Improvement of an *in vitro* test for *Haemonchus contortus* resistance diagnosis in small ruminant (RESISTA-Test©)
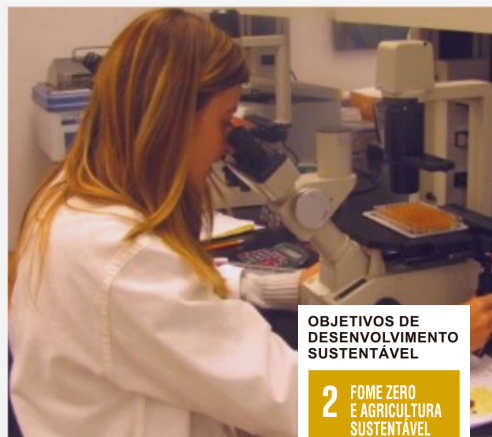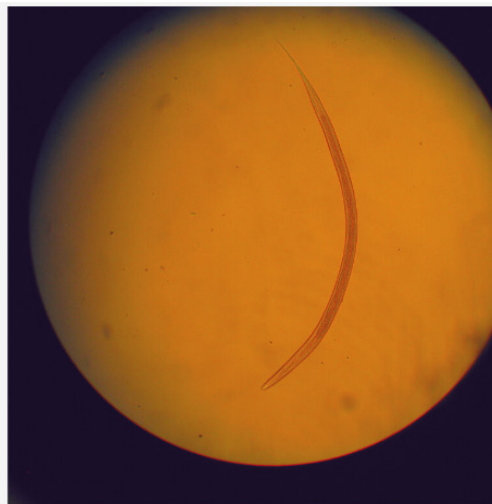

OBJETIVOS DE
DESENVOLVIMENTO
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SUSTENTÁVEL

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E DESENVOLVIMENTO
48

Improvement of an *in vitro* test for
Haemonchus contortus resistance diagnosis
in small ruminant (RESISTA-Test©)

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Sumário

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Apresentação

Esta publicação apresenta uma contribuição para o Objetivo de Desenvolvimento Sustentável 2 "Fome zero e agricultura sustentável". O ODS2 abrange a melhoria da produtividade e da renda dos pequenos produtores de alimentos com foco na agricultura familiar, onde a produção de pequenos ruminantes se destaca, em especial no Nordeste do Brasil. Nessa região os rebanhos são pequenos e utilizados em geral na subsistência familiar (produção de carne, leite e couro). Em outras regiões do Brasil a produção é mais tecnificada, mas a verminose continua a ser o principal gargalo sanitário do sistema de produção. Na lógica da redução do uso de insumos, o desenvolvimento de técnicas precisas de recomendação de uso de vermífugos mais eficazes para o controle parasitário representa um avanço em direção à sustentabilidade da produção pecuária, visto que permite o uso mais racional dos anti-helmínticos, com resultados mais eficazes e assertivos. Como consequência, ocorre desaceleramento no estabelecimento da resistência anti-helmíntica, redução de gastos com vermífugos, maior produtividade animal e menores riscos de resíduos de antiparasitários na

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carne e no leite. O RESISTA-Test© é um teste para diagnóstico laboratorial de baixo custo e bem menos laborioso que o teste tradicional realizado nas propriedades que, justamente em função disso, é raramente feito no Brasil. É um processo agropecuário inédito no país que, inclusive, pode reduzir o uso de animais em experimentos. Essa técnica permitirá, no futuro, que pequenos produtores, técnicos e médicos veterinários possam enviar uma amostra de fezes do rebanho, para a obtenção de um laudo laboratorial com a recomendação do melhor anti-helmíntico a ser utilizado pelos próximos 12 meses.

Presentation

This publication presents a contribution to the Sustainable Development Goals 2 "Zero Hunger". SDG2 covers the improvement of productivity and income of small food producers with a focus on family farming, where the production of small ruminants stands out, especially in the Northeast of Brazil. In this region, flocks are small and are generally used for family subsistence (meat, milk and leather production). In other regions of Brazil, production is more technical, but worms remain the main sanitary constrain in the production system. In the logic of reducing the use of inputs, the development of precise techniques for recommending the use of more effective dewormer for parasitic control represents an advance towards the sustainability of livestock production, since it allows more rational use of anthelmintics, with more effective and assertive results. As a consequence, there is a slowdown in the establishment of anthelmintic resistance, reduction of expenses with deworming agents, higher animal productivity and lower risks of antiparasitic residues in meat and milk. The RESISTA-Test[©] is a test for laboratory diagnosis of low cost and much less laborious than the traditional test performed on the properties which, due to this reason, is rarely performed in Brazil. It is an unprecedented agricultural process in the country that can even reduce the use of animals in experiments. This method will allow, in the future, small farmers, technicians and veterinarians to be able to send animal stool samples from the flock, to get a laboratory report with the recommendation of the best anthelmintic to be used for the next 12 months.

Improvement of an *in vitro* test for *Haemonchus contortus* resistance diagnosis in small ruminant (RESISTA-Test©)

Abstract

Parasitic resistance monitoring by the larval development test (LDT) can be a tool to delay its occurrence in flocks and preserve the efficacy of anthelmintics. The aim of this study was to optimize an *in vitro* diagnostic test (RESISTA-Test©) of *Haemonchus contortus* resistance to the main commercially anthelmintics available in Brazil. The efficacy of thiabendazole (TBZ), levamisole (LEV), ivermectin-monosaccharide (IVM-M), monepantel (MPT) and Zolvix® (ZLV) in the larval development test (LDT) was evaluated in susceptible (Echevarria1991-HcEc91) and resistant (Botucatu-HcBot) *H. contortus* isolates, in 24- and 96-well culture plates. Complementary tests were subsequently performed with ivermectin aglycone (IVM-A). After standardization of concentration ranges in HcEc91 and HcBot, *H. contortus* isolated from fecal samples of five sheep flocks (HcFlocks) were also evaluated. LDT data were analyzed using logit dose-response by the Probit model and the degree of parasitic resistance was expressed by the resistance factor (RF). Resistance factors above 3 indicated that the test was able to differentiate susceptible and resistant isolates to TBZ, LEV, MPT, IVM-A, and in a less consistent way for IVM-M. The high similarity profile of dose-response curves between plates and interaction plate*concentration (R^2 from 98.4 to 99.0% for HcEc91/HcBot and from 99.4 to 99.0% for HcFlocks), as well as low differences in mean efficacy (0.02 to 2.26% for HcEc91/HcBot and 0.02 to 4.90% for HcFlocks) for all anthelmintic, except MPT, indicated reliable LDT agreement in both plates, for the evaluation of HcEc91/HcBot and for HcFlocks. In the latter case, resistance detection by ZLV was clearer and more stable than by MPT. Results obtained with IVM-A were much more reliable than with IVM-M. Adapting the test to 96-well plates resulted in cost savings of at least 51.9%. The LDT validation by comparing the results with those of the Fecal Egg Count Reduction Test (FECRT) in flocks may make it available for routine laboratory use, supporting decisions in parasite control programs.

