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25   ABSTRACT

26   Cyathostomins are the most prevalent parasitic pathogens of <u>equids</u> worldwide. These

27   nematodes have been controlled using broad-spectrum anthelmintics; however, cyathostomin

28   resistance to each anthelmintic class has been reported and populations insensitive to more

29   than one class are relatively commonplace. The faecal egg count reduction test (FECRT) is

30   considered the most suitable method for screening anthelmintic sensitivity in horses, but is

31   subject to variation and is relatively time-consuming to perform. Here, we describe a larval

32   migration inhibition test (LMIT) to assess ivermectin (IVM) sensitivity in cyathostomin

33   populations. This test measures the paralysing effect of IVM on the ability of third stage

34   larvae (L3) to migrate through a pore mesh. When L3 from a single faecal sample were

35   examined on multiple occasions, variation in migration was observed: this was associated

36   with the length of time that the L3 had been stored before testing but the association was not

37   significant. <u>Half maximal effective concentration (EC50)</u> values were then obtained for

38   cyathostomin L3 from six populations <u>of horses or donkeys </u>that showed varying sensitivity

39   to IVM in previous FECRTs. Larvae from populations indicated as IVM resistant by FECRT

40   displayed significantly higher EC50 values in the LMIT than L3 from populations classified

41   as IVM sensitive or L3 from populations that had not been previously exposed to IVM or had

42   limited prior exposure. The analysis also showed that EC50 values obtained using L3 from

43   animals in which IVM faecal egg count reduction (FECR) levels had been recorded as <95%

44   were significantly higher than EC50 values obtained using L3 from animals for which FECR

45   was measured as >95%. For one of the populations, time that had elapsed since IVM

46   administration had an effect on the EC50 value obtained, with a longer time since treatment

47   associated with lower EC50 values. These results indicate that the LMIT has value in

48   discriminating IVM sensitivity amongst cyathostomin populations, but several factors were

49   identified that need to be taken into account when executing the test and interpreting the

50   derived data.

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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

78   perform and subject to less variability need to be investigated. For cyathostomins, such tests

79   should focus on ML anthelmintics. This is because these products hold the major market-share

80   worldwide and, as the prevalence of ML resistance is currently less advanced than with BZ and

81   THP anthelmintics (Molento et al., 2012), <u>and hence tests that inform on sensitivity</u> to ML are

82   likely to have most impact on mitigating the spread of resistance. Molecular mechanisms

83   leading to ML resistance in cyathostomins remain to be defined, so there are no molecular tests

84   available, leaving bench-based in vitro tests as the remaining option.

85   The larval development test has been investigated for measuring anthelmintic sensitivity in

86   cyathostomins; however, this test has not proven particularly informative in defining ML

87   sensitivity levels (Tandon and Kaplan, 2004; Lind et al., 2005; Matthews et al., 2012). An

88   alternative test that has been investigated for assessing anthelmintic sensitivity in ruminant

89   nematode (Demeler et al., 2010; 2012; 2013) and in cyathostomin (van Doorn et al., 2010)

90   populations is the larval migration inhibition test (LMIT). Here, we assessed the potential of the

91   LMIT for measuring IVM sensitivity in cyathostomin larvae derived from different equine

92   populations. This test was deemed appropriate for purpose because the major targets of IVM

93   are ligand-gated chloride channels, which, when bound, result in nematode paralysis (Shoop et

94   al., 1995) and hence will affect the ability of larvae to migrate through small pores of a filter.

95   The cyathostomin populations examined in the current study were derived from groups of

96   donkeys demonstrated previously, by FECRT, to exhibit differing levels of sensitivity to IVM in

97   vivo (Trawford and Burden, 2009), or from equids administered with minimal or no ML

98   treatments (Wood et al., 2013). The proportion of L3 that migrated through a pore filter at

99   increasing concentrations of IVM were measured and the data compared amongst individual

100   equids and between populations to inform on the value of this test.

