Advances and current challenges in the use of *in vitro* tests for gastrointestinal nematodes in Brazil

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Brazil has a large territory and it can be assumed that the production of small ruminants is still small, since there is importation of meat and dairy products from other countries. It is necessary to adopt technological solutions and health recommendations to make production more efficient because the existing herds are suffering from infections caused by gastrointestinal nematodes (GIN), particularly *Haemonchus contortus*. The suppressive parasite control has caused the development of parasite resistance and multiple anthelmintic resistance parasites are predominant in different regions of Brazil (ALMEIDA et al., 2010; CRUZ et al., 2010). Thus, currently the animal production in Brazil needs technical support in resolving important issues such as state the resistance of flocks to propose strategies for its prevention, monitoring and reduction, and provide new bioactives that are able to keep the population parasite at acceptable levels. According to Waller and Thamsborg (2004) this situation, together with the increasing market demand for ecological or "green" animal products has led to the development of the research of *in vitro* methods and in Brazil this is not different.

Usually, the diagnosis of animal parasite infection is carried out by counting the number of eggs per gram (EPG) and the evaluation of anthelmintic effectiveness by the Fecal Egg Count Reduction Test (FECRT). However, both techniques are not commonly used because they are very laborious and time-consuming since it is necessary to return to the properties for sample collections on two occasions. The major consequence is the lack of early resistance diagnosis, which can be an important tool for the rational management of chemical groups and maintenance of refugia in the population. Therefore, successful implementation of nematode control programs depends to a large degree on the availability of effective and sensitive methods for its detection and monitoring (TAYLOR et al., 2002).

Several research groups have established dose-response curves of different classes of anthelmintics to evaluate the inhibition of hatchability, motility and development of larvae for helminths of cattle, sheep, goats and horses (MOLENTO and PRICHARD, 2001; DEMELER et al., 2010). Many studies have demonstrated that *in vitro* results confirm the resistance situation detected in field (KAPLAN et al., 2007; TAYLOR et al., 2009) and there are still cases in which *in vitro* tests are more accurate than that detected *in vivo* (VÁRADY et al., 2006).

The available *in vitro* methodologies were not widely known within the research groups in Brazil. Thus, after training at research institutions abroad (Moredun Research Institute, University of Georgia), such techniques have been implemented in the routine at Embrapa Southeastern Region Animal Husbandry (CPPSE) and at the Federal University of Parana (UFPR) and taught to students and professionals from Brazil and other South American countries. The goal has been the dissemination of the techniques, training of researchers and technicians to perform diagnostic tests of parasite resistance and detection of new active substances on GINs.

Since 2008 there were three courses carried out at CPPSE and a course at UFPR to 25 people from Brazil, five from Argentina, three from Colombia and two from Uruguay, through lectures and practical classes. Recently a questionnaire was sent to the professionals who participated of the previous courses and was determined that 15 projects involving the *in vitro* techniques were submitted to funding agencies

(equipment, consumables). Moreover, these techniques were transferred to more 32 professionals in different institutions (A.C.S. CHAGAS, personal communication). In 2011, through the course Advances in Knowledge of Ruminant Parasite Resistance of Parasites and Hosts and the second course at UFPR, another 60 professionals will be trained in these methodologies, including molecular techniques for resistance diagnosis.

A major challenge in this area is the standardization of techniques between laboratories, so the results may be compared and validated. Some actions have been carried out in Brazil for this purpose, such as the publication of the "Practical Handbook: Methodologies for Resistance Diagnosis and Detection of Active Substances in Ruminant Parasites". In addition, projects for the establishment of research networks in this theme have been produced. Within them it is expected to provide a collection of susceptible and resistant isolates and their establishment on donor hosts for joint use within the research networks. Recent efforts have been made in this direction in which the *H. contortus* isolated from CPPSE (Embrapa2010, CARS) was characterized. It was found that, compared to the EPG, the efficacy for six groups of anthelmintics was: 99% (S) -26% (R) 12% (R) 88% (R) 93% (R) and 91% (R) respectively for trichlorfon, cobalt sulphate + albendazole, ivermectin, moxidectin, closantel and levamisole phosphate. Regarding the number of nematodes, the efficacy was 100% (S), 20% (R) 52% (R) 85% (R) 100% (S) and 93% (R), respectively. It was demonstrated that the *H. contortus* isolate is resistant to benzimidazoles, macrocyclic lactones and imidazotiazois. The result of susceptibility and resistance was obtained by RESO program (CHAGAS et al., 2010).

