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Original Research

Cyathostominae Egg Reappearance Period After Treatment With Major Horse Anthelmintics in Donkeys

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ABSTRACT

The Egg Reappearance Period (ERP) is considered an early indicator of anthelmintic resistance. The aims of the present study were to determine the field efficacy and evaluate the ERP of four broad-spectrum anthelmintic drugs administered at horse dose rate in donkeys naturally infected by Cyathostominae. The trials were conducted in two farms (A and B). Forty-eight female crossbreed donkeys, 24 animals for each farm, were selected on the basis of Fecal Egg Count (FEC) > 300 eggs per gram and allocated to four treatment groups of six animals: pyrantel group (PYR), fenbendazole group (FBZ), ivermectin group (IVM), and moxidectin group (MOX). FEC was performed from the first to the 12th week after treatment. In the farm A at 2 weeks after treatment, the Fecal Egg Count Reduction Test (FECRT) showed high efficacy for all drugs (PYR 99.3%, FBZ 99.8, and IVM/MOX 100%), and ERP rates were not shorter than those expected. In the farm B at 2 weeks after treatment, FECRT showed high efficacy for IVM/MOX (100%), suspect resistance (86.3%), and resistance (83.9%) to PYR and FBZ, respectively; only in the MOX group a shortened ERP was detected (9 weeks). No adverse reactions were observed at clinical examination. The results demonstrate that the major anthelmintic classes, administered orally at horse dose, are effective and safe for treatment of Cyathostominae in donkeys, although resistance development is possible and could be correlated to the high treatment frequency and the extra-label use of anthelmintic licensed for ruminants. Furthermore, a shortened ERP may be the early indicator of developing anthelmintic resistance in donkeys.

1. Introduction

Cyathostominae, or small strongyles, are the most common parasites in donkeys such as in horses; although in donkeys, massive parasitic infections often are subclinical, their impact on donkey's clinical status is unclear, and anthelmintic treatments are the main strategy to control these internal parasites [1,2]. However, in the last years, the overuse of anthelmintic drugs has resulted in development of parasite resistance in horses [3]. In donkeys, very few data are available on resistance. Anthelmintic drugs commercially registered for use in donkeys are very few and in practice are used those licensed for horses or ruminants at the dose rate suggested for these latter species [4]. In horses, for many years, anthelmintics were administered to all animals following an "interval dose program", originally based on 2 months interval treatment and currently developed on the expected strongyle Egg Reappearance Period (ERP) [5]. However, these treatment protocols have led to a high selection pressure for resistant alleles within equine

Animal welfare/ethical statement: The study was approved by the Ethical Animal Care and Use Committee of the University of Naples "Federico II," and all operations on the donkeys were performed with the owner's consent.

Conflict of interest statement: None of the authors of this article has a financial or personal relationship with other people or organizations that could inappropriately influence or bias the content of the article.

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nematode populations [3]. Resistance is the ability of worms in a population to survive treatments that are generally effective against the same species and stage of infection [6]. Two factors should be considered in vivo assessment in the evaluation of anthelmintic drug efficacy: Fecal Egg Count Reduction Test (FECRT), performed 2 weeks after treatment and ERP that measures the interval time between the last effective anthelmintic treatment and the reappearance of eggs in fecal samples [3,6]. According to the American Association of Equine Practitioners (AAEP) Parasite Control Guidelines, anthelmintic drug resistance should be considered if FECR is <95% for ivermectin (IVM) and moxidectin (MOX), <90% for fenbendazole (FBZ) and <85% for pyrantel (PYR). Normally, when the drug is effective, the ERP is 4–5 weeks for PYR and FBZ, 6–8 weeks for IVM, and 10–12 weeks for MOX. The ERP cutoff value is 80% for FBZ and PYR and 90% for IVM and MOX [6]. Shortened ERP is considered a predictive and early indicator toward resistance [7].

In the last decade, some studies have focused on the ERP value in horse strongyle infections as a more sensitive parameter in the assessment of drug resistance [8–14].

