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Journal of Threatened Taxa

10.11609/jott.2023.15.10.23931-24150
www.threatenedtaxa.org

26 October 2023 (Online & Print)
15(10): 23931–24150
ISSN 0974-7907 (Online)
ISSN 0974-7893 (Print)

Open Access

ISSN 0974-7907 (Online); ISSN 0974-7893 (Print)

Publisher
Wildlife Information Liaison Development Society
www.wild.zooreach.org

Host
Zoo Outreach Organization
www.zooreach.org

43/2 Varadarajulu Nagar, 5th Street West, Ganapathy, Coimbatore, Tamil Nadu 641006, India
Registered Office: 3A2 Varadarajulu Nagar, FCI Road, Ganapathy, Coimbatore, Tamil Nadu 641006, India
Ph: +91 9385339863 | www.threatenedtaxa.org
Email: sanjay@threatenedtaxa.org

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Cover: Orange Oakleaf *Kallima inachus* with colour pencils and watercolor wash by Elakshi Mahika Molur adapted from a workshop by Lenin Raj.

Journal of Threatened Taxa | www.threatenedtaxa.org | 26 October 2023 | 15(10): 24079–24085

ISSN 0974-7907 (Online) | ISSN 0974-7893 (Print)

https://doi.org/10.11609/jott.8236.15.10.24079-24085

#8236 | Received 24 October 2022 | Final received 07 June 2023 | Finally accepted 27 August 2023

OPEN ACCESS

COMMUNICATION

Efficacy of levamisole and oxyclozanide treatment on gastrointestinal nematodes of ungulates at the Central Zoo, Nepal

Pratik Kiju [1], Amir Sadaula [2], Parbat Jung Thapa [3] & Chiranjibi Prasad Pokheral [4]

[1] B.V.Sc. and A.H, Institute of Agriculture and Animal Science, Tribhuvan University, Paklihawa Campus, Siddharthanagar, 32900, Nepal.
[2,3,4] National Trust for Nature Conservation, P.O. Box 3712, Khumaltar, Lalitpur, 44600, Nepal.
[1] pratikkiju2@gmail.com (corresponding author), [2] naturalamir@gmail.com, [3] parbatjungthapa26@gmail.com, [4] cppokharel@ntnc.org.np

Abstract: The efficacy evaluation of levamisole and oxyclozanide treatment on the gastrointestinal nematodes of ungulates at the central zoo, Nepal was carried out from June—August 2021. A total of 40 fecal samples were collected from 10 species of ungulates from the central zoo for determining the efficacy of the anthelmintic given at day 0 of pretreatment and post-treatment analysis on day 07 and day 14. The concentration method (floatation concentration) was used for the microscopic examination of eggs, and quantitative examination (EPG) of nematode eggs was carried out with the help of modified McMaster slides. The identification was done using an optic micrometer and fecal egg culture. Anthelmintic resistance status was evaluated by the Fecal Egg Count Reduction Technique (FECRT) based on the method described by the World Association for the Advancement of Veterinary Parasitology (WAAVP) guidelines and with the Bayesian hierarchical model. Out of 40 samples, nematode prevalence was found to be 68%, in which single infection was detected in 48% and double infection in 52%. The efficacy of Zanide L forte (levamisole-0.75 g and oxyclozanide-1.00 g) was found to be 85% (UI 80-89) at day 07 and 89% (UI 85-92) at day 14 by using Hierarchical Modelling of Fecal Egg count based on 'eggCounts-2.3 on R version 3.6.1 and 86% (CI 61.51–95%) at day 07 and 90% (CI 74.18–95%) at day 14 by WAAVP guidelines. This study represents the first documented case of ineffectiveness of anthelmintic treatment resulting in anthelmintic resistance in the central zoo. Thus, there is a requirement for a suitable and efficacious anthelmintic program.

Keywords: Anthelminthic, captive wild ungulates, efficacy, FECRT %, nematodes.

Editor: B.R. Latha, Madras Veterinary College Chennai, India.

