class has been reported and populations insensitive to more than one class are relatively commonplace. The faecal egg count reduction test (FECRT) is considered the most suitable method for screening anthelmintic sensitivity in horses, but is subject to variation and is relatively time-consuming to perform. Here, we describe a larval migration inhibition test (LMIT) to assess ivermectin (IVM) sensitivity in cyathostomin populations. This test measures the paralysing effect of IVM on the ability of third stage larvae (L3) to migrate through a pore mesh. When L3 from a single faecal sample were examined on multiple occasions, variation in migration was observed: this was associated with the length of time that the L3 had been stored before testing but the association was not significant. Half maximal effective concentration (EC50) values were then obtained for cyathostomin L3 from six populations of horses or donkeys that showed varying sensitivity to IVM in previous FECRTs. Larvae from populations indicated as IVM resistant by FECRT displayed significantly higher EC50 values in the LMIT than L3 from populations classified as IVM sensitive or L3 from populations that had not been previously exposed to IVM or had limited prior exposure. The analysis also showed that EC50 values obtained using L3 from animals in which IVM faecal egg count reduction (FECR) levels had been recorded as <95% were significantly higher than EC50 values obtained using L3 from animals for which FECR was measured as >95%. For one of the populations, time that had elapsed since IVM administration had an effect on the EC50 value obtained, with a longer time since treatment associated with lower EC50 values. These results indicate that the LMIT has value in discriminating IVM sensitivity amongst cyathostomin populations, but several factors were identified that need to be taken into account when executing the test and interpreting the derived data.

1. Introduction

Cyathostomins are highly prevalent and potentially pathogenic parasitic nematodes found in the large intestine of horses and other equids worldwide. The cyathostomin group comprises around 50 species (Lichtenfels et al., 2008); however, little is known of the ecology of the individual species or how they interact with one another in the host or in external environment. Anthelmintic resistance (AR) is a major issue in this group of nematodes: resistance to benzimidazoles (BZ) is widespread and, in some areas, resistance to pyrantel, a member of the tetrahydropyrimidine (THP) class, is highly prevalent (Kaplan, 2002). Reduced sensitivity to the macrocyclic lactone (ML) anthelmintics, ivermectin (IVM) and moxidectin (MOX), has also been recorded in cyathostomin populations (Trawford et al., 2005; Trawford and Burden, 2009; Molento et al., 2008; Traversa et al., 2012; Relf et al., 2014). Multi-class resistance in single populations to BZ and THP class anthelmintics is also commonly reported (Kaplan et al., 2004; Canever et al., 2013; Lester et al., 2013). As no new anthelmintic classes are being developed for use in horses in the short to medium term, and reversion to anthelmintic sensitivity does not seem to readily occur in resistant nematode populations (Jackson and Coop, 2000), it is essential to preserve efficacy of the currently effective products. For these reasons, tests that facilitate decisions regarding anthelmintic treatment in horses will play an increasingly important role in control (Matthews, 2014). In this context, it is important to identify AR as soon as practically possible so that measures can be taken to prevent its spread (Tandon and Kaplan, 2004). The faecal egg count reduction test (FECRT) is currently the 'gold standard' non-invasive test for assessing anthelmintic efficacy in horses (Vidyashankar et al., 2012). This test is relatively labour intensive to implement: faecal samples for analysis need to be obtained on at least two occasions and it is often a challenge to obtain adequate numbers of horses with a faecal egg count (FEC) of sufficient magnitude to perform the test with high accuracy. The non-uniform distribution of eggs within and between faecal samples further complicates data analysis (Denwood et al., 2010). For these reasons, AR detection methods that are more efficient to

perform and subject to less variability need to be investigated. For cyathostomins, such tests should focus on ML anthelmintics. This is because these products hold the major market-share worldwide and, as the prevalence of ML resistance is currently less advanced than with BZ and THP anthelmintics (Molento et al., 2012), and hence tests that inform on sensitivity to ML are likely to have most impact on mitigating the spread of resistance. Molecular mechanisms leading to ML resistance in cyathostomins remain to be defined, so there are no molecular tests available, leaving bench-based in vitro tests as the remaining option.

The larval development test has been investigated for measuring anthelmintic sensitivity in cyathostomins; however, this test has not proven particularly informative in defining ML sensitivity levels (Tandon and Kaplan, 2004; Lind et al., 2005; Matthews et al., 2012). An alternative test that has been investigated for assessing anthelmintic sensitivity in ruminant nematode (Demeler et al., 2010; 2012; 2013) and in cyathostomin (van Doorn et al., 2010) populations is the larval migration inhibition test (LMIT). Here, we assessed the potential of the LMIT for measuring IVM sensitivity in cyathostomin larvae derived from different equine populations. This test was deemed appropriate for purpose because the major targets of IVM are ligand-gated chloride channels, which, when bound, result in nematode paralysis (Shoop et al., 1995) and hence will affect the ability of larvae to migrate through small pores of a filter. The cyathostomin populations examined in the current study were derived from groups of donkeys demonstrated previously, by FECRT, to exhibit differing levels of sensitivity to IVM in vivo (Trawford and Burden, 2009), or from equids administered with minimal or no ML treatments (Wood et al., 2013). The proportion of L3 that migrated through a pore filter at increasing concentrations of IVM were measured and the data compared amongst individual equids and between populations to inform on the value of this test.

