



# Pathogenicity of Drug-resistant Canine Isolate of *Trypanosoma brucei brucei* in Rats

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## ABSTRACT

**Background:** Trypanosomosis is a neglected tropical disease. Drug resistance in trypanosomosis has been on the increase in recent years. It is presumed that drug resistant trypanosomes are less pathogenic and may be very suitable for rodent models of human African trypanosomosis (HAT).

**Methods:** The *T. b. brucei* used was locally isolated from a dog and characterized by PCR. Drug resistance trait of the isolate was evaluated using diminazine aceturate and isometamidium chloride. Healthy rats were inoculated with the isolate, IP. Some haemato-biochemical parameters were evaluated. Three rats were euthanized on day 70 PI and necropsied. Data generated from the study were analyzed by student's *t*-test and significance was accepted at probability,  $p < 0.05$ .

**Result:** The isolate was confirmed to be drug resistant *T. b. brucei* and caused no mortalities in the infected rats. The low pathogenicity in rats was discussed and the typical CNS lesions of chronic-stage HAT produced by the isolate makes it a suitable candidate for rodent models of HAT.

**Key words:** Drug resistance, Human African trypanosomosis, Pathogenicity, Rodent model, *Trypanosoma brucei brucei*.

## INTRODUCTION

Trypanosomes are single-celled protozoa, transmitted by tsetse fly (*Glossina spp*) vector, that cause severe disease known as trypanosomosis in both humans and livestock (Morrison, 2011; Aksoy *et al.*, 2017). The disease is generally devastating in susceptible animals and is characterized by pyrexia, anemia, immunosuppression, emaciation, organomegalies and inflammatory reactions in different organs (Ikede and Losos, 1972). Anaemia is, however, the most important clinicopathological finding in trypanosomosis (Naessens, 2006) and death usually occurs as a result of the combined effects of anaemia, secondary infections due to immunosuppression and physiologic collapse (Sternberg, 2004; Naessens, 2006; Kennedy, 2013).

*Trypanosoma brucei gambiense* and *Trypanosoma brucei brucei* are known to cross the blood-brain barrier, producing a variety of psychoneuroendocrine abnormalities as well as brain histopathological changes in both humans and animals (Masocha *et al.*, 2004). Many studies using rodent models have contributed immensely to what is known about chronic – stage human African trypanosomosis (HAT) caused by *T. b. gambiense*. The rodent studies are usually done using *T. b. brucei* (Keita *et al.*, 1997). However, a major challenge is that many isolates of *T. b. brucei* run sub-acute course in most susceptible animal species including rodents with death in untreated cases, within 4 weeks of onset of parasitaemia. Therefore, it is difficult to produce a chronic disease course in rodents naturally.

Drug resistance by trypanosomes has become a serious concern in sub-Saharan Africa due to enormous increase in treatment failures recorded with even the previously very effective trypanocides such as diminazine aceturate and isometamidium (Egbe-Nwiyi *et al.*, 2003). Previous reports

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on drug-resistant trypanosomes presumed that they are less virulent (Egbe-Nwiyi *et al.*, 2003) and that diminazine aceturate resistant *T. b. brucei* carry such reduced virulence from one host to another (Ihedioha *et al.*, 2010). It is projected that the existence of drug resistant isolates of *T. b. brucei*, which are able to run a chronic disease course without treatments and also cross the blood-brain barrier, would mimic both humoral and histopathological findings in the meningoencephalitic form of HAT and, therefore, may serve as more convenient candidates for rodent models of HAT. The present study was conducted to evaluate the pathogenesis of drug resistant isolate of *T. b. brucei* in rats.

## MATERIALS AND METHODS

This study was conducted in University of Nigeria, Nsukka between 2019 and 2020. Eighteen male (18) albino mice and Ten (10) male Sprague Dawley® rats were used for the study. They were acclimatized for two weeks in metal cages and given feed and water *ad libitum*. Wet mount and

Leishman's staining techniques on thin blood films and buffy coat smears were used to rule out the presence of haemoparasites in the mice and rats. Complete blood count was done to further ascertain the health condition of the mice and rats. The parasite used was initially isolated from a mongrel dog within the study area and identified as *Trypanosoma brucei* species based on morphological characteristics, following wet mount and thin blood smear (Soulsby, 1982). It was further characterised by ITS1 PCR following DNA extraction using Chelex method (Eisler *et al.*, 2001).

