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Symptoms and Response to Treatment with Diminazene Aceturate and Mebendazole in Dogs Infected with Single *Trypanosoma congolense, Ancylostoma caninum* and Combination of *Trypanosoma congolense* and *Ancylostoma caninum*

R. I. O. Nwoha[1*] and B. M. Anene[2]

[1]Department of Veterinary Medicine, Michael Okpara University of Agriculture, Umudike, Nigeria.
[2]Department of Veterinary Medicine, University of Nigeria, Nsukka, Nigeria.

Authors' contributions

This work was carried out in collaboration between both authors. Author BMA designed the study and wrote the protocol. Author RION wrote the first draft of the manuscript, managed the literature searches, analyses of the study performed the spectroscopy analysis and managed the experimental process. Both authors read and approved the final manuscript.

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ABSTRACT

The economic losses associated with diseases caused by *Trypanosoma congolense* and the devastating effect of *Ancylostoma caninum* (*A. caninum*) in dogs' necessitated the present study. Sixteen dogs grouped into 4 of 4 members each were used in the study. GROUP I was uninfected dogs (control), GROUP II was infected with *Trypanosoma congolense* (*T. congolense*) infection, GROUP III was mixed infections of *Trypanosoma congolense and Ancylostoma caninum*

**Corresponding author: E-mail: rosemarynwoha@yahoo.com;*

(*T. congolense /A. caninum*) and GPIV was infected with *Ancylostoma caninum.* At first *Ancylostoma caninum* infection was done on GPIII and GPIV. Two weeks later *T. congolense* infections was done on GPII and superimposed on GPIII. Three weeks post trypanosome infection; GPII and GPIII were treated with diminazene aceturate. Mebendazole was used on GPIII and GPIV and treatment repeated 2 weeks later. The prepatent period of *T. congolense* infection was 14.00±1.40 days in single infection and 9.00±1.10 days in conjunct infection of *T. congolense* and *A. caninum.* Persistent parasitaemia resulted in repeated treatment with diminazene aceturate at 7 mg/kg and mebendazole at 100 mg twice daily for 3 days*.* The predominant signs revealed include; lethargy, vomition, enlargement of popliteal lymphnodes, pyrexia, oedema of fore and hind limbs and ocular discharges, anaemia, and slight emaciation. The symptoms were more severe in GPIII compared to GPII and GPIV. The egg per gram of faeces (EPG) in (GPIV) was significantly higher than the conjunct infection (GPIII). Treatment only slightly improved clinical manifestations. In conclusion, conjunct infections of *T. congolense / A. caninum* would result to more severe disease condition than in single infection of either disease in dogs. The severity of symptoms of the diseases were more in conjunct *T. congolense* / *A. caninum* as evidenced by high mortality compared with the single infections. Therefore symptoms of the diseases could serve as a surrogate diagnostic tool in diagnosis and vigorous treatment of infected dogs.

Keywords: Clinical signs; Trypanosoma congolense; A. caninum; Diminazene aceturate; mebendazole.

1. INTRODUCTION

Trypanosoma congolense is the major species of trypanosome responsible for the disease "Nagana" in livestocks [1]. Its effect on the health of susceptible animals is dependent on the infecting strain of the parasite [2]. In dogs, clinical symptoms of the disease show great diversification, ranging from slight swelling due to accumulation of inflammatory exudates, pyrexia, anorexia, generalized lymphadenopathy, diarrhea, myocarditis, ascites, cardiac arrhythmia, neurological manifestations, lethargy and death [3-7]. Pathologically, fibrosis and separation of muscle cells are evident in more advanced stages especially in the cardiac muscles. Death rate due to cardiac degeneration is often high in endemic areas [3]. Often there are cases of depressed immunity [8] and increased susceptibility to infection such as helminthosis. Helminthosis is a common disease problem of dogs worldwide and is mostly subclinical especially in adult animals probably due to acquired or innate resistance. It is an important cause of anaemia, and impairs the healthy wellbeing and productivity of infected dogs. Mixed infections of helminthosis and trypanosomosis are common in the field [9]. There may be a severe disease condition in dogs with mixed infections of trypanosomosis and *Ancylostoma caninium*. This work thus evaluates the clinical impact and chemotherapeutic effectiveness of diminazene aceturate and mebendazole in dogs infected with single *T. congolense* and in combination with *A. caninum*.