2. Materials and methods

2.1. Populations

Parasites from six equine populations were used: four consisted of donkeys and two of ponies. Three donkey herds (Populations A-C) were based at the UK Donkey Sanctuary (Devon, England, UK). The three populations were grazed separately on geographically distinct farms. Donkeys in Populations A and B had been demonstrated previously, by FECRT, to harbour cyathostomins that exhibited reduced sensitivity to IVM and MOX (Table 1, Trawford et al., 2005; Trawford and Burden, 2009). In Population C, the cyathostomins were deemed IVM sensitive as indicated by the finding of a mean FECR of >95% in treated animals 14 days after IVM administration (Table 1); however, the strongyle egg reappearance period for some of the donkeys in this population was below the standard egg suppression period of IVM and MOX (F. Burden, pers comm.), described previously as 8 weeks (Lyons et al., 1992, Bello, 1996) and 13 weeks (DiPietro et al., 1997), respectively. These observations are generally accepted as an early indicator of AR (Molento et al., 2012). In all three Donkey Sanctuary populations, IVM or MOX subcutaneous injection preparations registered for use in cattle had previously been administered orally (F. Burden, pers comm.). This may have predisposed the nematodes in these populations to reduced sensitivity to ML anthelmintics. Population D comprised a privately owned herd of donkeys grazed in Cheshire, UK. These donkeys had not received IVM in the 5 years preceding this study and had only received MOX and anti-cestode treatments during this time. No anthelmintic had been administered in the preceding 12 months. As part of this study, Population D was subjected to an IVM FECRT following World Association for the Advancement of Veterinary Parasitology guidelines (Coles et al., 1992) using a double centrifugation FEC technique sensitive down to 1 egg per gram (Christie and Jackson, 1982). In Population D, IVM FECR was found to be 100% in all donkeys tested. Populations E and

F comprised two groups of ponies used for conservation purposes in the Fens of East Anglia, South East England (Wood et al., 2013). Population E comprised Dartmoor ponies grazed on grassland fen. At the start of the study, all ponies had grazed the Fens for two years. During this time, these ponies were not administered with ML products. Population F consisted of Konik ponies grazed on grassland fen since the mid-1990s. These animals did not receive ML products during this time and were unlikely to have ever received this class of anthelmintic. Due to potential eco-toxicological risks (McKellar, 1997), IVM FECRT were not performed at the sites grazed by Populations E and F, as the use of ML products is not permitted. Given the lack of ML treatments in these populations, it is assumed that the resident cyathostomins in Populations E and F are highly sensitive to IVM and MOX.

2.2. Preparation of third stage larvae for LMIT analysis

Freshly voided faecal samples were collected from identified individuals and immediately placed into labelled plastic bags, which were sealed to exclude as much air as possible to retain anaerobic conditions. All samples were sent on the day of collection. Each sample was weighed, homogenised thoroughly and a 10 g sub-sample removed for FEC analysis (Christie and Jackson, 1982). The remainder was cultured under aerobic conditions by transferring faeces to plastic trays, which were placed inside perforated plastic bags. Faeces were incubated at 15^{o}C for up to 22 days, after which, the trays were flooded with lukewarm tap water for 4 h. The supernatants, containing strongyle L3, were poured over a Baermann filter (MAFF, 1986) and the filter placed in the neck of a jam jar filled with tap water. The filter was left overnight, removed the next morning and the remaining volume reduced to 20 ml. The L3 were transferred to culture flasks and enumerated in 10 x 10 µl aliquots and classified as small or large strongyle L3 on the basis of gut cell morphology (Thienpont et al., 1986). No large strongyles were observed in any samples from Populations A-E. Very low numbers of large strongyle larvae (<1%) were observed in samples from Population F. A

156 complete lack of ivermectin or moxidectin treatments in Population F are likely to explain
157 why this was the only population that was identified as positive for large strongyle larvae.
158 The L3 were stored in tap water in vented flasks at 4°C at a maximum concentration of 2,500
159 L3 ml^{-1} for up to 60 days. At this time point, viable L3, as assessed by motility and the
160 presence of intact gut cells, were observed. The water was replenished weekly.
161
162 *2.3. Larval migration inhibition test*
163 The LMIT described here is an adaptation of the method developed for assessing
164 anthelmintic sensitivity of ruminant nematode L3 by Demeler et al. (2010; 2012; 2013). This
165 protocol utilises a migration system that enables physical separation of motile from non-
166 motile L3 through a filter mesh (Nytal mesh). The pore diameter allows active larvae, but
167 not dead larvae, to pass through the mesh. For each test, approximately 2,500 L3 were
168 removed from each culture derived from an individual animal. The L3 were exsheathed in
169 700 µl, 2% w/v sodium hypochlorite solution for 3.5 min at room temperature and washed
170 thoroughly three times by centrifugation for 2 min at 203 x *g* in phosphate buffered saline,
171 pH 7.4 (PBS: 150 mM sodium chloride, 150 mM sodium phosphate). Exsheathed L3 were
172 subjected to Baermannisation for 2 h at 26°C immediately before the test was run. The L3
173 were collected by centrifugation, recounted and re-suspended in 1,200 µl PBS. As IVM had
174 to be dissolved in 100% dimethyl sulphoxide (DMSO) for the test, the effect of DMSO on L3
175 motility was tested over a range of concentrations (0, 5, 6, 7, 8, 9, 10% w/v DMSO/PBS).
176 Each dilution was assessed in duplicate using L3 from Population C and the experiment
177 repeated on three occasions. The derived data indicated that 5% DMSO w/v PBS was the
178 highest concentration at which < 2% adverse effect was observed on migration compared to
179 PBS-only control wells (data not shown). The impact of the diameter of the pores within the
180 mesh was then assessed. This was performed by killing the L3 by incubation at 70°C for 20
181 min, then adding them in PBS-only or 5% DMSO w/v PBS to the upper side of filters of pore

