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# Natural Resistance of Avian Sera to *Trypanosoma brucei brucei* (Plimmer & Bradford)

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**Key words:** *In vitro* anti-*Trypanosoma brucei brucei* activity, Survival time, Birds, Innate immunity, Xanthine oxidase.

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## Abstract

This study evaluated the natural anti-*Trypanosoma brucei brucei* activity of sera from some birds viz: Broiler chicken (*Gallus gallus domesticus*), Shika brown chicken (*Gallus gallus domesticus*), Parent Stock White chicken (*Gallus gallus domesticus*), Parent Stock brown chicken (*Gallus gallus domesticus*), Guinea fowl (*Numida meleagris*), Domestic pigeon (*Columba livia domestica*), Goose (*Plectropterus gambensis*), Crowned crane (*Balearica regulorum*), Peacock (*Pavo cristatus*), Spotted eagle owl (*Bubo africanus*), Hooded vulture (*Necrosyrtes monachus*) and Buzzard (*Buteo buteo*). To ensure that the donor birds were not infected with trypanosomes, their blood samples were screened using the thick film and haematocrit centrifugation technique (HCT). *Trypanosoma brucei brucei* (Federe strain) was suspended in RPMI supplemented with FCS and the motility of the parasite was used as an index of viability after the addition of each test serum. Bird sera generally exhibited some degree of anti-*Trypanosoma brucei brucei* activities *in vitro*. Serum from broiler hen had the highest anti-*Trypanosoma brucei brucei* activity showing an inhibition index of 0.83, with a marked and significant reduction ($P<0.05$) in survival time of 5.00±0.00 hours compared with that of all the sera from other birds including the control (30.00±0.00 hours). The *in vitro* anti-trypanosomal activity in all the serum samples was cidal in nature and appeared to be mediated by xanthine oxidase (XO), except in the serum of buzzard. The highest anti-*Trypanosoma brucei brucei* activity in the serum of broiler hen also coincided with the highest XO content. This study shows that sera from the selected birds have innate immunity against *T. brucei brucei*.

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## Introduction

Trypanosomiasis is one of the most common and devastating diseases affecting humans and livestock worldwide. Animal trypanosomiasis (Nagana) remains a major constraint to ruminant livestock production in Africa, Asia, and South America (ILRAD, 1990; Gutierrez *et al*., 2006). African animal trypanosomiasis is caused by several species such as *Trypanosoma vivax*, *Trypanosoma congolense* and *Trypanosoma brucei brucei*. The disease is manifested by increased heat production and raised body temperature, intermittent fever, anaemia, lymphatic enlargement with hepatosplenomegally and progressive cachexia (Dam *et al*., 1996). Some haematological changes (Omotainse and Anosa, 1995; Onah *et al*., 1996) and reproductive disorder (Sekoni, 1994) have also been observed in animals infected with trypanosomes. Cattle rearing being among the major occupation in Africa is greatly affected; the disease causes about 3 million deaths annually in cattle, causing annual production losses of about US$ 1.2 billion (Chamond *et al*., 2010).

The control measures adopted for the disease revolved around the use of trypanocidal drugs since the antigenic variations often exhibited by the parasite rendered the production of vaccine (using antibody) a very difficult task. Nonspecific factors from natural trypano-resistant animals have been investigated as a means of developing new control approaches against trypanosomiasis, especially in natural resistant species. Xanthine oxidase, a nonspecific factor, is a trypanocidal protein isolated from Cape buffalo serum (Muranjan *et al*., 1997).

Hydrogen peroxide ($H_2O_2$) generated during catabolism of hypoxanthine and xanthine by the serum enzyme inhibits trypanosome glycolysis, leading to a precipitous decline in cellular ATP (Black *et al*., 1999). Also, a cross-section of birds has been reported to be resistant against *T. cruzi* (Kierszenbaum *et al*., 1981). Considering these facts, our study aimed at evaluating the natural anti-trypanosomal properties of sera of some birds on *T. brucei brucei in vitro*.