2. MATERIALS AND METHODS

2.1 Experimental Animals

Sixteen mongrel breed of dogs of both sexes weighing between 4.0 and 8.0 kg were used in this experiment. The dogs were acclimatized for 4 weeks before commencement of the experiment during which they were screened for blood parasites and confirmed negative by Giemsa-stain, thin blood smears and haematocrit buffy coat method [10]. They were dewormed with tablets of mebendazole (Vermin®, Janssen-Cilag Ltd 50 - 100 Holmers Farm Way, High Wycombe, Bucks, HP12 4EG UK) at the dose of 100mg twice daily for 3 days and also treated with sulfadimidine at the dose of 48 mg/kg intramuscularly against systemic opportunistic bacterial infections. The experiment commenced a week post treatment. The animals were kept in clean cages in a fly proof house and fed twice daily. Water was given *ad libitum*.

2.2 Ethical Approval

The care of the animals was in conformity with the guideline for animals' experimentation of Council for International Organization of Medical Sciences (CIOMS) for biomedical research involving animals. The dogs were humanely cared for and treated throughout the study. They were comfortably housed in properly ventilated pens in good hygienic condition and provided good and adequate feeding with clean portable drinking water. All authors hereby declare that all experiments have been examined and approved

by the appropriate ethics committee and have therefore been performed in accordance with the ethical standards laid down in the 1964 Declaration of Helsinki.

2.3 Parasites and Infections

2.3.1 *Trypanosoma congolense*

Kilifi strain of *T. congolense* obtained from the National Institute of Trypanosomosis and Oncocerciasis Research (NITOR), Nigeria was used. The parasite was a primary isolate from a cow in Kaduna. It was maintained in white albino rats, and subsequently passage in a donor dog from where parasites were collected for infection of the experimental dogs.

Estimated 2.5×10^6 of *T. congolense* suspended in 1ml of normal saline was used to infect each experimental dog in the group. The quantity of parasites inoculated was estimated using the rapid matching method of [11].

2.3.2 *Ancylostoma caninum* Infection

The concentration of larval suspension was estimated using an automatic pipette (Biotht Peoline®), and counted under the microscope according to the method of [12]. Small doses of 20 µL larval suspensions were placed as drops on a microscope slide and counted under ×4 objective of a light microscope (Ozympu®). Dogs were starved prior to infection so as to establish infection. The selection criterion of the dose of the dogs was determined from infective dose from previous experiments conducted and quantity of infective dose was estimated under the microscope as described by [12]. A dose of 200 infective L_3 suspended in 1 mL of distilled water were delivered *per os* to each of the experimental dogs, using a 2 mL syringe without needle.

2.3.3 Reconstitution of *Diminazene aceturate*

A 2.36 g Veribin® a brand of trypanocide containing 1.05 g of diaminazene aceturate was reconstituted with 15 mL distilled water according to manufacturer's recommendation. The volume of diminazene acetutate administered to individual dog in GPII and GPIII, were calculated from their weight at the dose of 7 mg/kg via the intramuscular route.

2.3.4 Administration of mebendazole

Tablets of mebendazole (Vermin®, Janssen-Cilag Ltd 50 - 100 Holmers Farm Way, High Wycombe, Bucks, HP12 4EG UK) was given at the dose of 100 mg *per os* twice daily for 3 consecutive days. Treatment was repeated 2 weeks later.

2.4 Experimental Design

Dogs were randomly divided into 4 groups of 4 members in each group. GROUP I was uninfected dogs (control), GROUP II was infected with *Trypanosoma congolense* infection, GROUP III was co infections of *Trypanosoma congolense and A. caninum.* GROUP IV was with single *A. caninum.*

Post acclimatization, *Ancylostoma caninum* infection was done on GPIII and GPIV alone. Two weeks later, *Trypanosoma congolense* infection was done on GPII and GPIII.

Three weeks post trypanosome infection; GPII and GPIII were treated with diminazene aceturate. Mebendazole was used only on GPIII and GPIV and a repeat treatment given 2 weeks later.