Date of publication: 26 October 2023 (online & print)

Citation: Kiju, P., A. Sadaula, P.J. Thapa & C.P. Pokheral (2023). Efficacy of levamisole and oxyclozanide treatment on gastrointestinal nematodes of ungulates at the Central Zoo, Nepal. *Journal of Threatened Taxa* 15(10): 24079–24085. https://doi.org/10.11609/jott.8236.15.10.24079-24085

Funding: None.

Competing interests: The authors declare no competing interests.

Author details: Pratik Kiju is a veterinarian and currently pursuing an Erasmus Mundus Joint master's degree in Infectious Disease and One Health program. He is also working as a research assistant in the Center for One Health Research and Promotion. Amir Sadaula is a wildlife veterinarian at the National Trust for Nature Conservation (NTNC) - Biodiversity Conservation Center and has more than a decade of experience in treating wild animals all over Nepal. Parbat Jung Thapa is a zoo and wildlife veterinarian at the National Trust for Nature Conservation (NTNC) - Central Zoo Jwalakhel Nepal. Chiranjibi Prasad Pokheral is the Project Manager at National Trust for Nature Conservation – Central Zoo, Nepal.

Author contributions: PK—conceptualization, lab works, manuscript writing, editing, data compilation and analysis. AS, PJT & CPP—conceptualization, field methodology, review and editing.

Acknowledgements: My utmost gratitude towards Shambhu Shah, PhD, member secretary of the Internship Advisory Committee 2021 and prof. Hari Bahadur Rana for his commendable suggestions and guidance. My humble thanks to Dr. Subash Rimal, Dr. Persia Carrol Thapa, Dr. Swochhal Prakash Shrestha, Prof. Hari Bahadur Rana and Ms. Shristy Buddha Magar for their continuous guidance and support. Similarly, I would also like to express my appreciation towards zoo keepers; Mr. Ram Bahadur Shrestha and Mr. Kishor Bista who assisted me in the sample collection.

INTRODUCTION

Zoos are centers in which wild animals are kept for aesthetic, educational and conservation purposes (Thawait et al. 2014). The Central Zoo of Nepal was established in 1932. Ungulates cover the major population of the zoo animals in the Central Zoo, which includes Spotted Deer, Sambar Deer, Four-horned Antelopes, Himalayan Goral, Blue Bull, Barking Deer, One-horned Rhinoceros, Wild Boar, and Wild Water Buffalo.

Parasitic infection is one of the causes of morbidity and mortality in captivity, along with improper diet and poor husbandry practice (Singh et al. 2006; Mir et al. 2016; Kolapo & Jegede 2017). In the wild, animals generally have a natural resistance to parasites due to their diverse habitat and food, but due to the confinement and change in living conditions, captive wild animals might be more susceptible to many diseases caused by viruses, bacteria, rickettsia and parasites (Goossens et al. 2005; Thawait et al. 2014).

Nematodes are generalist parasites of a wide range of hosts (Walker & Morgan 2014). Generally, ungulates are infected by nematodes by ingesting infective larvae from the pasture, and in some species, larvae also penetrate through the skin (Walker & Morgan 2014). Zoo ruminants are particularly vulnerable to gastrointestinal nematodes due to high stocking density without the possibility of pasture rotation, leading the pasture to heavy exposure to infective nematode larvae or eggs (Goossens et al. 2006).

The epidemiology of nematodosis in domestic ruminants is well studied, but there are limited studies and reports that directly address parasite control programs in captive wild ruminants (Isaza et al. 1990; Goossens et al. 2006). Regular parasite load examination, anthelmintic efficacy, and resistance evaluation are not frequently done in many zoological gardens and parks. Furthermore, there is no published data on the efficacy of anthelmintics in captive wild ungulates in Nepal. Fecal egg count reduction (FECR) is the simplest, most effective, and most widely used method to evaluate the efficacy of anthelmintics (Coles et al. 1992; Cabaret & Berrag 2004) and has been used in captive wild animals (Nalubamba & Mudenda 2012; Pawar et al. 2020). Anthelmintic resistance is becoming a threatening issue in every livestock class and in every anthelmintic class globally (Kaplan 2004). Idiosyncrasies are also one of the major factors that contribute to the efficacy of anthelmintics on different wild animals on certain occasions (Ortiz et al. 2001). Thus, this present study will aim to contribute to establishing the prevalence of gastrointestinal nematode parasites and the anthelminthic efficacy of oxyclozanide and levamisole administration in the ungulates in Central Zoo, Nepal.

