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## Research Note

# A controlled study on efficacy and egg reappearance period of Ivermectin in donkeys naturally infected with small strongyles

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### Summary

The aim of the present study was to investigate the efficacy and the egg reappearance period (ERP) of ivermectin (IVM) in donkeys during a 13-week period. The study involved a total of 14 adult Amiata breed donkeys, 7 – 13 years of age, and naturally infected with small strongyles. A group of 10 donkeys was treated with IVM oral paste at a dose rate of 200 mcg/kg BW. Another group of 4 donkeys was kept as untreated control group. Faecal samples were collected and examined for strongyle eggs on day 0 before treatment. IVM efficacy was based on the faecal egg count reduction test (FECRT) on day 14 post-treatment. Then individual faecal samples were collected and examined by FECRT at weekly intervals. A FECRT of 100 % was found after treatment with IVM and its ERP, defined as the week when the mean FECRT decreased until to become lower than 90 % efficacy, was estimated to be 11 weeks without signs of developing anthelmintic resistance. No adverse reactions were observed during the study period. Our findings may be useful to veterinary practitioners and breeders as they show that IVM, at the recommended dose rate, can be still considered a highly effective and safe pharmacological tool for the treatment of small strongyles in donkeys. Therefore, it is strongly recommended that all possible strategies are undertaken to avoid the risk of emergence of anthelmintic resistance to IVM in donkeys.

**Keywords:** donkeys; small strongyles; ivermectin; efficacy; egg reappearance period

### Introduction

Donkeys (*Equus asinus*) are very important working animals as they provide vital support to human beings in many developing countries. Moreover, they are increasingly used for recreational activities (agritourism, trekking), onotherapy (Borioni *et al.*, 2012), meat production (Polidori *et al.*, 2008), and mostly milk production, since jenny's milk can be used in children with allergy to cow's

milk (Polidori *et al.*, 2013). In addition, the potent anti-proliferative activity of whey protein of donkey milk against human lung cancer cells has been reported (Mao *et al.*, 2009). Therefore, improving the donkey health status is important to owners, farmers, and consumers.

Small strongyles, also commonly called cyathostomins, are among the most important intestinal parasites of donkeys due to the high prevalence values commonly reported in several countries around

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the world (Matthee *et al.*, 2000; Bu *et al.*, 2009; Getachew *et al.*, 2010; Matthews *et al.*, 2013; Ismail *et al.*, 2016; Jajere *et al.*, 2016; Dibaba *et al.*, 2017). They include more than 40 species and several genera, they belong to the subfamily Cyathostominae and to the family Strongylidae, and they are found in the cecum and colon of domestic equids. It is known that small strongyles can be a threat to donkeys' health and welfare (Matthee *et al.*, 2002; Yoseph *et al.*, 2005; Orian *et al.*, 2015; Waqas *et al.*, 2015).

The control of endoparasites, including small strongyles, in donkeys depends mainly on the use of anthelmintics at doses determined for horses (Grosenbaugh *et al.*, 2011). These include macrocyclic lactones (i.e. Ivermectin and Moxidectin), benzimidazoles (i.e. Fenbendazole), and tetrahydropyrimidines (i.e. Pyrantel embonate) (Grosenbaugh *et al.*, 2011; Matthews *et al.*, 2013). However, the first report of resistance to moxidectin in cyathostomins was observed in donkeys (Trawford *et al.*, 2005). High levels of widespread resistance to fenbendazole have been reported in cyathostomins worldwide (Matthews *et al.*, 2013), including in a donkey farm in Italy (Buono *et al.*, 2018). Cases of pyrantel resistance of cyathostomins have been reported in two herds of donkeys in the UK (Lawson *et al.*, 2015) and in one donkey farm in Italy (Buono *et al.*, 2018). Conversely, it has repeatedly been shown that the efficacy of ivermectin (IVM) against intestinal strongyles in donkeys is as high as 100 % (Buono *et al.*, 2018; Seri *et al.*, 2005; Imam *et al.*, 2010; Arias *et al.*, 2013; Fangama *et al.*, 2013).

