Short Communication

In vitro evaluation of ivermectin, moxidectin, albendazole and pyrantel against cyathostomins of horses

Avaliação *in vitro* da ivermectina, moxidectina, albendazole e pirantel contra ciatostomíneos de equinos

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Abstract

Cyathostomins are the most prevalent nematodes of horses, and multidrug resistance has been reported worldwide. There is a need to implement alternative drug monitoring analytical tests. The objective of this study was to determine the consistency (5 repetitions) of the larval migration on agar test (LMAT) using ivermectin, moxidectin, pyrantel or albendazole against cyathostomin infective-stage larvae in eight different concentrations. LMAT showed a strong coefficient of determination ($R^2 > 0.91$), between the test repetitions (n=5). The average 50% effective concentration (EC50) for ivermectin, moxidectin, pyrantel and albendazole were 0.0404, 0.0558, 0.0864 and 0.0988 nMol, respectively. The results of the EC50 for albendazole showed the greatest range of concentration. Ivermectin and moxidectin had the lowest in between-test variation. In the future, internationally certified susceptible isolates could be used for screening new drug candidates, or to follow up the pattern of drug efficacy from field populations.

Keywords: Horse, Anthelmintics, *In vitro* test, parasites.

Resumo

Ciatostomíneos são os nematodas mais prevalentes em equinos e a resistência múltipla foi relatada em todo o mundo. Existe a necessidade de implementar o monitoramento dos produtos com testes analíticos alternativos. O objetivo deste estudo foi determinar a consistência (5 repetições) do teste de migração larval em ágar (TMLA) usando ivermectina, moxidectina, pirantel e albendazole contra larvas infectantes de ciatostomíneos em oito concentrações diferentes. O TMLA demonstrou um coeficiente de determinação (R^2) acima de 0,91 entre as repetições do teste. A concentração efetiva para 50% (CE50) para ivermectina, moxidectina, pirantel e albendazole foi de 0,0404; 0,0558; 0,0864 e 0,0988 nMol, respectivamente. A CE50 do albendazole demonstrou a maior amplitude entre os testes. A ivermectina e a moxidectina tiveram as menores variações das doses entre as repetições. No futuro, isolados certificados susceptíveis poderão ser testados com o TMLA para indicação de novos produtos e mesmo para acompanhar o perfil de eficácia de populações do campo.

Palavras-chave: Cavalos, anti-helmínticos, teste *in vitro*, parasitos.

Cyathostomins, are the most prevalent helminth pathogens of horses (PEREGRINE et al., 2014). The attention toward these parasites has increased over the past decade, following the intense and efficient control of large strongyles with anthelmintic compounds. Although anthelmintic resistance has been broadly disseminated, it was restricted to phenothiazine, benzimidazoles (BZs) and pyrantel (PYR) (WIRTHERLE et al., 2004). The macrocyclic lactones, ivermectin (IVM) and moxidectin (MOX), are highly effective against cyathostomins, however, a number of reports have described cases of cyathostomin resistance to both compounds (MOLENTO et al., 2008; TRAVERSA et al., 2009; CANEVER et al., 2013).

Nielsen et al. (2006) stated that it is essential to monitor any sustainable parasite control program, but the only practical test under field conditions is the fecal egg count reduction test (FECRT). It has now been two decades since the start of consistent use of *in vitro* tests as an alternative to the FECRT, and as a screening step in the search for new compounds (OLIVEIRA et al., 2017). The *in vitro* egg hatch test (EHT), larval development test (LDT) and the larval migration test (LMT) are tools that can also aid the diagnosis of resistance, and to screen for alternative medicines (KOTZE et al., 2006; PEACHEY et al., 2016; LÉGARÉ & OUELLETTE, 2017). Although the tests have some particularities, they have been largely used in ruminant nematodes, particularly sheep (GILL et al., 1995; VÁRADY & CORBA, 1999; MOLENTO & PRICHARD, 2001) and cattle (DEMELER et al., 2010). A few studies have used these *in vitro* methods for horses (CRAVEN et al., 1999;

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KÖNIGOVÁ et al., 2003; LIND et al., 2005; PEACHEY et al., 2016). Craven et al. (1999) reported a comparison between the FECRT and EHT for detecting anthelmintic resistance in strongyles. Resistance to BZD was demonstrated in 33 out of 42 farms (79%), when using FECRT, and in 32 out of 52 farms (62%), when using the EHT. The larval migration on agar test (LMAT) is a variation of the LMT, and allows the observation of ruminant nematode larvae that pass through a mesh and an agar gel layer, which serve as a barrier (MOLENTO & PRICHARD, 2001). Thus, with the advancement of cyathostomin multidrug resistance, the standardization of another *in vitro* test may become an important aid to drug monitoring. The objective of this study was to determine the consistency of the LMAT using four anthelmintic drugs against cyathostomins.

