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Effect of experimental single *Ancylostoma caninum* and mixed infections of *Trypanosoma brucei* and *Trypanosoma congolense* on the humoural immune response to anti-rabies vaccination in dogs

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ABSTRACT

Objective: To determine the effect of *Ancylostoma caninum* (*A. caninum*) and trypanosome parasites on the immune response to vaccination in dogs in endemic environments.

Methods: Sixteen dogs for the experiment were grouped into 4 of 4 members each. Group I was the uninfected control one, and GPII was infected with *A. caninum*; GPIII was infected with *A. caninum*/*Trypanosoma congolense* (*T. congolense*), and GPIV was infected with *Trypanosoma brucei* (*T. brucei*)/*A. caninum*. The dogs were first vaccinated with antirabies vaccine before infecting GPII, GPIII and GPIV with *A. caninum* which were done 4 weeks after vaccination. By 2-week post-vaccination, trypanosome parasites were superimposed on both GPIII and GPIV. A secondary vaccination was given to GPI, GPII, GPIII, and GPIV by Week 12 of the experiment (4 weeks post treatment).

Results: The prepatent period was (3.00 ± 1.40) days, in the conjunct infection of *T. brucei*/*A. caninum*. It was (9.00 ± 1.10) days, in conjunct *T. congolense*/*A. caninum*. The prepatent period of *A. caninum* was (14.0 ± 2.0) days in the single *A. caninum* group and (13.0 ± 1.0) days in the conjunct trypanosome/*A. caninum*. At the 1st week after vaccination, the antibody titer in all the vaccinated groups (GPI, GPII, GPIII, and GPIV) significantly increased ($P < 0.05$) and peaked at the 3rd week after vaccination. Following infections, there were marked significant decreases ($P < 0.05$) in the antibody production against rabies in GPII, GPIII and GPIV. The significant decrease ($P < 0.05$) in antibody titer was highest in the conjunct groups (GPIII and GPIV) compared to the single infection (GPII). Treatment with diminazene aceturate and mebendazole did not significantly improve antibody response in the dogs. A secondary vaccination administered at the 12th week after the primary vaccination significantly increased ($P < 0.05$) the antibody titer with a peak at the 3rd week after the secondary vaccination.

Conclusions: It was therefore concluded that *A. caninum*, *T. brucei* and *T. congolense* induced immunosuppression in antirabies vaccination in dogs.

1. Introduction

Ancylostoma caninum (*A. caninum*) is a highly pathogenic helminth parasite that commonly affects dogs in Nigeria[1]. Hookworm is a natural check in canine population, as it is frequently lethal to young puppies[2]. The disease is most commonly seen in dogs under one year old due to their low iron reserve[3]. Following infection, blood loss starts about the 8th day after infection when the immature adult has developed the toothed buccal capsule which enables it to grasp pulps of mucous containing arterioles[4]. It is voracious blood sucking activity that results in anaemia. In addition, ancylostomosis precipitates a gradual state of secondary immunodeficiency which corresponds to the degree of anaemia[5]. The immunodeficiency is dependent on the quantity of iron present in the affected animal which could be reversed by administering iron supplements[5]. On the other hand, trypanosomosis is a disease of great health, social, economic and biological implications in the affected regions[1,6]. Natural infections of *Trypanosoma brucei* (*T. brucei*) and *Trypanosoma congolense* (*T. congolense*) are transmitted through tsetse bites producing severe disease conditions in dogs[7,8]. There have been records of failure in immune response in animals infected with trypanosomosis[9]. In Nigeria, there is prevalence of concurrent African trypanosome and gastrointestinal helminth infections in endemic areas[8]. Concurrent infections of trypanosomes and *A. caninum* may lead to failure of immune response to vaccination and also enhance susceptibility and severity of secondary infections. These challenges necessitated the

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study of experimental single and conjunct infections of *A. caninum*, *T. brucei* and *T. congolense* and their effects on treatment and immune response in vaccinated dogs.

2. Materials and methods

2.1. Experimental animals

Sixteen indigenous breed of dogs of both sexes weighing between 4.0 and 8.0 kg were used in the experiment. They were acclimatized for 3 months during which they were screened for blood and gastrointestinal parasites and confirmed negative before used in the experiment. The dogs were kept in cages in a fly-proof and well-ventilated kennel. The dogs were cared for, fed and watered *ad libitum*.