Otimização de um teste *in vitro* para diagnóstico da resistência de *Haemonchus contortus* em pequenos ruminantes (RESISTA-Test©)

Resumo

O monitoramento da resistência anti-helmíntica pelo teste de desenvolvimento larvar (TDL) pode ser uma ferramenta para atrasar sua ocorrência nos rebanhos e para preservar a eficácia dos anti-helmínticos. O objetivo deste estudo foi otimizar um teste diagnóstico (RESISTA-Test©) *in vitro* da resistência de *Haemonchus contortus* aos principais anti-helmínticos comercialmente disponíveis no Brasil. A eficácia do tiabendazol (TBZ), levamisol (LEV), ivermectina-monossacárida (IVM-M), monepantel (MPT) e Zolvix® (ZLV) no teste de desenvolvimento larvar (TDL) foi avaliada em isolados de *H. contortus* susceptível (Echevarria1991 - HcEc91) e resistente (Botucatu - HcBot), em placas de cultura de 24 e 96 poços. Testes complementares foram posteriormente realizados com a ivermectina aglicona (IVM-A). Após padronização dos intervalos de concentração em HcEc91 e HcBot, *H. contortus* isolados de amostras de fezes coletadas de cinco rebanhos ovinos (HcRebanhos) também foram avaliados. Os dados do TDL foram analisados usando logit dose-resposta pelo modelo Probit e o grau de resistência anti-helmíntica foi expresso pelo fator de resistência (FR). FRs acima de 3 indicaram que o teste foi capaz de diferenciar os isolados susceptível e resistente a TBZ, LEV, MPT, ZLV e IVM-A, e de maneira menos consistente para IVM-M. O alto perfil de similaridade das curvas dose-resposta entre as placas e interação placa*concentração (R^2 de 98,4 a 99,0% para HcEc91/HcBot e de 99,4 a 99,0% para HcRebanhos), bem como baixas diferenças na eficácia média (0,02 a 2,26% para HcEc91/HcBot e 0,02 a 4,90% para HcRebanhos) para todos os anti-helmínticos, exceto MPT, indicaram concordância confiável do TDL em ambas as placas, tanto para a avaliação de HcEc91/HcBot quanto para HcRebanhos. No último caso, a detecção de resistência pelo ZLV foi mais clara e mais estável do que pelo MPT. Os resultados obtidos com a IVM-A foram muito mais confiáveis do que com a IVM-M. A adaptação do teste para placas de 96 poços resultou em economia de

pelo menos 51,9%. A validação do TDL por meio da comparação de seus resultados com os do teste de redução da contagem de ovos nas fezes (TRCOF) em rebanhos podem torná-lo disponível para uso laboratorial de rotina, apoiando a tomada de decisão em programas de controle parasitário.

Termos para indexação: teste diagnóstico, RESISTA-Test©, nematoides gastrintestinais, resistência, ovinos.

Introduction

The breeding of small ruminants is a global activity for meat, milk, and leather production (Sargison, 2016). Brazil has approximately 18 million of sheep (ANUALPEC, 2018), a number that is growing due to factors such as better management and genetic improvement (AQUINO et al., 2016). However, sheep meat consumption in Brazil is still low (ANUALPEC, 2018). Human population growth has prompted the conversion of many pasture areas into farmland for increased food production. Breeding large ruminants is becoming more difficult due to the lack of grazing land. Properties in densely populated areas may be smaller than 0.5 ha. In these places, the importance of sheep and goats in relation to meat and milk supply has been recognized. In this sense, in the subsistence sector, farmers and breeders depend more on small ruminants. This generates income and allows the sale of surpluses, improving the quality of life. Thus, any intervention that increases sheep and goat productivity is important (HIRPA; ABEBE, 2008).

The main health problem of small ruminants is gastrointestinal nematode infections (GIN). Animals suffer reduced weight gain, decreased fertility rate and increased mortality, causing significant economic losses (NOVA et al., 2014). Parasites such as *Haemonchus* and *Trichostrongylus* cause huge economic losses each year to the sheep production chain. In a survey conducted in the southern region of the state of Rio Grande do Sul, Brazil, the economic losses were estimated at US$ 500,000/year (OLIVEIRA et al., 2017). Excessive reliance on anthelmintic for parasite control has led to the development of multiple parasite resistance, so this approach is not considered sustainable for GIN control (SUTHERLAND; LEATHWICK, 2011; VAN WYK; REYNECKE, 2011). Several studies report GIN resistance to multiple drugs in sheep flocks from all regions of Brazil. The first report of resistance to benzimidazole was in Rio Grande do Sul (DOS SANTOS; GONÇALVES, 1967), where the first record of ivermectin-resistant nematodes also occurred (ECHEVARRIA; TRINDADE, 1989). In the northeastern of Brazil, resistance in goat nematodes was reported in Pernambuco to levamisole, albendazole and parbendazole (CHARLES et al., 1989) and in the state of Bahia to albendazole and ivermectin (BARRETO; SILVA, 1999). In Ceará, resistance occurred in goats using oxfendazole and levamisole (VIEIRA; CAVALCANTE, 1999), in sheep and goats using closantel, oxfendazole and ivermectin (MELO et al., 1998),

and so on. Monepantel belongs to the group of aminoacetonitrile derivatives (AAD) (KAMINSKY et al., 2008a, b) and its commercial product Zolvix® is the most recently released anthelmintic in Brazil. However, resistance to this group has already been reported in Brazil (CINTRA et al., 2016; ALBUQUERQUE et al., 2017; CIUFFA et al., 2017) as well as in other countries (SCOTT et al., 2013; MEDEROS et al., 2014; VAN DEN BROM et al., 2015).

Therefore, when a new anthelmintic is launched and widely used, nothing prevents the rapid development of resistance (CHAGAS, 2015). This makes the validation of practices for monitoring and controlling anthelmintic resistance in flocks extremely important. The main method to detect resistance *in vivo* is the fecal egg count reduction test (FECRT), which can be performed with all chemical groups. However, this method is expensive, laborious and time consuming (VÁRADY et al., 2007). When the resistance diagnosis is not routinely adopted in flocks, the main consequence is a lack of information about the anthelmintic efficacy and of the proper management of the chemical groups.

Compared to the FECRT, *in vitro* tests are less expensive, faster, and more accurate, and in many cases, are a reproducible alternative (LACEY et al., 1991; HAZELBY et al., 1994; VÁRADY et al., 1996). Depending on the method adopted, the test can generate linear dose-response curves for the chemical groups (HUBERT; KERBOEUF, 1992). The larval development test (LDT) is considered sensitive and practical, allowing the evaluation of different chemical groups at the same time (KAPLAN et al., 2007). The results can be compared with reference isolates (susceptible and resistant) so that the lethal concentration (LC_{50}) can be determined and the inter-assay interferences eliminated (CRAVEN et al., 1999). Many studies have shown good correlation between the FECRT and the discriminatory LC50 for resistance obtained *in vitro* (TAYLOR, 1990; LACEY et al., 1991; KÖNIGOVÁ et al., 2003; VÁRADY et al., 2006; KAPLAN et al., 2007; DÍEZ-BAÑOS et al., 2008; TAYLOR et al., 2009). Gill et al. (1995) improved the LDT to detect resistance to avermectins and milbemycins, which led to the launch of a commercial test kit, DrenchRite® (KAPLAN et al., 2007). However, this kit is expensive, difficult to import to Brazil and has been used mainly by parasitological diagnostic laboratories in the US.

Due to this situation, we sought to develop an *in vitro* diagnostic test (RESISTA-Test©), for *H. contortus* resistance to the main commercially available anthelmintic groups in Brazil. The diagnostic test was designed to perform a liquid-based test using drug standards representing different chemical groups. Some procedures were refined by adapting the diagnostic test to 96-well plates and checking which drugs could be used to obtain a reliable and affordable laboratory diagnosis. The validation of the test in the future may allow more rational and guided parasite control in sheep and goats, preserving chemical classes.

Material and methods

Anthelmintics

In order to establish lethal concentrations (LC) of anthelmintics in susceptible and resistant *H. contortus* isolates and in *H. contortus* collected from flocks, *in vitro* tests were standardized for thiabendazole - TBZ (Sigma-Aldrich T8904), levamisole - LEV (Sigma-Aldrich 31742), ivermectin monosaccharide - IVM-M (Sigma-Aldrich I8898), monepantel - MPT (PGS P11144) and Zolvix® - ZLV (Novartis). Additional tests were latter performed with ivermectin aglycone – IVM-A (Bioaustralis I1151-1) as well.