MATERIALS AND METHODS

Time and place of research

The research was carried out at the Central Zoo from 19 June 2021 and ended on 19 August 2021.

Sample collection

Pooled fecal samples were collected from the fresh feces of the ungulates early in the morning from different spots of the enclosure with the help of zoo keepers. The fresh sample was randomly taken on the basis of the number of ungulates in each enclosure. The sample was labelled accordingly and the method was followed as per Soulsby (2005). The sample of around 15 gm was kept in an airtight plastic zipper bag and transported in a cool box to the laboratory at the Department of Animal Science, Institute of Agriculture and Animal Science (IAAS), Tribhuvan University. Macroscopic examination of the helminths, if present, was done from the feces. The concentration method (floatation concentration) was used for the microscopic examination of eggs, and quantitative examination of eggs was carried out with the help of modified McMaster slides. The size of the eggs was measured using an optic micrometer. The sample containing more than one species of nematodes were kept as mixed infection sample and while samples with only one species were labelled as single infection.

For further confirmation, the fecal culture method using the 'Falcon tube method of fecal culture' for nematode larva was also carried out in accordance with the method provided by Soulsby (2005) and Zajac & Conboy (2012). One gram of feces was wrapped up in a blotting paper making a pouch. In a falcon tube, water was placed up to the circular rim at the distal end of the tube. The pouch was attached to the distal interior end of the Falcon tube using a long piece of the same blotting paper. The tube was now made airtight and left in a dark place for up to 7–8 days for incubation of nematodes larvae. After about 7–8 days, the blotting paper and the sample were removed. Twenty microliters of water was transferred to a glass slide with the help of a micropipette, which was then examined for the larvae of nematode under a microscope.

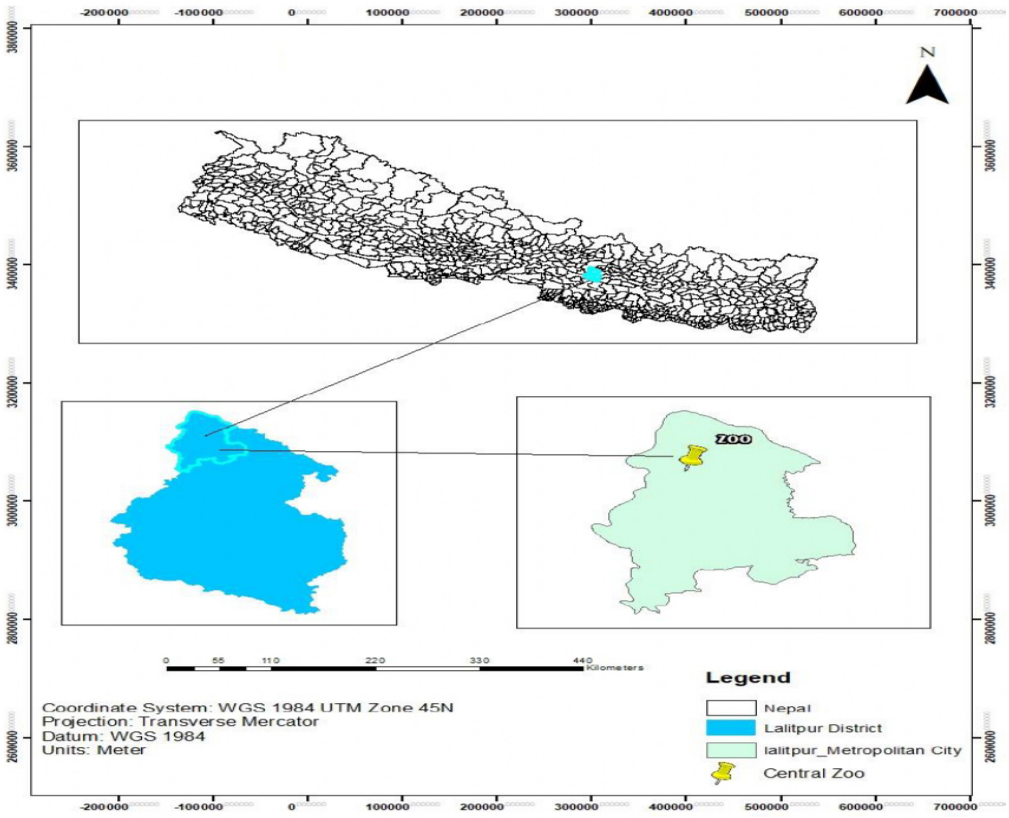

Figure 1. The location of the Central Zoo, Nepal.