2.2. Parasites and infections

2.2.1. Trypanosomes

2.2.1.1. T. brucei isolate/T. congolense isolate

T. brucei used in the study was a local isolate obtained from a clinically infected dog from Nsukka area of Enugu State. The isolate was typed and confirmed in the department of Veterinary Parasitology and Entomology, University of Nigeria, Nsukka. The parasites were maintained in rats and subsequently passaged in a donor dog from which the experimental dogs were inoculated.

Kilifi strain of *T. congolense* was obtained for use from the National Institute of Trypanosomosis and Oncocerciasis Research, Nigeria. The strain was first isolated from a cow in Kaduna, maintained in rats and subsequently passaged in a donor dog from which parasites were collected for infection of the experimental dogs.

Estimated 2.5×10^6 of *T. brucei* suspended in 1 mL of normal saline was used to infect each experimental dog in groups, and 1 mL of whole blood containing an estimated 2.5×10^6 *T. congolense* were given to each dog in groups via the intraperitoneal route (*i.p.*). The quantity of parasites inoculated was estimated by using the rapid matching method of Ezeokonkwo *et al.*[10].

2.2.1.2. A. caninum

Infective L_3 larvae of *A. caninum* were isolated and confirmed at the Department of Veterinary Parasitology and Entomology, University of Northumbria at Newcastle from positive faecal samples obtained from dogs in the local market of Nsukka area of Enugu State.

A dose of 200 infective L_3 suspended in 1 mL of distilled water was delivered *per os* to each of the experimental dogs, using a 2 mL syringe.

2.2.2. Reconstitution of diminazene aceturate

Diminazene aceturate (Veribin®, CEVA Sante Animale-La Ballasteiére 33501 Libourne Cedex, France), a generic brand of trypanocide, was used. A sachet of 2.3 g granules containing 1.05 g of diminazene aceturate was reconstituted with 15 mL of distilled water according to manufacturer's recommendation.

The volume of diminazene acetutate administered to individual dog in GPIII and GPIV for both *T. brucei* and *T. congolense* infections was calculated from their weights at the dose of 7 mg/kg via the intramuscular route.

Tablets of mebendazole (Vermin® made in UK by Janssen-Cilag Ltd 50-100 Holmers Farm Way, High Wycombe, Bucks, HP12 4EG) were given at the dose of 200 mg/kg *per os* for 3 consecutive days. Treatment was repeated 2 weeks later.

2.3. Care of experiment animals

The care of the animals was in conformity with the guideline for animals' experimentation of Council for International Organization of Medical Sciences 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 with good and adequate feeding and clean potable drinking water.

2.4. Experimental design

Dogs were randomly divided into 4 groups with 4 members in each group. Group I was uninfected dogs (control), and Group II was with *A. caninum* infection alone, while Group III was with *T. congolense*/*A. caninum* infection and Group IV was with *T. brucei*/*A. caninum* infection.

All the experimental groups including the control group were initially administered with antirabies low egg passage [(National Veterinary Research Institute) NVRI] Vom Nigeria. Four weeks after vaccinations, *A. caninum* infection was done and trypanosome infections followed 2 weeks after *A. caninum* infection. The infected groups were treated with both diminazene aceturate and mebendazole at 3 weeks post-infection. At 4 weeks post-treatment (12 weeks post primary vaccinations), secondary vaccinations were administered to the experimental dogs. The prepatent period of trypanosomes and parasitaemia was determined by using the wet mount and the haematocrit buffy coat method as described by Dempsay and Mansfield[11].

Also, the prepatent period of *A. caninum* infection was determined by daily faecal examination starting from Day 10 after infection by using simple floatation technique[12].

2.4.1. Preparation of rabies virus for reverse passive heamagglutination assay

Red blood cells (RBCs) were prepared as described by Wosu[13]. A local vial of low egg passage rabies vaccine from NVRI, Vom Nigeria was used as viral antigen and was reconstituted according to manufacturer's prescription.

Washed RBCs were sensitized with rabies virus using hydrated chromic chloride as the coupling agent as described by World Health Organization[14].

2.4.2. Rabies passive haemagglutination test

With a micropipette, 0.03 μL of 0.86% saline was deposited into each well of the rows in the microtitre plate. In the first row, a serial dilution of 0.03 μL of the inactivated test serum was made in the wells and the last aliquot discarded. In addition, an equal volume of 0.03 μL of the sensitized RBCs was added in all the wells in the row. In the second row, a serial dilution of 0.03 μL of a known antirabies serum (NVRI, Vom Nigeria) plus an equal volume of 0.03 μL of sensitized RBCs was added into each of the wells. In the third row, a serial dilution of 0.03 μL of washed sheep RBCs plus an equal volume of 0.03 μL of sensitized RBCs was made in each of the wells. The entire set-up was covered with a cellulose paper and incubated in the refrigerator at 4 ˚C for 1 h. The set-up had a known anti-serum against

Table 1

Antibody response to rabies vaccination in dogs with experimental single *A. caninum* and mixed infections of *T. brucei* and *T. congolense* and treatments with diminazene and mebendazole.