Tests for drug susceptibility or resistance were performed in mice. Trypanocidal drugs used were diminazine aceturate (DA) and isometamidium chloride (IMC). Three groups of mice comprising the infected control (n = 6), DA-treated group (n = 6) and IMC-treated group (n = 6) were infected by intraperitoneal (IP) inoculation of  $10^5$  *T. b. brucei* isolate per mice and treated (except the control) with DA @ 20 mg/kg BW, IP and IMC @ 1 mg/kg BW, IP at 24 h post-infection. Parasitaemia was monitored in the three groups weekly for 60 days (Singh *et al.*, 2018).

The rats used for the pathogenicity study were randomly assigned into two (2) groups, A and B, comprising 5 rats each. Group A served as the control and group B was the infected group. Infection of group B was achieved by IP injection of  $10^6$  trypanosomes /rat. Baseline (day 0) values for the parameters evaluated were obtained before infecting group B. Blood samples for determination of parasitaemia were routinely collected from the tail vein, but blood samples for haematology and hormonal assay were collected from the retro-bulbar plexus on days 12, 20, 26, 33, 40, 47, 54, 61 and 68. Blood for haematology was collected in ethylene diamine tetraacetic acid (EDTA)- coated tubes. Serum samples were obtained by centrifuging clotted blood samples at 3000 rpm for 10 minutes and decanting clear sera into clean tubes for hormonal assay. The packed cell volume (PCV) was determined by microhaematocrit method (Thrall and Weiser, 2002). Haemoglobin concentration (HbC) was measured by cyanomethaemoglobin method (Higgins *et al.*, 2008). Red blood cell (RBC) count and total white blood cell (TWBC) count were done by haemocytometer method (Thrall and Weiser, 2002). Two reproductive hormones were assayed; serum levels of luteinizing hormone (LH) and testosterone by microplate enzyme-immunoassay technique using commercial kits (Gitonga *et al.*, 2017). On 70<sup>th</sup> day post-infection, 3 rats from each group were humanely sacrificed. Their brains, pituitary glands, spleen and testes were carefully dissected. Tissue samples from these organs were fixed in 10% neutral-buffered formalin for 48 hours and routinely processed, sectioned with a rotary microtome at 5  $\mu$ m thickness and stained with haematoxylin and eosin (H&E). Data generated from the study were analyzed by Student's *t*-test using SPSS version 15.0. The level of significance was accepted at probability  $P < 0.05$ . The results were presented as means for each group with standard error (SE).

## RESULTS AND DISCUSSION

### Identification of parasites

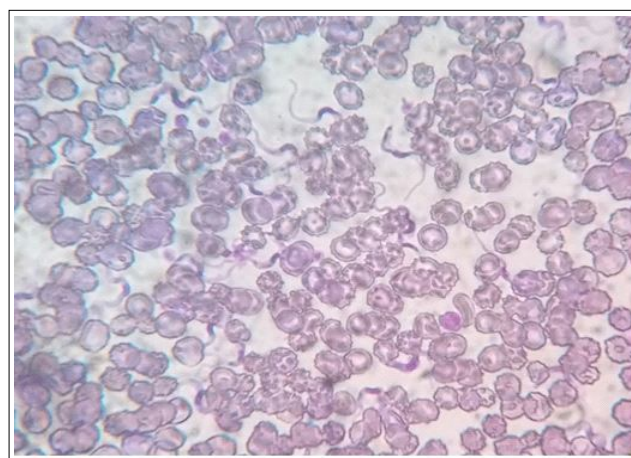
In the infected group, Giemsa-stained blood smear revealed numerous slender forms of *T. b. brucei* with their characteristic elongated body measuring 20-30  $\mu$ m in length, pointed posterior end, prominent undulating membrane and free flagellum (Fig 1). The blood samples on FTA® cards were positive for *T. b. brucei* on ITS1-PCR gel at 500bp, under a positive control (Fig 2).

Some of the structural characteristics of *T. brucei* species may be revealed in Giemsa-stained thin blood films and may tentatively identify the parasite (Giordani *et al.*, 2016). These features were earlier used in the study for initial identification of the isolate in addition to a nested PCR, ITS1 PCR, which permits multiple trypanosome species to be detected from a single reaction (Cox *et al.*, 2005). Ribosomal RNA (rRNA) genes in trypanosomes contain both a non-transcribed spacer (NTS) region and internal transcribed spacer (ITS) regions (Ahmed *et al.*, 2013). The ITS regions show variability in length among related species and this makes it useful for differentiating species of trypanosomes (Desquesnes *et al.*, 2001; Desquesnes and Davila, 2002).

