Nigerian Veterinary Journal 2009 Vol 29(4): 34 - 42

BERENIL(R)-RESISTANT *Trypanosoma brucei brucei* INFECTION IN A DOG IN NSUKKA AREA OF ENUGU STATE, NIGERIA

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SUMMARY

A 13-month old female dog was presented at the University of Nigeria Veterinary Teaching Hospital, Nsukka, with primary complaints of blindness, weakness and inability to stand. General examination of the dog revealed lateral recumbency, dehydration and emaciation, enlarged peripheral lymph nodes, swollen face, bottle jaw (submandibular oedema), bilateral corneal opacity and discharge, pale mucous membranes, high respiratory rate (panting) and body temperature (40°C) as well as bruises on the body. Examination and analysis of the blood sample revealed numerous trypanosomes and a packed cell volume of 11%. The parasite was identified as *Trypanosoma brucei brucei*. Repeated treatment of the dog with diminazene aceturate (Berenil®) at 7 mg/kg body weight failed to eliminate the parasites from the peripheral blood. Furthermore, experimental infection of mice with the parasite and treatment with various doses of berenil at various stages also failed to cure the infection, confirming that the isolate is berenil-resistant. The case showed the implications of long-term use of berenil in the treatment of trypanosomosis and the increasing occurrence of drug-resistant trypanosome isolates in the area.

KEY WORDS: Dog, *Trypanosoma brucei*, Natural infection, Berenil-resistance, Nsukka

INTRODUCTION

Canine trypanosomosis characterised by pyrexia, weakness, weight loss and anaemia is a frequently diagnosed disease condition in the Nsukka area of Enugu State, Nigeria. It usually presents in three clinical forms: ocular, lymphadenopathic and cerebral (Omamegbe *et al.*, 1984). The ocular and lymphadenopathic forms almost invariably occur together but all three clinical forms have been observed concurrently in some cases. *Trypanosoma brucei* and *T. congolense* are incriminated in the ocular and lymphadenopathic forms but the former is the main cause of the three forms of the disease, accounting for approximately 70 percent of such cases (Omamegbe *et al.*, 1984). However, all reported natural cases of cerebral trypanosomosis in the area have been due to T. brucei (Adewunmi and Uzoukwu, 1979; Omamegbe *et al.*, 1984; Onah and Uzoukwu, 1991).

Since 1970, the treatment of clinical cases of canine trypanosomosis in Nsukka area has invariably been with diminazene aceturate (Berenil™, Hoechst AG, Frankfurt am-Main, Germany), an aromatic diamidine that was first introduced in 1955 as a therapeutic agent for the treatment of animal trypanosomosis (Mamman *et al.*, 1995). This practice is against the manufacturer's instructions that diminazene aceturate is contraindicated in dogs and camels. This notwithstanding, it has been indicated that berenil might be used for the treatment of *T. brucei*, *T. congolense* and *T. evansi* infections in dogs. Although it was also noted that while it is curative for *T. congolense* and *T. evansi*, it may not be so for *T. brucei* (Holmes, 2005).

Only quinapyramine sulphate and suramin were indicated as curative for canine *T. brucei trypanosomosis* (Holmes, 2005). The long-term routine use of berenil as the only trypanocide, not just in dogs but also in other animals in Nsukka area, has resulted in serious problems in the treatment and management of the disease. Over the years, records at the University of Nigeria Veterinary Teaching Hospital (UNVTH) show a steady increase in the number of failures in the treatment of natural cases of trypanosomosis using berenil (Adewunmi and Uzoukwu, 1979; Omamegbe *et al.*, 1984; Onah and Uzoukwu, 1991). In addition, results of several experimental studies with *T. brucei* isolates from the Nsukka area have suggested the existence of resistance by *T. brucei* to berenil and other trypanocidal drugs in the area (Anika *et al.*, 1987; Anene, 1997; Anene *et al.*, 1999a; 1999b; 2006).

In this work, we report an a typical case of natural *T. brucei* trypanosomosis in a local dog in which repeated treatments with berenil failed to produce a cure. Experimental murine infections with the isolate and treatment with various doses of the trypanocide at various times also failed to cure the infection, thus establishing resistance of the isolate to berenil.

CASE REPORT

Case History

A 13-month-old female local (mongrel) dog weighing 11.5 kg was presented at the UNVTH sick and recumbent by a client from Orba in Udenu Local Government area of Enugu State. The primary complaints were a month-old blindness, bruises on the body, lethargy and inability to stand up. The dog was used for hunting by the client's brother who returned her as a result of her deteriorating condition and blindness. The dog had never been reported sick and never received medication and/or vaccination for any disease. The client also reported that he previously brought a different dog to the UNVTH with similar symptoms, which was treated but became sick again after three months and died. Hospital records confirmed that the client indeed presented a local male dog in which trypanosomosis due to *T. brucei* and ancylostomosis were diagnosed and treated the previous year.