182 diameter 25 and 28 μm. After 2 h, the underside of the filters was examined to assess if L3
183 'fell through' by gravity: L3 were not observed on the underside of filters of pore diameter 25
184 μm or 28 μm. Subsequently, 25 μm pore diameter filters were selected for use in the test.
185 For assessment of the effect of IVM on L3 migration, analytical grade IVM (Sigma Aldrich,
186 cat. no: I8898) was dissolved in 100% DMSO to give a stock solution of 3,000 μgml^{-1} IVM.
187 Before each batch of tests, this stock was serially diluted in PBS/DMSO to give working
188 dilutions of 5, 20, 60, 300, 3,000 μgml^{-1} IVM/5% DMSO w/v PBS (final molarity used in the
189 test ranged from 1.12 $x10^{-5}$ - 6.73 x 10^{-3} M). In all tests, L3 migration was assessed in a
190 positive control well containing only 5% DMSO w/v PBS and IVM test concentrations and
191 controls were set up in duplicate (approximately 100 L3 analysed per well). The L3 were
192 pre-incubated at 26°C for 2 h in the dark in 10 μl IVM at each test concentration in 5%
193 DMSO w/v PBS. After this, L3 in IVM solution were transferred to the upper side of filters
194 in corresponding duplicate wells on a migration plate, containing 1,910 μl of each test
195 concentration. The samples were incubated for 2 h at 26°C in the dark. After this, migration
196 chambers were lifted out and 600 μl PBS used to wash the outside of the chambers so that
197 any adhering (but migrated) L3 were washed into the corresponding well. The upper
198 chamber was inverted and, using 2 x 1,000 μl PBS, L3 that had not migrated were washed
199 into the corresponding well in the row below. The effect of IVM on the ability of the L3 to
200 migrate through the mesh pores was confirmed by observations, prior to fixing, that the
201 worms that had come through the mesh were motile and moving in classical sinusoidal
202 movements, whilst those retained above the mesh moved slowly or not at all, or assumed
203 angular postures and performed jerky movements of the head and tail regions. The L3 were
204 fixed with 200 μl, 100% molecular grade ethanol and migrated and non-migrated L3
205 enumerated at x100 magnification using an inverted stereomicroscope. To study
206 repeatability of the test and the potential effect of L3 storage time on migration in the LMIT,
207 L3 derived from a single culture from a donkey in Population B were analysed on seven

separate occasions. This donkey had not received IVM in the 780 days preceding sample collection. The test was run using L3 that had been stored from 22 to 55 days at 4°C. Finally, to compare the value of the LMIT in defining IVM sensitivity among cyathostomin populations of varying sensitivity to the anthelmintic in vivo, L3 derived from single time point faecal samples from each Population: A (n=4), B (n=5), C (n=6), D (n=5), E (n=6) and F (n=6) were assessed. All samples were tested at each IVM concentration in duplicate. Only when (un-scaled) mean migration of the L3 in the two positive control wells exceeded 70%, was migration in the presence of IVM subjected to further data analysis.

2.4. Statistical analysis and modelling

For data analysis, the percentage migration was calculated for each replicate, with migration in the PBS/DMSO-only well scaled to 100%. For data exploration, dose-response curves (DRC) of the proportion of L3 migrating versus Log10(IVM conc + 0.01) were plotted for each sample. The small additive component, 0.01, allowed the inclusion of data at zero IVM concentration and was found empirically to have minimal impact on estimated EC50 values. The DRC were statistically modelled using a four-parameter logistic dose response model on the natural logarithm of IVM concentration (Demeler et al., 2010; 2012) permitting estimation of EC50. Summary EC50 values were compared using the Kruskal Wallis test with post-hoc analysis using the method of Siegel and Castellan (1988). The association between EC50 and time since last IVM treatment was tested with a linear model predicting EC50 from time since last IVM treatment and population membership. DRC modelling, hypothesis testing and post-hoc analysis were preformed in the R statistical system (R Development Core Team, 2012) using the packages “drc”, “lme4” and “pgirmess”.

3. Results

3.1. Value of the LMIT results in defining IVM sensitivity amongst cyathostomin populations

When the test was run on seven occasions on different days using L3 derived from a single donkey, it was found that L3 stored for shorter periods in culture generally exhibited higher migration in the presence of IVM, but the differences observed between storage time points were not significant. The L3 migration values were then compared amongst the six cyathostomin populations. The percentage of times that < 70% migration in the control wells was observed was 10%. When < 70% migration was observed, the test was repeated with L3 from the same individual; however, < 70% migration was achieved in all subsequent tests with these samples, so the LMIT data from these L3 were not used in subsequent analyses and are not included in the numbers quoted for each equid population, above. The range of EC50 values obtained for each population (A-F) is shown in Table 2 and the derived DRC's are depicted in Figure 1. Resistance ratios were generated by dividing single EC50 estimates from data for each Population (A-E) by the EC50 value obtained from data from Population F (Table 2). Highest EC50 values were obtained using cyathostomin L3 from populations A and B, which had been demonstrated to be resistant to IVM in vivo. The next highest EC50 value was obtained using L3 from the population for which a reduced ERP had been demonstrated following IVM and MOX treatment (Population C). Lower EC50 values were obtained using L3 from populations shown to be sensitive to IVM by FECRT (Population D) or populations in which IVM treatments had been minimal or non-existent (Populations E and F).

