Evaluation of resistance in a selected field strain of *Haemonchus contortus* to ivermectin and moxidectin using the Larval Migration on Agar Test[1]

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ABSTRACT.- Fortes F.S., Kloster F.S., Schafer A.S., Bier D., Buzatti A., Yoshitani U.Y. & Molento M.B. 2013. **Evaluation of resistance in selected field strain of *Haemonchus contortus* to ivermectin and moxidectin using the Larval Migration on Agar Test.** *Pesquisa Veterinária Brasileira 33(2):183-187*. Laboratório de Doenças Parasitárias, Universidade Federal do Paraná, Rua dos Funcionários 1540, Curitiba, PR 80035-050, Brazil. E-mail: fortesfs@gmail.com

   Haemonchus contortus is one of the most common and economically significant causes of disease in small ruminants worldwide, and the control programs of parasitic nematodes - including *H. contortus* - rely mostly on the use of anthelmintic drugs. The consequence of the use of this, as the sole sanitary strategy to avoid parasite infections, was the reduction of the efficacy of all chemotherapeutic products with a heavy selection for resistance. The widespread of anthelmintic resistance and the difficulty of its early diagnosis has been a major concern for the sustainable parasite management on farms. The objective of this research was to determine and compare the ivermectin (IVM) and moxidectin (MOX) effect in a selected field strain of *H. contortus* with a known resistance status, using the *in vitro* larval migration on agar test (LMAT). Third stage larvae of the selected isolate were obtained from faecal cultures of experimentally infected sheep and incubated in eleven increasing diluted concentrations of IVM and MOX (6, 12, 24, 48, 96, 192, 384, 768, 1536, 3072 and 6144µg/mL). The dose-response sigmoidal curves were obtained using the R^2 value of >0.90 and the lethal concentration (LC_{50}) dose for the tested anthelmintic drugs using a four-parameter logistic model. The LC_{50} value for MOX was significantly lower than IVM (1.253µg/mL and 91.06µg/mL), identifying the *H. contortus* isolate as considerably less susceptible to IVM compared to MOX. Furthermore, the LMAT showed a high consistency ($p<0.0001$) and provided to be a useful diagnostic tool for monitoring the resistance status of IVM and MOX in *H. contortus* field isolate, as well as it may be used for official routine drug monitoring programs under the Ministry of Agriculture (MAPA) guidance.

INDEX TERMS: *Haemonchus contortus,* anthelmintics, ivermectin, moxidectin, sheep, macrocyclic lactones, *in vitro*, nematodes, efficacy.

RESUMO.- [**Avaliação da resistência em um isolado de campo selecionado de *Haemonchus contortus* à ivermectina e moxidectina usando o Teste de Migração de Larvas em Ágar.**] *Haemonchus contortus* é uma das causas mais comuns e economicamente significativas de doença em produções de pequenos ruminantes em todo o mundo, e os programas de controle de parasitas nematoides - incluindo *H. contortus* - baseiam-se principalmente no uso de drogas anti-helmínticas. A consequência da utilização desses compostos, como sendo a única estratégia sanitária para evitar infecções por parasitas, tem sido a redução da eficácia de todos os produtos quimioterápicos, selecionando fortemente para resistência. O desenvolvimento generalizado da resistência anti-helmíntica e a dificuldade de seu diagnóstico precoce têm sido uma grande preocupação para o manejo sustentável de parasitas no campo. O obje-