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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

130   F comprised two groups of ponies used for conservation purposes in the Fens of East Anglia,

131   South East England (Wood et al., 2013). Population E comprised Dartmoor ponies grazed on

132   grassland fen. At the start of the study, all ponies had grazed the Fens for two years. During

133   this time, these ponies were not administered with ML products. Population F consisted of

134   Konik ponies grazed on grassland fen since the mid-1990s. These animals did not receive

135   ML products during this time and were unlikely to have ever received this class of

136   anthelmintic. Due to potential eco-toxicological risks (McKellar, 1997), IVM FECRT were

137   not performed at the sites grazed by Populations E and F, as the use of ML products is not

138   permitted. Given the lack of ML treatments in these populations, it is assumed that the

139   resident cyathostomins in Populations E and F are highly sensitive to IVM and MOX.

140

141   *2.2. Preparation of third stage larvae for LMIT analysis*

142   Freshly voided faecal samples were collected from identified individuals and immediately

143   placed into labelled plastic bags, which were sealed to exclude as much air as possible to

144   retain anaerobic conditions. All samples were sent on the day of collection. Each sample

145   was weighed, homogenised thoroughly and a 10 g sub-sample removed for FEC analysis

146   (Christie and Jackson, 1982). The remainder was cultured under aerobic conditions by

147   transferring faeces to plastic trays, which were placed inside perforated plastic bags. Faeces

148   were incubated at 15°C for up to 22 days, after which, the trays were flooded with lukewarm

149   tap water for 4 h. The supernatants, containing strongyle L3, were poured over a Baermann

150   filter (MAFF, 1986) and the filter placed in the neck of a jam jar filled with tap water. The

151   filter was left overnight, removed the next morning and the remaining volume reduced to 20

152   ml. The L3 were transferred to culture flasks and enumerated in 10 x 10 μl aliquots and

153   classified as small or large strongyle L3 on the basis of gut cell morphology (Thienpont et al.,

154   1986). No large strongyles were observed in any samples from Populations A-E. Very low

155   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

7

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

208 separate occasions. This donkey had not received IVM in the 780 days preceding sample

209 collection. The test was run using L3 that had been stored from 22 to 55 days at $4^{o}C$.

210 Finally, to compare the value of the LMIT in defining IVM sensitivity among cyathostomin

211 populations of varying sensitivity to the anthelmintic in vivo, L3 derived from single time

212 point faecal samples from each Population: A (n=4), B (n=5), C (n=6), D (n=5), E (n=6) and

213 F (n=6) were assessed. All samples were tested at each IVM concentration in duplicate. Only

214 when (un-scaled) mean migration of the L3 in the two positive control wells exceeded 70%,

215 was migration in the presence of IVM subjected to further data analysis.

216

217 *2.4. Statistical analysis and modelling*

218 For data analysis, the percentage migration was calculated for each replicate, with

219 migration in the PBS/DMSO-only well scaled to 100%. For data exploration, dose-response

220 curves (DRC) of the proportion of L3 migrating versus Log10(IVM conc + 0.01) were

221 plotted for each sample. The small additive component, 0.01, allowed the inclusion of data at

222 zero IVM concentration and was found empirically to have minimal impact on estimated

223 EC50 values. The DRC were statistically modelled using a four-parameter logistic dose

224 response model on the natural logarithm of IVM concentration (Demeler et al., 2010; 2012)

225 permitting estimation of EC50. Summary EC50 values were compared using the Kruskal

226 Wallis test with post-hoc analysis using the method of Siegel and Castellan (1988). The

227 association between EC50 and time since last IVM treatment was tested with a linear model

228 predicting EC50 from time since last IVM treatment and population membership. DRC

229 modelling, hypothesis testing and post-hoc analysis were preformed in the R statistical

230 system (R Development Core Team, 2012) using the packages "drc", "lme4" and "pgirmess".