The predictive value of the LMIT for assessing relative IVM sensitivity was further considered by comparing EC50 values obtained using L3 derived from populations demonstrated to be IVM resistant (IVM-R) on the basis of mean FECR <95% after IVM treatment (Populations A and B), with those values obtained using L3 from the population

(C) for which the mean FECR was reported as >95%, but the ERP was reduced following IVM administration (IVM-RERP), and with populations that were highly sensitive to IVM (i.e. FECR 100%) or had received no IVM treatments (Populations D, E and F), combined here as IVM-sensitive (IVM-S, Figure 2). The data analysis indicated that EC50 values obtained with IVM-R L3 were significantly higher than EC50 values obtained using IVM-S L3 ($p < 0.05$), but not the EC50 values obtained using IVM-RERP L3. In addition, EC50 values obtained using L3 from the IVM-RERP population were significantly higher than those obtained with L3 from the IVM-S populations ($p < 0.05$).

3.2. Association between derived EC50 values and data derived from IVM-FECRT results in individual animals

EC50 values obtained using L3 from Populations A, B and C (i.e. those populations that had been subjected previously to IVM FECRT analysis) were used to investigate the hypothesis that there would be a negative association between the percentage reduction in FEC observed 2 weeks after IVM administration and the EC50 values obtained in the IVM-LMIT (Figure 3). The analysis indicated that there is indeed a relationship, with individuals measured as having a FECR of >95% having lower EC50 values in the IVM-LMIT than those for which FECR was <95% (two-way ANOVA, EC50 and FECR>95%, p(FECR) p=0.000284).

3.3. Analysis of IVM sensitivity in single populations over time since last IVM treatment

The EC50 values obtained using L3 from Populations A, B and C (i.e. those populations that had a relatively recent IVM treatment) were used to examine if there was a relationship between the proportion of L3 that migrated in the presence of IVM with the number of days since the last recorded IVM administration (Figure 4). The time since IVM treatment for each population ranged as follows: Population A (n=4) – 40-102 days (mean, 57.5 days),

Population B (n=5) – 41-780 days (mean, 250.25 days), and Population C (n=5, one animal of the original 6 animals was not treated with IVM) – 65-194 days (mean, 143.50 days). The analysis indicated that there is a relationship, with higher proportions of migration observed with L3 derived from samples obtained nearer to IVM treatment: i.e. the EC50 value obtained was negatively associated with time since last IVM treatment. This association was significant in the case of the L3 that were derived from Population C ($p=0.028$)

4. Discussion

Macrocyclic lactone anthelmintics, such as IVM, that paralyse nematode somatic muscles, among other modes of action, have been assessed in vitro via their effect on larval motility or migration. Such tests are potential options for detecting anthelmintic resistance because they are cheap, relatively quick to perform, preclude host influences and, as they can be run over a concentration range, may provide reproducible parameters with which to measure phenotype (Demeler et al., 2013). Several studies utilising ruminant parasitic nematodes have indicated that motility and migration tests are useful tools for informing on the ML sensitivity of single species populations (Martin and Le Jambre, 1979; Folz et al., 1987; Sangster et al., 1988; Demeler et al., 2010, 2012, 2013). Here, we examined the value of the LMIT for use with cyathostomin larvae obtained by culture from equine faeces. We assessed utility of the test for informing on IVM sensitivity of cyathostomins obtained from populations for which FECRT data was available or populations in which IVM treatments had not been applied or had been limited. Here, we used cyathostomin larvae from donkeys in a comparison with larvae derived from horses because it is problematic to obtain populations of small strongyles from horses for which an IVM FECR of <95% has been demonstrated. For example, shortened IVM ERP has been identified several times in cyathostomins in horse populations (for example, Relf et al., 2013), but populations exhibiting a mean FECR of less than 95%

have been reported only sporadically in horses and primarily in South America (Canever et al., 2013). In our comparisons, the LMIT was found to discriminate IVM sensitivity amongst cyathostomin populations in agreement with results that had been previously generated using the FECRT. For example, the derived EC50 values obtained with L3 from IVM-R (Populations A and B) and IVM-RERP (Population C) populations were significantly higher than EC50 values obtained using L3 from all IVM-S populations (D, E and F). These results concur with studies on *Haemonchus contortus*, where correlations were identified between the results of the LMIT and the in vivo anthelmintic resistance status (Gill and Lacey, 1998). In the *Haemonchus* study, the association was found to vary depending on how the nematode strains were selected, with no correlations found when using strains that had been selected experimentally using sub-optimal doses of anthelmintic. In agreement with the current study, though, good correlations between migration in the test and the results of prior FECRT analysis were found when resistant strains isolated from the field (i.e. selected with therapeutic doses of anthelmintic) were compared.

One observation from the current study was that although there was a significant difference observed in EC50 values between IVM-R or IVM-RERP and IVM-S populations, there was variation within each population in the EC50 value obtained using L3 derived from individual animals (Table 2). This was particularly noticeable in the IVM-R isolates. Although this concurs with variation in ML FECR levels obtained by FECRT in the IVM resistant populations here, this level of variability could affect the value of this test if pooled samples were to be assessed from a given population in the field, where it could be impractical to run the test on many individuals. Two further confounding factors were identified. One of these was the length of time that L3 had been stored in the laboratory before the test. An effect of culture age on migration was observed, even though L3 were Baermannised just prior to running the test. In previous publications using ruminant nematode larvae, the effect of L3 storage time was not detailed: for example, Demeler et al.