Third-stage infective larvae (L3) were obtained from fecal cultures that contained 96-100% cyathostomins (2-4% of large strongyles). The compounds albendazole (ABZ), PYR, IVM and MOX were diluted to eight different concentrations (Table 1) and tested in five repetitions, under the same conditions. A control without any compound was included. The concentrations (in triplicates) were prepared by dissolving each drug concentrations in distilled water and DMSO (3%). Fresh larvae (30 days old) were exsheathed with 0.3% sodium hypochlorite for 30 min before the test (MOLENTO & PRICHARD, 2001). A total of 400 larvae were placed in the compound solution in 24-well plates at a volume of 1 ml/well. For the first incubation period, the plates were incubated at 28°C for 6 h. The second incubation began with the addition of 1 ml of 1.4% agar into the solution. This 2-ml solution was then transferred into a previously prepared individual apparatus (MOLENTO & PRICHARD, 2001) at 28 °C for 18h. During this period, the larvae were exposed to a 150 W light bulb to stimulate L3 migration. At the end, the

solution was transferred to 50-ml tubes which were centrifuged, then the supernatant was removed. The L3 solution (150 μl) was examined under a microscope (20x) to count the number of migrating L3. Data ($X = log^X$) were analyzed to obtain the 50% effective concentration (EC50) values, 95% confidence interval and the coefficient of determination (R^2). The dose-response curves were generated using non-linear fit of the normalized transformed data using the PRISM 5 software.

Figure 1 shows the dose-response curves after the LMAT. The EC50 and R^2 values for the five tests are presented in Table 2. The results of the EC50 for ABZ had the greatest range of concentration [1.49 (log -7.69) to 20 (log -8.66) nMol] between the tests. The EC50 value of PYR varied from 2.44 to 11.44 nMol, and all tests had a R^2 above 0.94. The tests with IVM and MOX showed the lowest variation in EC50 values between the tests, with an average R^2 of 0.95 for IVM and 0.91 for MOX.

Attempts to develop and regularly use *in vitro* assays for the diagnosis of anthelmintic resistance in equine nematodes has shown limited success (VAN DOORN et al., 2010). The data obtained from the current study show that low concentrations of IVM and MOX were sufficient to inhibit the migration of 50% of L3, with little variability between the samples.

The results obtained for ABZ showed a wide variation (+/- 0.156), and it was not possible to determine a safe cut-off value to the EC50. Considering the purpose of the LMAT, and the way in which inhibition of L3 migration occurs, we suggest that the LMAT is a good tool for detecting variation to IVM and MOX, but not so much for the BZs (ROTHWELL & SANGSTER, 1993) as they share a similar mode of action (blocking calcium channel in the nervous cell). In the present study, the ABZ results did not show consistency, and even at very high concentrations, it did not prevent L3 migration. The most probable hypothesis would be that ABZ did not impede larval movement.

The results for PYR showed a high EC50 for the cyathostomin populations. In all tests, the R^2 was higher than 0.90. The results showed good applicability of the test for this drug due to its mode of action. One possible hypothesis to explain the reported results is the nicotinic action on the neuromuscular junction causing spastic paralysis; however, further tests are necessary to establish concentration limits for resistant populations.

Matthews et al. (2012) reported that the performance of *in vitro* assays for anthelmintic resistance in parasites of horses require further refinement, and several factors need to be taken into account when conducting LMT before interpreting the derived data (McARTHUR et al., 2015). We also believe that before running

Table 1. Drug concentrations (nMol/ml, log^{-1}) for albendazole, pyrantel, ivermectin and moxidectin used for the larval migration on agar test against cyathostomins of horses.

Albendazole	Pyrantel	Ivermectin	Moxidectin
1,0e-010	1,0e-010	1,0e-010	1,0e-010
3,12e-009	3,5e-009	5,0e-010	5,0e-010
6,25e-009	7,0e-009	1,0e-009	1,0e-009
1,25e-008	1,4e-008	2,0e-009	2,0e-009
2,5e-008	2,8e-008	4,0e-009	4,0e-009
5,0e-008	5,6e-008	8,0e-009	8,0e-009
1,0e-007	1,12e-007	1,6e-008	1,6e-008
2,0e-007	2,24e-007	3,2e-008	3,2e-008

Table 2. Effective concentration dose of 50% (Log EC$_{50}$ in nMol) and the coefficient of determination (R^2) of the five tests for albendazole, pyrantel, ivermectin and moxidectin using the larval migration on agar test against cyathostomins of horses.