The time interval between the last effective anthelmintic treatment and the resumption of significant strongyle egg shedding is called the egg reappearance period (ERP). The ERP is calculated as weeks post-treatment and this parameter differs for each drug (Nielsen *et al.*, 2019). Thus, it is important to know the ERP of each anthelmintic drug after treatment in the different equine species. However, the ERP after treatment with IVM in donkeys still remains poorly investigated. Indeed, the majority of experimental studies evaluating the efficacy of IVM in donkeys did not investigate the corresponding ERP (Seri *et al.*, 2005; Imam *et al.*, 2010; Fangama *et al.*, 2013). To the best of the authors' knowledge, only two studies have reported data about the ERP in donkeys treated with IVM so far (Buono *et al.*, 2018; Arias *et al.*, 2013). Thus, in order to give further insights, the aim of the present study was to investigate the efficacy and ERP of IVM in donkeys found naturally infected by intestinal small strongyles.

## Materials and Methods

### Research site and animals

The study was carried out at the Veterinary Teaching Hospital "Mario Modenato" of the University of Pisa (geographical coordinates: latitude 43° 25' 00" N, longitude 10° 43' 00" E) in Tuscany, Central Italy. The site was chosen as having animals, facilities, and management suitable to carry out this type of study. The donkeys were selected from a herd bred at the same site to minimize differences attributable to management. The herd had a history of

strongyle infections with lack of clinical signs and ivermectin had been irregularly used in previous years. Ten female and 4 male donkeys of Amiata breed were randomly selected for the trial. The animals were 7 to 13 years of age (median age=10 years) and had not been treated with anthelmintics in the previous 14 months. Selected donkeys were enrolled based on confirmed natural infection with intestinal strongyles on day 0 of the study and were randomly allocated to two groups of 10 (treated group) and 4 (control group) animals, respectively. According to the classification of egg shedding given by Kaplan and Nielsen (2010) for horses, donkeys with a faecal egg per gram count (FEPGC) of 1–≤200 eggs were considered as low shedders, with a FEPGC of >200–≤500 eggs as moderate shedders, and with a FEPGC of >500 eggs as high shedders. All animals had been kept together in paddock throughout the year, but the two groups were kept in two separate pens throughout the study period. Donkeys were fed alfa-alfa hay *ad libitum* and a commercial equine feed (Equifloc®, Molitoria Val di Serchio Srl, Lucca, Italy). Drinking water was supplied *ad libitum* from the same source for both groups.

### Collection of faecal samples

Individual faecal samples were collected from rectum when possible, otherwise a sample of freshly voided faeces was collected from the ground in the paddock (only faeces that were seen to be passed from individual donkeys were taken). Fresh faecal samples were collected from each donkey on day 0 before treatment, on day 14 post-treatment, and at weekly intervals thereafter for 11 weeks until the end of the trial (day 91). After collection, each faecal sample was placed into a separate polythene bag, labelled properly for identification, and transported to the laboratory within 1–2 h. All samples were stored at +5°C and examined within 48 hours after collection to reduce the effect of egg hatch, based on general recommendations suggested by Nielsen *et al.* (2010) for horse faecal samples.

### Faecal egg per gram counts

The samples were examined for strongyle FEPGC using a commercial sodium nitrate solution with specific gravity of 1.200 (Coprocol®, Candioli Farmaceutici S.p.A., Beinasco (TO), Italy) and the Mini-FLOTAC technique in combination with Fill-FLOTAC which has a sensitivity of 5 EPG of faeces, as previously described (Rinaldi *et al.*, 2014; Bellaw *et al.*, 2018).

### Faecal cultures

On days 0 and 70, pooled faecal samples were obtained using 10 g of faeces from each enrolled donkey and were incubated at 27°C for 7–10 days for larval development (MAFF, 1986). Third stage larvae were collected with the Baermann technique. One hundred larvae per culture were identified, based on the keys described by Cernea *et al.* (2008) and Kornas *et al.* (2009). If fewer than 100 larvae were present, all larvae were identified.

### Treatment

After the initial FEPGC was performed on day 0, all the donkeys were weighted to obtain the body weight (BW) using an electronic scale (Meini Bilance Srl, Fornacette (PI), Italy). These donkeys were administered IVM (Eqvalan® 1.87 % paste, Merial) at the recommended dose rate of 200 mcg/kg BW. A 6.42 gr syringe is useful for treatment of horses of weight up to 600 kg. On day 0, treated donkeys were observed for approximately 5 min after treatment to verify dose retention, as all treatments were given orally, and then periodically for three times in about three hours to record adverse reactions. Animals in the control group remained untreated and were kept in the trial until the end of the study.

