

DOI 10.24425/pjvs.2021.136803

Original article

Survey of anthelmintic resistance in a Romanian horse stud using three different methods

G. Dărăbuș, C. Badea, I. Oprescu, S. Morariu, N. Mederle, M. Ilie, T. Suici,
M. Imre

Banat University of Agricultural Sciences and Veterinary Medicine of Timișoara,
Faculty of Veterinary Medicine, 300645, Calea Aradului no. 119, Timișoara, Romania

Abstract

Resistance of cyathostomins to benzimidazole (BZ) anthelmintics is widespread in horses in many parts of the world. This study compared three methods for the determination of benzimidazole resistance of Cyathostominae in 18 horses from a stud farm in Romania. The horses were treated with Fenbendazole. The resistance test was performed by FECRT, ERP and PCR. On Day 0, larvae of species belonging to the Cyathostominae subfamily, types A, B, C, D and *Gyalocephalus*, as well as *Strongylus vulgaris* species of the Strongylinae subfamily, were identified. At 42 days post treatment with fenbendazole only larvae of Cyathostominae, types A and D were identified. Resistance to Fenbendazole was found in one horse, using the FECRT and ERP tests. Both genetic resistance and susceptibility to BZ anthelmintics was observed in 13 samples (72.22%) using the PCR test. However, three samples (16.67%) showed only the BZ-susceptibility gene. In 2 samples, (11.11%) only the resistance gene to BZ anthelmintics was identified. Several inconsistencies in the evidence of resistance to benzimidazole were observed between the PCR test and the other two methods, which indicates that several methods for determining and controlling the resistance should be used in practice.

Key words: horse, cyathostomins, anthelmintics, benzimidazole, resistance

Introduction

The most widespread helminths in equines are the Strongylidae family. Of these, with great importance for horses, due to the high prevalence, pathogenicity and resistance to benzimidazole developed over time, are those belonging to the Cyathostominae subfamily.

Although infestations with Cyathostominae are

generally tolerated by horses, massive infestations may express clinical signs, sometimes even causing death (Love et al. 1999). Asymptomatic forms can also influence the welfare and physical performance of horses.

Frequent use of anthelmintics has favored the emergence of chemical resistance over the last few years. The major causes of chemoresistance are especially the use of the same molecules for consecutive years, re-

peated intensive treatments, underdosing, uncontrolled use of anthelmintics, etc. (Osterman Lind et al. 2005, Traversa et al. 2007).

Various methods have been used to determine the chemoresistance to different anthelmintics. None of the methods used is perfect. In this study, the Egg Reappearance Period Test (ERP), Fecal Egg Count Reduction Test (FECRT) and the PCR Test were compared.

Materials and Methods

Horses

A total of 31 horses were tested in the study but only 18 horses, with an EPG higher than 200 (McMaster Method) (Coles et al. 1992, Cosoroabă et al. 2002, Lester and Mattheus 2014) were included in the comparative testing of the three methods for the determination of Fenbendazole resistance. They were aged between 6 months and 18 years, belonged to Arabian purebred and the English purebred breeds, and came from a stud farm in Timis County. Horses that had not received deworming in the last 12 months prior to day 0 were included in the study.

Strongyle species determination

For the determination of strongyle species, larval cultures were performed according to the technique described by Roberts and O'Sullivan (1950), modified by Reinecke (1973) (Reinecke 1973). The identification of strongyle species was performed according to determination keys by Madeira de Carvalho (1999).

Treatment protocol

Each horse was treated on day 0 with 10% fenbendazole paste, in a 7.5 mg/kg dose, for 5 days consecutively.

FECRT

The FECRT was performed on the basis of the World Association for the Advancement of Veterinary Parasitology (WAAVP) and AAEP Parasite Control Guidelines methods for the detection of anthelmintic resistance in nematodes of veterinary importance (Coles et al. 1992). EPG (Egg Per Gram) was determined on day 0 and on day 14 post-treatment according to the formula:

$$\text{FECRT} = \frac{\text{EPG (pre-treatment)} - \text{EPG (day 14 post-treatment)}}{\text{EPG (pre-treatment)}} \times 100$$

ERP test

The EPG for the ERP test was established using the McMaster method modified by Wetzel with a15 EPG detection limit (Lester and Mattheus, 2014) on day 0 and days 14, 28, 42 and 56 post-treatment. Strongyle species were detected through larval cultures on day 0 and on day 42 after treatment, when most eggs appeared in the faeces.

