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Research Article

In vivo and *In vitro* Efficacy of Albendazole Against Canine Ancylostomosis: A Possible Presence of Anthelmintic Resistance in Nigerian Local Breed of Dogs

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Abstract

Faecal Egg Count Reduction Test (FECRT) and Egg Hatch Assay (EHA) were used to evaluate the efficacy of albendazole (ALB) in the treatment of canine ancylostomosis in naturally infected Nigerian local dogs. The FECRT was determined in 30 naturally infected dogs that were randomly assigned into 4 groups (A-D). Groups, A (n = 8), B (n = 8) and C (n = 8) were treated with 2.5% ALB per day for one, two and three consecutive days respectively while group, D (n = 6) served as the untreated control. The EHA was conducted using *Ancylostoma caninum* eggs harvested from group A before and at 10 day post ALB treatment (PT). The ALB treatment produced 84% reduction of the pre-treatment FEC of dogs in groups A, 93% in group B and 98% in group C following the FECRT. The result of the EHA showed that ALB had LC_{50} values of 0.343 and 1.36 μg mL^{-1} on the worm eggs collected before and at day 10 PT. The data obtained in both the FECRT and EHA suggest a possible presence of ALB resistant hookworms in the study area. The study also showed that the EHA could reasonably determine the efficacy of albendazole against ancylostomosis in the dogs.

Key words: Albendazole, efficacy, *Ancylostoma caninum*, Nigerian dogs, anthelmintic resistance

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Data Availability: All relevant data are within the paper and its supporting information files.

INTRODUCTION

Ancylostomosis is a widespread and very important parasitic disease of dogs, particularly in the tropical and subtropical areas (Bethony *et al*., 2006; Peregrine, 2014). The disease is caused mainly by *Ancylostoma caninum* and to a lesser extent, *A. braziliense*; the former being the more common and pathogenic of the two species. Control of the disease in dogs relies mainly on periodic administration of one or a combination of anthelmintics belonging the following classes, namely, benzimidazole (e.g., albendazole, mebendazole, fenbendazole, febantel, oxfendazole), tetrahydropyrimidines (e.g., Pyrantel pamoate) and avermectin (e.g., Ivomec). Among these anthelmintic classes, the benzimidazoles, especially albendazole are among the most frequently used anthelmintics against hookworms in dogs and man, especially in developing countries probably due to their affordability and availability (Stepek *et al*., 2006; WHO., 2008). However, despite the widespread availability of these affordable and supposedly efficacious anthelmintics, *A. caninum* infection still remains a common and serious problem in dogs, making frequent anthelmintic treatment a routine in dog keeping. One possible outcome of such frequent treatment with anthelmintics among other things is that it could lead to the development of anthelmintic resistance (Shalaby, 2013). A guideline by the European Scientific Counsel on Companion Animal Parasites (ESCCAP) on worm control in dogs and cats stated that the traditional anthelmintic treatment of dogs would always have many parasite stages outside the final host that are unselected for resistance by treatment but if the frequency of treatments increases, then this could increase the selection pressure for resistance (ESCCAP., 2010).

Although, anthelmintic resistance in companion animals is not a widely recognized issue as it is in livestock, reports are beginning to emerge of their existence in dogs (Kopp *et al*., 2008; Lalchhandama, 2010). There are also other reports of reduced efficacy of Albendazole along with other benzimidazoles such as mebendazole, against hookworms of humans (Adugna *et al*., 2007; Soukhathammavong *et al*., 2012). It was also noted that only few proven cases of anthelmintic resistance in dogs exist and that the Faecal Egg Count Reduction Test (FECRT) remains the only way of detecting anthelmintic resistance in dogs (ESCCAP., 2010). Although the FECRT can be used with any available anthelmintic for detecting resistance, it is nevertheless, limited by the cost of its application and lack of sensitivity when less than 25% of the helminth population carries resistance gene (Areskog *et al*., 2013). It is therefore, desirable that more sensitive and cost effective *in vitro* tests are developed to allow continued monitoring of efficacy of anthelmintics in dogs. The egg hatch assay on the other hand is comparatively inexpensive, easy and a quick procedure. It also has the benefit to remove the role of host variation as basis of experimental error. However, the test has only been developed and used to identify resistant nematodes in livestock other than dogs.