Assessment of drug efficacy and anthelmintic resistance

Anthelmintic resistance status was evaluated by FECRT based on the method described by the World Association for the Advancement of Veterinary Parasitology (WAAVP) guidelines (Coles et al. 1992; McKenna 1994; Storey 2015). The FECRT has been the most recommended method so far being broadly utilized for field or research studies (Coles et al. 1992). FECRT assesses the anthelmintic resistance of a given compound by comparing worm egg counts from animals before and after treatment.

All the individuals who were positive for nematode eggs were subjected to EPG on day 0 before treatment, day 07 and day 14 after treatment.

FECR (%) = 100 %*(1-[T2/T1])

Here, T1 is the pre-treatment EPG

T2 is post-treatment EPG.

(Coles et al. 1992; McKenna 1994; Cabaret & Berrag 2004; Pawar et al. 2020)

Resistance is present when two criteria are met:

I. The percentage reduction in egg count is less than 95%.

II. The lower limit of its 95% confidence interval is equal or below 90%.

Treatment

At 10 mg per kg, ZANIDE- L Forte Bolus (Levamisole Hydrochloride BP 0.75 g and Oxyclozanide BP (Vet) 1 g) was given to the ungulates. There was a specific deworming practice at the zoo of changing the anthelminthic drug types regularly at the interval of 4-months. Ivermectin was used 4-months prior to this research and four months before Ivermectin, albendazole was used. So, this time it was the turn of ZANIDE- L Forte Bolus (Levamisole Hydrochloride BP 0.75 g and Oxyclozanide BP (Vet) 1 g). So, in accordance with that schedule, ZANIDE-L Forte was used. This research showed the deworming status of levamisole and oxyclozanide in the nematodes. After the determination of pre-anthelmintic EPG at Day 0, the post-anthelmintic EPG at Day 07 and Day 14 were also determined using the same procedure as mentioned earlier. The mean EPG of Day 7 and Day 14 is used to determine the efficacy of the respective days.

Data analysis

Fecal egg count in EPG is determined from a sample taken on day 0 prior to treatment with an anthelmintic drug, as well as on days 07 and 14 following treatment. The data were entered into a spreadsheet and

imported into IBM SPSS version 25 to test for statistical significance.

Egg count data on FECRT was analyzed for fecal egg count reduction (%FECR) using 'eggCounts-2.3' on R version 3.6.1. (Young et al. 2000; Torgerson et al. 2014; Wang et al. 2018)

For analysis of drug efficacies, a 'z'-test (Sample size > 30) was done to analyze the significance of the pretest and the posttest group on different days. Similarly, to determine the association within different groups, a chi-square test was done.

A p-value of less than 0.05 at 95% CI was considered statistically significant. Finally, tables and charts were used to present the results generated from SPSS and the graphical presentation was completed in MS Excel 2016.

RESULTS

During the study, out of 40 samples examined by the floatation concentration method, 27 samples were positive for the presence of nematode eggs as given in Table 1. Thus, the prevalence was found to be 67.5%. Single parasitic infection was detected in 13 (48.15%) and mixed parasitic infection in 14 (51.85%) samples. The intensity of eggs belonging to eight different types of nematodes, i.e., *Bunostomum* spp., *Strongyloides* spp., *Trichuris* spp., *Ostertagia* spp., *Haemonchus* spp., *Capillaria* spp., *Ascaris* spp., and *Oesophagostomum* spp., varied from + to +++ in the study. The eggs were identified on the basis of their sizes using the calibrated optic micrometer (Soulsby 2005). Further confirmation was done by the fecal culture method with reference to Soulsby (2005) and Zajac & Conboy (2012).