GIN evaluation

All procedures involving parasite donor animals were approved by the Committee on Ethical Use of Animals (CEUA) of Embrapa Pecuária Sudeste (Protocol No. 04/2017). Four Santa Inês lambs, aged between three and four months with average weight of 27 kg, were treated with Zolvix® (monepantel, 2.5 mg/kg LW) to eliminate natural infection by GIN parasites still susceptible to that drug (CHAGAS et al., 2013). On the 7th, 10th and 15th days after treatment, egg counts per gram of feces (EPG) were performed using the McMaster X 50 technique (UENO; GONÇALVES, 1998) to confirm that the animals were worm-free. On the 15th day after treatment, two animals were artificially infected with 4000 third stage larvae (L$_3$) of the *H. contortus* Echevarria1991 isolate (HcEc91: isolate from Rio Grande do Sul State, Brazil, susceptible to all anthelmintic (ECHEVARRIA et al., 1991)) and two other animals with the *H. contortus* Botucatu isolate (HcBot: resistant to monepantel (ALBUQUERQUE

et al. (2017)). The animals were kept in pairs in separate pens, supplemented daily with 400 g of corn silage and with free access to water and mineral salt. With the establishment of infection, feces were collected for egg recovery to perform in vitro tests.

Samples were also collected from five sheep-producing properties in the state of São Paulo, Brazil, where the animals were not subjected to deworming for at least 40 days and presented EPG>200 in a previous analysis (day before the experiment). In each flock, samples were collected from 49 animals individually, placed in vacuum-sealed plastic bags, identified with the animal's number and immediately taken to the laboratory (D0). Fecal cultures (pool) were performed for GIN identification (VAN WYK et al., 2004) and EPG for egg recovery for LDT. The flocks were visited 14 days later (D14) to collect feces to perform a new LDT (independent repetitions).

Egg recovery

Feces collected directly from the animals' rectal ampoule were dissolved in water and filtered using sequential sieves. A saturated NaCl solution was added to the eggs retained in the last sieve and centrifuged at 3,000 rpm for five minutes. The supernatant was washed with distilled. The eggs were then divided into five aliquots, and their suspensions were added to the 24- and 96-well culture plates (Kasvi®) (COLES et al., 1992).

Larval development test (LDT)

The LDT, adapted from Hubert; Kerboeuf (1992), was performed in 24- and 96-well plates. Solutions containing 100 and 70 *H. contortus* eggs were added to each well in 24- and 96-well plates, respectively, which also received culture medium (*Escherichia coli* [EC11303] and amphotericin B [A9528] - Sigma-Aldrich). This culture medium allows the development from first-stage larvae (L_1) to L_3. The plates were identified, sealed with PVC film and kept in an incubator for 24 h (27°C, RH ≥ 80%) for larval development (L_1). After this period, each well received serial anthelmintic dilutions and the plates were incubated again for six days, when larvae L_1, L_2 and L_3 from each well were quantified with an inverted microscope. For the tests with HcEc91/HcBot, all anthelmintic concentrations and the negative control with water were tested in six replicates (six wells) and in three independent experiments (on

different dates). For the tests with HcFlocks, all anthelmintic concentrations and the negative control were tested in two replicates (two wells following the commercial kit model, Drenchrite®) and in two independent experiments (D0 and D14).

Regarding the HcFlocks analyses, plates were examined to estimate the critical well for each anthelmintic (well in which 50% of parasites did not develop to L_3; interpolation between 2 wells was performed when necessary) (KAPLAN et al., 2007). All larvae in the control wells, and above and below the critical well, were counted and identified to determine the species composition of the sample. *H. contortus* resistance (estimated by the resistance factor - RF) was determined when the predominant parasite (L_3) in the control and in the critical wells consisted of this species (HcFlocks) (ANONYMOUS, 1996), which occurred in all flocks.

Statistical analysis

The anthelmintic efficacy against each isolate was determined based on the arithmetic mean of larval development according to the following equation (COLES et al., 1992), where: $Inhibition(\%) = 100(\overline{X}_{test} / \overline{X}_{total})$, $\overline{X}_{test}$ refers to the number of larvae that did not reach the L_3 stage, and $\overline{X}_{total}$ corresponds to the number of $L_1 + L_2 + L_3$.

LDT results were analyzed by Probit logistic regression model to determine LC_{50} and LC_{99} values, which were defined as the anthelmintic concentrations at which 50% and 99% of L_1 to L_3 development was inhibited. The degree of anthelmintic resistance was expressed as the resistance factor (RF), calculated as the LC_{50} and LC_{99} values of the resistant isolate divided by the respective susceptible isolate values (DOLINSKÁ et al., 2013). Analyses were performed with XLSTAT-Premium 2019.2.2 (Addinsoft 2019 – XLSTAT, Boston, USA) and a significance level of $p \leq 0.05$ was considered.

Two-way ANOVA was applied by the SAS GLM procedure (SAS, 2010) considering the effects of plate (24 and 96 wells), concentrations (1 to 12) and the interaction plate*concentration. LSMEANS was adopted for multiple comparison between average effects of the plates and of the interaction, using the Tukey test and the F-test, respectively, with a significance level of 5% ($p \leq 0.05$).

Results

Echevarria1991 and Botucatu isolates

Table 1 summarizes the mean efficacy ($\overline{X}$ ± SE) for each of the isolates compared between the 24- and 96-well plates by two-way ANOVA. For TBZ, LEV, IVM-M and ZLV there were subtle differences (DIF) between the plates. Although low, these differences were significant (p ≤ 0.05) according to the plate p-value, as well as for some concentrations, expressed by the plate x concentration interaction. However, biologically these differences (DIF) could be neglected (did not exceed 2.26%). In the case of MPT, the differences between the plates were higher (42.06 and 22.49% for HcEc91 and HcBot, respectively), with a significant (p ≤ 0.001) plate effect and plate x concentration interaction. The values of R^2 (98.4 to 99.9%) indicated the accuracy of the analysis, i.e., the higher R^2, the more explanatory the model is and the better it fits the sample. In Figure 1 the dose-response curves reinforce the confidence of the results obtained for TBZ, LEV, IVM-M and ZLV in both plates, since the results were very similar for these drugs. The inhibition percentage of larval development was thus very evident.

Table 1. Results of the larval development inhibition of *H. contortus* isolates Echevarria1991 (HcEc91) and Botucatu (HcBot), in 24 and 96-well plates, for thiabendazole -TBZ, levamisole - LEV, ivermectin monosaccharide - IVM-M, monepantel - MPT and Zolvix® - ZLV.