Clinical Examinations

A systematic examination of the dog was carried out according to specified routine clinical examination practice (Radostits *et al.*, 1994). Blood and faecal samples were also collected for parasitological examination and diagnosis. The faecal sample could not be examined immediately and was stored overnight at 4°C before being processed and examined.

Parasitology and Packed Cell Volume

The blood sample was examined routinely for parasitological diagnosis using wet blood film, Giemsa-stained thin blood film, haematocrit centrifugation (HC) and animal inoculation techniques. The packed cell volume (PCV) was determined from microcapillary tube preparations used for the HC technique. Examination of parasite motility in positive wet blood preparations and their morphological characteristics in Giemsa-stained thin blood films as described by Soulsby (1982) were used to identify the trypanosomes. In addition, 2 donor Wistar rats were each infected with 0.2 ml of the blood for additional characterisation of the trypanosome using the blood incubation infectivity test (BIIT) as previously described (Rickman and Robson, 1970; Onah and Ebenebe, 2003) and for obtaining parasites for drug sensitivity assay in mice. These rats were monitored daily for parasitaemia and blood was taken from them for the assays when they had attained fulminating parasitaemia. Furthermore, some of the infected blood was spotted on to Whatman FTA Gene Cards and sent to the University of Glasgow for additional confirmatory identification of the trypanosome isolate by PCR analysis (Anene *et al.*, 2006). The faecal sample was examined for helminth ova by direct faecal smear, salt floatation and the McMaster egg counting techniques (Soulsby, 1982).

Drug resistance test in mice

A drug resistance test on the trypanosome isolate was conducted using 40 mice following the report that the dog had died of the infection. The forty (40) outbred albino mice were each infected with 10^5 trypanosomes from the donor rats. They were then divided into four groups of 10 mice each. Animals in groups 1 and 2 were each treated with berenil at 7 mg/kg body weight on day 4 and 12 post- infection (p.i.) respectively, while those in groups 3 and 4 each received 14 mg/kg body weight on day 4 and 12 p.i., respectively. Although it was intended to monitor all the animals daily for evidence of relapse for a total of 100 days post-treatment (p.t.), monitoring ceased for each group when all the members of the group had relapsed.

Statistical analysis

Data from the drug resistance assay were analysed by descriptive statistics and differences between treatment-means determined by the Student's *t*-test using the Microsoft Excel statistical package. Differences with P values = 0.05 were considered significant.

RESULTS

Clinical findings

Systematic clinical examinations of the dog revealed the following: bruises on the ears and body, emaciation, dehydration (hide-bound) and lateral recumbency (Plate 1), copious bilateral ocular discharges with matting of the peri-orbital hairs and bilateral corneal opacity (Plates 2, 3 and 4), swollen face (Plate 3), submandibular oedema (Plate 4), enlarged popliteal, prescapular and submandibular lymph nodes, pale mucous membranes, high respiratory rate (panting) and body temperature (40°C) with pulse and heart rates of 108 and 92 beats/min, respectively. The animal weighed 11.5 kg and had a PCV of 11%. Based on the above findings, a tentative diagnosis of trypanosomosis with possible secondary bacterial infection as a result of the wounds was made.

PLATE 1: Dog in lateral recumbency on presentation

PLATE 2: Head oedema (swollen face) and bilateral corneal opacity. Also note the periocular matting of hairs by copious bilateral ocular discharges.

PLATE 3: Dog able to stand a day after treatment but the swollen face, bilateral corneal opacity and ocular discharges persisted

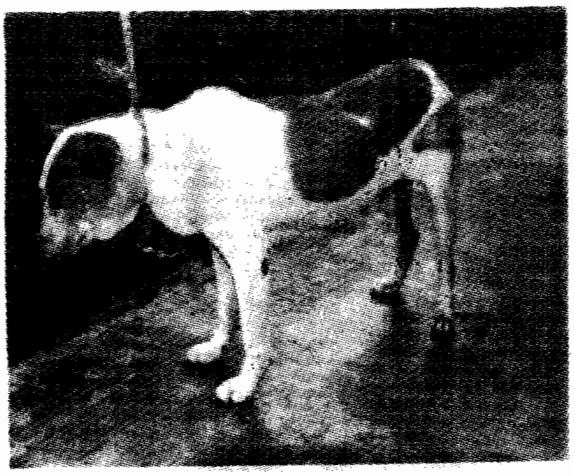

PLATE 4: The *T. brucei*-infected dog showing brisket and submandibular oedema (bottle-jaw, arrows)