### Data analysis

Individual FEPGCs and arithmetic means (AMs) of FEPGCs were determined at each sampling time (from day 0 to day 91) both for the treated and the control group. Differences between the AMs of FEPGCs in the two groups were compared by Student's t test and a P value  $\leq 0.05$  was considered statistically significant.

In accordance with guidelines recommended by Matthews and Burden (2013) for the control of common helminth infections in donkeys, on day 14 after treatment the efficacy of IVM was evaluated by individual and mean faecal egg count reduction tests (FECRTs) for the treatment group. The following formula was used:  $\text{FECRT (\%)} = \frac{\text{FEPGC before treatment} - \text{FEPGC after treatment}}{\text{FEPGC before treatment}} \times 100$ . According to methods proposed by the American Association of Equine Practitioners (AAEP) for parasite control in horses (Nielsen *et al.*, 2019), the results of FECRTs were interpreted as follows: FECRT  $>98\%$  = efficacy, FECRT between 95 and 98 % = suspected resistance, FECRT  $<95\%$  = resistance. To assess the persistence of efficacy of the treatment with IVM and its ERP in donkeys, individual and mean FECRTs were then scheduled at weekly intervals from day 21 till the end of the study (day 91). For the purpose of this study, the ERP was calculated by the method recommended by guidelines of the AAEP to assess the emergence of anthelmintic resistance (AR) to IVM in horses (Nielsen *et al.*, 2019). Thus, the ERP was calculated as the first week post-treatment when the FECRT falls below a cutoff value of 90 % efficacy (Nielsen *et al.*, 2019).

### Ethical Approval and/or Informed Consent

For this study, formal consent is not required. The research related to animals complied with all the relevant national regulations and institutional policies for the care and use of animals.

### Results

On day 0 of the study, all the 14 selected donkeys resulted to be coprologically positive for strongyle eggs and were classified as low (n=2), moderate (n=7), or heavy (n=5) shedders. Their FEPGCs ranged from 180 to 770 eggs in faeces of treated donkeys and

from 210 to 640 eggs in untreated ones with AMs of 446 and 385 eggs, respectively (Table I).

Neither adverse drug reactions nor other clinical signs were observed in any of the donkeys treated with IVM during the study period. Table 1 shows individual and mean FEPGCs with percent of efficacy of IVM as determined by results of FECRTs at each sampling time. Overall, IVM showed FECRT of 100 % and, thus, 100 % efficacy in each of the treated donkeys from day 14 to day 42 (6 weeks) post-treatment. Later, the efficacy of IVM started to slightly decrease but, nonetheless, the drug still continued to be highly effective (i.e. efficacy  $>95\%$ ) during four additional weeks. Mean FECRTs varied as follows: 97.7 % (individual FECRT range = from 97.9 to 100 %), 97.4 % (from 89.6 to 100 %), 97.5 % (from 94.8 to 100 %), and 96.7 % (from 93.2 to 99.2 %) on days 49, 56, 63, and 70 after treatment, respectively. Thereafter, IVM progressively lost its efficacy since the mean percent of FECRTs drastically dropped to 89.8 % (from 75 to 95.1 %), 80.9 % (from 64.6 to 93.9 %), and finally 70.8 % (from 38.8 to 85.4 %) on days 77, 84, and 91, respectively. On day 49, 2/10 of the treated donkeys were found coprologically positive and then the number of donkeys becoming coprologically positive slowly increased to 6/10 on day 56, 8/10 on day 63, and finally 10/10 from day 70 onwards. The ERP after treatment was determined to be 11 weeks, as the mean percent of FECRT decreased below the ERP-threshold chosen in the present study, i.e. a cutoff value of 90 % efficacy, on day 77 of faecal sampling.

All the donkeys of the untreated control group were constantly found coprologically positive for strongyle eggs throughout the study period, with individual FEPGCs ranging from 195 to 1575 eggs and mean FEPGCs ranging from 285 to 1043.7 eggs (Table I). No one of them showed clinical signs referable to intestinal strongylosis or to any other pathologic condition. Statistical analysis by Student's t test showed that differences between the AMs of FEPGCs in the treated group and those in the control group after treatment were extremely significant ( $P=0.0000$ ) at each sampling time.