## Materials and methods

*Ethical statement*

This research had ethical approval from the Committee on Animal Use and Care, Directorate of Academic Planning & Monitoring, Ahmadu Bello University, Zaria, with the Approval No: ABUCAUC/2017/007. Local approvals were given by the Director of Kano Zoological Garden and the Assistant Director of Research, NAPRI, Ahmadu Bello University, Zaria. Recommendations on guidelines for the accommodation and care of animals used, based on Directive 86/609/EEC (EU Directive 2010/63/EU for animal experiments), were taken into consideration in the course of this study.

*Sampling sites*

Zaria is located on the high plains of Northern Nigeria, 652.6 meters above the sea level, some 950 km away from the coast (112 031N 7Q 42E). Zaria is a city of Kaduna state which extends from the tropical grassland known as the Guinea Savannah to Sudan Savannah.
(https://abu.edu.ng/zaria-at-a-glance.html).

Kano, the capital of Kano state is located on Latitude 12°N and Longitude 8.3°E. Kano is 481 metres (1,578 feet) above sea level. The city lies within the semi-arid region of the Soudan savannah zone of West Africa about 840 kilometers from the edge of the Sahara desert. Kano has a mean height of 472.45m above the sea level and has two seasonal periods categorized based on moisture as dry and rainy seasons. The temperature usually ranges between 33°C and 15.85°C although sometimes during the harmatan it drops to as low as 10°C (Karamba and Mukhtar, 2012).

*Donors birds*

Blood was collected, with the assistance of veterinary assistants, from some birds reared in areas free of trypanosomes (Zaria and Kano in Nigeria) and were also certified trypanosome free by the thick film and haematocrit technique (Woo, 1989). Broiler chicken (*Gallus gallus domesticus*), Shika brown chicken (*Gallus gallus domesticus*), Parent Stock White

chicken (*Gallus gallus domesticus*), Parent Stock brown chicken (*Gallus gallus domesticus*) were obtained from the National Animal Production Research Institute (NAPRI), Ahmadu Bello University, Zaria, Kaduna State, Nigeria. Guinea fowl (*Numida meleagris*) was purchased from Samaru market, Zaria, Nigeria. Domestic pigeon (*Columba livia domestica*), Goose (*Plectropterus gambensis*), Crowned crane (*Balearica regulorum*), Peacock (*Pavo cristatus*), Spotted eagle-owl (*Bubo africanus*), Hooded vulture (*Necrosyrtes monachus*) and Buzzard (*Buteo buteo*) were provided by the Kano Zoological Garden, Kano State, Nigeria.

*Blood sample collection and processing*

Five millilitres (5ml) of the blood sample was aseptically collected from the donor animals (birds) through the wing vein; 1 ml of this was dispensed into EDTA (ethyl-diamine-tetra-acetic acid) coated tube placed in a beaker containing iceblocks while the remaining (4 ml) was dispensed into the sterile plain glass test tube and allowed to clot for 2-3 hours at room temperature from which serum was collected after centrifugation at 4,000 g for 5min (Black *et al.*, 1999). Sera samples obtained were stored in a freezer at -20°C until required for further analysis. Blood samples in the EDTA tubes were used to screen for the presence of trypanosomes based on which the serum of trypanosome free samples was used for the anti-trypanosomal activity.

*Screening of blood for trypanosomes*

Each blood sample in the EDTA treated tubes was screened for trypanosomes by the thick films and haematocrit centrifugation technique (HCT) (Woo, 1989).

*Parasite*

*Trypanosoma brucei brucei* (Federe strain), was obtained from the Nigerian Institute of Trypanosomiasis Research (NITR), Kaduna, Nigeria. The parasite was maintained through serial passaging in Wistar rats. Blood was collected from the tail of infected rats by capillary action into heparinized microhaematocrit tubes up to three-quarter their length, one end of each capillary tube was sealed with plastiseal. The sealed capillary tubes were placed in a microhaematocrit centrifuge and spun at 9000 g for 5 min. The parasites were extracted from the buffy-coat and used for the experiment.

