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Original Article

Evaluation of Benzimidazole Resistance in Equine Cyathostomins in the Kermanshah Province of Iran Using Coprological Analysis and Allele-Specific PCR

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Received 18 May 2025
Accepted 11 Aug 2025

Keywords:
Horse;
Benzimidazole;
Cyathostomins;
Anthelmintic resistance;
PCR

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Abstract

Background: Resistance to benzimidazole (BZ) by cyathostomin nematodes has become a major threat to equine health around the world. We aimed to evaluate the efficacy of BZ drugs against small strongyle nematodes in horses of western Iran using coprological and molecular examination.

Methods: Faecal egg count reduction tests were performed on 398 horses kept in 16 stables in western Iran (Kermanshah Province), to detect benzimidazole resistance in small strongyle nematodes. Allele-specific PCR was used to identify the F200Y (TAC/TTC) SNP in the beta-tubulin gene codon in cyathostomin larvae.

Results: There was a 96.1%-98.3% (90% CI) reduction in faecal egg counts following mebendazole treatment and a 96.6% - 98.7% (90% CI) reduction in faecal egg counts following fenbendazole treatment. The allele-specific PCR showed BZ-susceptible homozygote genotypes in all examined samples (Two pools of 10 to 50 L_3 of cyathostomin nematodes from each of the 18 selected horses).

Conclusion: Benzimidazole resistance in equine small strongyles has been reported globally, and the results of the present study were unexpected. The probable reasons for the slow development of BZ-resistance are climatic conditions in the country that have significant negative effects on the ability of free stages of the resistant strongyle nematode to survive and develop.

Introduction

Small strongyles, referred to as cyathostomins, are the most prevalent gastrointestinal nematodes infecting grazing equines across the world, causing high morbidity and mortality (1-3). The control of equine cyathostomins relies largely on the use of benzimidazole, tetrahydropyrimidine, and macrocyclic lactone anthelmintic drugs; hence is compromised by the global emergence of resistance (2). Anthelmintic resistance (AR) to phenothiazine was first described in equine small strongyles (4). Benzimidazole drugs (BZ) were first introduced for use in horses in 1960, and field studies indicate that BZ resistance in cyathostomins is now widespread worldwide, and is virtually ubiquitous in some European regions (5). This has been attributed to widespread use of interval treatment regimens aimed at suppressing strongyle egg reappearance (6). There are also reports of resistance to the tetrahydropyrimidine drug, pyrantel, while shortened egg reappearance times after macrocyclic lactone drug treatments indicate emerging resistance to ivermectin and moxidectin in some countries (7). Local knowledge of the AR status is important to ensure that the control of equine cyathostomins is effective (8, 9). Early detection encourages the use of alternative and more sustainable strategies to prevent further emergence and spread of resistant worms (10).

The faecal egg count reduction test (FECRT) is the most widely used method to describe the AR phenotype, based on the reduction in the arithmetic mean faecal egg counts (FECs) of groups of animals between the time of treatment and about 14 days after treatment. The World Association for the Advancement of Veterinary Parasitology (WAAVP) provides guidelines for the interpretation of FECRTs (11), which take into account equine-specific challenges such as small groups of horses and over-dispersed FECs (12). *In vitro* egg hatch tests correlate well with the FECRT to identify benzimidazole resistance in equine cyathostomins (13, 14), but require access to laboratory facilities and materials. Allele-specific PCR has been developed to describe the benzimidazole resistance genotypes of equine cyathostomins, based on the detection of point mutations in the isotype 1 beta-tubulin gene at codon 200 (14, 15) and 167 (16).

The horse population in Iran is about 155,000. About 50% are kept under semi-intensive systems. About 40% are kept traditionally by nomads and villagers. The remaining animals are kept in intensive breeding centers (www.maj.ir). Iran has a diverse climate, which influences horse grazing management and exposure to cyathostomins. In the northern provinces, with a humid climate, free grazing of horses is common; in mountainous areas such as Kermanshah, there is pasture grazing in the spring and autumn; and in the southern and central provinces, with a desert climate, there is no free grazing. Despite the widespread use of anthelmintic drugs in Iranian horses (17), there are very few reliable data on the AR status of equine cyathostomins.

We aimed to evaluate the efficacy of the BZ anthelmintics, fenbendazole and mebendazole, that are routinely used in helminth control in horses in the Kermanshah Province in the west of Iran, using the FECRT and isotype 1 beta-tubulin codon 200 gene allele-specific PCR assay.