Recently, Porr et al [2] determined the efficacy and ERP of IVM and MOX every 2 weeks for 12 weeks after treatment against intestinal strongyles in 46 horses. FECRT was 100% for IVM and MOX even if a shorter ERP was detected for MOX. Similarly, Tzelos et al [3] confirmed a shorter ERP after MOX treatments against small strongyles in 261 horses bred in the United Kingdom. Moreover, Bellaw et al [14] verified a failure of FBZ (FECRT 52.1%) and an ERP of 5 weeks for MOX following treatment in 36 ponies infected by Cyathostominae.

In donkeys, few clinical trials were performed on the efficacy of anthelmintics, administered at horse dose rate, to control strongyle infections. Oral administration of mebendazole (MBZ), FBZ, and PYR pamoate was effective against Cyathostominae in donkeys naturally infected [4,15,16]. Trawford et al [17] evidenced the first treatment failure of MOX against Cyathostominae in two donkey herds at the Donkey Sanctuary in Dorset, England. Lawson et al [18] reported PYR resistance against small strongyles in two donkey farms located in the United Kingdom. Recently, in a farm from Northern Italy, Veneziano et al [19] reported a shorter Cyathostominae ERP in donkeys treated with MOX.

The present study reports the results of 2 field trials conducted in Italy to evaluate the efficacy of the main broad-spectrum horse anthelmintic classes, calculating the FECRT, and to investigate a possible development of drug resistance, determining the ERP, in donkeys naturally infected by Cyathostominae.

2. Material and Methods

2.1. Farms and Animals

The trials were conducted between September and December 2015 in 2 commercial donkey farms (farm A and farm B) in Italy. The farm A, located in Southern Italy (Campania region), consisted of approximately 50 animals, mainly belonging to Ragusana breed or crossbreds. The anthelmintic treatment was performed annually in the autumn using an IVM oral paste. The farm B, located in Central Italy (Lazio region), consisted of approximately 60 animals, mainly belonging to Amiata breed or crossbreds. The anthelmintic treatments were performed three times/year (spring, summer, and autumn), mainly using benzimidazole and IVM injectable and oral drench formulations licensed for cattle and small ruminants.

In both farms, fecal analysis (individual Fecal Egg Counts [FECs] and pooled fecal cultures), performed before the beginning of the trials (day 2), showed high intestinal strongyle FECs in almost all donkeys (n = 32–farm A and n = 42–farm B) and total prevalence (100%) of Cyathostominae. On the basis of the positive FECs (donkey selective therapy cutoff >300 eggs per gram [EPG]) [1], for each farm, 24 female crossbreed donkeys, weighing 254.3 ± 40.3 kg (farm A) and 230.5 ± 52.4 kg (farm B), respectively, were selected for the study protocol. The body weight (BW) of each animal was estimated 2 days before the treatment (day 2), using the nomogram proposed by The Donkey Sanctuary [20]. The animals had a mean age of 9.1 ± 4.6 (farm A) and 4.1 ± 2.9 (farm B) years. Furthermore, they had a history of grazing on pasture contaminated with equine nematode parasites and have not been treated with any anthelmintics during the previous 3 months. On both farms, no movement of animals was performed before or during the study.

2.2. Experimental Groups

On day 2, the experimental animals had an average of 709 ± 381 EPG in the farm A and 824 ± 624 EPG in the farm B.

In each farm, the animals were ranked from the lowest to highest EPG counts. Based on increasing EPG counts, replicates of four animals were formed. Within each replicate, animals were randomly assigned to treatment groups. The 24 selected donkeys, per each farm, were assigned consecutively to the following treatment groups of six animals each: PYR paste (PYR group), FBZ paste (FBZ group), IVM paste (IVM group), and MOX oral gel (MOX group).

The study animals were tagged for identification using a numbered head collar. During the entire experimental period, the donkeys were housed communally in an outdoor pen with a permanent and hilly pasture; supplementary feeding was given such as hay and concentrate (barley, wheat bran, beet pulp, and carob). The four groups were maintained together under the same conditions, and they co-grazed throughout on the same pasture; there was no change of diet during the study, and water was provided ad libitum.

2.3. Treatment Procedures and Adverse Reactions

Commercial formulations licensed for horses were administered orally to donkeys at the horse dosage: PYR (6.94 mg/kg BW, Strike Oral Paste, Acme), FBZ (7.5 mg/kg BW, Panacur Oral Paste, MSD Animal Health), IVM (200 mcg/kg BW, Eqvalan Oral Paste, Merial Italia–Boehringer Ingelheim Animal Health), and MOX (400 mcg/kg BW, Equest Oral Gel, Zoetis Italia).