*In vitro detection of anti-trypanosomal activity of the test sera*

This was done using microtitre plates as follows:

Trypanosomes from the buffy coat were suspended in RPMI 1640 (Rossvelt Park Memorial Institute 1640) medium supplemented with 2% glucose, 2mM sodium pyruvate, 10% complement - inactivated (56°C for 30 min) fetal bovine serum (Black and Van-deweerd, 1989), sodium bicarbonate and sodium pyruvate, and antibiotics (streptomycin 100µg/ml, penicillin 100U/ml). A total of 50µl of each bird serum sample was introduced into one of the 96 wells of a microtitre plate and 50µl of *T. brucei brucei* suspension 8 per field ($31.62\times10^6$/ ml of blood) was added to it, rocked gently to mix and incubated at room temperature. Thereafter, a drop of about 5µl of each mixture was examined microscopically on an hourly basis using the wet film method. Cessation in the motility of parasites was taken as an indication of inhibitory/cidal serum activity against the parasites (Atawodi *et al.*, 2003; Bulus *et al.*, 2008). The motility of *T. brucei brucei* in each well was compared with the motility of the same parasites in the control well without test serum. A formula was derived (Djieyep *et al.*, 2017) to determine the Anti-Trypanosomal Activity Index (ATI) of each serum sample.

$$ATI = \frac{T_c - T_s}{T_c}$$

Where: $T_c$ is the survival time of the parasites in the control medium

$T_s$ is the survival time of the parasites in the sample.

*Infectivity assessment*

80µl of the mixture of the *in vitro* affected parasite with effective serum was inoculated into mice and monitored by microscopy for trypanosomes on daily basis for 10 days to determine if the observed anti-trypanosomal activity was inhibitory or cidal.

*Detection of Anti-Trypanosomal Antibodies in Serum Samples*

This was done using a slightly modified Ouchterlony Double Diffusion test (Ouchterlony and Nilsson, 1978).

*Detection of the xanthine oxidase content of the selected sera*

The technique of Black *et al.* (1999) was adopted to detect the xanthine oxidase content of the test sera. Accordingly, aliquots (100 µl) of each serum was added to 900 µl of $H_2O_2$-assay buffer (0.5 mM xanthine and 1 mM 2, 4, 6 tribromo-3-hydroxybenzoic acids in 0.1 mM 4-amino-antipyrine with a final concentration of 8 units horse-radish peroxidase per ml). The mixture was incubated at 25°C for 30 min, immediately chilled in an ice bath, and absorbance read at 512 nm wavelength was recorded after zeroing the spectrophotometer with an equivalent mixture lacking horseradish peroxidase. A serial dilution of the commercial cow's milk xanthine oxidase was done; absorbance was also recorded at 512 nm wavelength and used to plot a standard curve. It has been established that detection of $H_2O_2$ produced in serum by this assay is not affected by other enzymes in serum, including catalase (Le Tissier *et al.*, 1994).

The xanthine oxidase content of serum was determined by reading the serum value against the cow's milk xanthine oxidase standard curve.

*Data analysis*

The software Statistical Package for Social Sciences (SPSS) version 20 was used. One-way analysis of variance (ANOVA) was used to analyze the variations in the survival time of trypanosomes exposed to test sera as well as the xanthine oxidase content of the test sera. The significant difference was at the level of probability 0.5. All data were expressed as means ± Standard Error.

**Results**

*Trypanosome Infection Status of Sera from Selected Birds*

All the blood samples from the selected birds were negative for trypanosomes by thick blood film and haematocrit centrifugation technique (HCT).