Parasitaemia was determined using the wet mount method and the haematocrit buffy coat method [10]. The prepatent period of infection in the individual dogs were also determined.

2.5 Parameters Monitored

The parasitaemia, egg per gram of faeces, symptoms manifested and temperature changes were determined at daily intervals.

2.6 Evaluation of Symptoms

Symptoms presented were evaluated using the "score method" essentially as described in [13]. Briefly, range of variation in the lesions was divided into ordinal classes viz: Absent (0), Mild (+), Moderate (++) or Severe (+++).

Data obtained from the temperature and EPG were presented as mean ± standard error of mean (SEM). Statistical significance was analyzed using one way analysis of variance (ANOVA) and Duncan's multiple range test with SPSS version 16 soft ware package. The acceptance of level of Significance was at $p \leq 0.05$ [14].

3. RESULTS

The prepatent period of *Trypanosoma congolense* was 14.00±1.40 and 9.00±1.10 days,

respectively, in single *T. congolense* and conjunct *T. congolense* / *A. caninum*. The faecal egg output (egg per gram, EPG) in the single *A. caninum* (GPIV) was significantly ($p < 0.05$) higher than in the conjunct infection (GPIII).

3.1 Parasitaemia

The results on parasitaemia are shown in Table 1. By day 24 of the experiment only two out of the four dogs in conjunct *T. congolense* and *A. caninum* group (GPIII) were patent with trypanosome infection. By day 25, all the trypanosome infected groups (GPII and GPIII) became patent with infection. Treatment with both diminazene aceturate and mebendazole was commenced on day 42 post infection. By day 49 there was complete disappearance of parasitaemia in the groups (GPII and GPIII) and mortality recorded in GPIII. By day 56, there were relapses in GPII and GPIII, and by day 63, a repeat treatment cleared parasitaemia in all the groups. By day 70, there was complete mortality of all the members in GPIII.

3.2 Feacal Egg Count

The results of faecal egg output are presented in Table 2. The prepatent period of *A. caninum* was established by 13 to 14 and 14 to 16 days, respectively in the conjunct *T. congolense*/*A. caninum* and single *A. caninum* groups. Following *A. caninum* infection, EPG was obtained in the experimental groups (GPIII and GPIV) by day 14 post infection. By day 28, EPG in GPIV was significantly ($p<0.05$) higher than that in GPIII up till day 42. Treatment with mebendazole on day 42 cleared EPG in both groups by day 49. By day 56, there was a recrudescence of EPG in both groups and a repeat treatment made same day. By day 63, there was complete elimination of EPG up till day 85 post infection.

3.3 Symptom Manifestations

The symptoms manifested in the infected dogs are shown in Table 3. The symptoms of *T. congolense* and *A. caninum* were only observed by day 42 post infections. Symptoms shown include: Dark coloured foal smelling faeces in GPIII, enlargement of popliteal lymphnodes, pyrexia, ocular discharges and emaciation. Treatment on day 42 with diminazene aceturate at 7 mg/kg im and mebendazole at 100 mg twice daily per os for 3 days did not improve manifested symptoms. By day 56, there was further treatment due to additional manifestation of symptoms such as dullness in GPIII, severe pale mucous membrane in GPIII compared to

Table 1. Parasitaemia of dogs with single *T. congolense, A. caninum* and conjunct infections of *T. congoense* and *A. caninum* and treatment with diminazene aceturate and mebendazole

Experimental period (days)	GP I control	GP II *T. congolense*	GP III *T. congolense/ A. caninum*	GP IV *A. caninum*
0	0/4	0/4	0/4	0/4
1 ⇧	0/4	0/4	0/4	0/4
7	0/4	0/4	0/4	0/4
21 ⇳	0/4	0/4	0/4	0/4
24	0/4	2/4	4/4	0/4
25	0/4	4/4	4/4	0/4
26	0/4	4/4	4/4	0/4
27	0/4	4/4	4/4	0/4
28	0/4	4/4	4/4	0/4
35	0/4	4/4	4/4	0/4
42+*	0/4	4/4	4/4	0/4
49	0/4	0/4	0/3	0/4
56+*	0/4	1/4	1/3	0/4
63*	0/4	0/4	0/3	0/4
70	0/4	0/4	0/0	0/4
77	0/4	0/4	0/0	0/4
84	0/4	0/4	0/0	0/4