The *Strongyloides* spp. were major nematode eggs seen during the study with 44.44% prevalence, followed by *Bunostomum* spp. 22.22% and *Trichuris* spp., *Ostertagia* spp., *Haemonchus* spp., *Capillaria* spp., *Ascaris* spp., *Oesophagostomum* spp., with 5.56% each as shown in Figure 2.

The efficacy of Zanide L forte (levamisole-0.75 g and oxyclozanide-1.00 g) was found to be 85.3% (CI 80.4–89) at day 07 and 89.2% (CI 85–92.3) at day 14 by using hierarchical modelling of fecal egg count based on 'eggcounts-2.3 on R version 3.6.1 and 85.47% (CI 61.51–94.48%) at day 07 and 89.67% (CI 74.18–95.61%) at day 14 by WAAVP guidelines.

Since, the P value is less than 0.05 in both the days, the pretest at day 0 and post test data at day 07 and day 14 are statistically significant respectively. So, we reject the null hypothesis, i.e., there is a statistical difference between the mean of the two data sets. The anthelminthic treatment at day 0 has a significant effect on the EPG count of day 7 and day 14.

DISCUSSION

This study shows the overall prevalence of 67.5% of nematode infection in the total of 40 samples taken,

Table 1. Prevalence of gastro intestinal nematode infection in captive ungulates of the Central Zoo.

	Ungulate species	No. of sample collected	No of sample positive for nematodes	Sample demonstrating single infection	Sample demonstrating mixed infection	Types of infection
1	Spotted Deer *Axis axis*	12	6(50%)	3	3	*Bunostomum* spp., *Strongyloides* spp., *Trichuris* spp.
2	Blue Bull *Boselaphus tragacamelus*	4	2(50%)	0	2	*Ostertagia* spp., *Strongyoides* spp.
3	Black Buck *Antelope cervicapra*	2	2(100%)	0	2	*Bunostomum* spp., *Haemonchus* spp., *Strongyloides* spp.
4	Barking Deer *Muntiacus muntjak*	9	7(77.78%)	4	3	*Bunostomum* spp., *Trichuris* spp., *Strongyloides* spp.
5	Sambar Deer *Rusa unicolor*	1	0	0	0	-
6	Himalayan Goral *Naemorhedus goral*	2	2(100%)	1	1	*Trichuris* spp., *Strongyloides* spp.
7	Four-horned Antelop *Tetracerus quadricornis*	1	1(100%)	1	0	*Strongyloides* spp.
8	Wild Boar *Sus scrofa*	2	2(100%)	0	2	*Ascaris* spp., *Oesophagostomum* spp.
9	Wild Water Buffalo *Bubalus arnee*	4	3(75%)	2	1	*Bunostomum* spp., *Strongyloides* spp., *Capillaria* spp.
10	One-horned Rhino *Rhinocerous unicornis*	3	2(66.67%)	3	0	*Strongyloides* spp.
	Total	40	27(67.50%)	13(48.15%)	14(51.85%)	

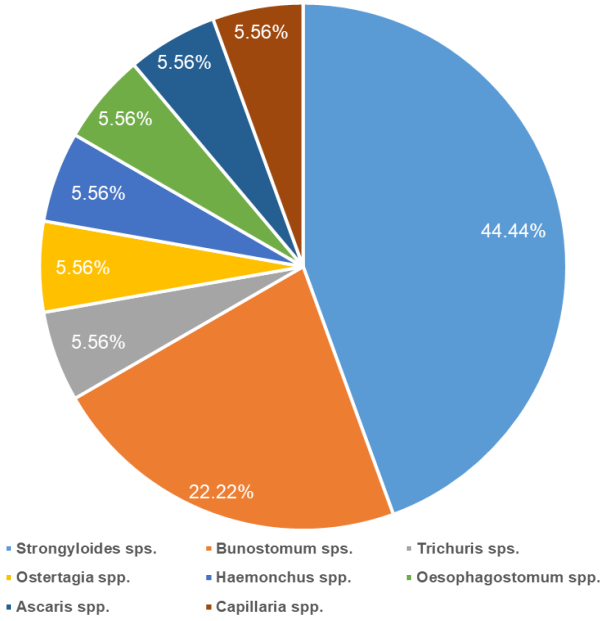

Figure 2. Overall prevalence of nematode species found in ungulates of the Central Zoo.