**Table 1.** Survival time of *Trypanosoma brucei brucei* in test sera with the anti-trypanosoma activity index of selected birds.

| Source of serum | Survival time (Hr) | ATI |
|---|---|---|
| *Gallus gallus domesticus* (Broiler cock) | $21.00\pm0.00^{de}$ | 0.3 |
| *Gallus gallus domesticus* (Broiler hen) | $5.00\pm0.00^{a}$ | 0.83 |
| *Gallus gallus domesticus* (Shika brown cock) | $19.67\pm4.04^{cde}$ | 0.34 |
| *Gallus gallus domesticus* (Shika brown hen) | $11.33\pm1.15^{b}$ | 0.62 |
| *Gallus gallus domesticus* (Parent Stock White cock) | $21.33\pm1.15^{e}$ | 0.29 |
| *Gallus gallus domesticus* (Parent Stock White hen) | $16.67\pm2.89^{cd}$ | 0.44 |
| *Gallus gallus domesticus* (Parent Stock Brown Cock) | $16.00\pm1.73^{c}$ | 0.47 |
| *Gallus gallus domesticus* (Parent Stock Brown Hen) | $10.00\pm2.00^{b}$ | 0.67 |
| *Numida meleagris* (Guinea fowl) | $9.33\pm1.15^{b}$ | 0.69 |
| *Columba livia domestica* (Domestic pigeon) | $20.67\pm1.15^{de}$ | 0.31 |
| *Plectropterus gambensis* (Goose) | $16.00\pm3.46^{c}$ | 0.47 |
| *Balearica regulorum* (Crowned crane) | $18.67\pm4.16^{cde}$ | 0.37 |
| *Pavo cristatus* (Peacock) | $18.33\pm3.78^{cde}$ | 0.39 |
| *Bubo africanus* (Spotted eagle owl) | $11.33\pm1.15^{b}$ | 0.62 |
| *Necrosyrtes monachus* (Hooded vulture) | $20.67\pm1.15^{de}$ | 0.31 |
| *Buteo buteo* (Buzzard) | $11.33\pm1.15^{b}$ | 0.62 |
| RPMI 1640* | $30.00\pm0.00^{f}$ | 0 |

Values are mean ± standard deviation. Values with different superscripts down the column are significantly different ($P<0.05$). ATI: Anti-trypanosomal Activity Index.

*Culture medium without serum (control).

*Effect of the Selected Avian Sera on Trypanosoma brucei brucei*

*Survival time of T. brucei brucei in the selected sera*

The survival times of *T. b. brucei* in the all the test sera ranched from 5 – 21 Hr, which were significant reductions ($P<0.05$) compared with the 30 Hr survival time observed in the control (Table 1). Serum from broiler hen exerted the most inhibitory effect by reducing the survival time to 5.00±0.00 hr, which was the least survival time with the highest ATI of 0.83 compared to the rest of the serum samples tested. This was followed by the serum from Guinea fowl with a survival time of 9.33±1.15 hr and an ATI of 0.69. The serum of parent stock white cock exhibited the highest survival time of 21.33±1.15 and the least ATI of 0.29 (Table 1).