Isolates	Anthelmintic	Plates (well)	$\overline{X}$ ± SE (%)	Min. (%)	Max. (%)	DIF (%)	Plate (p-value)	Plate x Conc. (p-value)	R²
HcEc91	TBZ	24	93.91±0.09	69.07	100	0.44	0.02	0.05	0.984
		96	93.47±0.16	66.66	100				
	LEV	24	86.13±0.16	46.54	100	0.02	0.94	0.0003	0.984
		96	86.11±0.28	48.40	100				
	IVM-M	24	47.60±0.18	0.00	100	1.85	0.006	0.006	0.997
		96	49.45±0.30	0.00	100				
	MPT	24	83.64±0.07	41.30	100	42.06	10^{-9}	10^{-11}	0.999
		96	41.58±0.12	0.00	100				
	ZLV	24	70.82±0.11	5.73	99.95	0.48	0.03	0.05	0.997
		96	70.34±0.19	5.63	100				
HcBot	TBZ	24	36.88±0.20	0.00	95.64	2.26	0.004	0.004	0.994
		96	34.62±0.34	0.00	89.62				
	LEV	24	63.73±0.16	1.93	100	0.19	0.54	0.88	0.996
		96	63.54±0.27	2.27	100				
	IVM-M	24	52.06±0.13	1.70	99.95	0.33	0.20	0.003	0.997
		96	51.72±0.23	1.57	97.64				
	MPT	24	22.18±0.71	1.09	100	22.49	10^{-11}	10^{-11}	0.927
		96	12.81±1.23	0.00	66.67				
	ZLV	24	39.21±0.10	0.00	91.39	0.85	0.003	0.55	0.998
		96	40.06±0.17	0.00	91.74				

* Significant at 5% (p ≤ 0.05) by ANOVA and the Tukey test. ± SE: Mean efficacy ± standard error; Min: minimum efficacy; Max: maximum efficacy; DIF: Difference in mean efficacies between 24-well and 96-well plates; R_2 (coefficient of determination, 0 to 1): measure of adjustment of the statistical model to the observed values.
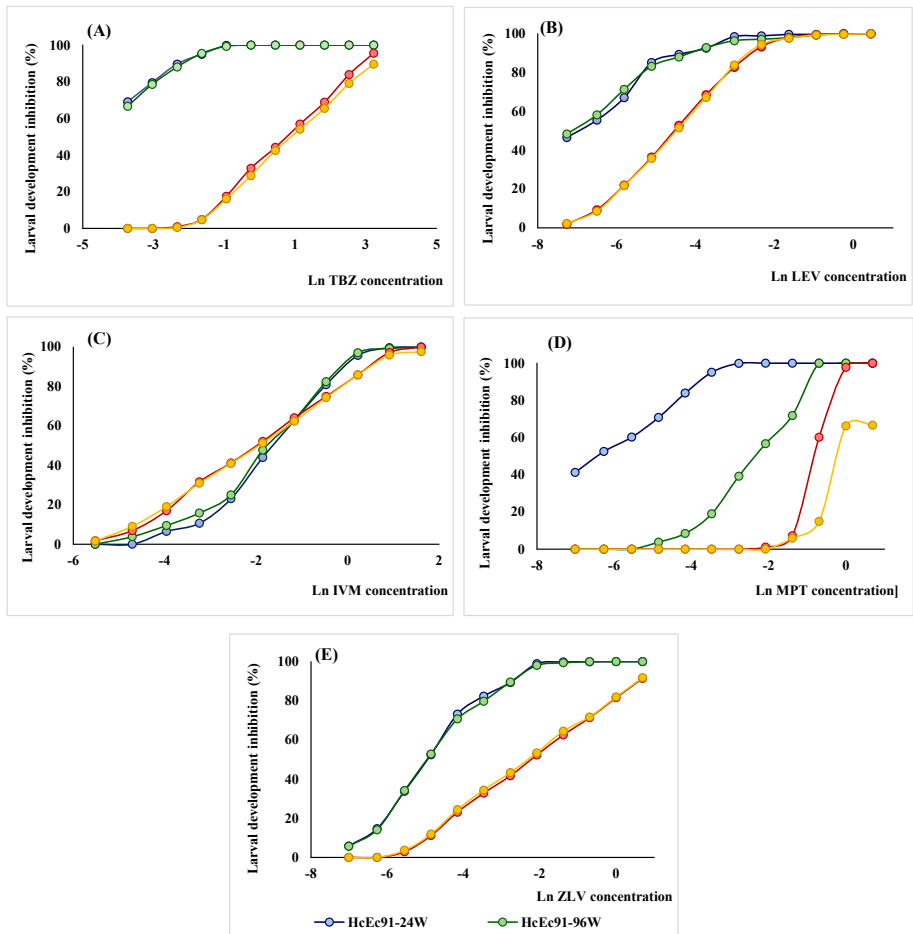
Figure 1. Log-dose and logit-response curves for *H. contortus*: Echevarria1991 susceptible isolate in 24-well (HcEc91-24W) and 96-well (HcEc91-96W) plates, and Botucatu resistant isolate in 24-well (HcBot-24W) and 96-well plates (HcBot-96W) to Thiabendazole-TBZ (A), Levamisole-LEV (B), Ivermectin-IVM-M (C), Monepantel-MPT (D) and Zolvix-ZLV (E).

Another criterion that reinforces these results was the LC_{50} (Table 2), the usual cutoff that separates resistant from susceptible isolates. The results obtained for each drug and isolate in the 24-well plates were within the confidence interval (95%) of the 96-well plate results. This only did not occur for MPT, since 0.086 µg/mL was not within the confidence range of 0.0009 to 0.0011 µg/mL and 1.041 µg/mL did not fit within the confidence interval of 0.437 to 0.453 µg/mL. In the comparison between isolates for IVM-M, LC_{50} obtained for the susceptible isolate did not differ much from the resistant isolate. This can also be seen in Figure 1, where the curves of both isolates intersect close to the development inhibition of 50% of the parasites. Such results indicate the reliability of the results obtained in both plates, except for MPT and IVM-M.

The LC results were used to determine the RFs of the isolates in both plates (Table 2). RF_{99} and RF_{50} values were above 3 for all drugs in both plates and only the RF_{99} values for LEV (1.88 and 1.32 in the 24- and 96-well plates, respectively) and RF_{50} for IVM-M (0.83 to 0.99 in 24- and 96-well plates, respectively) were below this level. MPT allowed better differentiation of isolates in 24-well plates, although the values obtained in 96-well plates were also reliable. RFs above 3 demonstrate that the test was able to distinguish isolates, which therefore did not occur only for IVM-M considering the RF_{50}.

Table 2. Lethal concentrations LCs, confidence limits (95%, μg/mL) and resistance factors (*RFs) in LDT to *Haemonchus contortus* susceptible (Echevarria1991-HcEc91) and resistant (Botucatu-HcBot) isolates, in 24- and 96-well plates, for thiabendazole -TBZ, levamisole - LEV, ivermectin monosaccharide - IVM-M, monepantel - MPT and Zolvix® - ZLV.

Anthelmintic	LCs	HcEc91		HcBot		RF_{50}		RF_{99}	
		24-well	96-well	24-well	96-well	24-well	96-well	24-well	96-well
TBZ	LC_{50}	0.011 (0.0110-0.0120)	0.010 (0.0060-0.0140)	2.166 (2.0856-2.2501)	2.650 (2.4440-2.8851)	191.52	253.15		
	LC_{99}	2.325 (2.0583-2.6402)	0.621 (0.3678-1.3548)	95.871 (86.2741-107.0918)	157.244 (123.8728-204.9480)			41.23	253.32
LEV	LC_{50}	0.002 (0.0017-0.0019)	0.001 (0.0004-0.0011)	0.011 (0.0103-0.0111)	0.011 (0.0101-0.0117)	5.85	14.58		
	LC_{99}	0.175 (0.1491-0.2071)	0.251 (0.1325-0.6083)	0.328 (0.3021-0.3576)	0.331 (0.2797-0.3967)			1.88	1.32
IVM - M	LC_{50}	0.131 (0.1244-0.1380)	0.158 (0.1346-0.1869)	0.129 (0.1243-0.1345)	0.132 (0.1211-0.1443)	0.99	0.83		
	LC_{99}	3.115 (2.6900-3.6539)	3.978 (2.7511-6.2582)	9.603 (8.6688-10.6836)	13.260 (10.5670-16.9982)			3.08	3.33
MPT	LC_{50}	0.001 (0.0009-0.0011)	0.086 (0.0743-0.1008)	0.445 (0.4365-0.4528)	1.041 (0.9749-1.1145)	447.76	12.03		
	LC_{99}	0.058 (0.0513-0.0669)	1.306 (0.9414-1.9630)	1.216 (1.1672-1.2709)	9.668 (7.7975-12.5113)			20.86	7.40
ZLV	LC_{50}	0.008 (0.0074-0.0079)	0.008 (0.0066-0.0095)	0.112 (0.1067-0.1171)	0.103 (0.0935-0.1141)	14.65	12.95		
	LC_{99}	0.206 (0.1903-0.2244)	0.244 (0.1669-0.3939)	21.715 (18.7480-25.3428)	21.433 (15.7926-30.1104)			105.27	87.68

*LC_{50} and LC_{99} of the resistant isolate (Botucatu) divided by the respective value of the susceptible isolate (Echevarria1991). RF values greater than 3 show that the test was able to distinguish susceptible and resistant isolates.