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232

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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

260   (C) for which the mean FECR was reported as >95%, but the ERP was reduced following

261   IVM administration (IVM-RERP), and with populations that were highly sensitive to IVM

262   (i.e. FECR 100%) or had received no IVM treatments (Populations D, E and F), combined

263   here as IVM-sensitive (IVM-S, Figure 2). The data analysis indicated that EC50 values

264   obtained with IVM-R L3 were significantly higher than EC50 values obtained using IVM-S

265   L3 (p < 0.05), but not the EC50 values obtained using IVM-RERP L3. In addition, EC50

266   values obtained using L3 from the IVM-RERP population were significantly higher than

267   those obtained with L3 from the IVM-S populations (p < 0.05).

268

269   *3.2. Association between derived EC50 values and data derived from IVM-FECRT results in*

270   *individual animals*

271   EC50 values obtained using L3 from Populations A, B and C (i.e. those populations that

272   had been subjected previously to IVM FECRT analysis) were used to investigate the

273   hypothesis that there would be a negative association between the percentage reduction in

274   FEC observed 2 weeks after IVM administration and the EC50 values obtained in the IVM-

275   LMIT (Figure 3). The analysis indicated that there is indeed a relationship, with individuals

276   measured as having a FECR of >95% having lower EC50 values in the IVM-LMIT than

277   those for which FECR was <95% (two-way ANOVA, EC50 and FECR>95%, p(FECR)

278   p=0.000284).

279

280   *3.3. Analysis of IVM sensitivity in single populations over time since last IVM treatment*

281   The EC50 values obtained using L3 from Populations A, B and C (i.e. those populations

282   that had a relatively recent IVM treatment) were used to examine if there was a relationship

283   between the proportion of L3 that migrated in the presence of IVM with the number of days

284   since the last recorded IVM administration (Figure 4). The time since IVM treatment for

285   each population ranged as follows: Population A (n=4) − 40-102 days (mean, 57.5 days),

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286   Population B (n=5) – 41-780 days (mean, 250.25 days), and Population C (n=5, one animal

287   of the original 6 animals was not treated with IVM) – 65-194 days (mean, 143.50 days). The

288   analysis indicated that there is a relationship, with higher proportions of migration observed

289   with L3 derived from samples obtained nearer to IVM treatment: i.e. the EC50 value

290   obtained was negatively associated with time since last IVM treatment. This association was

291   significant in the case of the L3 that were derived from Population C (p=0.028)

292

293   **4. Discussion**

294

295   Macrocyclic lactone anthelmintics, such as IVM, that paralyse nematode somatic muscles,

296   among other modes of action, have been assessed in vitro via their effect on larval motility or

297   migration. Such tests are potential options for detecting anthelmintic resistance because they

298   are cheap, relatively quick to perform, preclude host influences and, as they can be run over a

299   concentration range, may provide reproducible parameters with which to measure phenotype

300   (Demeler et al., 2013). Several studies utilising ruminant parasitic nematodes have indicated

301   that motility and migration tests are useful tools for informing on the ML sensitivity of single

302   species populations (Martin and Le Jambre, 1979; Folz et al., 1987; Sangster et al., 1988;

303   Demeler et al., 2010, 2012, 2013). Here, we examined the value of the LMIT for use with

304   cyathostomin larvae obtained by culture from equine faeces. We assessed utility of the test

305   for informing on IVM sensitivity of cyathostomins obtained from populations for which

306   FECRT data was available or populations in which IVM treatments had not been applied or

307   had been limited. Here, we used cyathostomin larvae from donkeys in a comparison with

308   larvae derived from horses because it is problematic to obtain populations of small strongyles

309   from horses for which an IVM FECR of <95% has been demonstrated. For example,

310   shortened IVM ERP has been identified several times in cyathostomins in horse populations

311   (for example, Relf et al., 2013), but populations exhibiting a mean FECR of less than 95%

312   have been reported only sporadically in horses and primarily in South America (Canever et

313   al., 2013). In our comparisons, the LMIT was found to discriminate IVM sensitivity amongst

314   cyathostomin populations in agreement with results that had been previously generated using

315   the FECRT. For example, the derived EC50 values obtained with L3 from IVM-R

316   (Populations A and B) and IVM-RERP (Population C) populations were significantly higher

317   than EC50 values obtained using L3 from all IVM-S populations (D, E and F). These results

318   concur with studies on *Haemonchus contortus*, where correlations were identified between

319   the results of the LMIT and the in vivo anthelmintic resistance status (Gill and Lacey, 1998).