Given the pathogenesis and zoonotic potential of hookworm disease in dogs as well as the widespread use of albendazole in treating hookworm disease in the study area, there is need to monitor on a regular basis the effectiveness of control programmes. Therefore, this study was designed to determine the efficacy of albendazole, a commonly used anthelmintic drug in the study area, against *A. caninum* using both *in vivo* and *in vitro* models.

MATERIALS AND METHODS

Study area and study population: The study was carried out in Nsukka area of Enugu State, Nigeria. Thirty male Nigerian local breed of dogs aged 6-7 months and weighing between 6.5 and 7.2 kg were used in the study. The dogs were purchased from dog markets around Nsukka and only those with up to 2000 hookworm eggs per gram of faeces were included in the study.

Study design: The *in vivo* Faecal Egg Count Reduction Test (FECRT) and *in vitro* Egg Hatch Assay (EHA) were used to determine the efficacy of albendazole. The studies were carried out between April and June, 2015.

Faecal Egg Count Reduction Test (FERCT): Faecal Egg Counts (FEC) were performed on faecal samples obtained from the dogs prior to anthelmintic treatment using the modified McMaster technique and the pre-treatment FEC determined. Thereafter, the dogs were evenly assigned into four groups, namely, A, B, C and D on the basis of their pre-treatment FEC (PFEC). The results of the pre-treatment FEC were then used to compute the group arithmetic mean pre-treatment FEC. Groups, A (n = 8), B (n = 8) and C (n = 8) were treated with 2.5% albendazole each day (Shanuzole®, Lagos, Nigeria) for a period of one, two and three consecutive days respectively while group, D (n = 6) served as the untreated control. Another FEC was carried out on the dog 10 days after the last treatment of each group and the arithmetic mean post-treatment FEC for each group

determined. The Faecal Egg Count Reduction (FECR) was thereafter determined using the formula:

$$FECR = 100 \times \left[1 - \left(\frac{T2}{T1}\right)\left(\frac{C1}{C2}\right)\right]$$

(Dash *et al*., 1988), where T1 and T2, respectively represent mean pre and post-treatment FEC of a treated group and C1 and C2 the mean pre and post-treatment FEC of the untreated control group, respectively. Any anthelmintic which did not produce more than 95% reduction of the pre-treatment FEC was considered ineffective (Coles *et al*., 1992). The experimental protocol used in this study was approved by the University of Nigeria Senate Committee on the care and ethical use of laboratory and experimental animals for medical and applied research and the procedures contributing to the work complied with the ethical conditions governing the use and conduct of experiments with life animals.

Egg Hatch Assay (EHA): Egg hatch assay was carried out using the methods of Coles *et al*. (1992) with minor modifications. The test was conducted on fresh hookworm eggs obtained from the faeces of dogs in group A, before and 10 days after treatment with albendazole. One mL (25 mg mL^{-1}) of 2.5% albendazole (Shanuzole®, Lagos, Nigeria) was reconstituted in 500 mL de-ionized water to get a stock solution (conc. 50 μg mL^{-1}). The stock solution was used to prepare twelve different concentrations of albendazole (0.00076-12.5 μg mL^{-1}) using 0.1% NaCl (prepared with de-ionized water) as diluents, by two fold serial dilution (Table 1 and 2) to enable the calculation of the dose required to prevent 50% of the viable eggs from hatching (LC_{50}). Four hundred and fifty microliter of the different concentrations of albendazole was added to 12 different wells of a 24-well flat bottomed plate (Dynatech Immulon) while, the control wells received only the diluents (0.1% NaCl). Fifty microlitre, of worm egg suspension containing approximately 100 eggs were taken in each of the experimental and control wells. The plates were incubated at 27°C for 48 h. After incubation, a little drop of Lugol's iodine was added into each well. The remaining eggs (dead and embryonated) and hatched out larvae were counted. All experiments were undertaken in triplicate on three separate occasions (n = 9). The percentage inhibition of egg hatching was calculated using the formula adopted from Coles *et al*. (1992) and modified by Ademola and Eloff (2011) as follows:

Table 1: Percent egg hatch at different concentrations of albendazole before treatment with albendazole @ 25 mg kg^{-1} b.wt.