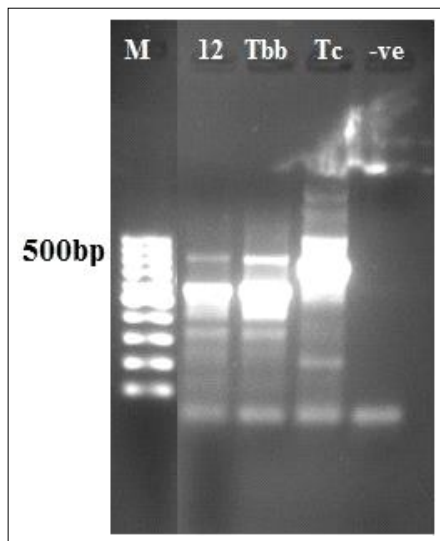
**Table 1:** Absolute parasitaemia scores/ml blood of *T. brucei brucei* infected rats.

Days post infection	Absolute parasitaemia scores/ml blood
5	1, 000, 000
14*	125, 900, 000
21	63, 100, 000
28	158, 500, 000
35*	316, 200, 000
42	63, 100, 000
49	79, 430, 000
56	79, 430, 000
63	100, 000, 000
70*	158, 500, 000

\*Parasitaemia peak days.



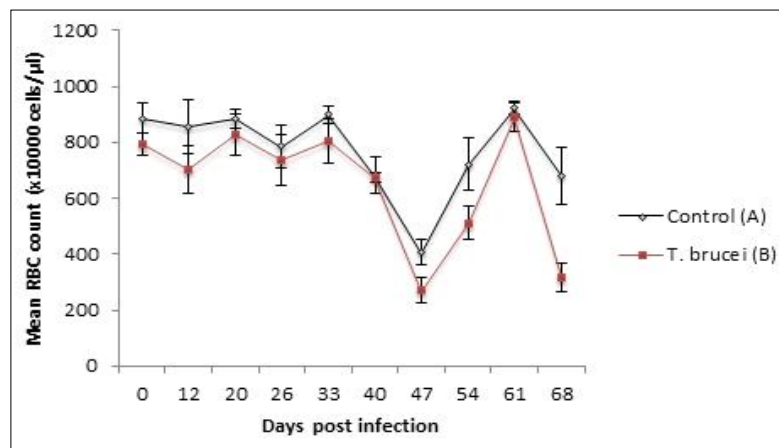
**Fig 1:** Giemsa- stained blood smear from the infected rats showing slender forms of *Trypanosoma brucei brucei*.



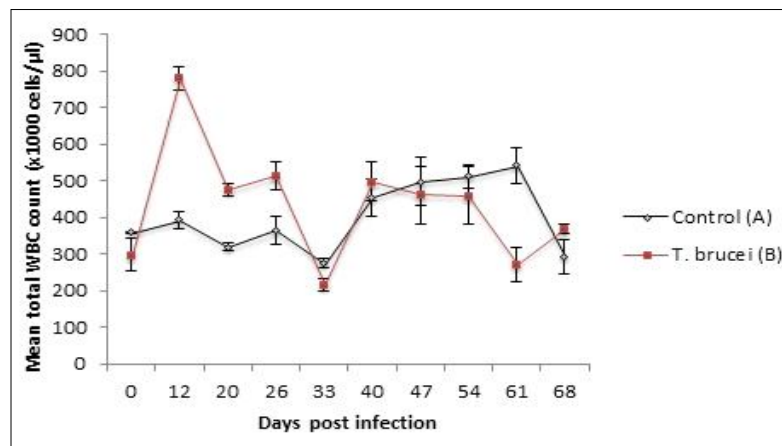
**Fig 2:** Agarose gel showing the *Trypanosoma brucei brucei* (lane 12) used in the study following IT IS – PCR. Lane Tbb: *Trypanosoma brucei brucei* – positive control. Lane Tc: *Trypanosoma congolense* (savannah) – positive control. Lane –ve: Negative control.