tivo desta pesquisa foi determinar e comparar o efeito da ivermectina (IVM) e da moxidectina (MOX) em um isolado de campo selecionado de *H. contortus* com um estado de resistência conhecido, utilizando o teste *in vitro* de migração de larvas em ágar (LMTA). Larvas de terceiro estágio de um isolado de *H. contortus* selecionado foram obtidas a partir de culturas de fezes de ovinos infectados experimentalmente e incubadas em onze concentrações diluídas crescentes de IVM e MOX (6, 12, 24, 48, 96, 192, 384, 768, 1536, 3072 e 6144μg/mL). As curvas sigmoides de dose-resposta foram obtidas utilizando o valor de R^2 >0,90 e a dose de concentração letal (CL_{50}) para as drogas anti-helmínticas testadas, utilizando um modelo logístico de quatro parâmetros. O valor de CL_{50} para MOX foi significativamente menor do que para IVM (1,253μg/mL e 91,06μg/mL), identificando o isolado de *H. contortus* como consideravelmente menos suscetível à IVM em comparação à MOX. Além disso, o LMTA mostrou uma alta consistência (*p*<0,0001) e pode ser uma ferramenta útil de diagnóstico para monitorar o status de resistência de IVM e MOX em isolado de campo de *H. contortus*, assim como ser utilizado de forma oficial e em rotina para programas de monitoramento das drogas sob a demanda do Ministério da Agricultura (MAPA).

TERMOS DE INDEXAÇÃO: *Haemonchus contortus*, anti-helmínticos, ivermectina, moxidectina, ovinos, lactonas macrocíclicas, *in vitro*, nematoides, eficácia.

INTRODUCTION

Haemonchus contortus is one of the most important gastrointestinal nematodes of small ruminant production worldwide due to its high prevalence and pathogenicity, causing serious economic losses (Kaplan 2004) and anthelmintic drugs are commonly used as the sole method to control this parasite. Nonetheless, the intensive use of all the available compounds has led to the widespread development of anthelmintic resistance in all major sheep producing countries (Van Wyk et al. 1999, Papadopoulos 2008, Molento et al. 2011, Kaplan & Vidyashankar 2012, Veríssimo et al. 2012).

Among the most widely used drugs, the macrocyclic lactones (MLs) such as ivermectin (IVM), an avermectin class compound, and moxidectin (MOX), a milbemycin, have a broad spectrum of activity against parasitic nematodes in livestock and humans. Resistance to these compounds has been detected in different nematodes in Brazil, also *H. contortus* (Echevarria et al. 1996, Thomaz-Soccol et al. 2004, Rosalinski-Moraes et al. 2007, Almeida et al. 2010, Cruz et al. 2010, Sczesny-Moraes et al. 2010, Veríssimo et al. 2012).

There is an urgent need to determine the drug efficacy status to continue to support farm planning and the sustainable use of the remaining effective anthelmintics, reducing the spread of resistant parasites (Coles et al. 2006, Demeler et al. 2010). The gold standard method for detecting anthelmintic resistance is the controlled efficacy test based on the reduction in worm counts after the sacrifice of animals. Although the most reliable test available to date to detect drug resistance, the test is extremely expensive, laborious and time consuming. The faecal egg count reduction test (FECRT), which compares the egg counts performed pre-

and post-treatment can define the percentage of efficacy by reducing egg numbers. Although the method is employed on all anthelmintic classes and has been commonly used to monitor resistance in nematodes, it does not measure the reduction of adult parasite number (Coles et al. 2006, De Graef et al. 2012, Kaplan & Vidyashankar 2012).

Therefore, a rapid and more cost effective diagnosis method for anthelmintic resistance is needed. Various *in vitro* methods have been developed in the last 30 years (Gill et al. 1995, d'Assonville et al. 1996, Molento & Prichard 2001, Taylor et al. 2002, Coles et al. 2006, Demeler et al. 2010), having the main characteristic of avoiding the "animal – *in vivo*" effect (i.e. breed, age, body fat/score), which can significantly alter the drug pharmacokinetics, as a consequence of different rates of absorption, metabolism and the time to reach the target tissues.