PCR

The chemoresistance to fenbendazole was also determined by the polymerase chain reaction (PCR) for benzimidazole. In cyathostomins, the mechanism of resistance to benzimidazole involves more than one mutation (TTC / TAC), so beta-tubulin isotype 1 codons 167 and 200 are considered to be important for resistance (Von Samson-Himmelstjerna et al. 2002, Lake et al. 2009, Ishii et al. 2017;).

The standard Isolate II Genomic DNA Kit was used for the larval DNA extraction. Larvae were collected from larval cultures by centrifugation of the culture liquid at 1500 rpm for three minutes.

DNA amplification was performed by polymerase chain reaction (PCR) according to von Samson-Himmelstjerna et al. (2002) Coles et al. (2006) modified. The primers used were:

Reverse - CN30R (non-allele-specific) with the sequence 5' AGC AGA GAG GGG AGC AAA GCC AGG 3'

Forward - CN24FS (allele specific) with the sequence 5' GGT TGA AAA TAC AGA CGA GAC TTT 3'

Forward - CN25FR (allele specific) with the sequence 5' GGT TGA AAA TAC AGA CGA GAC TTA 3'

The identification of the **benzimidazole-resistant** strongyles was performed using the CN25FR/CN30R primers set while the CN24FS/CN30R primers set was used for the **benzimidazole-susceptible** strongyles.

This method is used to identify seven species of cyathostomins: *Cylicocyclus nassatus*, *Cylicocyclus insigne*, *Cylicocyclus elongatus*, *Cylicocyclus radiatus*, *Cyathostomum pateratum*, *Cyathostomum catinatum* and *Cyathostomum coronatum* (Cirak et al. 1996, Lyons et al. 2001).

The amplicons obtained from DNA amplification were evaluated using agarose gel electrophoresis.

Statistical analysis

The statistical evaluation was performed by t test and GraphPad Software, QuickCalcs - Fisher's exact test (<https://www.graphpad.com/quickcalcs/>).

Table 1. Individual data regarding horses included in the study.

Crt. No.	Sample	Age	Gender	EPG day 0	EPG day 14	EPG day 28	EPG day 42	EPG day 56	Gene
1.	Ruby	6 mth	F	1900	0	0	500	1000	s+r
2.	Saturn	8 mth	F	500	0	0	250	750	s+r
3.	Shansa de Holby	5 yr	F	200	0	0	100	250	r+r
4.	Diva de Holby	6 yr	F	550	0	0	50	500	s+r
5.	Daisy	7 yr	F	300	0	0	50	750	s+r
6.	Nadira	7 yr	F	250	0	0	0	300	s+r
7.	Sonyador	7 yr	F	1250	0	0	150	1500	r+r
8.	Olde	8 yr	F	2500	250	250	300	2350	s+s
9.	Alma	9 yr	F	450	0	0	50	450	s+r
10.	Tora	13 yr	F	850	0	0	200	900	s+r
11.	Nisa	17 yr	F	550	0	0	50	750	s+r
12.	Sabrina	18 yr	F	1250	0	0	150	1000	s+r
13.	Tequila	1 yr	M	450	0	0	150	750	s+r
14.	Black	2 yr	M	250	0	0	100	450	s+r
15.	Dark	3 yr	M	250	0	0	150	500	s+r
16.	Flame	6 yr	M	200	0	0	0	250	s+r
17.	Namid	17 yr	M	400	0	0	50	450	s+s
18.	Taifun	17 yr	M	250	0	0	0	0	s+s

^{mth} months

^{yr} years

^s benzimidazole- susceptible gene

^r benzimidazole-resistant gene

Results

The results obtained in the present study are shown in Table 1. The summary of EPG values before and after fenbendazole treatment in 18-horses is presented. There were no statistical differences between males and females on days 0, 42 and 56 of the study correlated to the registered EPG. Statistically significant differences in EPG occurred between treatment day 0 and day 42 ($p < 0.001$), but not between day 0 and day 56.