Materials and Methods

Study area and sampling

The present study included 398 horses in 16 stables in the Kermanshah Province in western Iran (Fig. 1). The horses had not received any anthelmintic treatment for at least two months before the start of the study. The Kermanshah Province has a climate that is heavily influenced by the proximity of the

Zagros Mountains and is classified as a hot-summer Mediterranean climate (Csa). It rains mostly during the winter months (December, January, and February) and is warm (43-44°C) in summer (June, July, and August). The annual rainfall is 500 mm, and the average annual temperature is above 22 °C (Iran Meteorological Organization, 2015).

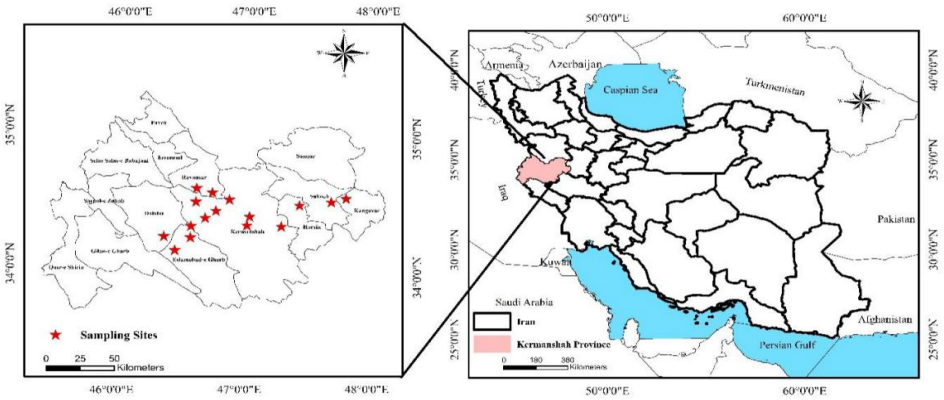

Fig. 1: Geographical location of the sampling sites, 16 stables in the Kermanshah Province in west Iran. The Kermanshah Province has a mountainous climate where winters are very cold and summers are very hot, especially in villages. Horses are taken to pasture in the spring and autumn

Parasitological methods

Faecal egg count reduction tests

Faecal samples were collected from 8 to 49 horses at each stable (398 horses in 16 stables) to determine the presence of strongyle nematode eggs. The samples were taken immediately after being voided and transported to the laboratory in plastic containers on the same day. The number of strongyle-type eggs per gram of faeces (EPG) was measured by a centrifugal Wisconsin sugar flotation technique with a detection threshold of 1 EPG, using Sheather's solution with a specific gravity of 1.2 (18). Eighteen horses with more than 150 EPG were selected to perform a FECRT to determine the efficacy of BZ treatment. The weights of the horses were estimated from their body measurements (19).

Horses were randomly assigned to 3 experimental groups with six animals per group: group 1, treated with orally with 8.0 mg/kg of body weight fenbendazole (FBZ) (Rooyandarou, Iran); group 2, treated orally with 8.8 mg/kg of body weight mebendazole (MBZ) (Zagros Pharmed, Iran); group 3, untreated controls. Faecal samples were collected from these horses 24 hours before treatment and again 14 days after treatment. Faecal egg count reductions for each treatment group were calculated based on the following formula (20):

FECR (%) = (mean EPG pretreatment – mean EPG 14 days post-treatment) × 100/mean EPG pretreatment

Cut-off values of the FECRT results suggested by the WAAVP (based on research protocol for farm/stable horses) were used to categorize the equine strongyles as: 1) susceptible (>99% FECR); 2) resistant (<95% FECR); 3) low resistant; and 4) inconclusive (11).

Statistical analysis of FECRT

The post MBZ and FBZ treatment FECs were compared with the corresponding FECs of the control group to evaluate of efficacy of the anthelmintic drugs (21). Two approaches were used to calculate 90% confidence (credible) intervals for the reduction of egg counts

in the feces of horses. First, for each treatment group 90% confidence interval (CI) for FEC reductions was calculated based on the WAAVP guidelines method (21) using the following formulas:

Mean & Sample variance of egg counts in treatment group (post-treatment):

$$\bar{X}_T = \frac{1}{n}\sum_{i=1}^{n} x_{T,i} \quad \& \quad S_T^2 = \sqrt{\frac{(x_i - X_T)^2}{n-1}}$$

- $\bar{X}_T$: Mean egg counts in the treatment group post-treatment.
- S_T^2: Sample variance of egg counts in the treatment group post-treatment.
- n: Number of samples.
- $x_{T,i}$: Egg count in the i-th sample.