**Table 2.** Xanthine oxidase concentration of sera from the selected birds.

| Source of serum | Concentration of the xanthine oxidase (µg/ml) |
|---|---|
| *Gallus gallus domesticus* (Broiler cock) | $2.60\pm0.02^{gh}$ |
| *Gallus gallus domesticus* (Broiler hen) | $3.03\pm0.16^{h}$ |
| *Gallus gallus domesticus* (Shika brown cock) | $2.42\pm0.03^{fgh}$ |
| *Gallus gallus domesticus* (Shika brown hen) | $1.59\pm0.58^{def}$ |
| *Gallus gallus domesticus* (Parent Stock White cock) | $1.99\pm0.15^{efg}$ |
| *Gallus gallus domesticus* (Parent Stock White hen) | $2.33\pm0.40^{fgh}$ |
| *Gallus gallus domesticus* (Parent Stock Brown Cock) | $1.62\pm0.05^{def}$ |
| *Gallus gallus domesticus* (Parent Stock Brown Hen) | $0.13\pm0.30^{ab}$ |
| *Numida meleagris* (Guinea fowl) | $1.63\pm0.15^{def}$ |
| *Columba livia domestica* (Domestic pigeon) | $2.38\pm0.24^{fgh}$ |
| *Plectropterus gambensis* (Goose) | $1.76\pm0.03^{ef}$ |
| *Balearica regulorum* (Crowned crane) | $0.68\pm0.96^{abc}$ |
| *Pavo cristatus* (Peacock) | $1.44\pm0.07^{cde}$ |
| *Bubo africanus* (Spotted eagle owl) | $0.85\pm0.15^{bcd}$ |
| *Necrosyrtes monachus* (Hooded vulture) | $1.91\pm0.44^{efg}$ |
| *Buteo buteo* (Buzzard) | $0.00\pm0.00^{a}$ |

Values are mean ± standard deviation. Values with different superscripts down the column are significantly different ($P<0.05$).

*Infectivity*

None of the parasites exposed to the effective sera *in vitro* was able to cause infection in mice up to the end of the experiment.

*Antibody activity of effective sera from selected birds against T. brucei brucei antigens*

No antibody activity against *T. brucei brucei* antigens was detected in the test sera of the selected birds.

*Xanthine oxidase content of sera from selected birds*The xanthine oxidase content of serum from each selected bird is presented in Table 2. No xanthine oxidase was detected in the serum of buzzard. Parent stock brown hen had the lowest concentration of serum xanthine oxidase (0.13±0.30 µg/ml) while serum from broiler hen had the highest xanthine oxidase content (3.03±0.16 µg/ml), which was significantly higher ($P<0.05$) than that of eleven (11) other selected birds, but at par with four (4) others (namely broiler cock, Shika brown cock, parent stock white hen and domestic pigeon) (Table 2).

**Discussion**

In this study, sera from selected birds generally exhibited significant anti-*Trypanosoma brucei brucei* activity *in vitro* with varying degrees of activity index. The results suggest that the activity was trypanocidal

as shown by the inability of the trypanosomes exposed to the test sera to infect mice thereafter. Furthermore, the trypanocidal activity was not mediated by antibodies as shown by the absence of antibodies in the test sera. Given that xanthine oxidase was detected in all the test sera, except that of the serum of buzzard, suggest a role for xanthine oxidase in the observed trypanocidal activity of the sera; xanthine oxidase is known for its anti-trypanosomal action; it is an enzyme that yields trypanocidal $H_2O_2$ during catabolism of hypoxanthine and xanthine to uric acid (Muranjan *et al.*, 1997)**.** The $H_2O_2$ generated by the serum enzyme inhibits trypanosome glycolysis, leading to a precipitous decline in cellular ATP (Black *et al.*, 1999). Indeed, according to Kierszenbaum *et al.* (1981), the natural resistance displayed by avian serum on virulent forms of *T. cruzi* is mediated by the non-specific factor(s) rather than antibodies and complements. This assertion is supported by the fact that the anti-trypanosomal activity of the test sera observed in this study related directly well with xanthine concentration.

This is well exhibited by the test serum of broiler hen, which exerted the most cidal effect with the corresponding highest ATI (0.83) and xanthine oxidase concentration. This trend was observed in other test sera that exhibited profound anti-trypanosomal activity.

It is of interest to note that the serum from buzzard, which exhibited a relatively significant reduction in survival time and high ATI against *T. b. brucei* lacked xanthine oxidase, suggesting that an unknown/non-specific factor(s) is/are at play.

**Conclusion**

This study revealed the presence of anti-*Trypanosoma brucei brucei* components in sera of the selected birds, with a corresponding nexus to the presence of xanthine oxidase rather than antibodies. These findings could be exploited for developing a new control strategy against *T. brucei brucei* infection.

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