(2010) used L3 from sheep faecal cultures stored for 'up to 3 months', but the impact of culture period was not specifically addressed. Other studies (for example, Sangster et al., 1988) do not mention the length of time that L3 were stored for prior to use in the LMIT. In others, the effect of L3 storage time has been assessed: for example, using the LMIT, Molan et al. (2000) compared the sensitivity to condensed tannins of *Trichostrongylus colubriformis* larvae stored for 1 month versus larvae stored for 7 months. Similar to the findings here, these authors found that the *T. colubriformis* L3 stored for longer periods in the laboratory were more sensitive to the xenobiotic tested than larvae stored for shorter periods ($p<0.001$). The differences observed in the current study were not significant; however, larvae were stored up to only 55 days as opposed to 7 months. On the basis of the results here and the observations made in other nematode species, it is recommended that L3 be used as fresh as possible when assessing IVM sensitivity in the LMIT.

The analysis also indicated that the time that elapsed between last IVM treatment and when the faecal samples were obtained for processing had an effect on the derived EC50 values in the LMIT. The effect was only found to be significant for Population C: this may have been because this population had the widest range in days since last IVM treatment in the donkeys that were selected for supply of L3 for the LMIT. This observation could be explained by the fact that the nearer to IVM treatment that the L3 are tested in the LMIT, the more likely it is that the parasites used are derived from nematodes that may have survived treatment. This is particularly problematical to investigate in cyathostomins because the exact length of the life cycle of different individual species is unknown and these parasites can undergo a variable period of encystment in the large intestinal wall (Love et al., 1999). Because of the effect of time since last anthelmintic treatment observed here, it is recommended that the impact of this parameter be assessed further in future.

Despite these various caveats, the results here showed clear differences in EC50 values measured amongst the cyathostomin populations that were tested. This was observed even

364 although the L3 samples tested were likely to comprise mixed cyathostomin species. It has
365 been indicated in preliminary studies that different cyathostomin species may vary in their
366 sensitivity to IVM in the LMIT (van Doorn et al., 2010). Also, in ruminant nematode studies
367 it has been observed that different species differ in ML sensitivity in the LMIT, which cannot
368 always be predicted from their relative sensitivity to MLs in vivo (Demeler et al., 2012). In
369 the van Doorn et al., (2010) study, where cyathostomin L3 were rendered more tolerant in
370 vitro though iterative selection by several cycles of migration in the presence of IVM, it was
371 identified that *Cyathostomin catinatum* became the predominant species in the two
372 populations that were tested. Furthermore, cyathostomin species composition in donkeys can
373 be different from species composition in horses (Matthee et al., 2004). For these reasons, the
374 authors will now examine the species of cyathostomin present in these populations using L3
375 recovered from the LMIT utilising species specific DNA probes that they have developed
376 based on intergenic spacer region nucleotide sequences (Cwiklinski et al., 2012).
377 Although the results here indicate that the LMIT has value in providing information on the
378 IVM sensitivity status of a cyathostomin population, it cannot be assumed that the
379 'resistance' mechanisms that affect the ability of the L3 to migrate in the LMIT are the same
380 as those present in parasitic stages that operate to allow these stages to survive treatment in
381 the host. As such, the LMIT provides only a gauge on the relative IVM sensitivity of a
382 population. There is some indication from the work of van Doorn et al., (2010), that the
383 mechanism at play in the LMIT involves glutamate, but this requires further study. Despite
384 the aforementioned limitations, significant differences in LMIT EC50 values were observed
385 here between cyathostomin larvae derived from IVM-R or IVM-RERP populations and
386 larvae obtained from populations that were shown to be, or assumed to be, IVM sensitive.
387 Moreover, at the individual equid level, a direct correlation was identified between the
388 percentage reduction in FEC measured in vivo using the IVM-FECRT and the LMIT EC50
389 value obtained using L3 from the same animal. The parasite isolates used here may be at the

extremes of IVM sensitivity and resistance (as indicated by the high resistance ratios generated for populations A and B when compared to population F) than may be found generally, and work now needs to be performed using samples derived from a wider range of populations for which the in vivo resistance phenotype is not so obvious, for example, cyathostomin populations for which IVM FECR is > 95% but the strongyle ERP is reduced.

Authors' contributions

JM, IH and CM designed the study. CM and AR performed the LMIT. FB and JH provided some of the faecal samples. CM and IH performed the data analysis. JM and CM drafted the manuscript, IH generated the figures and the other authors provided comments to the manuscript and approved the final version.

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Conflict of interest

None of the authors have an actual or potential conflict of interest, including financial, personal or other relationship that could inappropriately influence, or be perceived to influence, the work presented in this manuscript.

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Table 1. Details of the equid populations used to provide L3 for the larval migration inhibition test and their relative sensitivity to ivermectin as indicated by faecal egg count reduction tests. Details for population moxidectin sensitivity is also indicated where the data is available. ML; macrocyclic lactone, IVM; ivermectin, MOX; moxidectin, ERP; egg reappearance period, FECR; faecal egg count reduction, L3; third stage larvae.

Population name	Host species	Location	ML sensitivity or treatment history	Mean IVM-FECR measured in population from which L3 samples were derived[1] (lower confidence limits)
A	Donkey	South west England	IVM resistant [MOX resistant]	91% (0%)
B	Donkey	South west England	IVM resistant [MOX resistant]	82% (0%)
C	Donkey	South west England	IVM reduced ERP [MOX reduced ERP]	>95% (>90%)
D	Donkey	North west England	IVM sensitive	100%
E	Horse	South east England	No ML in last 2 years	ND[2]
F	Horse	South east England	ML not administered	ND[2]

[1] Where indicated, resident nematode populations were tested for ivermectin sensitivity using a faecal egg count reduction test (FECRT) method based on World Association for the Advancement of Veterinary Parasitology guidelines for ruminants (Coles et al. 1992). The mean reduction in faecal egg count at 14 days after administration is indicated here. [2] FECRT not performed, as macrocyclic lactone use was not permitted as the horses are graze on natural conservation sites.