For each animal, the dosage was calculated on the basis of the BW previously estimated (day 2). All groups received a single treatment administered by veterinary operators (F.B., C.R., V.L.B., B.N., and A.F.). All treated animals were intermittently observed for adverse reactions for 3 hours (three times) during the day of treatment (day 0) and then weekly until the end of the trial by veterinary operators (L.P., D.P., and V.V.).

2.4. Coprological Examinations: FECRT and ERP

According to general recommendations proposed by Nielsen et al [21], fecal samples were taken from the rectum of each study animal, stored in a refrigerator (+4°C), and examined within 48 hours to reduce the effect of egg hatch. Individual FECs were performed in all donkeys before the start of the trial (day 2), and at week 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, and 12 after treatment, using special modification of McMaster method, with a detection limit of 10 EPG and a Sheather's saturated sugar solution with a specific gravity of 1.250 [22]. On each sampling day, group pooled fecal samples were incubated at 27°C for 7–10 days for larval development, and third stage larvae were identified using the keys

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proposed by the Atlas of diagnosis of equine strongylidosis [23]. When fecal cultures had 100 or less third stage larvae, all were identified; when fecal cultures had more than 100 larvae, only 100 were identified. To determine the efficacy of the different drugs, the arithmetic mean (AM) of EPG was calculated at the second week, and the percent efficacy (%) for each group was considered in terms of FECRT using the formula: ([AM $EPG_{PRE\ TREATMENT}$–AM $EPG_{2\ WEEKS\ POST\ TREATMENT}$]/AM $EPG_{PRE\ TREATMENT}$) × 100, according to the AAEP guidelines [6]. Furthermore, in agreement with AAEP guidelines, the cutoff values used to interpret the results of FECRT in horses were the following: PYR efficacy > 90%, suspected resistance 85%–90%, resistant < 85%; FBZ efficacy > 95%, suspected resistance 90%–95%, resistant < 90%; IVM/MOX efficacy > 98%, suspected resistance 95%–98%, resistant < 95% [6]. The ERP was defined as the post-treatment week at which the percent reduction in FEC decreased below the efficacy cutoff value of 80% and 90% for PYR/FBZ and IVM/MOX, respectively [6].

Microsoft Office Excel 2010 was used for data recording and FEC reductions, expressed as percentage with 95% confidence intervals, were calculated using the Reso FECRT analysis program, version 4 (http://sydney.edu.au/vetscience/sheepwormcontrol/) for Excel. The resistance would be indicated if the lower confidence limit (LCL) was below 90% [3], and the mean percentage of FECR was below 85% for PYR, 90% for FBZ and 95% for IVM/MOX, according to the AAEP guidelines [6] . If both criteria were present, the resistance was confirmed, if only 1 of the 2 criteria was reported, resistance was suspected.

3. Results

For all animals, the dose was administered carefully, and no adverse reactions were observed in any of the treated donkeys during the study.

In the farm A, pretreatment mean EPG was 673, 725, 757, and 682 for PYR, FBZ, IVM, and MOX groups, respectively. At the second week, the FECRT was 100% for IVM and MOX, 99.8% (LCL 98.1%) for FBZ, and 99.3% (LCL 97.8%) for PYR, suggesting that all investigated drugs were effective against small strongyles. Furthermore, for all tested anthelmintics, the ERP rates were in accordance with those reported by the AAEP guidelines: 7 weeks for PYR and FBZ, 12 weeks for IVM and MOX (Figs. 1–4; farm A).

In the farm B, pretreatment mean EPG was 850, 640, 813, and 993 for PYR, FBZ, IVM, and MOX groups, respectively. At the second week, the FECRT showed high efficacy for IVM and MOX (100%), a suspected resistance to PYR (86.3%; LCL 64.3%) and resistance to FBZ (83.9%; LCL–4.6%). Moreover, the ERPs were 8 weeks for IVM and 9 weeks for MOX, suggesting a shortened ERP rate for MOX (Fig. 1–4; farm B).