Tested drugs	Albendazole		Pyrantel		Ivermectin		Moxidectin	
Repetitions	EC$_{50}$	R^2	EC$_{50}$	R^2	EC$_{50}$	R^2	EC$_{50}$	R^2
1	0.033	0.97	0.040	0.95	0.027	0.98	0.039	0.95
2	0.148	0.81	0.057	0.97	0.025	0.98	0.077	0.85
3	0.087	0.83	0.038	0.96	0.055	0.92	0.049	0.93
4	0.167	0.70	0.047	0.94	0.035	0.96	0.073	0.85
5	0.059	0.90	0.25	0.98	0.060	0.90	0.041	0.95
Average	0.0988		0.0864		0.0404		0.0558	
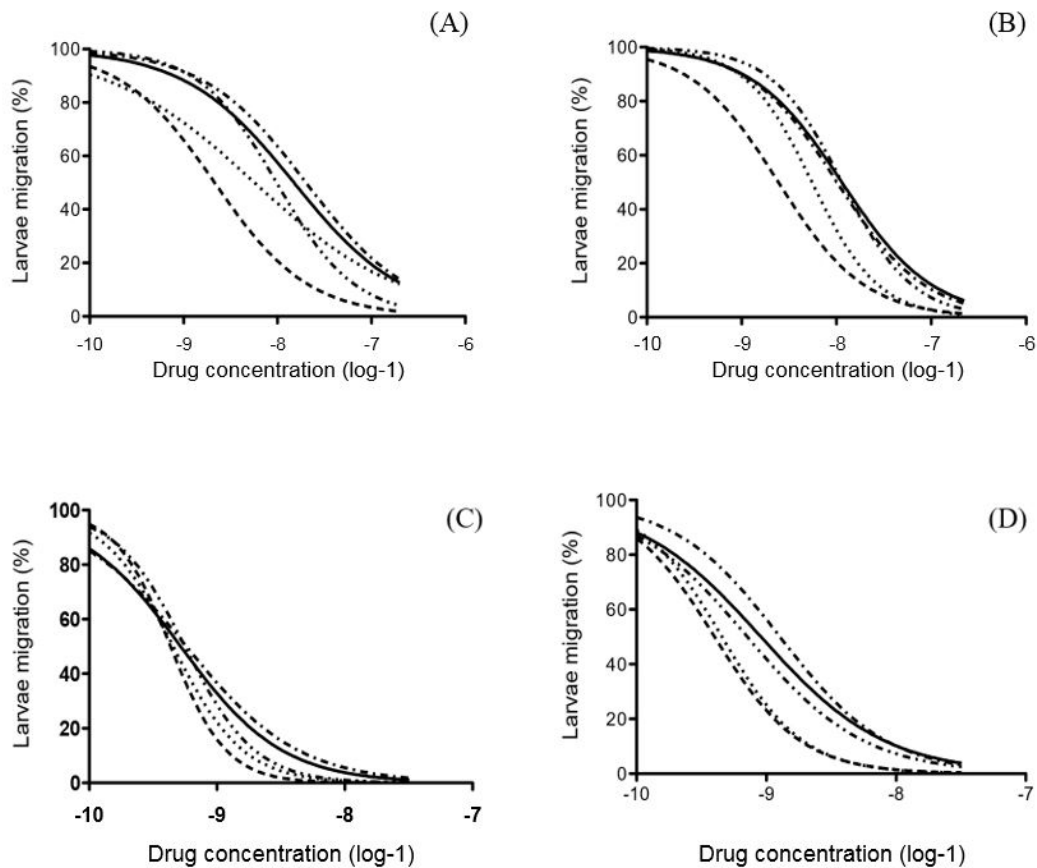
Figure 1. Effective drug concentration curves (log-1) for (A) albendazole, (B) pyrantel, (C) ivermectin and (D) moxidectin, after five independent larval migration on agar test against cyathostomins of horses.

in vitro test or comparing different efficacy methods (EHT, LM, FECRT), researchers must test the reliability of the assays, to validate the methods and calculate the level of variability. Although the reference values for each product tested in these experiments, still need further refinement (due to differences in ABZ efficacy), we have demonstrated the consistency of the LMAT method (among 5 repetitions). In the future, certified susceptible isolates could be used for screening new drug candidates, or to follow up the pattern of some anthelmintic efficacies from cyathostomin field populations.

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