Well No.	Hatching (%)	Probit (hatching)	Conc of albendazole (μg mL^{-1})
1	1.8	2.95	20
2	3.3	3.12	10
3	3.7	3.25	5
4	5.9	3.45	2.5
5	12.8	3.87	1.25
6	22.4	4.23	0.625
7	51.8	5.05	0.3125
8	68.0	5.47	0.156
9	86.8	6.13	0.078
10	92.1	6.41	0.039
11	96.4	6.75	0.0195
12	98.6	7.33	0.0097

LC_{50} : 0.303 μg mL^{-1} (Ineffective: Resistance suspected)

Table 2: Percent egg hatch at different concentrations of albendazole after treatment with albendazole @ 25 mg kg^{-1} b.wt.

Well No.	Hatching (%)	Probit (hatching)	Conc of albendazole (μg mL^{-1})
1	1.00	2.67	20
2	11.11	3.77	10
3	14.29	3.92	5
4	41.67	4.8	2.5
5	68.18	5.47	1.25
6	94.12	6.55	0.625
7	95.45	6.64	0.3125
8	95.40	6.75	0.156
9	96.00	6.75	0.078
10	98.00	7.05	0.039
11	99.00	7.33	0.0195
12	99.00	7.33	0.0097

LC_{50}: 1.86 μg mL^{-1} (Ineffective: resistance suspected)

$$\text{Inhabition of egg hatching}\,(\%) = \left(\frac{a}{b} \div \frac{a}{c}\right) \times 100\ (\%)$$

where, a is number of larvae, b is total number of larvae and eggs in wells with Albendazole and c is total number of larvae and eggs in control well. The Lethal Concentration (LC_{50}) of albendazole was calculated by log probit analysis (Finney, 1971). In the analysis, probabilities $p \leq 0.05$ were considered significant. Eggs having LC_{50} value in excess of 0.1 μg mL^{-1} were indicative of anthelmintic resistance (Le Jambre, 1976).

RESULTS

Faecal egg count reduction test: The FECRT results as presented in Table 3 shows the efficacy of Albendazole in the dogs. It could be seen that a single-dose treatment with Albendazole produced a percentage faecal egg count reduction of 84.0% (group A) with upper and lower confidence levels of 64 and 93, respectively. Similarly, once daily treatment for 2 (group B) consecutive days produced percentage reduction of 93.0% with lower and upper

Table 3: Mean pre- and post-treatment faecal egg counts of Nigerian local dogs naturally infected with hookworm and treated with 2.5% albendazole

Groups	Pre-treatment FEC ($\times 10^3$ epg)	Post Treatment FEC ($\times 10^3$ epg)	Percentage reduction in FEC	Lower confidence level	Upper confidence level	Remark
A (n = 8)	47.28±12.39	7.22±2.00	84	64	93	Ineffective
B (n = 8)	42.29±9.190	3.04±2.20	93	66	99	Ineffective
C (n = 8)	45.39±8.490	0.88±0.67	98	89	100	Effective
D (n = 6)	48.34±12.05	45.17±12.19	NA	NA	NA	NA