Mean & Sample variance of egg counts in control group (end of experiment):

$$\bar{X}_C = \frac{1}{n}\sum_{i=1}^{n} x_{C,i} \quad \& \quad S_c^2 = \sqrt{\frac{(x_i - X_c)^2}{n-1}}$$

- $\bar{X}_C$: Mean egg counts in the control group at the end of the experiment.
- S_c^2: Sample variance of egg counts in the control group at the end of the experiment.
- n: Number of samples.
- $x_{C,i}$: Egg count in the i-th sample.

Percentage reduction in mean egg counts:

$$\%Reduction = 100 \times \left(1 - \frac{\bar{X}_T}{\bar{X}_C}\right)$$

Variance of reduction (log scale):

$$V^2 = \frac{S_T^2}{n_T \bar{X}_T^{\,2}} + \frac{S_C^2}{n_C \bar{X}_C^{\,2}}$$

- V^2: Variance of reduction on the log scale.
- n_T: Number of samples in the treatment group.
- n_C: Number of samples in the control group.

Upper & Lower confidence limits for percentage reduction:

$$UCL = 100 \times \left[\left(1 - \frac{\bar{X}_T}{\bar{X}_C}\right) Exp\left(-t_{1-\frac{\alpha}{2}(df=n1+n2-2)} \sqrt{V^2}\right)\right]$$

$$lCL = 100 \times \left[\left(1 - \frac{\bar{X}_T}{\bar{X}_C}\right) Exp\left(+t_{1-\frac{\alpha}{2}(df=n1+n2-2)} \sqrt{V^2}\right)\right]$$

- **UCL**: Upper confidence limit for percentage reduction.
- **LCL**: Lower confidence limit for percentage reduction.
- $t_{1-\frac{\alpha}{2}}$: Critical value from the t-distribution.
- df: Degrees of freedom (n1 + n2 - 2).
- α: Significance level.

In a second approach, data analysis was performed using the FECRT analysis web application of the bayescount R package (22). This online tool provided results based on a Bayesian approach, but did not offer directly calculated 95% confidence intervals. This method yielded independent estimates of treatment effects and the variances of those estimates, providing another perspective on drug efficacy. The CIs & CrIs were interpreted using the classification criteria outlined by Denwood et al. (23). Differences in the results obtained from the two approaches were noted.

Morphological identification of third stage larvae

Larval cultures from each of the 18 pre-treatment faecal samples were prepared by incubating 20-30 g of each sample at 26˚C for 10 days (24). The L_3 were recovered by Baermannisation and were examined and identified as cyathostomins, large strongyles or trichostrongyles according to morphological keys (25-27).

Molecular analysis

Allele-specific PCR assay was used to screen for the presence of the mutation in codon 200 of the isotype I beta-tubulin gene in the pre-treatment cyathostomin L_3 populations (14).

Template preparation

Two pools of 10 to 50 L_3 were prepared from each of the 18 horses with EPG above 150 before treatment. DNA extraction from each pool was performed using the tissue DNA extraction kit (Takapouzist, Tehran, Iran) following the manufacturer's instructions. The lysates were stored at -20 °C until used.

Allele-specific PCR

The isotype I beta-tubulin gene codon 200 allele-specific PCR was carried out as described previously (14). Cn24FS forward and Cn30R reverse primers were used to detect the base sequence coding for phenylalanine at codon 200 (TTC), and Cn25FR forward and the same Cn30R reverse primers were used to detect the base sequence coding for tyrosine (TAC). PCR reactions were performed in 50 µL volumes and contained 1X Mastermix PCR buffer (Pishgam, Tehran, Iran), 30 pmol of each primer (Pishgam, Tehran, Iran), and approximately 2 ng/4 µL of genomic DNA in Mastercycler nexus (Eppendorf, Hamburg, Germany) under the following thermal pattern: 10 min incubation at 95 °C to denature strand double-strand DNA, 40 cycles of 60 s at 94 °C (denaturing step) 60 s at 63 °C (annealing step), 60 s at 72 °C (extension step) followed by a final extension of 72 °C for 10 min. For all reactions, samples without DNA served as a negative control. Positive control (DNA of BZ-resistant cyathostomins, achieved from the feces of a grazing horse, Golestan Province in northern Iran) was run on each reaction. The PCR products were run on a 1.5% agarose gel in 0.5× TBE buffer and visualised using ethidium bromide and an ultraviolet light illuminator.

Results

Results of field analysis

No strongyle eggs were detected in 304 of the 398 horse faecal samples from the 16 stables.

The FECs of 75 horses were lower than 150 EPG and were excluded from the subsequent study. The remaining 19 horses with FECs above 150 EPG were randomly assigned to 3 experimental groups with six animals per group (18 horses) to perform the FECRTs (one horse was moved to another province) (Fig. 2).