Experimental period (Weeks)	GPI (Control)	GPII (*A. caninum*)	GPIII (*T. congolense*/*A. caninum*)	GPIV (*T. brucei*/*A. caninum*)
0[d]	130.00 ± 127.30[a]	36.00 ± 10.10[a]	26.00 ± 14.40[a]	448.00 ± 218.60[a]
1	337.00 ± 235.20[a]	96.00 ± 18.50[a]	112.00 ± 16.00[a]	1 312.00 ± 932.40[a]
2	1 188.00 ± 975.10[a]	480.00 ± 198.10[a]	288.00 ± 80.50[a]	2 624.00 ± 1 864.8[a]
3[e]	2 560.00 ± 886.80[a]	2 560.00 ± 886.80[a]	2 560.00 ± 886.80[a]	2 560.00 ± 886.80[a]
4[e]	2 560.00 ± 886.80[a]	2 560.00 ± 886.80[a]	2 560.00 ± 886.80[a]	2 560.00 ± 886.80[a]
5[f]	2 560.00 ± 886.80[a]	2 560.00 ± 886.80[a]	2 560.00 ± 886.80[a]	2 560.00 ± 886.80[a]
6[f]	2 560.00 ± 886.80[a]	1 100.00 ± 851.50[b]	340.00 ± 128.00[b]	1 437.00 ± 1 792.00[b]
7	2 560.00 ± 886.80[a]	480.00 ± 198.10[b]	144.00 ± 40.30[b]	131.00 ± 932.40[b]
8	2 048.00 ± 724.10[a]	12.00 ± 2.30[b]	72.00 ± 20.10[b]	64.00 ± 22.60[b]
9[gh]	1 536.00 ± 295.60[a]	8.00 ± 2.80[b]	4.00 ± 4.00[b]	4.00 ± 6.40[b]
10[h]	1 088.00 ± 367.70[a]	96.00 ± 2.50[b]	4.70 ± 1.80[c]	4.00 ± 1.00[c]
11[ghi]	1 010.00 ± 256.00[a]	100.00 ± 1.20[b]	5.30 ± 0.50[c]	4.00 ± 0.00[c]
12[hi]	1 005.00 ± 341.30[a]	150.00 ± 6.20[b]	8.00 ± 0.00[c]	6.50 ± 1.50[c]
13	2 731.00 ± 682.70[a]	2 569.00 ± 90.50[a]	-	128.00 ± 56.40[b]
14	8 192.00 ± 0.00[a]	4 608.00 ± 1288.5[ab]	-	4 437.00 ± 2 076.20[b]
15	13 653.00 ± 2 730.70[a]	12 288.00 ± 2364.80[a]	-	13 653.00 ± 270.70[a]

[d]: Primary vaccinations; [e]: Infection with *A. caninum*; [f]: Infection with *Trypanosomes*; [g]: Treatment with mebendazole; [h]: Treatment with diminazene aceturate; [i]: Secondary vaccination Superscripts; [a, b, c]: Represents the homogeneity between the experimental groups at probability $P \leqslant 0.05$. Data were expressed as mean ± SE.

rabies as control which must be positive and an RBC control that must settle at the bottom of wells before the results was read. Results were read as the reciprocal of the highest dilution factor that gave a reproducible titre.

2.5. Statistical analysis

Data obtained were analyzed with SPSS package 16.0 version using ANOVA. The results were presented as mean ± SE and were separated by using Duncan multiple range of test. The level of significance was accepted at $P < 0.05$[15].

3. Results

The prepatent period was (3.00 ± 1.40) days in the conjunct infection of *T. brucei*/*A. caninum*. It was (9.00 ± 1.10) days in the conjunct *T. congolense*/*A. caninum*. The prepatent period of *A. caninum* was (14.0 ± 2.0) days in single *A. caninum* group and (13.0 ± 1.0) days in conjunct trypanosome/*A. caninum* infected group.