⇧ *Ancylostoma caninum infection,* ⇳ *Trypanosome infection, Numerator-Number of aparasitaemic dogs, Denominator- Number of treated dogs, * Administration of diminazene aceturate, + Administration of mebendazole*

Table 2. Mean egg per gram (EPG) ± SE of dogs infected with single *T. congolense, A. caninum* and conjunct infections of *T. congoense* and *A. caninum* and treatment with diminazene aceturate and mebendazole

Experimental period (days)	GP I (control)	GP III *T. congolense*	GP III *T. congolense & A. caninum*	GP IV *A. caninum*
0 ⇧	ND	ND	ND	ND
1 ⇧	ND	ND	ND	ND
7	ND	ND	ND	ND
14	ND	ND	1.210±120[b]	1.050±110[b]
21	ND	ND	1.600 ±147 [b]	2.050±210[b]
28	ND	ND	1. 025±225[c]	2.850±247[b]
35	ND	ND	1. 250±222[c]	3.075±229[b]
42 +	ND	ND	1.633 ±593[c]	3.525±206[b]
49	ND	ND	ND	ND
56 +	ND	ND	1.833±167[b]	1.350±529[b]
63	ND	ND	ND	ND
70	ND	ND	ND	ND
77	ND	ND	ND	ND
85	ND	ND	ND	ND

Different superscripts (a,b) in a row indicate significant difference between the group means (p< 0.05)
⇧ *Ancylostoma caninum infection, + Treatment with mebendazole*

GPII and severity in previously manifested symptoms. Further treatments on day 63 improved clinical conditions in GPII and GPIV but not in GPIII. By day 65 there was oedema of the fore and hind limb in GPII, vomition in GPIII and anorexia and emaciation in both GPII and GPIII. By day 71, there was sudden robust appetite in both GPII and GPIII, dullness in GPIII which culminated in complete mortality in the group.

3.4 Change in Temperature

The result of change in temperature of the experimental groups was shown in Table 4. There was no significant ($p<0.05$) change in the temperature of *A. caninum* (GPIV) through out the period of experiment. There was a significant ($p<0.05$) increase in temperature in both GPII and GPIII by day 49 despite treatments with diminazene aceturate at 7 mg/kg im and mebendazole at 100 mg twice daily *per os* for 3 days. A repeat treatment on day 56 and 63 resulted in clinical improvement in temperature of both GPII and GPIII compared to GPI (control). By day 70, there was complete mortality of members of GPIII.

4. DISCUSSION

The prepatent period of single *T. congolense* and conjunct *T. congolense /A. caninum* infections in dogs falls within the normal period (Table 1). *Trypanosoma congolense* infection in dogs usually has a prolonged prepatent period ranging from 10-24 days [15,16]. More so the period varies directly with the host species. Shorter prepatent period in the conjunct groups may be due to antigenic competition with *Ancylostoma* infection [17]. *Ancylostoma* parasites may have suppressed immune response to secondary trypanosome infection in the conjunct group (GPIII) thus enhanced early parasitaemia compared to single infection where the immunity was higher. This agrees with the reports of [18] and [4] who associated prepatent period of species of trypanosome to immune status of the host. It also supports previous findings in *T. congolense*/*Haemonchus contortus* infection in cattle [19] where conjunct infection had a shorter prepatent period than single infection. The disappearance of parasitaemia occurred after 24-72 hours (Table 1) as recorded by other researchers on trypanosomes [20,21], thus confirms the potency of diminazene aceturate as a trypanocide. *Ancylostoma* infection was established by 13 -16 days post infection (Table 2). This was shorter than previous report of 15 - 18 days in young dogs [18]. The existence of both *T. congolense* and *A. caninum* in GPIII may have interacted in way that ameliorates immune response to *A. caninum* thus enhanced the