320   In the *Haemonchus* study, the association was found to vary depending on how the nematode

321   strains were selected, with no correlations found when using strains that had been selected

322   experimentally using sub-optimal doses of anthelmintic. In agreement with the current study,

323   though, good correlations between migration in the test and the results of prior FECRT

324   analysis were found when resistant strains isolated from the field (i.e. selected with

325   therapeutic doses of anthelmintic) were compared.

326   One observation from the current study was that although there was a significant

327   difference observed in EC50 values between IVM-R or IVM-RERP and IVM-S populations,

328   there was variation within each population in the EC50 value obtained using L3 derived from

329   individual animals (Table 2). This was particularly noticeable in the IVM-R isolates.

330   Although this concurs with variation in ML FECR levels obtained by FECRT in the IVM

331   resistant populations here, this level of variability could affect the value of this test if pooled

332   samples were to be assessed from a given population in the field, where it could be

333   impractical to run the test on many individuals. Two further confounding factors were

334   identified. One of these was the length of time that L3 had been stored in the laboratory

335   before the test. An effect of culture age on migration was observed, even though L3 were

336   Baermannised just prior to running the test. In previous publications using ruminant

337   nematode larvae, the effect of L3 storage time was not detailed: for example, Demeler et al.

338   (2010) used L3 from sheep faecal cultures stored for 'up to 3 months', but the impact of

339   culture period was not specifically addressed. Other studies (for example, Sangster et al.,

340   1988) do not mention the length of time that L3 were stored for prior to use in the LMIT. In

341   others, the effect of L3 storage time has been assessed: for example, using the LMIT, Molan

342   et al. (2000) compared the sensitivity to condensed tannins of *Trichostrongylus colubriformis*

343   larvae stored for 1 month versus larvae stored for 7 months. Similar to the findings here,

344   these authors found that the *T. colubriformis* L3 stored for longer periods in the laboratory

345   were more sensitive to the xenobiotic tested than larvae stored for shorter periods (p<0.001).

346   The differences observed in the current study were not significant; however, larvae were

347   stored up to only 55 days as opposed to 7 months. On the basis of the results here and the

348   observations made in other nematode species, it is recommended that L3 be used as fresh as

349   possible when assessing IVM sensitivity in the LMIT.

350   The analysis also indicated that the time that elapsed between last IVM treatment and

351   when the faecal samples were obtained for processing had an effect on the derived EC50

352   values in the LMIT. The effect was only found to be significant for Population C: this may

353   have been because this population had the widest range in days since last IVM treatment in

354   the donkeys that were selected for supply of L3 for the LMIT. This observation could be

355   explained by the fact that the nearer to IVM treatment that the L3 are tested in the LMIT, the

356   more likely it is that the parasites used are derived from nematodes that may have survived

357   treatment. This is particularly problematical to investigate in cyathostomins because the

358   exact length of the life cycle of different individual species is unknown and these parasites

359   can undergo a variable period of encystment in the large intestinal wall (Love et al., 1999).

360   Because of the effect of time since last anthelmintic treatment observed here, it is

361   recommended that the impact of this parameter be assessed further in future.