Species belonging to the Cyathostominae subfamily, A, B, C, D and *Gyalocephalus*, and the *Strongylus vulgaris* species from the Strongylinae subfamily were identified following the examination of the larval cultures obtained from the faeces collected on day 0. In the case of larval cultures from faeces collected at 42 days post-treatment with fenbendazole, only Cyathostominae larvae, types A and D, were identified. The lack of *Strongylus vulgaris* larvae and Cyathostominae B and C types reveals the lack of fenbendazole chemoresistance for these species/types.

The efficacy proved to be 100% in 17 out of the 18 horses taken into study, 14 days after treatment, as shown by the FECRT calculations. This indicates the lack of chemoresistance. However, the effectiveness of fenbendazole was 90% in one of the horses—a very

odd and confusing result. Moreover, the calculation of the FECRT value for the entire group of horses resulted in a 97 % efficacy rate, indicating the lack of resistance within the herd. According to the ERP test, it also appears that the same horse would exhibit chemoresistance, related to egg reappearance in faeces only 2 weeks after treatment (Table 1). One week post treatment, EPG values were 0 for all the horses. We chose to report the results every two weeks in order for them to resemble the FECRT.

PCR revealed the presence of either benzimidazole resistance or benzimidazole sensitivity genes but also the presence of both of them in the same individual. Thus, 13 samples (72.22%) were positive for *Cyathostomidae* with both genes, 3 samples (16.67%) only showed the benzimidazole- susceptible gene and 2 samples (11.11%) showed only the benzimidazole resistance gene. The frequency of homozygous resistance is significantly lower than the frequency of resistance alleles ($p < 0.001$). Also, the frequency of the homozygous susceptible gene is significantly higher in relation to the frequency of alleles responsible for sensitivity ($p < 0.01$).

Also, in two horses where only homozygous resistance genes by FECRT - considered a “gold standard” were identified (Vidyashankar et al. 2012, Peregrine

et al. 2014), without identifying eggs in the faeces two weeks post-treatment, appears not to be fenbendazole resistant.

ERP is considered by some authors (Borgsteede et al. 1993; Van Doorn et al. 2014) to be the time period between deworming until EPG is equal to or 10% higher than the initial one. For fenbendazole, ERP is 6-8 weeks. Analyzing the situation of two horses in which only homozygous resistance genes were identified by PCR, only one is considered resistant according to ERP (EPG greater by 20% than initial EPG, 8 weeks after treatment). Of the 13 horses found with heterozygous (R + S) genes by PCR, EPG was equal or greater than the initial one after 56 days, in 10 horses, indicating chemoresistance.

Discussion

The resistance of the nematodes, especially strongyles, is a topical issue. Chemoresistance has a slow onset, usually without clinical signs, only occurring when therapy has no results (Sangster et al. 1999). Causes of chemoresistance may vary in Romania, mainly due to sub-dosages and to the fact that benzimidazole treatments are available to every horse owner without the veterinarian's involvement.

Resistance of cyathostomins to fenbendazole has been reported worldwide. Thus, the phenomenon is described by Lyons et al. (2007) in the USA, Drögemüller et al. (2004) in Germany, Traversa et al. (2009) in Germany, Italy and the UK, Canever et al. (2013) in Brazil, Kumar et al. (2016) in India.

In Romania, fenbendazole resistance within the strongyle population has been reported by Cernea et al. (2015) and Buzatu et al. (2016).

This study aimed to identify not only the presence of chemoresistance in the population of *Strongylus* sp. in horses, but also the consistency of different determination methods and to reveal the existence of contradictions between the results obtained using the three methods a matter which is difficult to explain. Thus, in the horse (sample 8) in which only the sensitivity gene was identified through PCR, the other two methods showed benzimidazole resistance. The determination of resistance genes using the PCR method indicated the possibility of appearance of chemoresistance in the case of continuous use of treatment with benzimidazolics. It is also important to note that the EPG value was the highest at outset and that the initial EPG values were not reached throughout the experiment.