In all studied donkeys, pre-treatment and post-treatment fecal cultures revealed exclusively the presence of larvae of Cyathostominae (*Cyathostomum sensu lato*) in all experimental groups in both farms.

4. Discussion

This is the first comparative study on anthelmintic efficacy and Cyathostominae ERP rates in donkeys using major horse anthelmintic classes. At clinical examination, no adverse reactions were observed in any of the treated donkeys.

In the farm A, all drugs proved to be effective in reducing FECs at 2 weeks after treatment showing a FECRT of 99.3%, 99.8%, and 100% for PYR, FBZ, and IVM/MOX, respectively. The ERP rates were longer than usually expected. In accordance with the cutoff values reported by the AAEP guidelines when the drugs were introduced for the first time: 7 weeks for PYR and FBZ, 12 weeks for IVM and MOX. In the farm B, at 2 weeks after treatment, the FECRT was 86.3% for PYR, 83.9% for FBZ, and 100% for IVM and MOX. These data suggest, according to the AAEP guidelines, a high efficacy for macrocyclic lactones to control intestinal strongyles, while a resistance to FBZ was detected. Regarding PYR, a suspected resistance was reported (FEC range 85%–90%) and associated with an expected ERP of 5 weeks. Moreover, a shortened ERP was observed only in MOX group (9 weeks).

As regards the pyrimidine class, Gokbulut et al [16] evaluated the efficacy of two different oral formulations of PYR pamoate (paste and granule) in donkeys naturally infected by intestinal strongylidae. Two weeks after treatment, PYR paste and PYR granule groups showed a FECRT of 98.5% and 97.3% respectively, and the value of ERP was greater than 80% up to 5 weeks after treatment in accordance with the cutoff values of the AAEP guidelines. Lawson et al [18] investigated about PYR embonate paste resistance in two donkey herds in south England. In the studied herds, the mean FECRT was of 72% and 70%, and these results suggested the presence of a Cyathostominae population resistant to PYR in donkeys.

Regarding benzimidazoles, the results of the present study are different from those previously reported in Italy by Veneziano et al [15] in donkeys treated orally with FBZ 10% drench at horse dose

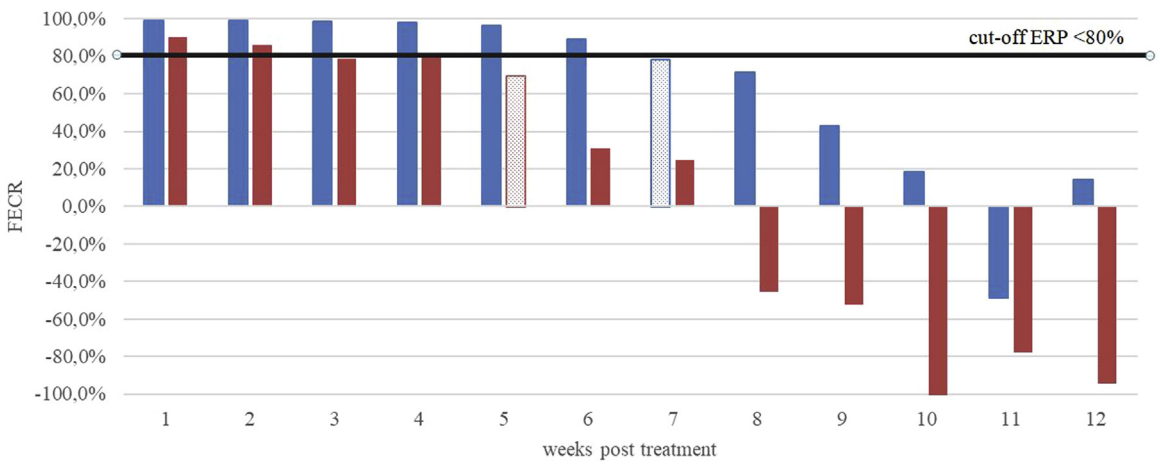

Fig. 1. Pyrantel pamoate: FECR and ERP in the studied donkeys. The blue bar represents the farm A; the red bar represents the farm B. The dotted bar shows the weeks at which ERP decreases under the cutoff value. ERP, egg reappearance period; FECR, Fecal Egg Count Reduction.

