

Bio-mathematical software applicable in pharmacological resistance tests

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Abstract. In case of in vitro and in vivo resistance assays (egg hatch assay - EHA; larval development assay - LDA; Fecal Egg Count Reduction Test - FECRT) data analysis can be made using original software (Anthelmintic Resistance Program - ARP, <http://www.pharma-logic.ro>). The software is used especially for the quantification and interpretation of the results of resistance assays. ARP allows the calculations of hatching percentage of eggs in various concentrations of benzimidazoles, the graphical representation of the reference curve of reduction, determination of the lethal dose 50(DL₅₀) establishing hatching percentage at the reference dilution, graphical analysis of the reduction curve, as well as the risk of resistance to the various tested substances.

Keywords: Egg Hatch Assay; Larval Development Assay; Fecal Egg Count Reduction Test; Pharmacological resistance.

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Introduction

In laboratory conditions, strongyle resistance to benzimidazole-based anthelmintic drugs is frequently assessed through Egg Hatch Assay (EHA) and Larval Development Assay (LDA). Regardless of the employed method, these assays have advantages as well as disadvantages. This is why various authors tried to adapt them in order to increase their precision and speed. In case of EHA, the

advantages are: speed, lower cost and reasonable precision (Coles and Simpkin, 1977; Hall et al., 1978). Therefore, Whitlock et al. (1980) considered EHA as the reference method for the confirmation of resistance to benzimidazoles (BZ) of parasitic nematodes in equines. Inconvenience of this method is the fact that the newest generation of benzimidazoles (fenbendazole and oxfendazole) are poorly soluble, hence hard to use. Boersema (1983) highlights the difficult

interpretation of this assay, in view of the DL₅₀ reference values obtained by various researchers. Kerbœuf and Hubert (1987) showed that DL₅₀ varies not only from laboratory to laboratory but even in the same sensitive variety, in view of the existence of a previous infestation as well as the length of the infestation. Due to these variations, the use of the value of DL₅₀ as reference for the calculation of the resistance factor must be re-considered. The major inconvenience is the difficult interpretation of the obtained results.

The LDA cannot provide information regarding the risk for the development of resistance and the choice of a sensitive variety is difficult, with a major impact on the correct evaluation of the data. This is the main reason why LDA is not frequently used in establishing resistance to benzimidazoles (Davies and Schwalbach, 2000; Pook et al., 2002; Tandon and Kaplan, 2004). It is also important to establish a correlation between in vivo (Fecal Egg Count Reduction Test - FECRT) and in vitro (EHA, LDA) assays.

All these inconveniences made us to design an original computer software, called Anthelmintic Resistance Program (ARP) intended to eliminate errors in data interpretation of resistance test in vivo and in vitro (<http://www.pharma-logic.ro>).

Materials and methods

The research carried out between December 2003 and January 2009 had the purpose of highlighting the phenomenon of equine strongyle resistance to BZ. The assay has been carried out in Romania and Portugal, on an equine population of 4434 individuals, from intensive and extensive farming systems, zoos and national parks. A total of 3174 tests were performed (8.88% of the total population), allowing the validation, correlation and interpretation of obtained data and finally the validation of the ARP software.

In vivo analysis of resistance to BZ by FECRT was carried between 2003 and 2008 on 992 horses belonging to 22 populations. Initial collection of samples was done before therapy (AT). Later on, Mebendazole (MBZ), Fenbendazole (FBZ) or Albendazole (ABZ)

based medications were given to the horses orally. Fecal samples were collected four times at 7 days intervals, post-therapeutically (PT 7; 14; 21 and 28 days). For both AT and PT samples, the intensity and prevalence of the infection with strongyles were calculated. Intensity was expressed as eggs per gram (EPG) using the McMaster method or as larva per gram (LPG) after incubation, using the adapted Stoll method for quantitative larval count. The data was statistically interpreted calculating the standard deviation, the reduction percentage and the 95% confidence interval (Coles et al., 1992; Cabaret and Berrag, 2004). Evaluation of EHA was made in comparison to a 0.15µg/ml TBZ considering as reference the population of strongyles with a hatching percentage equal or greater than 50% (as suggested by Madeira de Carvalho, 2001).

Results

The software enables the automatic calculation of the lethal dose 50, 90 or 100 (DL₅₀, DL₉₀ or DL₁₀₀). Even if done in vitro, the proper term would be lethal concentration (CL₅₀, CL₉₀ or CL₁₀₀). Using the same software, the minimal inhibiting concentration (MIC) can also be calculated for the case of larval development assay (LDA). Performing the assays, we noticed that the hatching or larval development percentage is not correlated with the fall in concentration of the tested BZ. Thus, we considered as inadequate and insufficient to interpret the assay results only by the data of EHA. At the reference concentration (0.15 µg/ml) or the concentration at which the first third stage larva appeared, regardless of the results obtained from higher and lower concentrations. Through APR, these errors of interpretation are completely eliminated.