in which single infection was detected in 48.15% and double in 51.85%. The findings of Pun (2014) were similar to the research conducted, i.e., prevalence of 59% of parasite infection in the central zoo, Kathmandu. The present findings in respect to overall prevalence in captive herbivores agreed with Bir Moti Bagh Mini Zoo, India (68%) (Mir et al. 2016), Dehiwala National Zoological Gardens, Sri Lanka (62.9%) (Aviruppola et al. 2016), Ljubljana Zoo, Slovenia (61%) (Kvapil et al. 2017), Rangpur Recreational Garden and Zoo, Bangladesh (60%) (Khatun et al. 2014) but disagreed with research at the Zoological gardens of Malaysia (45.7% of ungulates) (Lim et al. 2008), Maharajbag Zoo, Nagpur (50%) (Borghare et al. 2009), the Antwerp Zoo and the Animal Park Planckendael, Belgium (36.5%) (Goossens et al. 2005), and Mahendra Choudhury Zoological Park, Chhatbir, Punjab (25.17%) (Singh et al. 2006).

Strongyloides spp. (44.44%) were the major nematodes detected in the study, followed by *Bunostomum* spp. (22.22%) and *Capillaria* spp., *Haemonchus* spp., *Trichuris* spp., *Oesophagostomum* spp., *Ascaris* spp., and *Oestertagia* spp., were found at 5.65% each. Nematodes were the group of concern in this research because the majority of studies reported a null prevalence of parasitic infection with trematode and cestode (Atanaskova et al. 2011; Pun 2014; Mir et al. 2016; Pawar et al. 2020;). Cestodes and trematodes need intermediate hosts and are less likely to accumulate in captive and enclosed ecosystems (Atanaskova et al. 2011). On the contrary, nematodes are one of the most important veterinary helminths that have a negative impact on wildlife health as well as conservation ecology (Goossens et al. 2006; Singh et al. 2006).

Table 2. Nematode species identified from the size of their eggs.

	Nematode Species	Size of Egg	Reference value Soulsby (2005).
1	*Strongyloides* spp.	47 by 20 µm	40–60 by 20–25 µm
2	*Bunostomum* spp.	84 by 49 µm	79–97 by 47–50 µm
3	*Trichuris* spp.	67 by 34 µm	68–75 by 36–40 µm
4	*Haemonchus* spp.	78 by 36 µm	70–85 by 41–48 µm
5	*Ascaris* spp.	59 by 41 µm	50–75 by 40–50 µm
6	*Oestartagia* spp.	65 by 35 µm	60–85 by 40–45 µm
7	*Capillaria*.spp.	42 by 25 µm	45–50 by 22–25 µm
8	*Oesophagostomum* spp.	35 by 63 µm	35-45 by 60–80 µm

Many nematode parasites of veterinary importance have a huge genetic diversity and features that favor the development of anthelmintic resistance (Kaplan 2004). Similarly, the author has also stated that anthelmintic resistance has been reported in every anthelmintic class.

The present study reports the baseline study of the effectiveness of the levamisole and oxyclozanide treatment on the nematodes of the captive ungulates of the Central Zoo. The present study agrees with WAAVP guidelines (Coles et al. 1992) for the diagnosis of anthelmintic resistance without using a control group (pretreatment mean was used for comparison) and similar studies were also conducted by Young et al. (2000), Goossens et al. (2006), and Pawar et al. (2020).

The result (<90% FECR and lower CI <95%) indicated presence of anthelmintic resistance (Coles et al. 1992; McKenna 1994). Similar results were obtained from captive wild impala in Zambia, in which the efficacy using FECR % was around 90% showing low efficacy and suggesting anthelmintic failure (Nalubamba & Mudenda 2012).

The failure of an anthelmintic could be the result of resistance, either from the survival of existing nematodes or the establishment of a new infection. The unavailability of the correct dose for the specific wild captive animals with an improper route of anthelmintic (causing more wastage and low dosage) (Nalubamba & Mudenda 2012) and idiosyncrasies (Ortiz et al. 2001) may have contributed to the development of the anthelmintic resistance in the current study. Additionally, ZANIDE-L Forte Bolus (Levamisole Hydrochloride BP 0.75 gm and Oxyclozanide BP (Vet) 1 gm is not a specific drug for nematodes, especially the oxyclozanide may not be

Table 3. Egg per gram (EPG) counts and FECR% (Bayesian hierarchical model) of captive wild ungulates from Nepal's central zoo treated with Zanide-L forte* at 10mg/kg body weight.