Table 2. EC50 value ranges obtained in the larval migration inhibition test for L3 from individual equids in each population. IVM: ivermectin, R: resistant, S: sensitive. A: IVM FECR < 95%, B: IVM FECR <95%, C: reduced strongyle egg reappearance period after IVM and MOX treatment, D: IVM FECRT >95%, E: IVM not administered in previous two years, F: IVM never administered. Resistance ratios were generated by dividing the EC50 estimate for each population (using all dose response data) by the EC50 estimate for Population F.

Population IVM sensitivity status	**EC50 range (µg/ml) obtained using L3 from individual equids**	**EC50 (µg/ml) estimate for each population using all dose response data for each to fit a single best curve: [95% upper and lower confidence intervals]**	**Resistance ratio**
A IVM-R	3.07-13.19	6.24 [8.66, 3.81]	56.7
B IVM-R	1.33 - 6.14	2.31 [2.98, 1.65]	21.0
C ML-RERP	0.48 - 2.30	1.28 [1.60, 0.96]	11.6
D IVM-S	0.06 - 0.26	0.11 [0.13, 0.08]	1.0
E IVM-S	0.15-0.48	0.25 [0.33, 0.16]	2.3
F IVM-S	0.09-0.14	0.11 [0.15, 0.07]	-

559 **Legends to figures**

560

561 **Figure 1**. Dose response curves (% migration against concentration [log
562 10(concentration+0.01] in μg/ml) generated for each population (A-F) in the larval migration
563 inhibition test. A: IVM-R, B: IVM-R, C: ML-RERP, D: IVM-S, E: IVM-S, F: IVM-S.

564

565 **Figure 2.** Box plots of derived EC50 values from the ivermectin larval migration inhibition
566 test using L3 from equine populations grouped as harbouring cyathostomins that were
567 ivermectin resistant as assessed by FECRT (IVM-R), displayed a reduced strongyle egg
568 reappearance period post ivermectin treatment (IVM-RERP) or were sensitive to ivermectin
569 as assessed by FECRT or had limited or no treatments of ivermectin in the preceding decade
570 (IVM-S).