362   Despite these various caveats, the results here showed clear differences in EC50 values

363   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. <u>Furthermore, cyathostomin species composition in donkeys can</u>

373 <u>be different from species composition in horses (Matthee et al., 2004).</u> For these reasons, the

374 <u>authors will now examine the species of cyathostomin present in these populations using L3</u>

375 <u>recovered from the LMIT utilising species specific DNA probes that they have developed</u>

376 <u>based on intergenic spacer region nucleotide sequences (Cwiklinski et al., 2012).</u>

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

390 extremes of IVM sensitivity and resistance (as indicated by the high resistance ratios

391 generated for populations A and B when compared to population F) than may be found

392 generally, and work now needs to be performed using samples derived from a wider range of

393 populations for which the in vivo resistance phenotype is not so obvious, for example,

394 cyathostomin populations for which IVM FECR is > 95% but the strongyle ERP is reduced.

395

396
397 **Authors' contributions**

398 JM, IH and CM designed the study. CM and AR performed the LMIT. FB and JH provided

399 some of the faecal samples. CM and IH performed the data analysis. JM and CM drafted the

400 manuscript, IH generated the figures and the other authors provided comments to the

401 manuscript and approved the final version.

402

408

409 **Conflict of interest**

410 None of the authors have an actual or potential conflict of interest, including financial,

411 personal or other relationship that could inappropriately influence, or be perceived to

412 influence, the work presented in this manuscript.

413

414   **References**

415

416   Bello, T.R., 1996. Alternative antiparasitic treatment of horses with pyrantel pamoate

417   suspension and ivermectin oral solution compared with horses treated only with ivermectin

418   oral solution. J. Eq. Vet. Sci. 16, 18-21.

419   Canever, R.J., Braga, P.R., Boeckh, A., Grycajuck, M., Bier, D., Molento, M.B., 2013. Lack of

420   cyathostomin sp. reduction after anthelmintic treatment in horses in Brazil. Vet. Parasitol.

421   194, 35-39.

422   Christie, M., Jackson, F., 1982. Specific identification of strongyle eggs in small samples of

423   sheep faeces. Res. Vet. Sci. 32, 113-117.

424   Coles, G.C., Bauer, C., Borgsteede, F.H., Geerts, S., Klei, T.R., Taylor, M.A., Waller, P.J.,

425   1992. World Association for the Advancement of Veterinary Parasitology (W.A.A.V.P.)

426   methods for the detection of anthelmintic resistance in nematodes of veterinary importance.

427   Vet. Parasitol. 44, 35-44.

428   Cwiklinski, K., Kooyman, F.N., Van Doorn, D.C., Matthews, J.B., Hodgkinson JE. 2012. New

429   insights into sequence variation in the IGS region of 21 cyathostomin species and the

430   implication for molecular identification. Parasitology. 139,1063-1073.

431   Demeler, J. Küttler, U., El-Abdellati, A., Stafford, K, Rydzik, A., Varaday, M., Kenyon, F.,

432   Jackson, F., Vercruysse, J., von Samson-Himmelstjerna, G., 2010. Standardization of the

433   larval migration inhibition test for the detection of resistance of ivermectin in

434   gastrointestinal nematodes of ruminants. Vet. Parasitol. 174, 58-64.

435   Demeler, J., Kleinschmidt, N., Küttler, U., Koopmann, R., von Samson-Himmelstjerna, G.,

436   2012. Evaluation of the egg hatch assay and the larval migration inhibition assay to detect

437   anthelmintic resistance in cattle parasitic nematodes on farms. Parasitol. Int. 61, 614-618.

438   Demeler, J., Gill, J.H., von Samson-Himmelstjerna, G., Sangster, N.C. 2013. The in vitro assay

439   profile of macrocyclic lactone resistance in three species of sheep trichostrongyloids. Int J

440   Parasitol Drugs Drug Resist. 3, 109-118.

441   Denwood, M.J., Reid, S.W., Love, S., Nielsen, M.K., Matthews, L., McKendrick, I.J., Innocent,

442   G.T., 2010. Comparison of three alternative methods for analysis of equine Faecal Egg

443   Count Reduction Test data. Prev. Vet. Med. 93, 316-323.

444   DiPietro, J.A., Hutchens D.E., Lockc, T.F., Walkerd, K., Paul, A.J., Shipleyc, C., Rullie, D.,

445   1997. Clinical trial of moxidectin oral gel in horses. Vet. Parasitol. 72, 167-177.