GINs from flocks

The fecal cultures indicated *Haemonchus* as the predominant genre in the flocks on D0 and D14 (Figure 2).
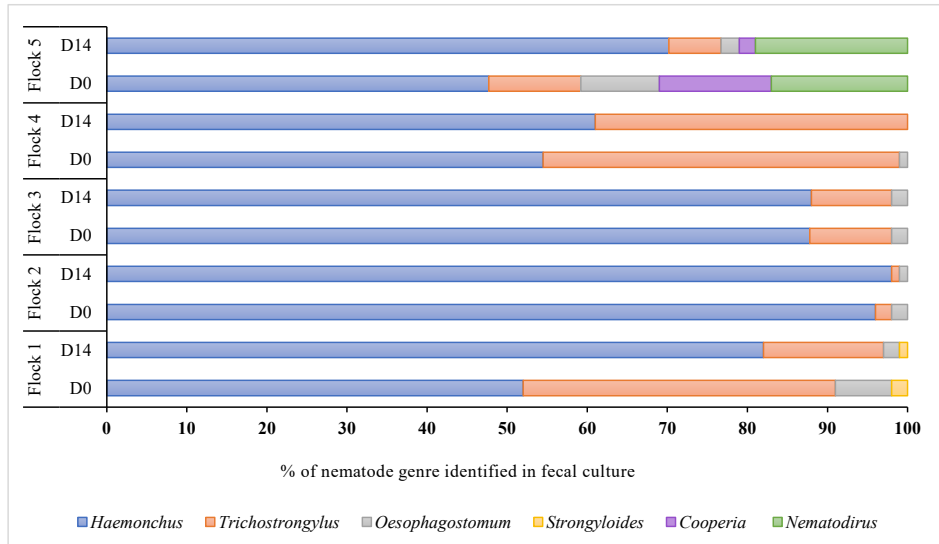

Figure 2. Percentage of nematode genre in fecal culture from flocks (Flock 1 to Flock 5) on days 0 and 14.

Table 3 shows again that although there were significant differences (p ≤ 0.05), the percentage of these differences (DIF) between plates and between days were subtle for all drugs (the lowest being 0.02% to ZLV HcFlock-3 and the highest 4.90% to LEV HcFlock-5), except for MPT, which was higher on HcFlock-3 (10.56% and 8.41% for D0 and D14, respectively) and on HcFlock-5 (8.03% and 6.35% for D0 and D14, respectively). Differences detected for the others anthelmintic can be neglected as they did not exceed 5.25% (HcFlock-1). Again, high R^2 values (99.4 to 99.9%) indicate the accuracy of the analysis. The results show the agreement of LDT when performed in different plates and days of field collection, being sensitive and functional, except for MPT.

Table 3. Results of the inhibition of *H. contortus* larval development by flock (HcFlocks 1 to 5), in 24- and 96-well plates, on days 0 and 14, for thiabendazole -TBZ, levamisole - LEV, ivermectin monosaccharide - IVM-M, monepantel - MPT and Zolvix® - ZLV.

Flock	Anthelmintic	Day	Plates (well)	$\overline{X}$ ± SE (%)	Min. (%)	Max. (%)	DIF (%)	Plate (p-value)	Plate x Conc. (p-value)	R²
HcFlock-1	TBZ	D0	24	34.48±0.20	0.00	100	0.98	0.00003	0.00003	0.999
			96	33.50±0.21	0.00	100				
		D14	24	35.07±0.18	0.00	100	4.77	0.00004	0.00004	0.999
			96	39.84±0.18	0.00	100				
	LEV	D0	24	85.81±0.16	43.50	100	1.55	0.00004	0.00004	0.999
			96	84.26±0.16	34.91	100				
		D14	24	90.52±0.16	52.53	100	1.11	0.00004	0.0005	0.998
			96	89.41±0.17	53.42	100				
	IVM-M	D0	24	47.82±0.27	0.47	100	3.64	0.00004	0.00003	0.999
			96	44.18±0.27	1.31	100				
		D14	24	47.98±0.21	0.00	100	4.13	0.00003	0.00003	0.999
			96	43.85±0.21	0.00	100				
	MPT	D0	24	33.76±0.14	0.00	100	5.25	0.00003	0.00003	0.999
			96	28.51±0.15	0.00	100				
		D14	24	37.02±0.17	0.00	100	4.14	0.00004	0.00003	0.999
			96	32.88±0.16	0.00	100				
	ZLV	D0	24	70.06±0.26	7.24	100	0.20	0.59	0.00003	0.999
			96	70.26±0.26	6.95	100				
		D14	24	71.35±0.19	11.27	100	1.74	0.00003	0.02	0.999
			96	73.09±0.21	14.96	100				

Table 3. continuação

Flock	Anthelmintic	Day	Plates (well)	$\overline{X}$ ± SE (%)	Min. (%)	Max. (%)	DIF (%)	Plate (p-value)	Plate x Conc. (p-value)	R^2
HcFlock-2	TBZ	D0	24	50.77±0.27	0.45	100	0.16	0.69	0.007	0.999
			96	50.93±0.26	0.00	100				
		D14	24	48.79±0.18	0.00	94.77	0.60	0.03	0.006	0.999
			96	48.19±0.17	0.00	95.03				
	LEV	D0	24	96.13±0.17	76.13	100	0.08	0.74	0.003	0.994
			96	96.05±0.17	79.17	100				
		D14	24	87.73±0.15	57.01	100	2.63	0.00003	0.00004	0.998
			96	90.36±0.15	62.03	100				
	IVM-M	D0	24	83.70±0.18	43.00	100	0.62	0.03	0.00004	0.999
			96	83.08±0.19	37.79	100				
		D14	24	79.72±0.20	36.79	100	0.85	0.007	0.609	0.999
			96	80.57±0.20	37.58	100				
	MPT	D0	24	17.48±0.13	0.00	100	1.39	0.00004	0.00003	0.999
			96	18.87±0.14	0.00	100				
		D14	24	18.26±0.14	0.00	76.36	0.96	0.0001	0.0001	0.999
			96	17.30±0.15	0.00	74.33				
	ZLV	D0	24	30.64±0.17	0.00	89.81	0.36	0.17	0.00003	0.999
			96	31.00±0.18	0.00	91.17				
		D14	24	29.80±0.13	0.00	89.96	0.06	0.74	0.02	0.999
			96	29.74±0.13	0.00	88.96				