number of adult worms and the presence of feacal eggs. This corroborates an inverse relationship between the number of adult worms and the number of eggs in the faeces [22]. The antigenic interaction between *T. congolense* and *A. caninum* resulted in low EPG observed in conjunct GPIII compared to single GPIV (Table 2). This contradicts previous findings of [23,19] and [24] who observed significant increases in EPG in animals with concurrent infections with trypanosomosis and helminthosis. Also [19] recorded high EPG in cattle with conjunct infections of *T. congolense* and haemonchosis compared with the single infections. The robust immunity of GPIII which prevented symptom manifestations was breached by day 49 subsequent upon secondary infection with *T. congolense* parasites. Superimposition of *Trypanosoma congolense* in *A. caninum* infected dogs may have damped the integrity of immunity in the groups and resulted in symptom manifestations (Table 3). The predominant signs in *T. congolense* infected groups were mainly lethargy, enlargement of popliteal lymphnodes, emaciation, pyrexia, oedema of fore and hind limbs, mortality, ocular discharges and anaemia (Table 3). These signs are consistent with previous records in trypanosomosis [25,7,26].

Table 3. Comparative symptom manifestations in dogs infected with single *T. congolense, A. caninum* and conjunct infections of *T. congoense* and *A. caninum* and treatment with diminazene aceturate and mebendazole

Days	Clinical signs	GPI (control)	GPII *(Tc)*	GPIII (*Tc/Ac)*	GPIV (*Ac)*
1	None	0	0	0	0
21	None		0	0	
42* +	Dark coloured foul- Smelling feaces	0	0	+++	0
	Enlargement of popliteal lymphnodes	0	+	++	0
	Pyrexia	0	++	++	0
	Ocular discharges	0	++	0	0
	Emaciation	0	0	0	0
56*	Emaciation	0	0	++	0
	Dullness	0	0	+	0
	Enlargement of popliteal lymphnodes	0	+++	+++	0
	Swollen abdomen	0	0	0	0
	Occular discharge	0	++	++	0
	Pale mucus membrane	0	++	+++	0
	Pyrexia	0	+	+	0
	Passage of dark coloured foul- Smelling feaces	0	0	+++	0
63*+	Dullness	0	0	+++	0
	Passage of dark coloured foul- Smelling feaces	0	0	+++	0
	Enlargment of popliteal lymphnodes	0	+++	+++	0
	Pyrexia	0	+	+	0
	Death	0	0	+	0
65	Vomition	0	0	++	+
	Oedema of fore and hindlimb	0	+	0	0
	Anorexia	0	+	+++	0
	Emaciation	0	++	+++	0
70	Robust appetite	+++	+++	0	+++
	Enlargment of popliteal lymphnodes	0	+	0	0
	Dullness	0	0	+++	0
	Occular discharges	0	0	0	0
	Oedema of fore and hind limbs	0	+	0	0
	Death	0	0	+++	0

Ac- A. caninum; Tc- T. congolense; Tb- T. brucei

Table 4. Mean±SE Temperature (ºC) in dogs with experimental single *T. congolense, A. caninum* and conjunct infections of *T. congoense* and *A. caninum* and treatment with diminazene aceturate and mebendazole

Experimental period (weeks)	GPI control	GPII (*Tc*)	GPIII (*Tc*/*Ac*)	GPIV (*Ac*)
0	37.8±0.10[a]	37.8±0.20[a]	38.0±0.10[a]	38.5±0.30[a]
1 ⇧	37.8±0.20[a]	37.3±0.60[a]	38.0±0.70[a]	37.8±0.50[a]
7	37.8±0.50[ab]	37.7±0.20[ab]	38.3±0.50[b]	37.7±0.40[ab]
21 ⇳	37.8±0.10[a]	37.5±0.20[a]	37.9±0.10[a]	37.3±0.60[a]
28	37.6±0.30[a]	37.8±0.50[a]	38.0±0.10[a]	38.0±0.70[a]
35	38.3±0.20[a]	37.5±0.20[a]	37.5±0.20[a]	37.8±0.20[a]
42 *	37.9±0.50[a]	37.4±0.80[a]	37.8±0.50[a]	38.3±1.00[a]
49	37.8±0.70[a]	38.2±0.40[ab]	39.10.40[bc]	37.2±0.90[a]
56 *+	37.6±0.30[a]	38.4±0.50[ab]	38.6±0.80[ab]	38.2±1.00[ab]
63 *+	38.2±0.50[a]	38.9±0.90[ab]	39.3±0.50[b]	38.2±0.70[a]
70	38.3±0.20[a]	37.8±0.50[a]	ND	38.2±0.90[ab]
77	38.0±0.40[a]	38.3±0.70[a]	ND	37.6±0.90[a]
84	38.0±0.60[a]	38.0±0.50[a]	ND	38.0±0.90[a]