	Ungulate species	No. of sample taken	EPG Day 0	EPG Day 7	EPG Day 14	FECR Day 7 UI	FECR Day 14 UI
1	Spotted Deer *Axis axis*	12	1050	150	150	82.1% (51%.9–95%)	82.1% (51%.9–95%)
2	Blue Bull *Boselaphus tragacamelus*	4	7950	1250	650	83.4% (75.2–89.3)	91.2% (85.3–95.1)
3	Black Buck *Antilope cervicapra*	2	4150	350	250	90.5% (81.5–95.7)	93% (85.3–97.3)
4	Barking Deer *Muntiacus muntjak*	9	1500	200	300	84.3% (62.1–94.9)	77.5 (51.4–91.1)
5	Sambar Deer *Rusa unicolor*	1	-	-	-	-	-
6	Himalayan Ghoral *Naemorhedus goral*	2	1400	250	200	78.9% (52.1–92.1)	82.6 (57.5–94.2)
7	Four-horned Antelop *Tetracerus quadricornis*	1	500	50	0	81.6% (24.7–97.77)	92.1% (48.6–99.7)
8	Wild Boar *Sus scrofa*	2	700	200	200	64.9% (16.3–88.7)	65% (16.6%–89.2%)
9	Wild Water Buffalo *Bubalus arnee*	4	450	100	50	69.7% (11.1–93.8)	80.07% (26.9–97.5)
10	One-horned Rhino *Rhinocerous unicornis*	3	200	50	50	56.3% (41.6–94.3)	56.3% (41.6–94.3)
	Total	40	17900 ± 2527.81	2600 ± 373.14	1850 ± 181.04	85.3% (80.4–89)	89.2% (85–92.3)

Table 4. 'Z' test two sample means for day 0 and day 7.

	Sample size	Treatment	Pre-treatment Day 0 (Mean EPG ± S.E)	Post- treatment Day 07 (Mean EPG ± S.E)	'z' value	p value
1	40	Levamisole and Oxyclozanide	17900± 2527.81	2600 ± 373.14	2.520991	0.012

Table 5. 'Z' test two sample for means of Day 0 and Day 14.

	Sample size	Treatment	Pre-treatment Day 0 (Mean EPG ± S.E)	Post- treatment Day 14 (Mean EPG ± S.E)	'z' value	p value
1	40	Levamisole and Oxyclozanide	17900± 2527.81	1850 ± 194.36	2.654587	0.007

effective to the extent required. So, the necessity in this case is to change the drugs used for the rotation, based on the infection that is prevalent.

CONCLUSION

Infection with nematodes is of major veterinary importance. Frequent, unnecessary, and under-dosing of anthelmintics has given rise to a major problem of anthelmintic resistance in animals. The serious problem of anthelmintic resistance is based on the fact that levels of resistance can increase rapidly and the development of new classes of drugs is less. The efficacy of levamisole and oxyclozanide is found to be less than 90% with a lower confidence limit of 95% confidence level less than 90% suggesting the presence of resistance of gastrointestinal nematodes against the anthelmintic at the Central Zoo. This study is the first documentation of the efficacy of the anthelmintic used in the captive wild animal setting, in the Central Zoo, Kathmandu. The low efficacy of the anthelmintic is a concerning factor that requires proper nutrition, sanitation, and periodic deworming strictly based on advanced scientific strategies with periodic checks on anthelmintic resistance, which will aid in combating the serious issue of anthelmintic resistance.

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NAAS rating (India) 5.64

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Journal of Threatened Taxa

www.threatenedtaxa.org

ISSN 0974-7907 (Online) | ISSN 0974-7893 (Print)

October 2023 | Vol. 15 | No. 10 | Pages: 23931–24150
Date of Publication: 26 October 2023 (Online & Print)
DOI: 10.11609/jott.2023.15.10.23931-24150

Publisher & Host

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