Pre-treatment coprocultured L_3 identification revealed only cyathostomins in all 18 horses. Data analysis using the WAAVP-recommended method (21) showed a 95.8% - 98%. 3% (90% CI) reduction in FECs for MBZ and 96.2% - 98.7% (90% CI) reduction in FECs for FBZ.

The FECRT analysis by the Bayesian approach showed a 96.1% - 98.3% (90% CrI) reduction in FECs for MBZ and a 96.6% - 98.7% (90% CrI) reduction in FECs for FBZ. Both approaches indicated similar low levels of resistance for MBZ and FBZ (Table 1).

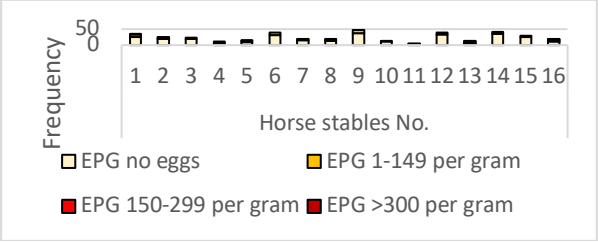

Fig. 2: Stacked bar chart of the frequency of horse faeces contamination with cyathostomin eggs by stables.

Table 1: Treatment (fenbendazole, FBZ, 8 mg kg^{-1} BW; mebendazole, MBZ, 8.8 mg kg^{-1} BW) on faecal egg count (eggs per gram - EPG) in 3 groups of horses in Kermanshah province, west Iran

Horse number	Therapeutic groups	Pre treatment EPG	Post treatment EPG	Percentage reduction
1	FBZ	324	13	95.98
2	FBZ	362	6	98.34
3	FBZ	154	6	96.10
4	FBZ	299	1	95.65
5	FBZ	188	3	98.40
6	FBZ	268	9	96.64
7	MBZ	285	13	95.43
8	MBZ	201	9	95.52
9	MBZ	189	3	98.41
10	MBZ	331	8	97.58
11	MBZ	240	11	95.41
12	MBZ	198	2	98.98
13	Control	291	303	-
14	Control	358	319	0.39
15	Control	406	375	0.31
16	Control	301	321	-
17	Control	219	189	0.3
18	Control	198	201	-

Results of allele-specific PCR

DNA was extracted from 36 pools (two from each horse pre-treatment), including 10 - 50 L_3 per pool from each infected horse before treatment. The BZ-susceptible Cn24FS/Cn30R primer combination amplified a single fragment of 308 bp in all examined pools. However, the BZ-resistant Cn25FR/Cn30R primer combination did not amplify any PCR product.

Discussion

This is the first combination of field coprological and *in vitro* molecular analysis in Iran to determine the efficacy of BZ drugs against gastrointestinal nematodes in horses. According to the newly published WAAVP guidelines, a single full therapeutic dose of a fully effective BZ drug should eliminate more than 99% of the susceptible gastrointestinal nematodes (11). The results of the present study could be interpreted as representing low levels of resistance. However, these levels of reduced efficacy of a correctly administered anthelmintic drug may also be due to pharmacokinetic effects. For example, they may be due to reduced absorption and bioavailability arising from poorer solubility of mebendazole compared to more modern BZ drugs, e.g., albendazole and oxfendazole, or due to concurrent gastrointestinal disorders (9). This consideration highlights the value of applying different tests for the detection of AR.

Various *in vitro* tests have proven valuable in some research settings, but these tests have limited application and usefulness for the diagnosis of AR at the farm level. Consequently, the *in vivo* FECRT remains the method of choice to assess drug efficacy, and hence the most commonly applied test for the phenotypic diagnosis of AR (28). In contrast to *in vitro* tests, the FECRT permits the assessment of drug efficacy for all anthelmintic classes in

multiple parasite species. Furthermore, it can be performed locally without the need for a reference diagnostic laboratory or specialized equipment. Performing FECRT on stabled horses often presents additional challenges not commonly encountered for ruminants (12). The most important of these are small group sizes of less than 10 horses to test and the overdistribution of low FECs amongst groups (29). Furthermore, 40 cyathostomin species infecting horses have been described (25), but resistance may only be present in one or two. Coprocultured ruminant L_3 can be speciated based on their morphology to differentiate FEC reductions by species (30); but L_3 morphology does not allow differentiation of equine cyathostomin species (27). Despite these limitations, the FECRT is the only reliable and suitable method to assess the BZ efficacy phenotype in live animals (24, 31-33).