Table 3. continuação

Flock	Anthelmintic	Day	Plates (well)	$\bar{X}$ ± SE (%)	Min. (%)	Max. (%)	DIF (%)	Plate (p-value)	Plate x Conc. (p-value)	R^2
HcFlock-3	TBZ	D0	24	35.39±0.21	0.00	88.43	1.18	0.03	0.0002	0.999
			96	34.72±0.21	0.00	90.64				
		D14	24	34.58±0.15	0.00	79.99	0.46	0.05	0.0005	0.999
			96	35.04±0.16	0.00	81.04				
	LEV	D0	24	54.77±0.19	2.37	99.06	1.99	0.00004	0.00004	0.999
			96	56.76±0.19	6.54	100				
		D14	24	41.64±0.20	0.00	89.76	0.99	0.002	0.00003	0.999
			96	42.63±0.20	0.65	96.10				
	IVM-M	D0	24	72.84±0.21	20.49	100	0.08	0.79	0.001	0.999
			96	72.76±0.20	21.13	100				
		D14	24	64.64±0.22	30.62	93.91	4.17	0.00003	0.03	0.999
			96	68.81±0.21	32.64	99.34				
	MPT	D0	24	50.03±0.16	0.00	100	10.56	0.00003	0.00003	0.999
			96	60.59±0.16	0.00	100				
		D14	24	51.43±0.14	0.00	100	8.41	0.00004	0.00003	0.999
			96	59.84±0.14	0.00	100				
	ZLV	D0	24	96.39±0.07	83.25	100	0.02	0.87	0.00003	0.998
			96	96.41±0.06	83.56	100				
		D14	24	94.60±0.08	76.92	100	0.37	0.005	0.0002	0.998
			96	94.97±0.08	75.89	100				

Table 3. continuação

Flock	Anthelmintic	Day	Plates (well)	$\overline{X}$ ± SE (%)	Min. (%)	Max. (%)	DIF (%)	Plate (p-value)	Plate x Conc. (p-value)	R^2
HcFlock-4	TBZ	D0	24	36.57±0.13	0.00	89.91	2.22	0.00004	0.00003	0.999
			96	34.35±0.13	0.00	91.11				
		D14	24	32.56±0.15	0.00	81.14	0.04	0.86	0.00002	0.999
			96	32.52±0.16	0.00	80.54				
	LEV	D0	24	39.68±0.14	0.00	95.35	0.34	0.10	0.00003	0.999
			96	39.34±0.14	0.00	96.73				
		D14	24	31.92±0.15	0.00	86.48	1.04	0.00003	0.001	0.999
			96	32.96±0.15	0.00	86.00				
	IVM-M	D0	24	50.51±0.17	2.84	94.39	0.36	0.15	0.07	0.999
			96	50.87±0.17	2.60	95.89				
		D14	24	39.40±0.11	0.00	87.56	2.83	0.00003	0.00002	0.999
			96	42.23±0.11	0.00	89.73				
	MPT	D0	24	17.27±0.10	0.00	100	2.32	0.00003	0.00003	0.999
			96	14.95±0.10	0.00	100				
		D14	24	32.31±0.11	0.00	100	1.62	0.00002	0.00002	0.999
			96	30.69±0.11	0.00	100				
	ZLV	D0	24	86.65±0.10	48.38	100	1.15	0.00004	0.00003	0.999
			96	87.80±0.10	51.00	100				
		D14	24	86.69±0.08	50.23	100	0.36	0.007	0.002	0.999
			96	87.05±0.09	50.70	100				

Table 3. continuação

Flock	Anthelmintic	Day	Plates (well)	$\overline{X}$ ± SE (%)	Min. (%)	Max. (%)	DIF (%)	Plate (p-value)	Plate x Conc. (p-value)	R^2
HcFlock-5	TBZ	D0	24	43.30±0.30	0.00	95.84	0.99	0.03	0.48	0.999
			96	42.31±0.30	0.00	97.22				
		D14	24	44.04±0.17	0.95	93.50	1.32	0.00003	0.00003	0.999
			96	42.72±0.18	0.00	95.18				
	LEV	D0	24	63.47±0.17	17.21	100	4.90	0.00004	0.00003	0.999
			96	68.37±0.18	20.81	100				
		D14	24	45.11±0.13	2.39	91.18	4.77	0.00003	0.00004	0.999
			96	49.88±0.14	8.06	92.83				
	IVM-M	D0	24	88.74±0.12	55.92	100	1.18	0.00003	0.00003	0.999
			96	89.92±0.11	50.68	100				
		D14	24	70.38±0.15	31.25	100	4.53	0.00004	0.00003	0.999
			96	74.91±0.15	34.94	100				
	MPT	D0	24	39.17±0.14	0.00	100	8.03	0.00003	0.00003	0.999
			96	47.20±0.14	0.00	100				
		D14	24	33.77±0.12	0.00	100	6.35	0.00002	0.00002	0.999
			96	40.12±0.12	0.00	100				
	ZLV	D0	24	85.36±0.10	42.05	100	0.41	0.001	0.00001	0.999
			96	84.95±0.10	47.94	100				
		D14	24	84.91±0.10	41.55	100	1.20	0.00003	0.00003	0.999
			96	83.71±0.10	40.29	100				

* Significant at 5% ($p \le 0.05$) level of probability by ANOVA and the Tukey test. $\overline{X}$ ± SE: Average efficacy ± standard error; Min: minimum efficacy; Max: maximum efficacy; DIF: Difference in mean efficacies between 24-well and 96-well plates; R^2 or coefficient of determination (0 to 1): measure of adjustment of the statistical model to the observed values.

The estimated LC_{50} for HcFlocks are shown in Table 4. The confidence intervals (95%) obtained in the 24-well plates on the same day of collection were within the confidence interval of the 96-well plates, except for TBZ D14 and MPT D0 and D14 for HcFlock-1, ZLV D14 HcFlock-2, LEV D0 and MPT D0 and D14 HcFlock-3, and again MPT D0 and D14 HcFlock-5. Most LC_{50} results were consistent and in agreement with their respective RF50 values (Table 5). RF above 3 demonstrates that the test was able to detect resistance in HcFlocks. For TBZ this occurred for all HcFlocks in both plates and collection days. For LEV, HcFlock-1 and 2 were susceptible (RF_{50} from 0.05 on HcFlock-2 to 2.10 on HcFlock-1), while the others were resistant (RF_{50} from 5.19 to 278.41 on HcFlock-5). For IVM-M, RF50 values rated all HcFlocks as susceptible, while RF_{99} presented opposite results for HcFlock 3, 4 and 5. Detection of resistance to MPT proved to be inconsistent. HcFlock-2 was clearly resistant. The others presented contradictory values, since results obtained in 24-well plates showed resistant status while in the 96-well plates the results indicated susceptible status for HcFlock 1, 3, 4 and 5. On the other hand, RF_{50} and RF_{99} values for ZLV rated HcFlock-2 as resistant (15.61 to 48.70) and the others as susceptible (0.06 on HcFlock-3 to 2.11 on HcFlock-1). These results indicate that with the use of ZLV the difference in status was clearer and more stable between collection days and plates than with MPT.

Table 4. Lethal concentrations LC$_{50}$ and confidence limits (95%, µg/mL) in LDT for HcFlocks in 24 and 96-well plates, on collection days zero (D0) and fourteen (D14), for thiabendazole -TBZ, levamisole - LEV, ivermectin monosaccharide - IVM-M, monepantel - MPT and Zolvix® - ZLV.