NA: Not applicable

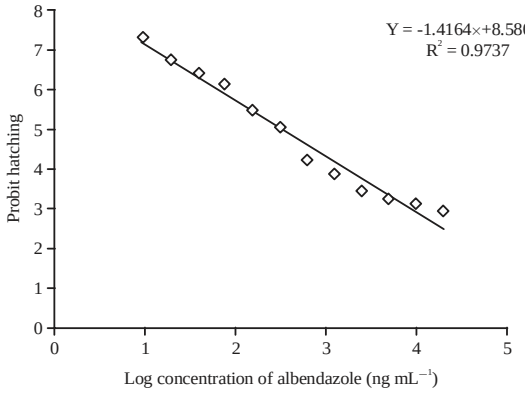

Fig. 1: Log-dose probit response line of albendazole before treatment

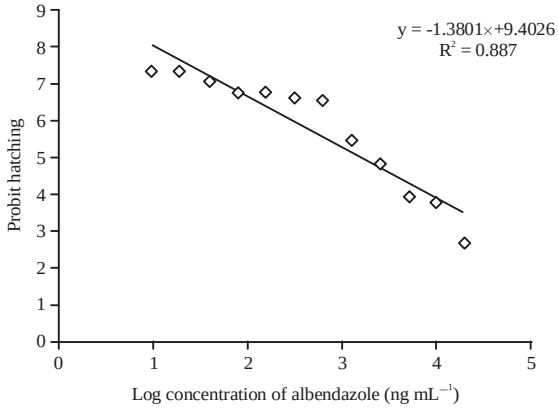

Fig. 2: Log-dose probit response line of albendazole after treatment

confidence levels of 66 and 93, respectively while 3 (group C) consecutive days treatment had 98.0% reduction with lower and upper confidence levels of 89 and 100, respectively.

Egg Hatch Assay (EHA): A probit analysis of the Log-dose response of albendazole on the hookworm eggs obtained from the dogs before treatment indicated an LC_{50} values of 0.30 μg mL^{-1} (Table 1 and Fig. 1). The results of the Egg Hatch Assay after treatment with albendazole as indicated in Table 2 and Fig. 2 showed that the albendazole had an LC_{50} value of 1.86 μg mL^{-1} against hookworm eggs.

DISCUSSION

The results of this study identified inefficacy of albendazole at the manufacturer's recommended therapeutic doses against hookworms when given as a single-dose treatment for one (84%) and two (93%) consecutive days on the basis of Faecal Egg Count Reduction Test (FECRT). There was however, an improved efficacy (98%) when albendazole was administered daily for three consecutive days though the lower and upper confidence levels were 89 and 100, respectively, suggesting a possible presence of anthelmintic resistance. The Faecal Egg Count Reduction Test (FECRT) is a standard used to monitor efficacy of anthelmintics against gastro-intestinal nematodes in field conditions (Coles *et al*., 1992; Pena-Espinoza *et al*., 2014). A reduction greater than 95% is required (both for worm and egg counts) for a claim of efficacy against any worm species (immature or adult) to be made (Coles *et al*., 1992; Relf *et al*., 2014). Kopp *et al*. (2008) reported a high level anthelmintic resistance following a substantial reduction in the efficacy of pyrantel to 25.7% from an earlier reported 75.1% against *Ancylostoma caninum* in dogs using the *in vivo* FECRT. Anthelmintic resistance in companion animals is not a widely recognized issue as it is in livestock but reports are beginning to emerge of their existence in dogs (Kopp *et al*., 2008; Lalchhandama, 2010). Similarly, a study by Vercruysse *et al*. (2011) to assess the anthelmintic efficacy of albendazole in school children in seven countries where soil transmitted helminths are endemic using the FECRT produced efficacy of 88.5, 87.4, 87.1, 74.7 and 86.8% against hookworms in Brazil, Cambodia, Cameroon, India and Tanzania, respectively.

The results of this study also showed that the single-dose regimen of albendazole for one and two consecutive days at the highest dose (25 mg kg^{-1}) recommended by the manufacturer is sub-therapeutic against hookworms in the dogs since they both produced less than 95% efficacy. The importance of this finding is that such treatment could precipitate anthelmintic resistance sequel to under dosing. Under dosing is generally considered an important factor in the development of anthelmintic resistance (Edwards *et al*., 1986; Ihler, 2010; Shalaby, 2013)

because sub-therapeutic doses might encourage the selection of benzimidazole-resistant alleles among the surviving worm populations and possibly the mutation of a gene that confers benzimidazole-resistance (Humbert *et al*., 2001; Silvestre and Humbert, 2002; Shalaby, 2013). It is also very important in the study area, given that it is a common practice to deworm dogs with single-dose regimen of albendazole without provision for repeat treatment or faecal monitoring. Prichard (1990) noted that every compound that is less than 100% effective will theoretically select for resistance.