Calculating CL₅₀ for the studied active substances requires an initial step, i.e. that a regression curve should be obtained, estimated in terms of a first degree equation ($ax + b$). The dynamics of the coordinates, that illustrates hatching percentage at various concentrations of active substances, contributes to the global statistical evaluation of these substances over the egg hatching. The regression curve represents an average of all the curves that pass through tow points on the chart. The ARP

software enables the analysis of the ratio and the relationship between the 13 different parameters of the egg hatching and larval development. Calculating coefficients for *a* and *b* necessary for the mapping of the regression curve are automatically obtained through ARP. The required data that must be inserted is the number of dilutions and the percentages of egg hatching or the larval development suitable for concentration of the active substance. The software automatically does all the necessary calculations between the 20 values obtaining for all individual values a coefficient *a* and *b*.

Regression mapping may be done for both in vitro assays (EHA and LDA), interpretation of the results being made in a similar way. In case that *a* value is grater (tends to infinite), DR tends to be positive. In this case there is a risk of resistance. This phenomenon is evident in equines that have received for too long a treatment with BZ and PBZ derivatives.

If the regression curve tends to be strongly positive correlated with a high percentage of egg hatching at the reference dilution of 0.15 µg/ml, BZ and PBZ resistance is certain (figure 1).

In vivo analysis by FECRT of resistance to BZ, conducted between 2003 and 2008, on 992 horses belonging to 22 populations, revealed installation of resistance phenomenon in 66.66% of cases (table 1, figure 1).

Mean prevalence value was 90.63%, while the mean intensity value was 2231.81 LPG, the limits being between 45.83% and 100%, 88 LPG and 5509 LPG, respectively. Also this data was analyzed by using ARP (figure2).