Among the *in vitro* methods, the migration tests consist in a simple, rapid and cost effective alternative for the analysis of the effects of a few classes of anthelmintics. The main mechanism to be tested is the muscle paralysis caused to the nematodes, which can be viewed as a reduction of migration when comparing the control and treated groups. The Larval Migration on Agar Test (LMIT) is being used as a diagnostic tool to detect and monitor the resistance to MLs in the Laboratory (d'Assonville et al. 1996, Molento & Prichard 2001, Demeler et al. 2010). Therefore, the objective of the present study was to evaluate and compare the IVM and MOX effect in a *H. contortus*, isolated from a sheep farm with a history of IVM resistance and MOX susceptibility (Veríssimo et al. 2012), using a modified migration test (Molento & Prichard 2001).

MATERIALS AND METHODS

Parasite field isolate and production of faecal culture from the isolate. The parasite field isolate used in this study was obtained from a sheep farm located in São Paulo State, Brazil, with reports of resistance to IVM (16%) and susceptibility to MOX (99%) by the FECRT conducted previously (Veríssimo et al. 2012). The original parasite population was composed by *Haemonchus* sp. (59%), *Cooperia* sp. (22%), *Trichostrongylus* sp. (11%) and *Teladorsagia* sp. (8%). Faecal samples containing nematode eggs were collected from sheep and were submitted to faecal cultures for 7 days to obtain third stage larvae (L_3). The infective L_3 obtained were stored in distilled water under refrigeration until used to artificially infect new parasite-free sheep in order to obtain pure cultures of *H. contortus* (see detail Protocol below).

Experimental infections and the production of faecal cultures 1 and 2 (isolate SP1503Ovi2011). Four lambs were selected and treated orally with oxfendazole 2.5mg/kg body weight (BW) (Oxfaden®, Biovet) and levamisole at 5.0mg/kg/BW (Ripercol® L, Fort Dodge) for three consecutive days, to remove every natural parasite infection. Faecal samples were collected for 4 weeks from the animals to confirm their parasite-free status. After that the animals were kept on worm-free paddocks until the experimental infections.

Two lambs (Group 1) were artificially infected with 8000 L_3 of gastrointestinal nematodes by oral gavage. The infection was allowed to establish for at least 28 days. From day 28 to 34 faecal samples were taken for EPG and larvae culture. The animals were euthanized on day 34 post-infection and the *H. contortus* adults were directly collected from the abomasum. The adult worms

were immediately placed into Petri dishes containing saline solution at 36ºC and kept in an incubator for a period of 2-12 hours for completion of the female oviposition. The eggs were harvested and placed in a sedimentation glass. Then, the eggs were mixed with autoclaved horse feces to obtain a monospecific faecal culture (Faecal culture 1).

Two lambs (Group 2) were artificially infected by the oral administration of 8000 L_3 of *H. contortus* obtained from the faecal culture 1. After the confirmation of the establishment of the infection, new faecal cultures were prepared to produce a pure *H. contortus* larval culture (Faecal culture 2). The infective larvae from the faecal culture 2 were identified and stored in distilled water up to 30 days until it was used with the *in vitro* assays. Although not included in the Parasite Isolate Database, the nomenclature code used for the present isolate (*SP1503Ovi2011*) was based on that of CARS (Consortium for Anthelmintic Resistance and Susceptibility) using the following code: state of origin/code number of the farm sampled/host animal/year of isolation.