Results of pre-treatment (day 0) and post-treatment (day 70) faecal cultures and morphological identification showed that only small strongyle larvae were present in samples from all the enrolled donkeys, both from those assigned to the treated group and from those assigned to the control group.

### Discussion

Results of our study provide information on the efficacy of IVM against small strongyle infection in donkeys. The Mini-FLOTAC technique in combination with Fill-FLOTAC was used for FEPGCs in the present study. This technique can be considered as the most accurate egg counting method nowadays available in Veterinary Medicine (Bosco *et al.* 2014) and, thus, it allowed an accurate interpretation of the results of FEPGCs which were very reliable. Our findings show that IVM efficacy was very high (100 %) against

Table 1. Efficacy of Ivermectin (200 mcg/kg BW) in donkeys naturally infected with small strongyles. Results are presented as individual and mean faecal egg per gram counts (FEPGCs) on treatment day (day 0) as well as individual FEPGC with faecal egg count reduction test (FECRT %) in the same cell, mean FEPGCs, and mean FECRTs in treated donkeys (I to X) examined at weekly intervals after treatment (days 14 to 91). In addition, individual and mean FEPGCs in untreated control donkeys (XI to XIV) at the same sampling times are also presented.

Days	Treated donkeys										Control donkeys						
	I	II	III	IV	V	VI	VII	VIII	IX	X	Mean FEPGCs	Mean FECRT	XI	XII	XIII	XIV	AM of FEPGCs
0	370	630	410	200	770	700	580	240	180	380	446	–	280	210	410	640	385
14	0	0	0	0	0	0	0	0	0	0	0 <sup>A</sup>	100%	365	280	460	400	376.2 <sup>A</sup>
	100%	100%	100%	100%	100%	100%	100%	100%	100%	100%							
21	0	0	0	0	0	0	0	0	0	0	0 <sup>B</sup>	100%	275	400	525	355	388.7 <sup>B</sup>
	100%	100%	100%	100%	100%	100%	100%	100%	100%	100%							
28	0	0	0	0	0	0	0	0	0	0	0 <sup>C</sup>	100%	465	355	525	485	457.5 <sup>C</sup>
	100%	100%	100%	100%	100%	100%	100%	100%	100%	100%							
35	0	0	0	0	0	0	0	0	0	0	0 <sup>D</sup>	100%	655	310	525	615	526.2 <sup>D</sup>
	100%	100%	100%	100%	100%	100%	100%	100%	100%	100%							
42	0	0	0	0	0	0	0	0	0	0	0 <sup>E</sup>	100%	205	335	325	275	285 <sup>E</sup>
	100%	100%	100%	100%	100%	100%	100%	100%	100%	100%							
49	5	0	0	0	0	0	0	5	0	0	1 <sup>F</sup>	99.7%	845	790	985	855	868.7 <sup>F</sup>
	98.6%	100%	100%	100%	100%	100%	100%	97.9%	100%	100%							
56	10	0	5	0	45	25	0	25	0	5	11.5 <sup>G</sup>	97.4%	505	520	770	660	613.7 <sup>G</sup>
	97.3%	100%	98.8%	100%	94.1%	96.4%	100%	89.6%	100%	98.7%							
63	5	5	10	0	40	25	5	5	0	15	11 <sup>H</sup>	97.5%	475	550	960	1010	748.7 <sup>H</sup>
	98.6%	99.2%	97.6%	100%	94.8%	96.4%	99.1%	97.9%	100%	96%							
70	25	5	5	5	35	20	15	15	5	15	14.5 <sup>I</sup>	96.7%	680	490	785	755	677.5 <sup>I</sup>
	93.2%	99.2%	98.8%	97.5%	95.4%	97.1%	97.4%	93.7%	97.2%	96%							
77	60	45	20	35	75	60	45	40	45	30	45.5 <sup>L</sup>	89.8%	360	390	195	300	311.2 <sup>L</sup>
	83.8%	92.8%	95.1%	82.5%	90.2%	91.4%	92.2%	83.3%	75%	92.1%							
84	80	150	25	35	140	145	85	85	55	50	85 <sup>M</sup>	80.9%	1575	1165	675	760	1043.7 <sup>M</sup>
	78.4%	76.2%	93.9%	82.5%	81.8%	79.3%	85.3%	64.6%	69.4%	86.8%							
91	195	195	60	40	160	125	175	125	110	115	130 <sup>N</sup>	70.8%	715	505	340	625	546.2 <sup>N</sup>
	47.3%	69%	85.4%	80%	79.2%	82.1%	69.8%	47.9%	38.8%	69.7%							