446   Folz, S.D., Pax, R.A., Thomas, E.M., Bennet, J.L., Lee, B.L., Conder, G.A., 1987.

447   Development and validation of an in vitro *Trichostrongylus colubriformis* motility assay.

448   Int.J. Parasitol. 17, 1441-1444.

449   Gill, J.H., Lacey, E., 1998. Avermectin/milbemycin resistance in trichostrongyloid nematodes.

450   Int J Parasitol. 28:863-877.

451   Jackson, F., Coop, R.L., 2000. The development of anthelmintic resistance in sheep nematodes.

452   Parasitology, 120 Suppl, S95-S107.

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

454   Kaplan, R.M., Klei, T.R., Lyons, E.T., Lester, G., Courtney, C.H., French, D.D., Tolliver, S.C.,

455   Vidyashankar, A.N., Zhao, Y., 2004. Prevalence of anthelmintic resistant cyathostomes on

456   horse farms. J. Am. Vet. Med. Assoc. 225, 903-910.

457   Lester, H.E., Spanton, J., Stratford, C.A., Bartley, D.J., Morgan, E.R., Hodgkinson, J.E.,

458   Coumbe, K., Mair, T., Swane, B., Lemone, G., Cookson, R., Matthews, J.B., 2013.

459   Anthelmintic efficacy against cyathostomins in horses in England. Vet. Parasitol. 197, 189-

460   196.

461   Lichtenfels, J.R., Kharchenko, V.A., Dvojnos, G.M., 2008. Illustrated identification keys to

462   strongylid parasites (strongylidae: Nematoda) of horses, zebras and asses (Equidae). Vet.

463   Parasitol. 156, 4-161.

464   Lind, E.O., Uggla, A., Waller, P., Höglund, J., 2005. Larval development assay for detection of

465   anthelmintic resistance in cyathostomins of Swedish horses. Vet. Parasitol. 128, 261-269.

466   Lyons, E.T., Drudge, J.H., Tolliver, S.C., Granstrom, D.E., Stamper, S., 1992. Evaluation of

467   exclusive use of ivermectin *vs*. alternation of antiparasitic compounds for control of internal

468   parasites of horses. Am. J. Vet. Res. 52, 97-104.

469   Martin, P.J., Le Jambre, L.F., 1979. Larval paralysis as an in vitro assay of levamisole and

470   morantel tartrate resistance in *Ostertagia*. Vet. Sci. Comms. 3, 159-164.

471   Matthee, S., Krecek, R.C., McGeoch, M.A. 2004. A comparison of the intestinal helminth

472   communities of Equidae in Southern Africa. J Parasitol. 90, 1263-1273.

473   Matthews, J.B., 2014. Anthelmintic resistance in equine nematodes. Int. J. Parasit. Drugs and

474   Drug Resistance. In Press. http://dx.doi.org/10.1016/j.ijpddr.2014.10.003.

475   Matthews, J.B., McArthur, C., Robinson, A., Jackson, F., 2012. The in vitro diagnosis of

476   anthelmintic resistance in cyathostomins. Vet. Parasitol. 185, 25-31.

477   McKellar QA., 1997. Ecotoxicology and residues of anthelmintic compounds. Vet. Parasitol. 72,

478   413-426.

479   Molan, A.L., Waghorns, G.C., Min, B.R., McNabb, W. C., 2000. The effect of condensed

480   tannins from seven herbages on *Trichostrongylus colubriformis* larval migration in vitro.

481   Folia Parasitologica 47, 39-44.

482   Molento, M.B., Antunes, J., Bentes, R.N., Coles, G.C., 2008. Anthelmintic resistant nematodes

483   in Brazilian horses. Vet. Rec. 162, 384-385.

484   Molento, M.B., Nielsen, M.K., Kaplan, R.M., 2012. Resistance to avermectin/milbemycin

485   anthelmintics in equine cyathostomins - current situation. Vet. Parasitol. 185, 16-24.