From Table 1, there was detectable antibody titer against rabies in all the experimental groups pre-vaccination. Following vaccination, antibody titer increased ($P < 0.05$) gradually and peaked at Week 3 after vaccinations. There was a significant decrease ($P < 0.05$) in antibody titer in all the *Ancylostoma* infected groups (GPII, GPIII and GPIV) starting from Week 6 to 12 in the single *A. caninum* group (GPII) and beyond in the conjunct groups (GPIII and GPIV). The decreases were significantly ($P < 0.05$) higher in the trypanosome infected groups (GPIII, GPIV) than in *Ancylostoma* group (GPII). It, however, did not significantly differ between the conjunct groups (GPIII and GPIV). At Week 12 after secondary vaccinations, there were appreciable increases in the groups which later did not differ from the control in the single *A. caninum* group (GPII) at Week 13 and in the other infected groups (GPIII and GPIV) at Week 15. Post treatment with mebendazole, the titer in the single *A. caninum* group (GPII) equalled that of the control unlike in the conjunct groups (GPIII and GPIV). Treatment with diminazene aceturate did not result in significant increase in the antibody titre except at Week 15.

4. Discussion

It appears that there has been an appreciable awareness on the importance of antirabies vaccination in canine population as indicated by the significant antibody titer against rabies in the experimental dogs prior to vaccination. It probably may suggest a reasonable level of antirabies vaccination practice in the area where the experimental dogs were sourced. This is supported by Schneider *et al*. having classified rabies vaccination as one of the core vaccines with a global distribution[16]. The antibody response against rabies virus increased at Week 1 after vaccinations and peaked at Week 3. It could be said that the primary vaccination may have engendered a positive effect on the memory B cell enhancing antibody production. This is as recorded in antirabies vaccination in dogs[17]. The subsequent decreases ($P < 0.05$) in antibody titer after infections with trypanosomes and *A. caninum* could be due to the immunosuppressive effect of the parasites on the host[5,18]. Trypanosomes alter immune response in infected hosts through diverse mechanism which includes the release of factors that induces production of interferon-g by the T cells which have immunomodulating effect on the synthesis of the immune elements[19]. Except the immunosuppressive effect of trypanosomes on the host, *A. caninum* induces immunosuppression through the mechanism of malnutrition and iron deficiency anaemia[20]. Hookworm parasites compete with the host for essential nutrients especially proteins necessary for the production of immunoglobulins as seen in cases of acute and chronic compensated hookworm[20]. Iron deficiency causes tissue defect in mammals and a significant *in-vitro* impairment of lymphocyte transformation and macrophage inhibition factor as in iron deficient patients suffering from microcytic, hypochromic anaemia[21]. Under field conditions, both trypanosomosis and ancylostomosis occur in mixed infections[8]. From the results, the levels of antibody depreciation in the mixed infected groups were significantly higher compared with single *Ancylostoma* infected group. This could have resulted from the synergic immunosuppressive effect of both parasites on the primary immune response. Treatment with mebendazole induced significant improvement in the antibody titer which peaked on secondary vaccination. Mebendazole has also been shown to possess immune

boosting and antioncogenic properties in treated individuals[22,23].

On the contrary, treatment with diminazene aceturate gave no significant improvement in the immune response in the mixed infected groups resulting in repeated treatment and eventual elimination of parasites. This probably explains the periods of persistent decrease in antibody production in the groups. Several doses of diminazene aceturate administered at weekly intervals may be the cause of mortality in GPIII probably due to toxicity. This finding does not correspond with previous report on trypanosome infection in mice and cattle[24,25] which reported rapid restoration of immune competence after treatment with trypanocidal drug. Nonetheless, despite the infectiveness of the trypanocide, the humoral immune response to the secondary vaccination was quite profound and ultimately attained the level of control at Week 3 after vaccination. This is somewhat similar to the report of Ballweber which showed that *T. congolense* infected cattle with suppressed primary immune response after trypanosomal infection mounted a secondary response following trypanocidal treatment[26]. It could therefore seem that both parasites had no effect on the memory B cells which were initially inhibited during infection or that the drugs administered aided in reviving the suppressed immunity as it were in mebendazole treated group (GPII). In conclusion, both trypanosomosis and ancylostomosis induced a significant decrease in antibody response to antirabies vaccination in dogs. Dogs with mixed infections of both disease conditions had a much suppressed antibody response. It is therefore pertinent that dogs in endemic areas be placed on effective control protocols against both disease conditions.

Conflict of interest statement

We declare that we have no conflict of interest.

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