Another recognized limitation of the FECRT is the failure to identify relatively low proportions of genotypically resistant gastrointestinal nematodes in a population (9). In contrast, PCR assays are sensitive tools for the early detection of BZ-resistant alleles in a population. Thus, in the present study, a previously published allele-specific PCR assay was used to screen for the presence of a point mutation in the beta-tubulin codon 200, which is linked with BZ-resistance (14). The homozygous TTC/TTC genotype is BZ-susceptible, and the homozygous TAC/TAC genotype is BZ-resistant. Only homozygous TAC/TAC individuals survive BZ treatment (34). Heterozygous TTC/TAC individuals are killed by the drug, but could be a source of BZ-resistant worms in a population (14). The allele-specific PCR assay can identify heterozygous TTC/TAC cyathostomins, but only homozygous BZ-susceptible genotypes were identified in this study. Anthelmintic resistance has been described in undifferentiated gastrointestinal nematodes of Iranian sheep (35-37). BZ-resistance (albendazole, mebendazole, and fenbendazole) in equine small strongyles has been reported in most global regions (13, 2, 33, 41), including neighboring Turkey (32). Although fenbendazole and mebendazole are widely used broad-spectrum anthelmintic drugs in sheep, horses, and cattle in Iran (42), available data suggest that the development of BZ-resistance has been slow in the country. There is only one new report of impaired efficacy of fenbendazole in only 4 horses in Tabriz and Hamedan cities in Iran (FECRT<90%, without molecular confirmation), which indicates a low level of resistance to this drug in these cities (43). To the best of our knowledge, the present study is the first evaluation of BZ-resistance in equine cyathostomins in the Kermanshah province, Iran.

An important question arises as to why, despite a long history of the use of BZ drugs in the Iranian horse population, BZ-resistance has not emerged. The hot and dry summer climate and snow cover during the cold winter climate in the Kermanshah Province would limit the development, survival, and availability of free gastrointestinal nematode stages for much of the year (44). This would reduce the need for frequent interval anthelmintic drug treatments, but would also result in low levels of refugia if treatments are given at the end of the summer or winter seasons. Conversely, it has been suggested that gastrointestinal nematode larval survival and development on pasture throughout the year might place a high selection pressure for anthelmintic resistance (45, 46), putatively due to the resultant need for more frequent interval treatments. An alternative explanation for the low levels of BZ resistance in the Kermanshah Province may be that the climatic conditions do not favor the development of the cyathostomin species in which resistance most frequently arises (47). However, it was not possible to identify the co-infecting cyathostomin species in the present study. Combinations of BZ and macrocyclic lactone anthelmintics are commonly used for the control of gastrointestinal nematodes

in the Kermanshah Province of Iran (Personal observation). This practice allows the effective control of nematodes along with slowing the development of AR (48-51). In most parts of Iran, as in the Kermanshah Province, many horses receive the anthelmintic treatments during the pasturing seasons in mid-spring and autumn (Personal observation). This strategic control would be expected to correspond with the presence of the largest size of the parasite refugium on the pastures; and therefore, would place the lowest selection pressure for AR. Studies have shown that in order to reduce the selection pressure for AR, a logical recommendation is to avoid or reduce treatments at times when parasite refugia are small (52). Further studies to explain the failure to detect equine cyathostomins that are resistant to BZ drugs in the Kermanshah Province would help to inform sustainable control strategies in other regions.

There were a number of limitations in the current work. The low cyathostomin burden in the examined horses was one of the current limitations that didn't allow us to include more horses (EPG above 150) in the FECRT exam. Another limitation was our inability to determine the genus and species of cyathostomins by microscopy. The next generation sequencing (NGS) approach might be useful for future studies in the analysis of horse nemabiome diversity (53).

Conclusion

Although the use of anthelmintic drugs in Kermanshah province, Iran, is prevalent, available data suggest that the development of BZ resistance has been slow and is not currently a major health threat. The reasons for this probably reflect the unique climatic conditions, which could influence the species composition of cyathostomin communities and the timing of anthelmintic treatments with regard to the size of refugia populations. Further investigations of anthelminthic resistance in equine nematodes in different geographical areas in Iran are highly recommended.

Acknowledgements

The authors wish to express their appreciation to Professor Theo de Waal (University College Dublin, Ireland) for his valuable comments.

Financial Support

This study was supported by the Razi University under the framework of the DVM thesis project of Rezvan Jamshidpour, and Bu-Ali Sina University, Hamedan, Iran (Grant Number 1400-10235 to RN).

Conflict of Interest

The authors declare that there is no conflict of interests.

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