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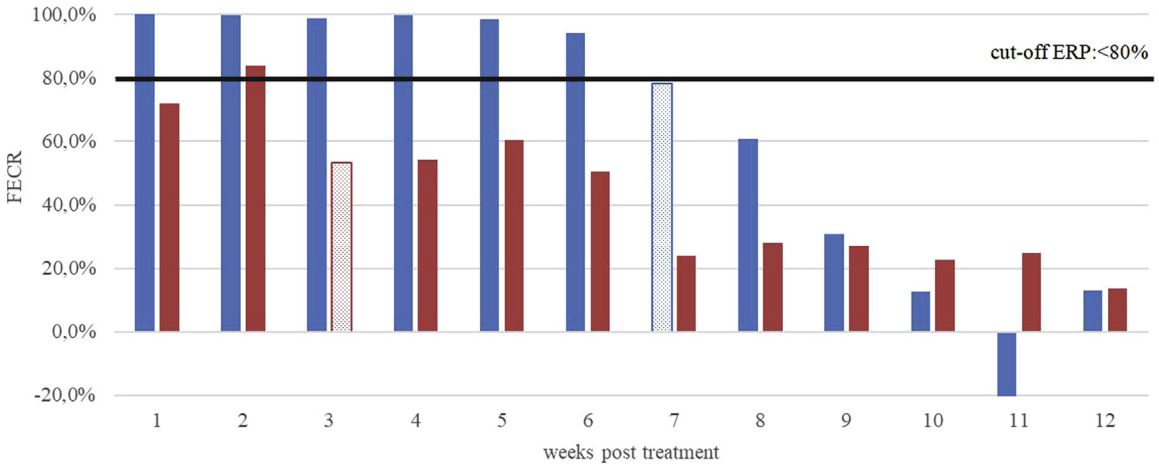

Fig. 2. Fenbendazole: FECR and ERP in the studied donkeys. The blue bar represents the farm A; the red bar represents the farm B. The dotted bar shows the weeks at which ERP decreases under the cutoff value. ERP, egg reappearance period; FECR, Fecal Egg Count Reduction.

rate. At 2 weeks after treatment, FBZ was totally effective (100%) to control infection by intestinal strongyles, and the ERP value was twice the expected one (4–5 weeks), reaching 8 weeks after treatment.

Similar results were reported by Gokbulut et al [4] in a clinical trial aimed to evaluate the efficacy of MBZ in donkeys naturally infected by Cyathostominae. The animals were treated using an oral paste formulation of MBZ, administered at the manufacturer's recommended horse dosage of 10 mg/kg BW (MBZ 1) and at the double horse dosage of 20 mg/kg BW (MBZ 2). Two weeks after treatment, FECRT was of 99.7% for MBZ 1 and 99.3% for MBZ 2, and the ERP value was 35 days (5 weeks) for both treated groups, suggesting that to control intestinal strongyle infection the donkeys should be treated at the same dose of horses (10 mg/kg BW).

Imam et al [24] in Sudan evaluated the efficacy of albendazole to control intestinal nematodes in donkeys, using drench formulation labeled for sheep. Two groups of animals were treated with a single oral dose of albendazole (10 mg/kg BW) and a double oral dose of albendazole (10 mg kg BW). The efficacy in both groups was of 100% up to 3 weeks after treatment, and no data are reported on the ERP.

With reference to macrocyclic lactones, most of the studies performed in donkeys report the use of formulations licensed for cattle or small ruminants, with different percentages of effectiveness.

Binev et al [25] evaluated the efficacy of IVM following subcutaneous injection to control intestinal strongyles on 263 donkeys from different regions of Bulgaria. The extra-label use of injectable IVM proved to be effective in reducing FECs at 2 weeks after treatment (FECRT 96%). Although the authors reported the treatment as effective, according to the AAEP suggested cutoff values, the FECRT value obtained for IVM could be indicative of suspected resistance.

Imam et al [24] evaluated in donkeys the efficacy of a single dose of a sheep IVM oral drench formulation (200 μg/kg BW). The FECRT was 100% until 3 weeks after treatment.