Overall, it can be stated that chemoresistance to benzimidazoles has not been noticed in the studied group of horses, despite several contradictory results.

The presence of high resistance genes may mean that in the future the use of fenbendazole in controlling the Cyathostominae population will contribute to the selection of resistance genes and the establishment of chemoresistance.

Based on the obtained results, we recommend the use of several methods to quantify any chemoresistance for Cyathostominae. The identification of benzimidazole resistance genes requires permanent monitoring of the chemoresistance phenomenon and requires specific measures, such as the rotation between the different types of anthelmintics. On the other hand, for the prevention of chemoresistance, it is necessary to monitor the elimination of eggs by faeces and to reduce the frequency of treatments (Carstensen et al. 2013). The use of rotational grazing systems and selective treatment can be considered (Francisco et al. 2012).

In conclusion, it can be stated that for an effective parasitological control and prevention of resistance to benzimidazoles, besides other measures, it is necessary to test the imminence of chemoresistance through more than one method.

Acknowledgements

This study was made using the support and infrastructure project "POSCCE Project SMIS No. 2669," code SMIS-CSNR 2669.

References

- Borgsteede FH, Boersema JH, Gaasenbeed CP, van der Burg WP (1993) The reappearance of eggs in faeces of horses after treatment with ivermectin. *Vet Q* 15: 24-26.
- Buzatu MC, Mitrea IL, Lyons ET, Ionita M (2016) A field survey to investigate the anthelmintic efficacy against equine strongyles in Romania. *J Equine Vet Sci* 39 (Suppl): S48-S49.
- Canever RJ, Braga PR, Boeckh A, Grycajuck M, Bier D, Molento MB (2013) Lack of Cyathostominae sp. reduction after anthelmintic treatment in horses in Brazil. *Vet Parasitol* 194: 35-39.
- Carstensen H, Larsen L, Ritz C, Nielsen MK (2013) Daily variability of strongyle fecal egg counts in horses. *J Equine Vet Sci* 33: 161-164.
- Cernea M, Cristina RT, Ștefănuț LC, Madeira de Carvalho LM, Taulescu MA, Cozma V (2015) Screening for anthelmintic resistance in equid strongyles (Nematoda) in Romania. *Folia Parasitol* 62: 2015.023.
- Cirak V Y, Hermosilla YC, Bauer C (1996) Study on gastrointestinal parasite fauna of ponies in northern Germany. *App Parasitol* 37: 239-244.
- Coles GC, Bauer C, Borgsteede FH., Geerts S, Klei TR, Taylor MA Waller PJ (1992) World Association for the Advancement of Veterinary Parasitology (W.A.A.V.P.) methods for the detection of anthelmintic resistance