Anthelmintic	Day	Plates	HcFlock-1	HcFlock-2	HcFlock-3	HcFlock-4	HcFlock-5
TBZ	D0	24	2.690 (2.325-3.125)	0.737 (0.631-0.861)	2.540 (2.161-3.002)	2.332 (1.964-2.787)	1.420 (1.184-1.711)
	D0	96	2.783 (2.386-3.257)	0.715 (0.597-0.854)	2.583 (2.146-3.132)	2.747 (2.251-3.386)	1.451 (1.177-1.800)
	D14	24	2.605 (2.228-3.060)	0.879 (0.748-1.033)	2.942 (2.411-3.642)	3.325 (2.778-4.023)	1.275 (1.061-1.538)
	D14	96	1.750 (1.452-2.118)	0.926 (0.762-1.124)	2.764 (2.214-3.500)	3.443 (2.748-4.397)	1.434 (1.158-1.787)
LEV	D0	24	0.001 (0.001-0.001)	0.001 (0.001-0.001)	0.043 (0.036-0.051)	0.084 (0.071-0.100)	0.009 (0.008-0.012)
	D0	96	0.002 (0.001-0.002)	0.001 (0.001-0.001)	0.018 (0.014-0.023)	0.086 (0.076-0.106)	0.006 (0.004-0.008)
	D14	24	0.001 (0.00-0.001)	0.001 (0.001-0.001)	0.073 (0.061-0.088)	0.172 (0.143-0.208)	0.055 (0.044-0.070)
IVM-M	D0	24	0.001 (0.00-0.001)	0.001 (0.001-0.001)	0.064 (0.051-0.080)	0.157 (0.127-0.197)	0.034 (0.026-0.046)
	D0	96	0.179 (0.156-0.205)	0.007 (0.006-0.009)	0.021 (0.017-0.026)	0.152 (0.127-0.184)	0.003 (0.002-0.004)
	D14	24	0.221 (0.189-0.258)	0.008 (0.006-0.010)	0.021 (0.016-0.027)	0.146 (0.117-0.182)	0.004 (0.003-0.005)
	D14	96	0.182 (0.158-0.209)	0.011 (0.009-0.013)	0.033 (0.024-0.043)	0.384 (0.319-0.467)	0.022 (0.017-0.027)
MPT	D0	24	0.242 (0.206-0.286)	0.010 (0.008-0.013)	0.022 (0.015-0.030)	0.311 (0.249-0.394)	0.014 (0.010-0.018)
	D0	96	0.164 (0.148-0.183)	0.722 (0.623-0.851)	0.044 (0.040-0.049)	0.666 (0.619-0.718)	0.108 (0.097-0.120)
	D14	24	0.257 (0.235-0.283)	0.631 (0.535-0.755)	0.018 (0.015-0.020)	0.798 (0.704-0.913)	0.055 (0.049-0.062)
	D14	96	0.127 (0.112-0.143)	0.683 (0.581-0.814)	0.067 (0.061-0.074)	0.186 (0.168-0.206)	0.166 (0.148-0.187)
ZLV	D0	24	0.177 (0.156-0.201)	0.733 (0.611-0.900)	0.019 (0.016-0.022)	0.218 (0.194-0.245)	0.098 (0.086-0.111)
	D0	96	0.008 (0.007-0.009)	0.233 (0.199-0.274)	0.001 (0.001-0.001)	0.001 (0.001-0.002)	0.002 (0.001-0.002)
	D14	24	0.008 (0.007-0.010)	0.228 (0.189-0.278)	0.001 (0.001-0.001)	0.001 (0.001-0.001)	0.002 (0.001-0.002)
	D14	96	0.007 (0.006-0.008)	0.119 (0.103-0.138)	0.001 (0.001-0.001)	0.001 (0.001-0.002)	0.002 (0.001-0.003)
			0.006 (0.005-0.007)	0.251 (0.208-0.306)	0.001 (0.001-0.001)	0.001 (0.001-0.002)	

Table 5. Resistance factors (*RFs) in LDT in 24 and 96-well plates, on different collection days (D0 and D14), for thiabendazole -TBZ, levamisole - LEV, ivermectin monosaccharide - IVM-M, monepantel - MPT and Zolvix® - ZLV.

Anthelmintic	Day	Plates	HcFlock-1		HcFlock-2		HcFlock-3		HcFlock-4		HcFlock-5	
			RF_{50}	RF_{99}	RF_{50}	RF_{99}	RF_{50}	RF_{99}	RF_{50}	RF_{99}	RF_{50}	RF_{99}
TBZ	D0	24	237.86	34.79	65.18	16.53	224.62	59.81	206.16	86.68	125.59	82.46
		96	265.46	59.90	68.16	42.96	246.40	189.31	262.03	278.38	138.41	235.89
	D14	24	230.37	49.43	77.70	23.83	260.17	237.45	293.99	173.19	112.75	80.39
		96	166.92	128.41	88.29	97.37	263.66	577.91	328.39	764.05	136.82	282.80
LEV	D0	24	0.61	0.35	0.36	0.05	23.48	25.01	45.86	44.27	5.19	16.65
		96	2.10	0.23	1.17	0.05	24.34	17.31	116.01	24.93	7.85	8.29
	D14	24	0.38	0.16	0.36	0.73	39.99	68.50	93.73	105.17	30.14	278.41
		96	0.99	0.18	0.88	0.29	85.78	38.68	211.53	67.43	46.27	206.49
IVM - M	D0	24	1.36	1.22	0.06	0.22	0.16	1.23	1.16	8.97	0.02	0.16
		96	1.40	0.89	0.05	0.12	0.13	1.07	0.92	5.93	0.03	0.05
	D14	24	1.38	1.41	0.08	0.36	0.25	41.53	2.93	19.45	0.16	3.44
		96	1.53	1.41	0.06	0.24	0.14	10.93	1.97	14.27	0.09	1.35
MPT	D0	24	165.18	18.98	727.42	238.28	44.43	4.21	671.18	28.05	108.64	10.91
		96	2.98	0.51	7.30	8.13	0.21	0.12	9.23	3.42	0.64	0.18
	D14	24	127.56	25.19	688.10	263.74	67.58	6.14	186.95	18.05	167.63	26.21
		96	2.04	0.89	8.48	11.69	0.22	0.21	2.52	0.80	1.13	0.47
ZLV	D0	24	1.04	2.11	30.50	48.70	0.07	0.08	0.16	0.56	0.22	0.40
		96	1.00	1.38	28.60	43.79	0.06	0.10	0.14	0.34	0.19	0.58
	D14	24	0.91	1.79	15.61	20.01	0.07	0.16	0.15	0.63	0.23	0.44
		96	0.75	1.26	31.51	46.78	0.06	0.11	0.14	0.49	0.25	0.48

* LC_{50} and LC_{99} of the HcFlock on the collection days divided by the respective value of the susceptible isolate (Echevarria1991). RF values greater than 3 show that the test was able to distinguish resistance to the Anthelmintic.

RF_{50} below 3 was obtained for IVM-M demonstrating that this molecule was not able to distinguish isolates. Thus, complementary tests were performed with IVM-A in 96-well plates to get LCs and RFs results. Comparative values between IVM-M and IVM-A are shown in Table 6 indicating that there are major differences between these drugs. RF_{50} and RF_{99} much higher than 3 obtained for IVM-A were unquestionably more reliable in the distinction of *H. contortus* resistant and susceptible isolates (640 and 6480.33, respectively).

Table 6. Lethal concentrations LC_{50}, confidence limits (95%, µg/mL) and resistance factors (RFs) for ivermectin monosaccharide - IVM-M and aglycone - IVM-A in LDT to *Haemonchus contortus* susceptible (Echevarria1991-HcEc91) and resistant (Botucatu-HcBot) isolates, in 96-well plates.

Anthelmintic	HcEc91		HcBot		RFs*	
	LC_{50}	LC_{99}	LC_{50}	LC_{99}	RF_{50}	RF_{99}
IVM-M	0.158	3.978	0.132	13.260	0.81	3.33
	(0.1346-0.1869)	(2.7511-6.2582)	(0.1211-0.1443)	(10.5670-16.9982)		
IVM-A	0.001	0.026	0.637	194.410	640	6480.33
	(0.0001-0.0004)	(0.0088-0.0446)	(0.5482-0.7394)	(135.1915-292.9334)		

*LC_{50} and LC_{99} of the resistant isolate (Botucatu) divided by the respective value of the susceptible isolate (Echevarria1991). RF values greater than 3 show that the test was able to distinguish susceptible and resistant isolates.