***In vitro* Larval Migration on Agar Test (LMAT).** The LMAT was performed following the technique modified by Molento & Prichard (2001). The L_3 collected from the faecal culture 2 were exsheathed using 0.3% sodium hypochlorite and when more than 90% of the motile larvae were exsheathed, they were washed three times in distilled water and subdivided in groups of 400 L_3 per well in triplicates. Each group of larvae was mixed with distilled water to make a total volume of 0.5mL while drug dilutions were prepared in separate tubes (total volume of 0.5mL). Eleven drug concentrations were tested (6, 12, 24, 48, 96, 192, 384, 768, 1536, 3072 and 6144μg/mL) for 1% ivermectin (Ivomec®, Merial Saúde Animal) and 1% moxidectin (Cydectin®, Fort Dodge). The L_3 plus the drug dilutions were placed at 27ºC for 6h (first incubation). After that, an 1.4% agar solution was added to the treated larvae plus drug solution and immediately transferred to a previously prepared apparatus made of a Petri dish containing a plastic mesh in the middle of the base, a metal mesh and a plastic cylinder on the top. The apparatus was previously filled with 22mL of distilled water and stored at -20°C allowing the frozen water to cover the mesh. After the addition of the whole content, the apparatus was placed in an incubator for 18h at 27ºC under a 150 MHz light bulb, to stimulate the larval migration - out the agar portion. After the second migration, the liquid portion containing the migrated larvae was transferred into a 50mL tube and centrifuged at 3000 rpm for 5 minutes where the supernatant was removed. The tubes were then vortex and triplicate samples of 1 mL were taken. The final counts were made by the average of the aliquots multiplied by 10.

All experimental procedures reported in this manuscript were examined and approved by the Animal Use Ethics Committee of the Agricultural Sciences Campus of the Federal University of Paraná, Brazil (Protocol 021/2010).

Statistical analysis. The 50% lethal concentration (LC_{50}) and the standard error were measured by a non-linear regression model which resulted in a sigmoidal concentration response curve (four-parameter logistic equation with a variable slope), using the program GraphPad Prism® 5.01. All analyses were carried out after transforming the data into their logarithm ($X = \log X$), before calculating the log LC_{50} values (LC_{50} values), 95% confidence intervals and R^2 values. In addition, the *p*-values for the LC_{50} values of IVM and MOX responses were calculated using the same program.

RESULTS

The nematode genera recovered from the faecal culture 2 showed >96% of *Haemonchus contortus* genus followed by <3% of *Cooperia* sp. and *Trichostrongylus* sp.. All *in vi-tro* tests were run with the *H. contortus* L_3 from the faecal culture 2. For the inclusion of experimental data, it was necessary to attend two criteria: a migration ratio of at least 80% in the negative control and R^2 values > 0.90. The dose-response curves for IVM and MOX drugs using the *in vitro* LMAT are presented in Figure 1.

The statistical analysis of the curves was performed using the LC_{50} values. The results correctly identified the *H. contortus* isolate resistant to IVM and susceptible to MOX, showing the IVM-LC_{50} value of 91.06μg/mL and the MOX-LC_{50} value of 1.253 μg/mL (Table 1). The results were statistically significant (*p*<0.0001) for both drugs.
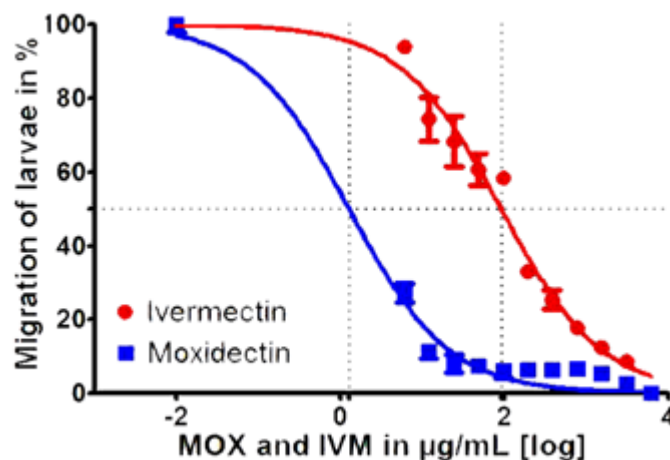

Fig.1. Dose-response curves obtained with the Larval Migration on Agar Test with the SP1503Ovi2011 isolate of *Haemonchus contortus* using ivermectin (IVM, solid line) and moxidectin (MOX, dashed line). Error bars indicate the standard error. The error bars are not seen where the differences between the values observed for all concentrations tested was 0 or very close to 0.