Recently, anthelmintic efficacy of IVM and MOX injectable formulations against helminthic infections of donkeys was evaluated in a trial by Fangama et al [26]. The experimental groups were treated with extra-label injection formulations registered for sheep as follows: the first one with a single subcutaneous dose of MOX at 200 μg/kg BW; the second one with a single intramuscular dose of MOX at 200 μg/kg BW, and the third one with a single subcutaneous dose of IVM at 200 μg/kg BW. Two weeks after treatment, the MOX was ineffective in both groups with a FECRT of 90.9% in the

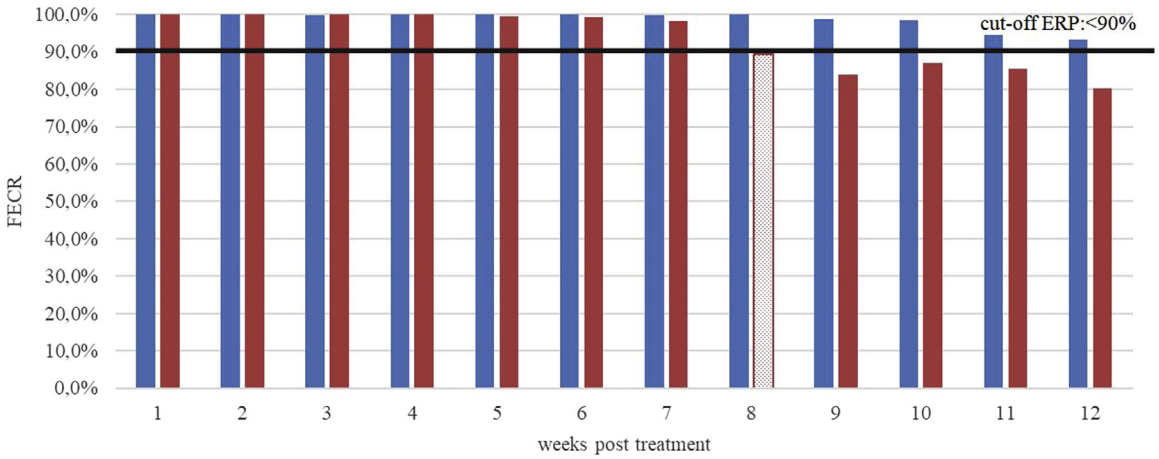

Fig. 3. Ivermectin: FECR and ERP in the studied donkeys. The blue bar represents the farm A; the red bar represents the farm B. The dotted bar shows the weeks at which ERP decreases under the cutoff value. ERP, egg reappearance period; FECR, Fecal Egg Count Reduction.

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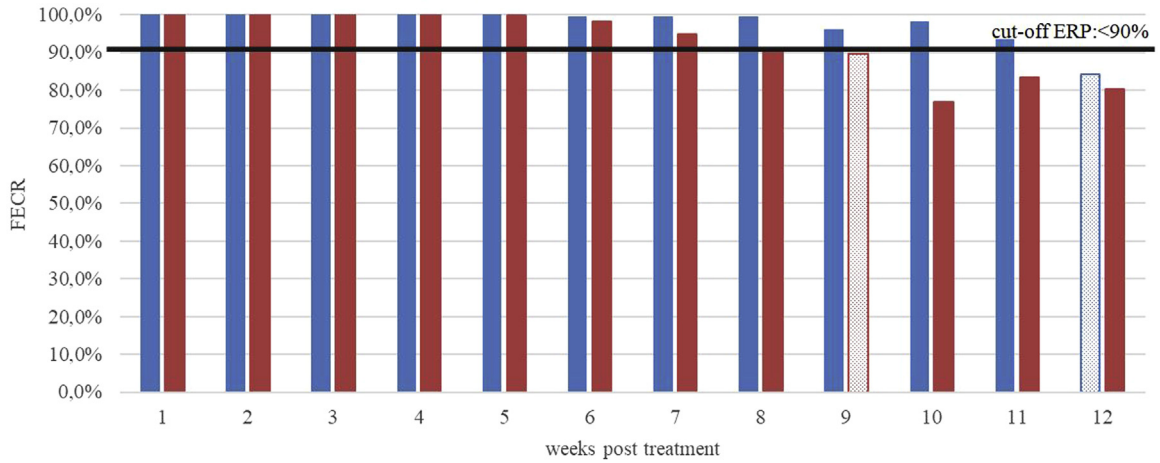

Fig. 4. Moxidectin: FECR and ERP in the studied donkeys. The blue bar represents the farm A; the red bar represents the farm B. The dotted bar shows the weeks at which ERP decreases under the cutoff value. ERP, egg reappearance period; FECR, Fecal Egg Count Reduction.

subcutaneous group and 78.5% in the intramuscular group; while IVM showed an efficacy of 100%.