486   Love S, Murphy D, Mellor D. 1999. Pathogenicity of cyathostome infection. Vet Parasitol.

487   85:113-121.

488 R Development Core Team. 2012. R: A language and environment for statistical computing. R

489 Foundation for Statistical Computing, Vienna, Austria. ISBN 3-900051-07-0, URL

490 http://www.R-project.org.

491 Relf, V.E., Lester, H.E., Morgan, E.R., Hodgkinson, J.E., Matthews, J.B., 2014. Anthelmintic

492 efficacy on UK Thoroughbred stud farms. Int. J. Parasitol., 44, 507-514.

493 Sangster, N.C., Riley, F.L., Collins, G.H., 1988. Investigation of the mechanism of levamisole

494 resistance in trichostrongylid nematodes of sheep. Int. J. Parasitol. 18, 813-818.

495 Shoop, W.L., Mrozik, H., Fisher, M.H., 1995. Structure and activity of avermectins and

496 milbemycins in animal health. Vet. Parasitol. 59, 139-156.

497 Siegel, S., Castellan, N. J. Jr., 1988. Nonparametric statistics for the behavioural sciences. In:

498 McGraw-Hill International Editions, New York, N.Y., 2nd ed., pp 213-214.

499 Tandon, R., Kaplan, R.M., 2004. Evaluation of a larval development assay (DrenchRite®) for

500 the detection of anthelmintic resistance in cyathostomin nematodes of horses. Vet. Parasitol.

501 121, 125-142.

502 Thienpont, D., Rochette, F., Vanparijs, O.F., 1986. Diagnosing helminthiasis by coprological

503 examination. 2nd ed. Janssen Research Foundation, Beerse, Belguim, 85 pp.

504 Traversa, D., Castagna, G., von Samson-Himmelstjerna, G., Meloni, S., Bartolini, R., Geurden,

505 T., Pearce, M.C., Woringer, E., Besognet, B., Milillo, P., D'Espois, M. 2012. Efficacy of

506 major anthelmintics against horse cyathostomins in France. Vet. Parasitol. 188, 294-300.

507 Trawford A.F., Burden F., Hodgkinson J., 2005. Suspected moxidectin resistance in two donkey

508 herds at The Donkey Sanctuary. Proceedings of the 20th International Conference of the

509 WAAVP, Christchurch, New Zealand.

510 Trawford, A.F., Burden, F., 2009. Ivermectin resistance in cyathostomes in four donkeys herds

511 at the Donkey Sanctuary, UK. Proceedings of the 22nd International Conference of the

512 WAAVP, Calgary, Canada.

513 van Doorn, D.C., Kooyman, F.N., Eysker, M., Hodgkinson, J.E., Wagenaar, J.A., Ploeger,

514   H.W., 2010. In vitro selection and differentiation of ivermectin resistant cyathostomin

515   larvae. Vet. Parasitol. 174, 292-299.

516 Vidyashankar, A.N., Hanlon, B.M., Kaplan, R.M., 2012. Statistical and biological

517   considerations in evaluating drug efficacy in equine strongyle parasites using fecal egg

518   count data. Vet. Parasitol. 185, 45-56.

519 Wood, E.L., Matthews, J.B., Stephenson, S., Slote, M., Nussey, D.H., 2013. Variation in fecal

520   egg counts in horses managed for conservation purposes: individual egg shedding

521   consistency, age effects and seasonal variation. Parasitology 140, 115-128.

522

523

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.