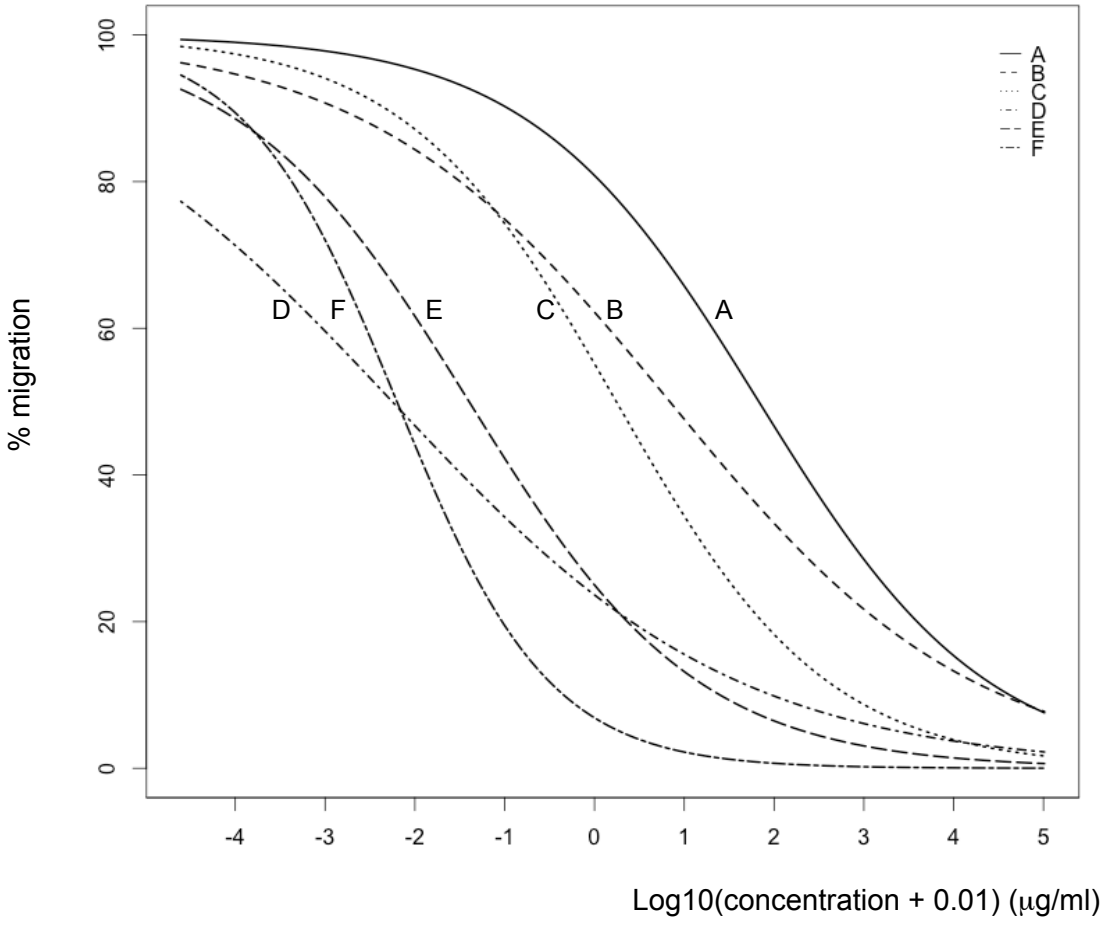
571

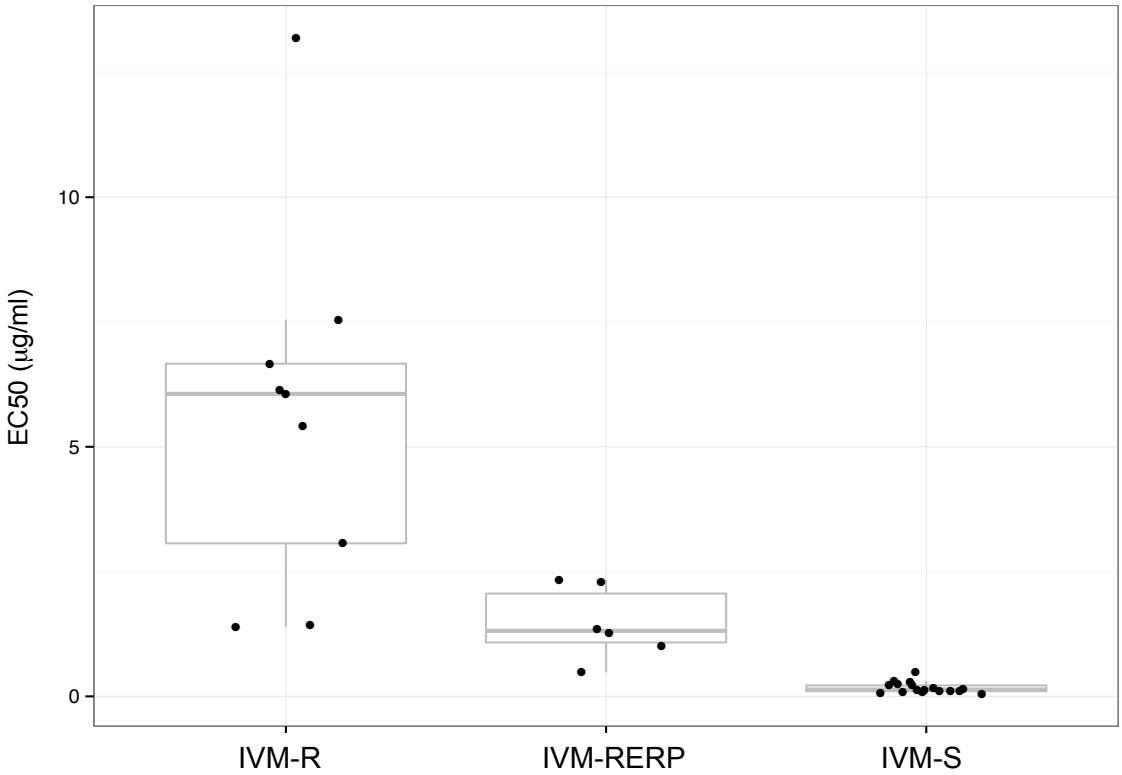
572 **Figure 3.** Comparison of EC50 values obtained using L3 from those individuals for which
573 ivermectin faecal egg count reduction test analysis had been performed. Individual equids
574 are separated into two groups: those for which a faecal egg count reduction of <95% (upper
575 chart) was recorded and those for which a faecal egg count reduction of >95% (lower chart)
576 had been obtained. Note that there was variation within populations in the level of
577 ivermectin faecal egg count reduction when the test was applied and hence one individual
578 from Population A (IVM-R) had an ivermectin faecal egg count reduction of > 95%. The y-
579 axis shows the frequency of individuals over the range of EC50 values that were measured.
580 The x-axis depicts the EC50 value obtained using L3 from individual equids.