### Drug resistance and parasite's pathogenicity

The drug resistant study in mice showed that the isolate was resistant to diminazine aceturate (DA) and isometamidium chloride (IMC) and the infected mice remained parasitaemic at the end of the study with death occurring in the DA-treated group but not in the IMC- treated group. The pathogenicity studies in rats showed that parasitaemia was first observed on day 5 PI, after which 2 peaks of parasitaemia were recorded on days 14 and 35 PI (Table 1). All the infected rats remained parasitaemic till the end of the study. There were no significant ( $P>0.05$ ) differences in the mean body weight, rectal temperature, PCV, HbC, serum LH and testosterone levels of the infected rats compared to the uninfected rats throughout the period of the experiment, although the infected group consistently had slightly higher rectal temperature and lower PCV values than the uninfected control. A significantly ( $P<0.05$ ) lower RBC count was observed only on days 47, 54 and 68 of the experiment in the infected group compared to the control (Fig 3). The TWBC count of the infected group was also significantly ( $P<0.05$ ) higher than that of the control group on days 12 and 20 followed by significant ( $P<0.05$ ) decreases on days 33 and 61 post-infection (Fig 4). No



**Fig 3:** Graph showing variations in the mean red blood cell (RBC) count of the rat groups.



**Fig 4:** Graph showing variations in the mean total white blood cell (TWBC) count of the rat groups.

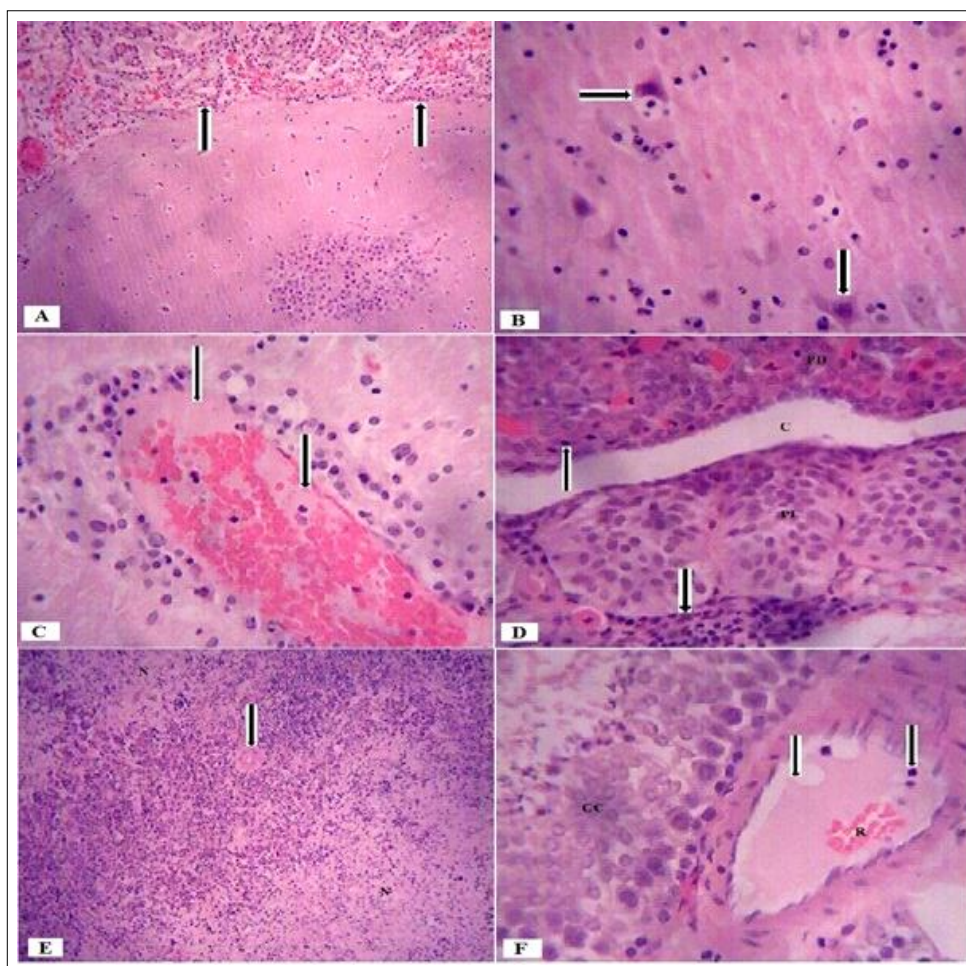
mortalities were observed in the infected group throughout the duration of the experiment.