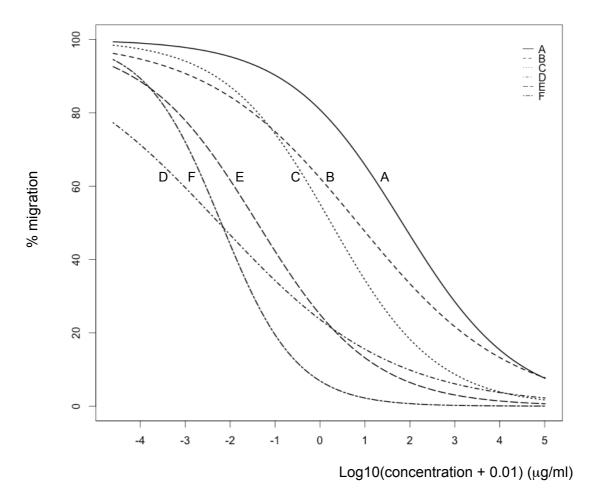| Population IVM sensitivity status	EC50 range (µg/ml) obtained using L3 from individual equids	EC50 (µg/ml) estimate for each population using all dose response data for each to fit a single best curve: [95% upper and lower confidence intervals]	Resistance ratio
A IVM-R	3.07-13.19	6.24 [8.66, 3.81]	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]	-

Legends to figures

561 **Figure 1**. <u>Dose response curves (% migration against concentration [log</u>

562 <u>10(concentration+0.01] in µg/ml)</u> 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.
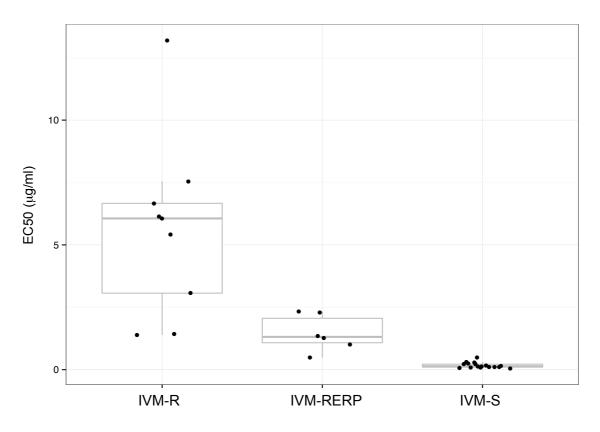
564

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.

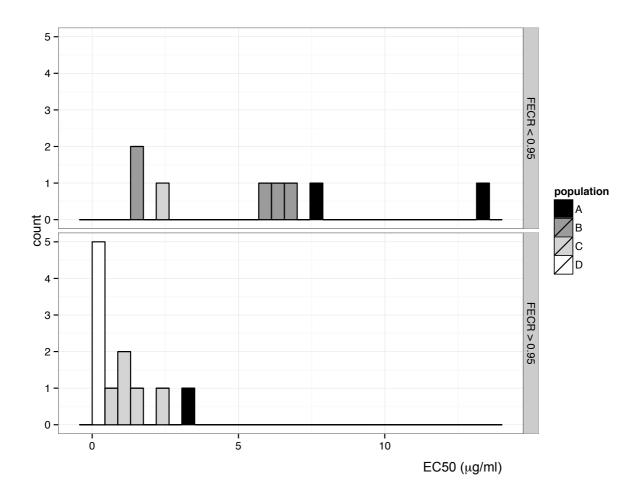580 The x-axis depicts the EC50 value obtained using L3 from individual equids.

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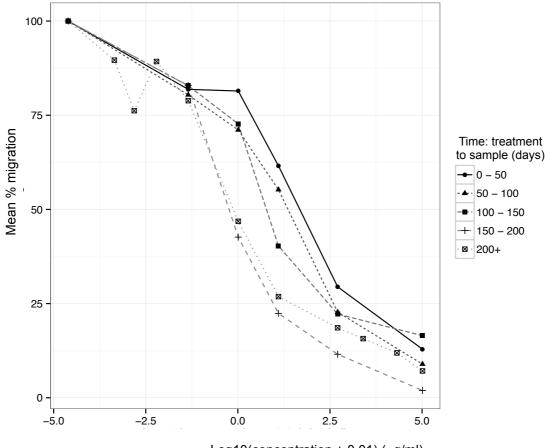
584   mean EC50 value obtained at each log concentration for each set of time to sample data is

585   shown.