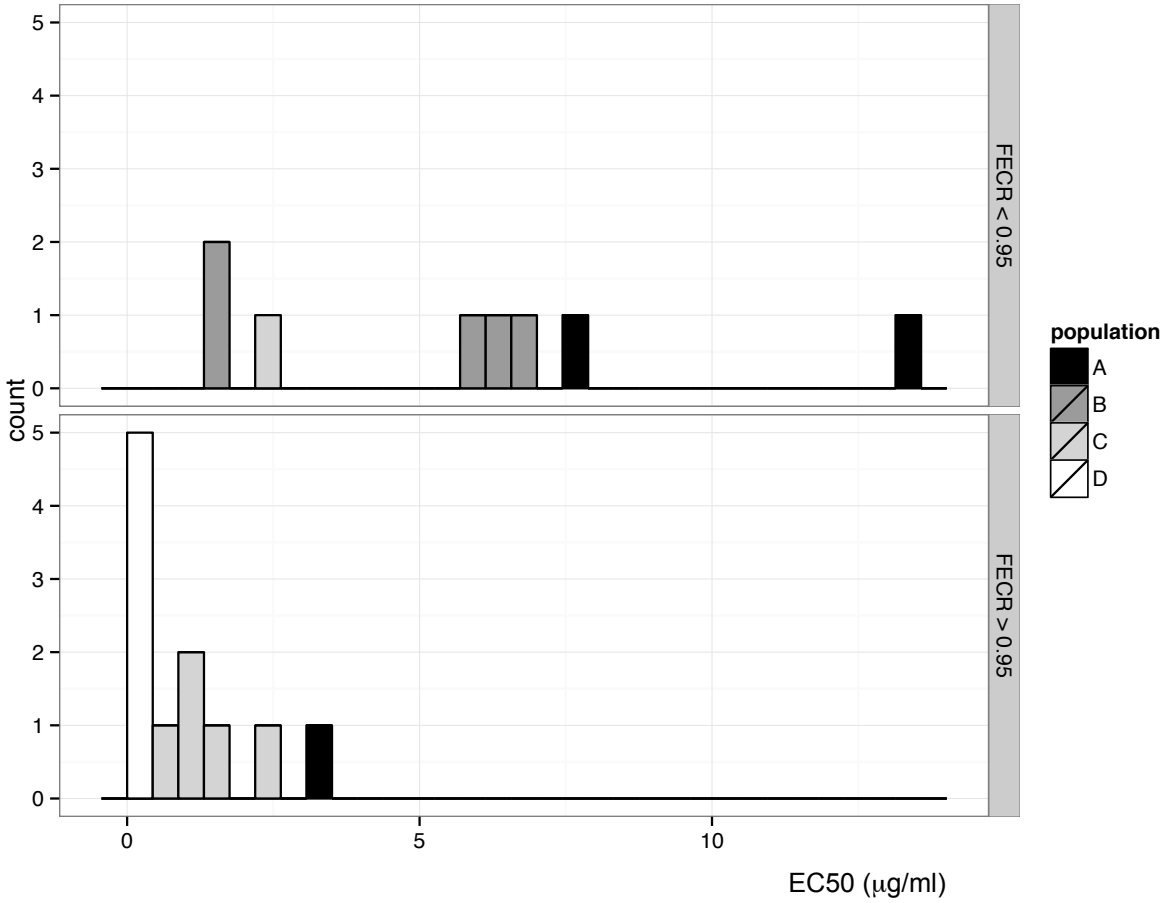
581

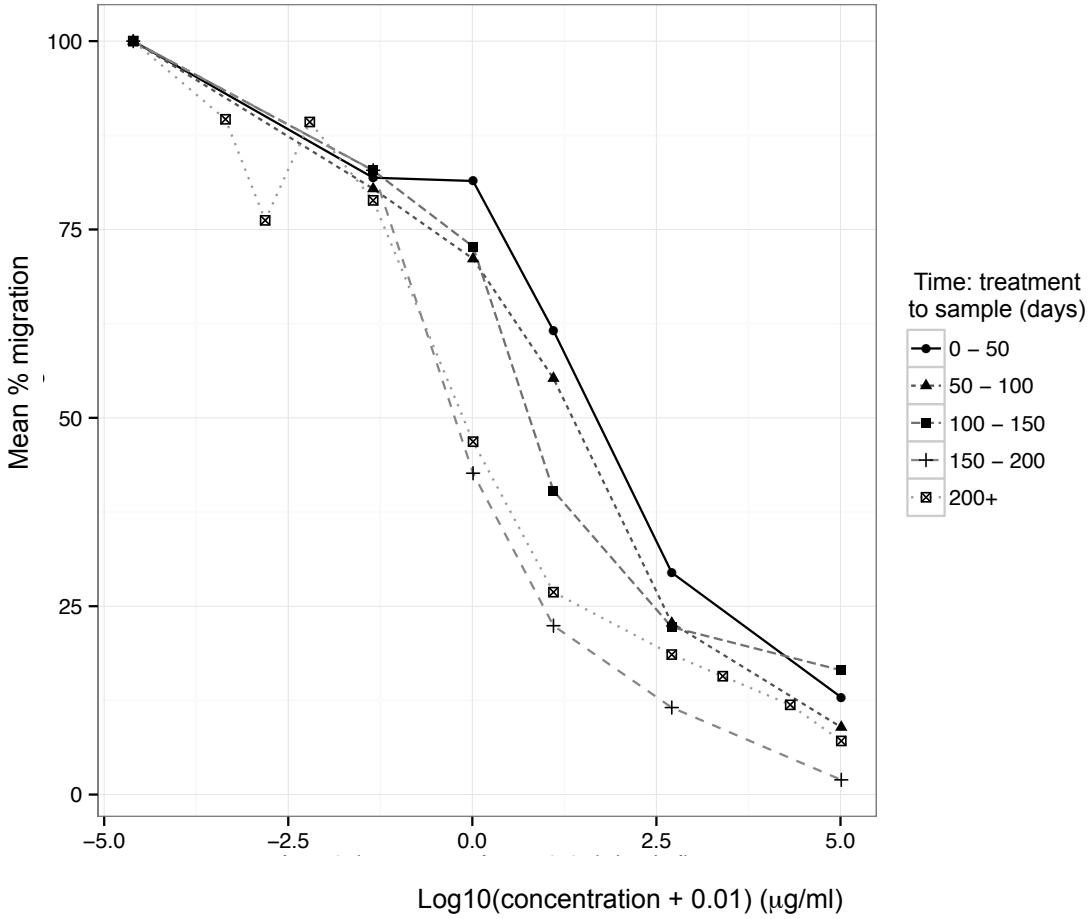
582 **Figure 4.** Percentage migration of L3 in the larval migration inhibition test, comparing time
583 since last ivermectin administration for larvae derived from Populations A, B and C. The

584 mean EC50 value obtained at each log concentration for each set of time to sample data is
585 shown.


% migration
100
80
60
40
20
0
-4
-3
-2
-1
0
1
2
3
4
5
A
B
C
D
E
F
D
F
E
C
B
A
Log10(concentration + 0.01) (μg/ml)


EC50 (μg/ml)
10
5
0
IVM-R
IVM-RERP
IVM-S
Phenotype as measured by FECRT


count
FECR < 0.95
FECR > 0.95
population
A
B
C
D
0
5
10
EC50 (μg/ml)


Mean % migration
100
75
50
25
0
−5.0
−2.5
0.0
2.5
5.0
Log10(concentration + 0.01) (μg/ml)
Time: treatment to sample (days)
0 – 50
50 – 100
100 – 150
150 – 200
200+