Parasitaemia level in this study was relatively low and may imply that the isolate was less pathogenic. More virulent strains produce higher peaks of parasitaemia (Morrison, 2011). The possible low pathogenicity of this isolate may account for the lack of significant variation in the haematobiochemical parameters assessed, which are usually significantly altered in infections with more virulent isolates (Turner, 1990). The significant decrease in the erythrocyte and leukocyte counts at the later stage of the infection suggests anaemia and immunosuppression, respectively, which are the hallmark of this disease in susceptible hosts (Naessens, 2006). In chronic infection, increased erythrophagocytosis and dyserythropoiesis characterize the bone marrow changes and are believed to play an important

role in the production of anaemia in trypanosomosis (Ojok *et al.*, 2001). However, the degree of anaemia and immunosuppression in the present study were mild and may account for the unusual survival of the infected rats for up to 70 days post-infection. Furthermore, the prolonged disease course without mortalities may also be due to previous exposure of the isolate to trypanocides or an inherent phenotypic feature of drug resistant isolates (Egbe-Nwiyi *et al.*, 2005; Ihedioha *et al.*, 2010). On the other hand, resistance of the isolate to both DA and IMC may also be due to genetic alterations in the parasite, also probably resulting from previous exposure to trypanocides (Steward *et al.*, 2010).

### Histopathology

Microscopic lesions in the brain comprised of non-suppurative meningoencephalitis (Fig 5A), gliosis and



**Fig 5:** Tissue sections of the brain, anterior pituitary, spleen and testis of *T. brucei brucei* infected rats. A - C: brain tissue sections, A - showing meningeal hyperaemia with severe infiltration of mononuclear inflammatory cells (arrows), B - gliosis of the neuropil with satellitosis and neuronophagia (arrows) and C - perivascular cuffing of cerebral blood vessel with lymphocytes, macrophages and glial cells. Note congested blood vessel (arrows). D - anterior pituitary gland showing moderate infiltration of mononuclear cells (arrows) at the base of the pars intermedia (PI) and in the interstitial spaces of the pars distalis (PD). E - splenic tissue showing massive lympholysis and necrosis of the splenic parenchymal tissue (N) and atrophy of the red pulp. Note the arteriole (arrow) in a depopulated germinal center of the white pulp. F - testicular tissue showing congested blood vessel with few erythrocytes (R) and mononuclear inflammatory cells (arrows). Note the germ cells of the seminiferous tubules (GC). H&E stain (A, x100; B-E, x400)

satellitosis/neuronophagia (Fig 5B) and perivascular cuffing of congested cerebral blood vessels (Fig 5C). The pituitary gland showed moderate infiltration of mononuclear inflammatory cells (Fig 5D). Severe parenchymal necrosis and lympholysis were evident in the spleen with atrophied red pulp (Fig 5E). Lesions in the testis were mainly vascular congestion with few mononuclear cells in the blood vessels and interstitial spaces (Fig 5F).

The microscopic lesions in the brain are consistent with histopathological findings in both the meningoencephalitic stage of HAT and CNS involvement in African animal trypanosomiasis (Ikede and Losos, 1972; Philip *et al.*, 1994; Galiza *et al.*, 2011). Inflammatory changes extended also to the pituitary gland, but were not sufficient to cause alterations in the pituitary-gonadal axis, hence the lack of significant changes in the luteinizing hormone and testosterone in the present study. Nevertheless, inflammation and congestion of testicular blood vessels observed in this study may be deleterious to spermatogenesis and may aid invasion of *T. b. brucei* into the testicular tissues. Testicular degeneration is a common finding in animal trypanosomiasis (Cnops *et al.*, 2015). Similarly, lympholysis and atrophy of the red pulp observed in the spleen in this study may be responsible for the significant reduction in the total leukocyte and erythrocyte counts especially at the later stages of the infection. These may explain the occurrence of immunosuppression and anaemia commonly observed in trypanosome infection in susceptible animal hosts and man (Vincendeau and Bouteille, 2006).

## CONCLUSION

The characteristic CNS lesions produced by this isolate and its low virulence, thereby prolonging the survival of the rats, would make it very suitable for rodent models of HAT. It was not clear whether this low virulence was as a result of phenotypic changes during passage across hosts or genetic alteration induced by possible previous treatments with trypanocides, but it appears that drug-resistant trypanosomes may generally be less pathogenic.

**Conflict of interest:** None.

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