The *in vitro* methods are also extremely useful in screening for substances of plant origin with activity against helminths. These can be done with eggs, larvae of the first and third stage and adults and are able to detect if plant extracts can inhibit the hatching, development, feeding and exsheathment of the parasites. One difficulty faced in handling plant extracts in the *in vitro* tests with GIN can be its solubilization. It would be necessary to increase the amount of solvent, but the parasites are very sensitive and that can cause false-positive results. So they should be used at low concentrations and sometimes it is not enough to get a good soluble solution. When a bioactive doesn't have a significant efficacy is necessary to increase a lot the concentrations to obtain a dose-response curve and to calculate the LC_{50}. So consequently the standard derivation for this substance presents a large variability. In some cases, the higher concentrations possess dark color and many particles that make impossible to visualize and count larvae or eggs (CARVALHO et al., 2011).

More comprehensive studies are needed to assess if the bioactive compounds are being disposed in faeces or which metabolites are being generated after administration. In addition, the egg hatch test (EHT) and larval development test (LDT) should be done using faeces from the animals that received the bioactive to generate more data. Inhibition on these stages can promote reduced re-infection and lighter worm loads by decreasing pasture contamination levels, which could be of significant epidemiological importance (MAX, 2010). These details will help to conduct a controlled experimental design to test substances or extracts from plants.

The development of strategies based on *in vitro* tests and less dependent on *in vivo* experiments is imperative. One of the biggest benefits of the herbal treatment is the reduction in the use of experimental animals, since the plant extracts can cause toxic effects at different levels. For sure it is necessary to investigate and scientifically validate phytotherapeutic alternatives for future use to GIN's control in ruminants. Therefore, it is important to follow all steps that come before the clinical experiment. Carvalho et al. (2011) evaluated extracts of *Piper tuberculatum, Lippia sidoides, Mentha piperita, Hura crepitans* and *Carapa guianensis* in the EHT and LDT on *H. contortus*. The best results were obtained with *P. tuberculatum* that reached LC_{50} and LC_{90} of 0.031 and 0.09 $mg.ml^{-1}$ in the EHT and of 0.02 and 0.031 $mg.ml^{-1}$ in the LDT. So only this extract was evaluated in *Rattus norvegicus* experimentally infected with *Strongyloides venezuelensis*. The doses of 150 and 250 mg/Kg of body weight (BW) did not cause any significant reduction ($P>0.05$) in worm burden and EPG, compared with the control group. Therefore the test with sheep was avoided.

The rescue of popular knowledge associated with scientific research, development of new analytical chemistry techniques, as well as alternative strategies (i.e. *in vitro* methods) are tool allies in this research line, which, given the diversity of plant species, has a vast field for surveys. In addition, test miniaturization through well assay plates allows millions of compounds to be tested (WOODS and KNAUER, 2010). However, it is a challenge to understand why there is so much difficulty in reaching the same significant results from the *in vivo* compared to those obtained in the *in vitro* tests. In some studies this can be due to destruction of the compounds in the rumen or maybe the *in vitro* results cannot be compared because the adequate adjuvant was not used on the *in vivo*.

Preliminary studies *in vitro* indicated some activity of limonene, but just when an adjuvant (used according to SQUIRES et al., 2010) was added the results were really significant in the study of Chagas et al. (2011). The formulation based on the commercial plant isolate R-(+)-Limonen was evaluated on sheep artificially infected with *H. contortus* Embrapa2010 isolate. On the eighth day after treatment there was a significant reduction in FEC in the treatment 3 (600 mg/kg R-(+)-Limonen in fasting BW) and 4 (500 mg/kg R-(+)-Limonen BW) attained efficacies of 81.3% and 98.7%, respectively. The average FEC on day zero was 4.458 and 3.875, decreasing to 833 and 50 on the day +8, respectively. The egg hatch inhibition was 8.7% in the negative control and 63.4% in treatment 3 for day +8. In treatment 4, there were not enough eggs to perform the test, since the efficacy was 98.7%. In treatment 3, toxicological effects were observed, mainly as the lack of motor coordination, head shaking and lack of interest in food. These effects were much less severe in treatment 4, in which only head shaking for 15 minutes was observed. In treatment 4, the animals received a lower dose and were not denied food. In conclusion, the treatment at 500 mg/kg/BW reached the efficacy recommend by the WAAVP and the EHT results indicated the possibility of a reduction in the pasture contamination. However, we believe this formulation can be improved in the future with a smaller dose per mg/kg, to reduce toxicity.

The R&D support from drug companies is essential, since problems with absorption through the gastrointestinal tract and drug solubility are the main obstacles from *in vivo* studies to obtain the same anthelmintic effect detected in the *in vitro* tests. However, the effort to discover new drugs should be side by side with a farm-based prevention approach. It is necessary to be aware that it is time to develop and apply tools for new drugs detection and also monitor and prevent parasite selection. Studies have been showing that the *in vitro* tests are reliable for these purposes.

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