- in nematodes of veterinary importance. *Vet Parasitol* 44: 35-44.
- Coles GC, Jackson F, Pomroy WE, Prichard RK, von Samson-Himmelstjerna G, Silvestre A, Taylor MA, Vercruysse J (2006) The detection of anthelmintic resistance in nematodes of veterinary importance. *Vet Parasitol* 136: 167-185.
- Cosoroabă I, Dărăbuș G, Oprescu I, Morariu S (2002) Diagnostic paraclinic și tehnici experimentale în parazitologie (in romanian), 1st ed., Ed. Mirton, Timișoara, pp 47-48.
- Drögemüller M, Schnieder T, von Samson-Himmelstjerna G (2004) Evidence of P-Glycoprotein sequence diversity in cyathostomins. *J Parasitol* 90: 998-1003.
- Francisco R, Paz-Silva A, Francisco I, Cortiñas FJ, Miguélez S, Suárez J, Filipa Cazapal-Monteiro C, Suárez JL, Sol Arias M, Sánchez-Andrade R (2012) Preliminary analysis of the results of selective therapy against strongyles in pasturing horses. *J Equine Vet Sci* 32: 274-280.
- Ishii JB, Arenal A, Felix A, Yoshitani U, Beech R, Molento MB (2017) Diagnosis of resistance alleles in codon 167 of the beta-tubulin (Cya-tbb-1) gene from third-stage larvae of horse cyathostomins. *Res Vet Sci* 115: 92-95.
- Kumar S, Garg R, Kumar S, Banerjee PS, Ram H, Prasad A (2016) Benzimidazole resistance in equine cyathostomins in India. *Vet Parasitol* 218: 93-97.
- Lake SL, Matthews JB, Kaplan RM, Hodgkinson JE (2009) Determination of genomic DNA sequences for beta-tubulin isotype 1 from multiple species of cyathostomin and detection of resistance alleles in third-stage larvae from horses with naturally acquired infections. *Parasit Vectors* 2 (Suppl): S6.
- Lester HE, Matthews JB (2014) Faecal worm egg count analysis for targeting anthelmintic treatment in horses: points to consider. *Equine Vet J* 46: 139-145.
- Love S, Murphy D, Mellor D (1999) Pathogenicity of cyathostome infection. *Vet Parasitol* 85: 113-21.
- Lyons ET, Tolliver SC, Collins SS (2007) Study (1991 to 2001) of drug-resistant Population B small strongyles in critical tests in horses in Kentucky at the termination of a 40-year investigation. *Parasitol Res* 101: 689-701.
- Lyons ET, Tolliver SC, Drudge JH, Collins SS, Swerczek TW (2001) Continuance of studies on population S benzimidazole-resistant small strongyles in a Shetland pony herd in Kentucky: effect of pyrantel pamoate (1992-1999). *Vet Parasitol* 94: 247-256.
- Madeira de Carvalho LM (1999) Horse strongyles L3 larval stages. Morphological characters relevant for their identification, 17th Conference WAAVP, Workshop Systematics of the Cyathostominae of horses, Copenhagen, Denmark, 19 August 1999, pp 6.
- Osterman Lind E, Uggla A, Waller P Höglund J (2005) Larval development assay for detection of anthelmintic resistance in cyathostomins of Swedish horses. *Vet Parasitol* 128: 261-269.
- Peregrine AS, Molento MB, Kaplan RM, Nielsen MK (2014) Anthelmintic resistance in important parasites of horses: Does it really matter? *Vet Parasitol* 201: 1-8.
- Reinecke HK (1973) The larval anthelmintic test in ruminants. Technical communication No.106, Department of Agricultural Technical Services, South Africa, pp 20.
- Sangster C (1999) Pharmacology of anthelmintic resistance in cyathostomes: will it occur with the avermectins/milbemycins? *Vet Parasitol* 85: 189-201.
- Traversa D, Klei TR, Iorio R, Paoletti B, Lia RP, Otranto D, Sparagano OA, Giangaspero A (2007) Occurrence of anthelmintic resistant equine cyathostome populations in central and southern Italy. *Prev Vet Med* 82: 314-320.
- Traversa D, von Samson-Himmelstjerna G, Demeler J, Mililo P, Schürmann S, Barnes H, Otranto D, Perrucci S, di Regalbono AF, Beraldo P, Boeckh A, Cobb R (2009) Anthelmintic resistance in cyathostomin populations from horse yards in Italy, United Kingdom and Germany. *Parasit Vectors* 2 (Suppl): S2.
- Van Doorn DC, Ploeger HW, Eysker M, Geurden T, Wagenaar JA, Kooyman FN (2014) *Cylicocyclus* species predominate during shortened egg reappearance period in horses after treatment with ivermectin and moxidectin. *Vet Parasitol* 206: 246-252.
- Vidyashankar AN, Hanlon BM, Kaplan RM (2012) Statistical and biological considerations in evaluating drug efficacy in equine strongyle parasites using fecal egg count data. *Vet Parasitol* 185: 45-56.
- Von Samson-Himmelstjerna G, von Witzendorff C, Sievers G, Schneider T (2002) Comparative use of faecal egg count reduction test, egg hatch assay and beta-tubulin codon 200 genotyping in small strongyles (Cyathostominae) before and after benzimidazole treatment. *Vet Parasitol* 108: 227-235.

*** American Association of Equine Practitioners. Parasite Control Guidelines. <https://aaep.org/guidelines/parasite-control-guidelines>.