Table 1. The LC_{50} values, with the 95% confidence interval (95% CI), log LC_{50} values, R^2 and *p*-values using ivermectin (IVM) and moxidectin (MOX) in the Larval Migration on Agar Test with the isolate SP1503Ovi2011 of *Haemonchus contortus*

	MOX	IVM
LC_{50}	1.253 μg/mL	91.06 μg/mL
95% CI	0.704–2.229 μg/mL	75.53–109.8 μg/mL
Log LC_{50}	0.099	1.959
R^2	0.97	0.96
p-value	<0.0001	

DISCUSSION

It is very important to monitor anthelmintic efficacy at regular intervals to detect subtle changes, as early as possible in order to avoid the establishment of anthelmintic resistance. This may be performed by accessible and sensitive *in vitro* diagnostic tests, determining even which drugs are still effective against a particular parasite population to enable the choice of an anthelmintic for therapeutic use in the field (Kaplan 2004, Demeler et al. 2010, Molento et al. 2011). The larval development tests (LDT) are the most commonly employed and was able to easily discriminate between susceptible and resistant parasite isolates.

The LDT showed ability to identify low levels of resistant worms in a population, but the execution of these exams present some difficulties (Dolinská et al. 2012). Another potential alternative that is still being studied for the detection of resistant parasites is the larval migration tests (LMT). The LMT has being used for the detection of drug resistance for IVM, MOX and the benzimidazoles in sheep, goats and cattle nematodes (Wagland et al. 1992, Rabel et al. 1994, Molento & Prichard 2001, Gatongi et al. 2003, Kaplan et al. 2007, Demeler et al. 2010, Borges et al. 2011, Almeida et al. 2012).

The development of IVM resistance and also the concomitant loss of sensitivity to MOX is an ongoing debate (Conder et al. 1993, Shoop et al. 1993, Molento et al. 1999, Ranjan et al. 2002, De Graef et al. 2012). El-Abdellati et al. (2010) suggest that when detecting resistance to IVM the use of any type of ML's is inappropriate. However, the rate of resistance differs between compounds and occurs slower to MOX than to IVM (Ranjan et al. 2002, Le Jambre et al. 2005). While the resistance to one anthelmintic can be detected, another drug of the same class may still remain highly effective (Kaplan & Vidyashankar 2012). Although increasing reports of resistance to MOX in nematodes throughout the world, severe multidrug resistance was detected, with MOX remaining as the drug with better effect (Mortensen et al. 2003, Kaplan et al. 2007). So, our results indicate that MOX could have some potential application in controlling *H. contortus* in sheep on that farm and this might be true to other gastrointestinal nematodes.

The disturbing anthelmintic resistance status previously detected by FECRT was confirmed by the *in vitro* test in the present trial, as shown by the different LD50's. This is in accordance with other reports, where sheep nematode populations showed high resistance against MLs *in vivo* and also were less susceptible *in vitro*, using larval development tests (Hubert & Kerboeuf 1992, Taylor et al. 2002). The problem of anthelmintic resistance, identified in both tests, is possibly the situation that predominates in most of the sheep farms in Brazil. Therefore, we suggest that the Ministry of Agriculture, MAPA could officially adopt *in vitro* tests to be used by drug-monitoring programs. Before that, it is essential to improve and adapt standard operating protocols for running and interpreting the LMAT in order to make this test applicable for routine practice. It is necessary to test different isolates with different status of drug resistance in different laboratories, using the identical protocol, to confirm the reproducibility and repeatability of the LMAT data.

In conclusion, the *in vitro* LMAT described here was successfully used to generate reliable ($p<0.0001$) dose-response curves for the tested drugs. The obtained LC_{50} value for MOX was 72.67 times lower than for the IVM, identifying this *H. contortus* isolate highly resistant to IVM. The present data also shows the potential of using the LMAT for the analysis of resistance to MLs and can provide a useful diagnostic tool for monitoring anthelmintic resistance.

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