Albendazole is known to have a very short residual effect in dogs and its metabolite, albendazole sulfoxide is only detectable in plasma for less than 12 h in this species as against three days in sheep and goat. Hence, it is routinely recommended that higher dose or more frequent treatments with albendazole may be required to achieve the desired efficacy (Junquera, 2004). However, it was noted in this study that a repeat treatment rather than increased dose was more effective in the removal of the hookworms since the upper limit of the recommended dose range was used. The reduced efficacy recorded in this study is also believed to be associated with the known pharmacokinetic profiles of benzimidazoles anthelmintics in dogs. Previous studies have suggested that only limited rates of dissolution and absorption of benzimidazole anthelmintics are achieved in cat, dog and human. Consequently, these compounds may need to be given at higher doses or as multiple administrations in order to provide adequate therapeutic concentrations to give an acceptable anthelmintic efficacy. However, increasing the frequency of treatment to achieve therapeutic efficacy as was observed in the present study may also contribute to the development of anthelmintic resistance because increased frequency of treatment is likely to impose selection pressure for anthelmintic resistant strains. Shalaby (2013) observed that frequent usage of the same group of anthelmintic may result in the development of anthelmintic resistance. Increasing the frequency of treatment has also other disadvantages such as increase in cost, tissue residues and toxicity.

The egg hatch assay was performed to determine the *in vitro* efficacy of albendazole against *A. caninum* in Nigerian local breed of dogs with a view to adapting it for the monitoring of anthelmintic resistance in dogs. The assay was performed on hookworm eggs obtained from the dogs before and after treatment with albendazole as it is believed that worms which survive treatment were probably more resistant than the ones that were eliminated. The results of the EHA as obtained in the present study showed the albendazole used in the FECRT had LC_{50} value of 0.30 μg mL^{-1} against the hookworm eggs before treatment which is greater than the discriminating dose of 0.1 μg mL^{-1}. This validates the result of the FECRT and indicates reduced efficacy of the albendazole or reduced susceptibility of the worms to the anthelmintics. Eggs having LC_{50} value in excess of 0.1 μg mL^{-1} are indicative of anthelmintic resistance (Le Jambre, 1976; Hamdullah *et al*., 2015) in accordance with the guidelines of the World Association for Advancement of Veterinary Parasitology (WAAVP) on EHA. A change in the ability of individual parasites to survive at the recommended therapeutic dose of an anthelmintic, has been described as resistance (Shalaby, 2013).

Similarly, the results of the EHA after treatment with the albendazoles gave LC_{50} value that was more than the value obtained before treatment, suggesting that the surviving worms were more resistant than those eliminated by the treatment. This finding therefore, confirms the a prori belief that worms which survive treatment were probably more resistant than those killed by the treatment. Therefore, the higher number of eggs that hatched in excess of the discriminating dose (0.1 μg mL^{-1} of the albendazoles) following treatment was attributed to a possible presence of resistant worms in the population. The implication of this finding is the possibility of the small number of surviving worms, which are the most resistant component of the population, to contaminate the environment with resistant offspring for subsequent generations. Observations similar to the results discussed in this study, have been made on benzimidazole resistance studies in small ruminants. Cawthorne and Whitehead (1983) in the United Kingdom, Easwaran *et al*. (2009) in India and Borgsteede *et al*. (1996) in the Netherlands recorded similar results on benzimidazole resistance in small ruminants with LD_{50} values >0.1 μg mL^{-1} of albendazole. However, in Romania, Cernea and Cernea (2009) using the egg hatch assay recorded dissimilar results with albendazole having an LD_{50} of -0.097 μg mL^{-1} in a study which assessed the efficacy of albendazole against *Ancylostoma caninum* eggs harvested from a group of 62 dogs. Cernea and Cernea (2009) attributed the absence of benzimidazole resistance in their study to the rational use of benzimidazole based therapy (once or twice a year in some of the dogs assessed) and also to lack of anthelmintic treatment in some of the dogs.

CONCLUSION

In conclusion, the data obtained from the *in vivo* and *in vitro* studies suggest a possible presence of albendazole resistant hookworms in the study area. The data obtained in the *in vitro* study suggest that the EHA could reasonably determine the efficacy of albendazole against ancylostomosis

in dogs. Hence, the test could be harnessed for use to effectively monitor the efficacy of albendazole as well as their hookworm resistance status in the dog population. It is therefore, recommended that careful consideration be given to worm control measures for dogs and regular monitoring of faecal egg output be conducted to evaluate the effectiveness of any control programme.

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