A, B, C, D, E, F, G, H, I, L, M, N Extremely statistically significant differences (P=0.0000)

Cyathostominae larvae in all orally treated donkeys. These findings are supported by the high FEPGCs constantly detected in untreated control donkeys which rule out any influence on the worms' burden in the examined donkeys caused by season or weather conditions occurred during the study period. The present findings are in agreement with those of other reports where an efficacy of 100 % was stated in donkeys at 14 days after administration of a dose of 200 mcg/kg of IVM both in oral (Buono *et al.*, 2018; Imam *et al.*, 2010; Arias *et al.*, 2013) and in injectable formulation (Seri *et al.*, 2005; Fangama *et al.*, 2013). Similar findings were also detected in horses (McFarlane *et al.*, 2010; Larsen *et al.*, 2011). Nevertheless, lower efficacy and complete AR of cyathostomins to IVM are important reasons for concern in horses (Canevar *et al.*, 2013; Molena *et al.*, 2018). Moreover, donkey breeders and equine veterinary practitioners working with donkeys have a reduced number of anthelmintics to use, which can lead to continuous and frequent use of a single drug, subjecting cyathostomins to higher selection pressure. This inappropriate approach is considered responsible for the rapid appearance of AR.

The control of small strongyles in donkeys depends largely on the routine administration of anthelmintic drugs at specific times of the year to remove adult parasites, to prevent or minimize contamination of the environment with eggs and larvae, and thereby to disrupt the seasonal cycle of transmission. Knowing the ERP of each anthelmintic drug is of pivotal importance for parasite control in equines, including donkeys, since anthelmintic treatments at intervals equal to or shorter than ERP can provide a selective advantage to resistant strongyle populations (Saeed *et al.*, 2019). Indeed, the frequent exposure of small strongyles to an individual anthelmintic may result not only in the elimination of susceptible adult worms but also in the lack of parasite stages in refugia (encysted larvae, free-living larval stages in environment) that escape the effects of the treatment, though they are still susceptible to the anthelmintic. The refugia subpopulation is not under selection for AR because it is not exposed to the drug at the time of treatment. This subpopulation of worms does not have genes for AR, as such it may dilute and delay the proliferation of those worm populations having resistance alleles and allow the effectiveness of an anthelmintic drug to be prolonged by providing a reservoir for drug-susceptible genes (Saeed *et al.*, 2019). Therefore, treatment intervals with any anthelmintic should be based on its ERP and a shortened ERP is the first indicator of the development of resistance to an anthelmintic drug (Nielsen *et al.*, 2019). Accurate knowledge of the ERP of an anthelmintic is also essential to assess the potential for shedding and contamination of eggs by each animal within any group of adult equines. For this purpose, indeed, individual fecal samples should be examined by FEPGC a minimum of 4 weeks beyond the ERP for the last drug used (Nielsen *et al.*, 2019). Unfortunately, ERP definitions have been reported exclusively for horses but researchers are not in agreement with a common threshold beyond which animals should be retreated, not even in horses. In fact, the criterion determining the ERP varies