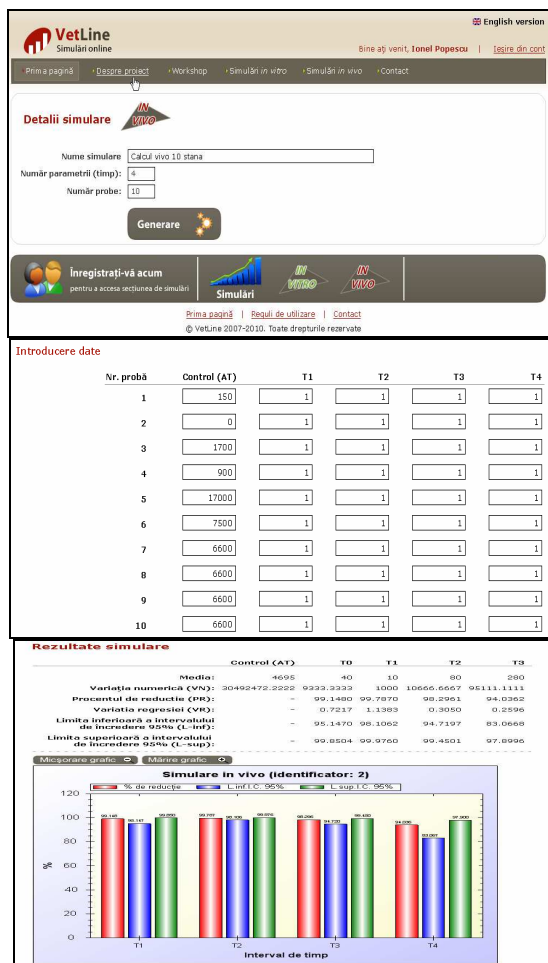


Figure 1. Software snapshot for the reference dilution

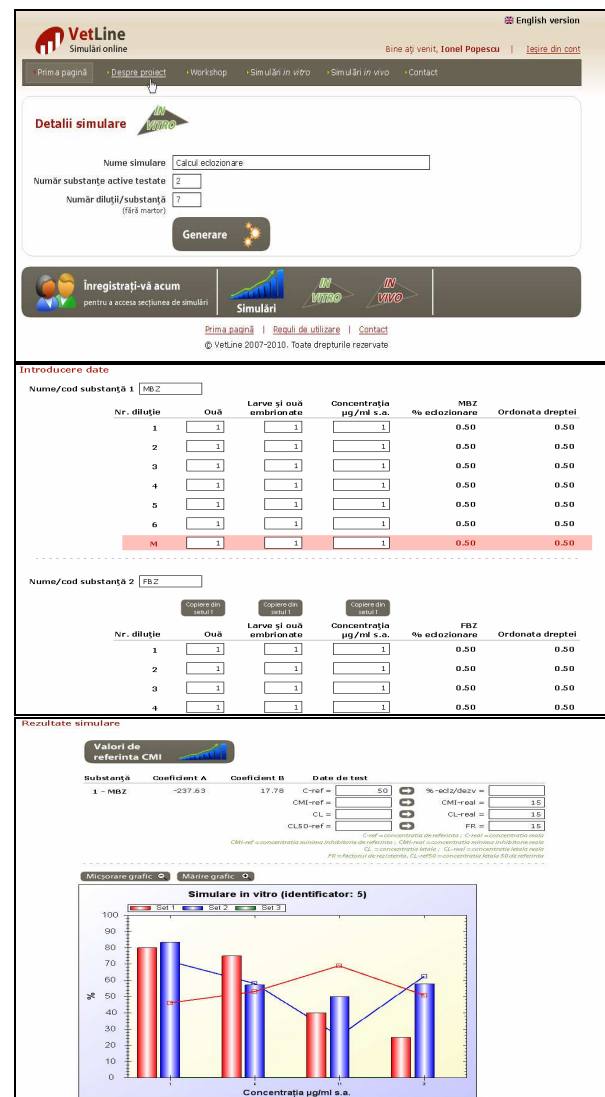


Figure 2. Software snapshot for an analysis of FECRT

Table 1. In vivo analysis by FECRT of resistance to benzimidazole in horse strongyles from Romania

Year	Horse population		Medication	Resistance	Resistant populations (%)
	County	Horses (n)			
2003	Satu Mare	34	ABZ	+	50.00
	Cluj	62	MBZ	-	
2004	Maramures	38	ABZ	-	50.00
	Bistrita Nasaud	187	ABZ	+	
	Salaj	39	FBZ	+	
	Bihor	36	MBZ	-	
	Arad	22	FBZ	-	
	Alba	102	FBZ	+	
2005	Cluj	34	MBZ	-	66.66
	Mures	56	ABZ	+	
	Sibiu	28	FBZ	+	
2006	Hunedoara	41	ABZ	+	100.00
	Brasov	54	ABZ	+	
	Harghita	12	ABZ	+	
	Covasna	45	FBZ	+	
2007	Mures	33	ABZ	+	66.66
	Satu Mare	32	FBZ	+	
	Bistrita Nasaud	65	ABZ	+	
2008	Bihor	20	ABZ	-	66.66
	Cluj	12	MBZ	+	
	Mures	21	MBZ	-	
2008	Alba	19	ABZ	+	66.66
Total	22	992		15	66.66

ABZ - albendazole; MBZ - mebendazole; FBZ - febendazole

Our analyses established that the correlation between FECRT and in vitro tests was 86.25%, quantified through the use of a bio-mathematical model (figure 3).

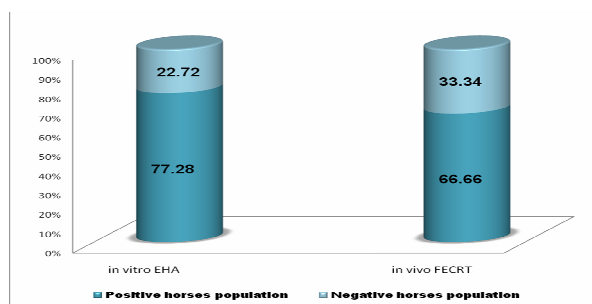


Figure 3. In vitro resistance to BZ drugs detected by EHA and LDA and percentage of correlation with in vivo assay

Discussions

In case of probenzimidazoles (PBZ) and BZ derivatives, the reference substance is TBZ in dose of 0.15 µg/ml. The interpretation suggested by Madeira de Carvalho (2001)

proved to be too simplistic, being disapproved by FECRT that showed low efficacy of the tested drug, while EHA and LDA showed no signs of resistance. From the assays we determined the fact that control samples had a decisive role in the precision of the results. In some cases the hydrochloric acid used to dilute the BZ influenced egg hatching, result showing that this may reduce the hatching percentage in a significant way. Through ARP these major inconveniences have been eliminated because all interrelations between the different hatching percentages and concentration are taken into account even the control samples, in view of which the software calculates the final data. Considering the *a* and *b* coefficients we ARP ca automatically calculate the desired hatching percentage at any given reference concentration value. For the mapping of the regression curve (DR) we must obtain first the values of the coefficients *a* and *b*. This way the curve equation is calculated automatically, which may be represented graphically and allows the interpretation of the risk of resistance in the studied strongyle population.

Due to the lack of correlations between the three commonly used three (FECTR, EHA and LDA) it was necessary to conceive a software (Anthelmintic Resistance Program) capable of interpreting as a global result the hatching and larval development percentage obtained from different concentrations used in the assays (see Cernea, 2007).

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