*Superscripts a b c represents the homogeneity between the experimental groups at probability P≤ 0.05. + Treatment with mebendazole, * Treatment with diminazene aceturate, ⇳ Infection with trypanosomes, ⇧ Infection with A. caninum, Ac Ancylostoma caninum, Tc Trypanosoma congolense*

except oedema of the fore and hind limbs. Oedema of subcutaneous tissue in GPII was immunologically induced leading to an increased vascular permeability and extravasation of fluid into extravascular spaces. Emaciation in the conjunct *T. congolense* / *A. caninum* group resulted from mobilization of body energy reserves due to deprivation of essential nutrients for the synthesis of ATP in the anorexic dogs. The absence of pyrexia in GPIV (Table 4) observed in this study disassociates pyrexia in ancylostomosis as previously noted by [26,25]. The significant increase ($p< 0.05$) in temperature observed mostly in GPIII could be dependent on the enhanced level of released pyrogens in the severely stressed dogs. Presence of dark foul smelling faeces in the *Ancylostoma* groups indicates haemorrhages associated with injuries caused by blood sucking adult hookworms to intestinal arterioles (Table 3). Vomition in most members of *Ancylostoma* group was induced by the presence of hookworm in the intestine. These signs are consistent with previous records [18]. The symptoms observed were more in the conjunct trypanosome / *A. caninum* group (GPIII) compared to the single infection (GPII). The severity of disease manifestations in GPIII was higher as shown by high rate of mortality by day 70 of the experiment (Table 3). Treatment with 7 mg/kg i/m of diminazene aceturate and mebendazole at 100 mg twice daily for 3 days did not improve clinical conditions in the groups (Table 3). This contradicts the reports of [4] and [6] who noted clinical improvement in dogs treated with diminazene aceturate [4,6]. Further manifestations of symptoms such as oedema of the fore/hind limb post treatments in single *T. congolense* group were due to encountered relapses in parasitaemia in the group. Several relapses in the dogs were due to resistant strains of *T. congolense* used in the study (Table 1). Incidences of relapse cases usually occur within 8-14 days post treatment [27,28], thus agree with the findings in this study. Also relapses may have been induced by late administration of treatment at day 42 post infection [29]. Nothwithstanding this [21] observed complete disappearance of *T. brucei brucei* infection at single dose of 7 mg/kg diminazene aceturate. Repeated doses were administered to enhance the therapeutic activity of diminazene aceturate and facilitate parasite clearance in relapse parasitaemia. Mortalities recorded post treatment especially in GPIII may have resulted from diminazene toxicity due to repeated administration (Table 3). This supports earlier record of high risk of diminazene toxicity in dogs [30]. It however contradicts [31], who recorded diminazene tolerance at the dose of 20 mg/kg i/m and at high dose of 50 mg/kg im daily for 5 days [32,31,33]. The confounding factor and toxicity recorded in the present research results from the degree of pathological effects of both *T. congolense* and *A. caninum* on liver function. Both parasites caused hepatic compromise and its inability to eliminate drug metabolites which built to toxic levels in the group. The use of mebendazole in the treatment of *A. caninum* was effective despite recrudesce of faecal eggs 2

weeks post treatment. The faecal eggs are essentially from immature migrating larvae that escaped treatment effect to mature into egg shedding adults in the intestine. A repeat treatment eliminated the newly matured adults and feacal eggs (Table 2).

5. CONCLUSION

The severity of symptoms of the diseases were more in conjunct *T. congolense* / *A. caninum* as evidenced by high mortality from the synergistic actions of both parasites on the liver compared with the single infections. Treatment with both mebendazole and diminazene aceturate did not produce appreciable clinical improvement in the treated groups probably due to effect of resistant strains of *T. congolense*. Nevertheless symptoms manifested were near consistent and therefore could serve as surrogate diagnostic tool for prompt treatment of the disease conditions in dogs.

CONSENT

It is not applicable.

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COMPETING INTERESTS

Authors have declared absence of competing interests.

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