between authors. Some authors have calculated the ERP as the first week after treatment when eggs reappear in the stool (Little *et al.*, 2013). Other authors have considered the ERP as the week after treatment when the AM of FEPGCs is equal to or greater than a fixed threshold such as 100 (Van Doorn *et al.*, 2012) or 200 (Mercier *et al.*, 2001) eggs. Unfortunately, using all these methods, results are highly biased by the pre-treatment FEPGC levels, since some imply that an anthelmintic must reduce the FEPGC of 100 % to be considered effective, whilst others do not take into consideration that low shedder donkeys can take longer to reach the fixed threshold or may never reach the threshold. A third group of authors have defined the ERP based on results of FECRTs. The ERP is thereby measured by performing FECRTs at weekly intervals and is calculated as the first week after treatment when the mean percent FECRT is lower than a predetermined cutoff level of 80 % (Tarigo-Martinie *et al.*, 2001) or 90 % (Larsen *et al.*, 2011) efficacy. In the present study, the ERP was considered as the week when a <90 % FECRT was observed. This definition was chosen among the different methods reported in literature to calculate ERP because we agree with other authors (Larsen *et al.*, 2011) that it represents a reasonable and reliable approach. Indeed, as the ERP is a measure of the efficacy of an anthelmintic during the weeks after treatment, this method is adapted to the drug under evaluation and sets the cutoff level approximately 10 % lower than the expected 99 – 100 % efficacy of IVM (Larsen *et al.*, 2011). The present study was performed for a 13-week period to determine the ERP of IVM in the treated donkey group as ERP rates of 9 – 13 weeks for IVM have been reported in horses (Nielsen *et al.*, 2019), and the ERP was determined to be 11 weeks. Thus, we found no evidence of shortened small strongyle ERP in donkeys as the ERP we achieved for IVM is consistent with guidelines of the AAEP for parasite control in horses. Moreover, our study with IVM indicates a much longer ERP for cyathostomins in donkeys than the range of ERP (6 – 8 weeks) usually reported in horses in the last ten years, when the drug results to be effective (Nielsen *et al.*, 2019). The present cyathostomin ERP for IVM in donkeys falls within the range of rates previously reported in other studies. One study relied on the first post-treatment positive coprological flotation and showed that the ERP after treatment with IVM plus Praziquantel was 2 and 3 months in a group of 6 European donkeys and 6 African donkeys kept in a zoological park in Spain, respectively (Arias *et al.*, 2013). Another study estimated the ERP based on FECRT calculation and reported that the ERP for IVM was 8 or 12 weeks in two groups of 6 farmed donkeys each in Italy (Buono *et al.*, 2018). Therefore, combining results of the present study with those of similar studies previously reported (Buono *et al.*, 2018; Arias *et al.*, 2013), it can be argued that the ERP after treatment with IVM in donkeys range from 8 to 12 weeks. This is very close to ERP rates (9 – 13 weeks) documented for IVM when the drug was first introduced in horses (Nielsen *et al.*, 2019). However, it is somewhat difficult to compare our results with those of other studies assessing the ERP after treatment with IVM in donkeys due to the lack of



a uniform method to calculate ERP. Moreover, additional reasons for different results may be due to level of parasite burden and age of donkeys as well as to season and weather conditions, since intestinal strongyles are known to reduce the egg shedding when environmental conditions are less favorable for their transmission.

## Conclusion

Small strongyles can be a threat to health and welfare of donkeys. The findings of the present study (i.e. 100 % efficacy in all treated donkeys on day 14 after treatment and ERP as long as 11 weeks) show that a single oral dose of IVM paste formulation administered at an estimated dose rate of 200 mcg/kg BW was highly effective and safe to control naturally acquired small strongyle infection in donkeys and ruled out any emerging resistance to IVM in the studied donkey group bred in Italy. This corroborates results of previous studies on efficacy of IVM and its ERP after treatment against small strongyles in donkeys. Therefore, our findings may be useful to veterinary practitioners and breeders as they show that IVM can be still considered as a valuable pharmacological tool to use for deworming programmes in donkeys. Given the increased number of reports of the emergence of AR worldwide (Tarigo-Martinie *et al.*, 2001; Kaplan and Nielsen, 2010; Van Doorn *et al.*, 2012; Canever *et al.*, 2013; Little *et al.*, 2013; Matthews *et al.*, 2013; Molena *et al.*, 2018), including cases in donkeys (Trawford *et al.*, 2005; Lawson *et al.*, 2015; Buono *et al.*, 2018), and mostly given the lack of new anthelmintics, our findings are a strong reminder that all strategies needed to prevent and minimize the development of AR to IVM in donkeys must be undertaken. Furthermore, since ERP may be an early indicator of suspected AR, it is advisable that researchers and practicing veterinarians reach an agreement on the definition of ERP in donkeys.

## Conflict of Interest

Authors state no conflict of interest.

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