The failure efficacy of parenteral formulations observed in this study could be attributed with the extra-label use of MOX.

Similarly, Trawford et al [17] reported an apparent treatment failure of macrocyclic lactones drugs against Cyathostominae in the Donkey Sanctuary in Dorset (England). During two clinical trials, donkeys were treated orally with a MOX formulation labeled for intramuscular administration in cattle. In the first trial, 600 donkeys were treated with MOX, and the monitoring after treatment indicated a value of 8 weeks. In the second trial, FECRT was performed on two groups of donkeys, 14 and 25 days following treatment with MOX. The mean reductions in FEC were 87% and 31%. The authors hypothesized that the reduced efficacy and shortened ERP were probably attributable to MOX resistance in Cyathostominae; however, the extra-label use of MOX could have affected the pharmacokinetics of this drug, thus influencing its effectiveness.

Recently, in a donkey farm from Northern Italy, Veneziano et al [19] reported a high efficacy of MOX oral gel formulation at horse dose rate (FECRT 99.7% at day 14), but a shorter ERP.

Similarly, in the present study, the shortened ERP value was observed only in the MOX group (9 weeks) in the farm B from central Italy, where injectable and drench formulations registered for ruminants were frequently used.

The ERP values detected in the farm A are similar to those that were found in the horses in the past when the different anthelmintic classes were introduced for the first time.

These were 7 weeks for PYR and FBZ and twelve weeks for IVM. For an equine parasitologist, the donkey can be considered similar to a horse of 40 years ago.

From this point of view, it is important to remember that the drug resistance and the ERP reduction could be repeated in donkeys too. If the rules that are important to prevent resistance will not follow in a few decades, the situation may be similar to that of horses.

The primary question is whether we have demonstrated in our experiment the reduced efficacy of anthelmintic drugs and the suspicion of the onset of macrocyclic lactone resistance in Cyathostominae in donkeys. This conclusion can be influenced by several factors. Firstly, there are no specific recommendations for a treatment in donkeys and similar studies used either horse recommended dose rate or dose suitable for ruminants. This means that we do not exactly know the threshold values to evaluate resistance. Secondly, the veterinarian and the farmers also did not know about the exact dose requirement for treatment of donkeys which may accelerate the development of resistance. Thirdly, dose response study or molecular detection in case of benzimidazole treatment is necessary to confirm the lack of the treatments. Additionally, in vitro tests may be useful for phenotyping of the Cyathostominae resistant to anthelmintic drugs.

5. Conclusion

This study reported for the first time the presence of anthelmintic resistance to FBZ and suspected resistance to PYR against Cyathostominae in donkeys in Italy.

Overall, our results confirmed that the efficacy of IVM and MOX against small strongyles remains high in donkeys.

The ERP rates were in accordance with the cutoff values reported by the AAEP guidelines for PYR, FBZ, and IVM; only MOX showed a shortened ERP in the farm from the central Italy. The resistance to FBZ and suspected resistance to PYR, and the decreased ERP for MOX, may be explained with the high treatment frequency and the extra-label use of drugs licensed for ruminants.

Based on our results to evaluate the phenomena of anthelmintic resistance, it is crucial to combine always the FECR test with the assessment of ERP.

Cyathostominae infections are widespread in donkey farms and can be correlated with poor management practices and lower frequency of endoparasite treatments. The intestinal strongyles control programs in donkeys are very different than those used in horses and the donkey can be considered as an animal in refugia.

Although in the world the presence of confirmed resistance in donkey farms is rare, the recent reports in Europe of anthelmintic treatment failure suggest that parasite control programs based on the extra-label use of drugs licensed for ruminants could contribute in future to the selection of resistance in donkeys. For this reason, a large scale survey on the presence of resistance and the ERP value in Cyathostominae populations in donkey farms is warranted.

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