Discussion

The validation of laboratory methods for parasite resistance diagnosis to anthelmintic in small ruminants is extremely important, since they may support adjustments of the drug management and preserve the effectiveness of active principles in the flocks (VON SAMSON-HIMMELSTJERNA et al., 2009). However, the dilution ranges of the chemical groups and the behavior of the reference isolates are not described, so not in vitro tests have been standardized and validated for routine use in Brazil. Thus, from national isolates of *H. contortus*, the present study was able to determine the dose-response curves to the main anthelmintic groups in 24 and 96-well plates. In addition, the responses of parasite samples from flocks were compared to HcEc91/HcBot, so that the correlation between both could be verified.

The observed DIFs were exceptionally low when comparing isolates between plates. Results indicated the reliability of the LDT when performed in both plates for all drugs, except for MPT. With respect to the dose-response curves, LC_{50} and RF values obtained from HcEc91/HcBot, we observed that they could be differentiated regarding MPT, especially in 24-well plates, which did not occur for IVM-M. Regarding the results of the HcFlocks, again the percentage of difference between plates was low, being more pronounced for MPT. ZLV was more effective in the detection of resistance than MPT (being able to replace the latter), while the results obtained for IVM-M were mostly inconsistent.

Kelly; Hall (1979) stated that RF values greater than 3 demonstrate that the test was able to distinguish susceptible and resistant isolates. The RF values obtained in the present study allowed this distinction, meaning that the results obtained for the HcFlocks are valid. However, this distinction was not possible for IVM-M considering the RF_{50}, which may be due to the use of monosaccharide IVM as standard substance. Dolinská et al. (2013, 2014) reported that the use of avermectin analogs and especially IVM-A significantly increased the ability of the LDT to differentiate isolates. This fact was confirmed by the RF values obtained for IVM-A in the present study. However, despite the strong potential of IVM aglycone in detecting resistance in the LDT, it is believed to have low sensitivity in cases of mixed parasitic populations (DOLINSKÁ et al., 2012). The lowest LC_{50} values in different IVM analogs were demonstrated in *H. contortus* (LACEY et al., 1991; DEMELER, 2005). These values were 2 to 4 times lower than those for *Ostertagia circumcincta* and *T. colubriformis* (DOLINSKÁ et al., 2012).

In the resistance diagnosis, we expected the larval development of both isolates and both plates for MPT would be similar to that observed for ZLV, since MPT is the chemical base of ZLV. However, this was not observed. The commercial product showed a more coherent and statistically similar dose-dependent curve for both isolates and in both plates. In the case of MPT, higher concentrations were required to obtain better efficacy. Figure 1 clearly shows the distinction between isolates for ZLV in both plates. Although for MPT, the behavior of the isolates was different between plates, their differentiation was possible, according to the RFs obtained: RF_{50} values of 447.76 and 12.03

and RF_{99} values of 20.86 and 7.40, in 24 and 96-well plates, respectively. For HcFlocks, however, 24-well plates gave opposite results regarding the resistance status from those obtained in the 96-well plates. Other researchers have reported difficulty in detecting *H. contortus* resistance status to MPT. Lecová et al. (2013) attempted to determine the *in vitro* efficacy of MPT by micro-agar LDT (MALDT) against resistant and susceptible isolates of *H. contortus*. Remarkably similar LC50 values were obtained for both isolates (0.0034 and 0.0037 µg/mL, respectively), making the resistance diagnosis unfeasible. Recently, Kotze et al. (2018) evaluated the resistance of isolates to this chemical group through LDT using ZLV, which allowed the isolates' differentiation (RF=6). In the present study, the RFs obtained were much higher than in the previous study. In other studies, in which MPT resistance was detected in *H. contortus*, this substance was subjected to previous chemical reactions in order to transform it into MPT-sulfone (KAMINSKY et al., 2008a; LECOVÁ et al., 2013; STUCHLÍKOVÁ et al., 2013, 2016). Perhaps the use of MPT for diagnostic tests requires structural modification, so use of ZLV may be a more efficient and less costly option. In addition, ZLV it is more stable than monepantel (ultra-pure reference substance) given its chemical composition. The pharmacologically active substance is monepantel with monepantel-sulfone as marker residue and excipients (RRR-α-tocopherol, beta-carotene, maize oil, propylene glycol, macrogolglycerol hydroxystearate, polysorbate 80, propylene glycol monocaprylate and propylene glycol dicaprylocaprate) that stabilize this commercial formulation (EPAR, 2009).

The results clearly detected strong resistance to TBZ in all HcFlocks, to LEV in HcFlock 3, 4 and 5 and to MPT and ZLV in HcFlock-2. Our RF_{50} results for TBZ are higher than those reported by Várady; Corba (1999) (RF_{50} =14.3). This leads us to believe that the resistant isolate of the present study, originally from a SP flock, was strongly selected for resistance (ALMEIDA et al., 2010). The same situation was detected in all flocks. This can be explained by the fact that in Brazil (especially São Paulo), benzimidazoles have been the anthelmintic most used in recent decades, with a high percentage of resistance in the flocks of that state (VERÍSSIMO et al., 2012). The LEV RF_{50} values obtained for the isolates (5.85 and 14.58 in 24 and 96-well plates, respectively) were lower than those reported by Várady; Corba (1999) (32.52) and those obtained for *H. contortus* LevR and Lawes resistant isolates reported by Sarai et al. (2013) (668 and 903, respectively). For HcFlock 3, 4 and 5, the

highest RF_{50} values were 85.78, 211.53 and 46.27, indicating resistance in more than 50% of the flocks evaluated. The low resistance reported for MPT was expected since it is the drug with the highest cost in the Brazilian market.

FECRT and LDT costs were investigated to check if the latter would be economically advantageous. FECRT costs US$ 220.45 for a trial of three chemical groups and a control group, with an additional cost of US$ 57.50 per extra group (LOVE; HUTCHINSON, 2003). In a seven-group experiment, the cost of FECRT would be US$ 392.95. The price for performing FEC on stool samples from seven experimental groups is US$ 538.60 (DPI, 2012). In the present study, considering FECRT costs related to the visits (travel expenses and costs of anthelmintics) were in average US$ 187.03 for a farm located in the São Paulo state, with the cheapest being US$ 169.37 and the highest being US$ 195.20. We also estimated the cost of resistance diagnosis for a flock by LDT in both plates (culture plates, chemical molecules and reagents of the culture medium), with two repetitions per concentration (according to the commercial test). For the 24-well plate, the costs would be US$ 166.06 and US$ 58.46 if the test was done with MPT or ZLV, respectively. In the 96-well plate, the costs would be US$ 46.06 and US$ 28.13, respectively. Therefore, by performing the test in the 96-well plate, cost reductions of 72.3% and 51.9% would occur, respectively, compared to the 24-well plate. In all those cases, the costs were calculated using the IVM-A since it presented higher LDT performance. It should be considered that the synthesis/production of MPT needs to be ordered from suppliers outside Brazil, which adds to the costs. In addition, the lowest cost to perform FECRT was greater than the highest cost to perform the LDT.

Conclusions

RF values allowed the differentiation of isolates, especially for TBZ, LEV, MPT, ZLV and IVM-A, and less significantly for IVM-M. The small differences in the average efficacy of anthelmintics indicated reliable agreement between plates, both in the evaluation of isolates and for *H. contortus* from flocks. Adapting the test to 96-well plates resulted in cost savings of at least 51.9% compared to the 24-well plates. RESISTA-Test© could be a diagnostic tool in the future, supporting the adjustments of the drug management and preserving chemical classes. The LDT results confirmed the reliability of this diagnostic test.

Acknowledgements

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