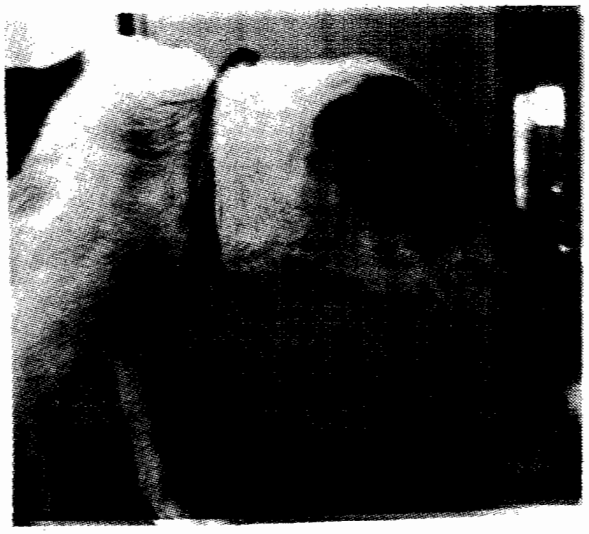

PLATE 5: Reduced swollen face and submandibular oedema following berenil treatment Note however that the bilateral corneal opacity and ocular discharges still persist

Parasitological findings

Examination of wet blood smear revealed numerous active parasites with snake-like or wriggling movements. Examination of the Giemsa-stained thin blood film revealed numerous polymorphic trypanosomes with prominent undulating membranes and subterminal kinetoplasts. When the trypanosome isolate was serially passaged in Wistar rats and their Giemsa-stained thin blood films examined, the trypanosomes lost their morphological polymorphism. Furthermore, when the parasites were incubated in human serum for 4 hours and then used to infect 10 Wistar rats none of the infected rats became parasitaemic; indicating that the isolate was human serum sensitive. Based on the findings above, the isolate was identified as *Trypanosoma brucei brucei*. This identification was confirmed when DNA extracted from the isolate was subjected to a series of PCR reactions with primer sets specific for *T. b. brucei* repeats (Anene *et al.*, 2006). Examination of the faecal sample by direct smear revealed numerous hookworm (strongyle-type) ova. When it was analysed by the egg floatation technique, more ova than could conveniently and accurately be counted were seen. The sample was then analysed by the McMaster egg counting technique and a total of 2800 eggs per gram of faeces was obtained. The dog was therefore finally diagnosed as suffering from a concurrent infection of trypanosomosis and ancylostomosis.

Clinical management and prognosis

Based on the tentative diagnosis on the first day, the following treatments were instituted: deep intramuscular (i.m.) injection of berenil at 7 mg/kg body weight repeated in 2 weeks, 2 ml penicillin-streptomycin (Combiotic®, Chas. Pfizer Inc) i.m. for 5 days (each 2 ml contained 400,000 units of penicillin-G procaine and 0.5 gm of dihydro-streptomycin), 1.0 ml fortified vitamin B complex (Sparhawk Laboratories, Leneka, Kansas) and 2 ml iron dextran injections i.m. for 3 days, and 125 ml 5% dextrose saline intravenously. Following these treatments the animal was able to stand and walk about the next day and the bottle-jaw, swollen face and ocular discharges reduced. The temperature was 37.2°C, the pulse, heart and respiratory rates were 105, 124 and 20/min, respectively.

The bilateral corneal opacity and swollen peripheral lymph nodes were still present but the animal ate well. However, trypanosomes were detected in the blood by direct blood smear examination. Based on the finding of numerous

hookworm eggs in the faeces, mebendazole at a single dose of 40 mg/kg body weight was prescribed and given on the second day in addition to the previous prescriptions. On the third day, the vitamin B complex and iron dextran treatments were completed. The animal continued to improve with the enlargement of the lymph nodes reducing, although the corneal opacity and scanty parasitaemia persisted. On the fifth day the antibiotic treatment was completed but the dog continued to lose weight and weighed 9.5 kg. The dog also started panting again, had a rectal temperature of 39.8°C and when the blood was examined trypanosomes were still present. It was again treated with 7 mg/kg of berenil i.m. and 50 ml 5% dextrose saline infusion subcutaneously (s.c.). On the sixth day, the dog weighed 9.4 kg but showed some improvement with a temperature of 39.2°C, PCV of 21%, good appetite and the swollen face and bottle jaw disappeared. However, the corneal opacity and ocular discharges were still persistent (Plate 5). On the 15th day the dog weighed 9.5 kg, had a temperature of 38.2°C and pulse and respiratory rates of 108 and 24/min, respectively. Ticks were seen on the body of the dog but there were no trypanosomes in examined wet smear and Giemsa-stained thin blood film preparations.

The final dose of berenil at 7 mg/kg body weight, 1.5 ml vitamin B complex and 0.5 ml ivermectin s.c. (Ivomec™: Merck, Sharp and Dohme, B.V., Haarlem) were administered. The client did not return after two weeks for the repeat dose of ivomec injection as directed. Six months later, we traced the client to his country home, Awhum Orba. On inquiry we were informed that the dog never recovered its sight even when it had apparently recovered from other signs and that subsequently, it fell sick again, lost weight drastically, could not stand and had died three weeks prior to our visit.

Drug Resistance test

Results of the drug resistance test in mice are presented in Table I. Although the higher dose of berenil (14 mg/kg body weight) significantly increased the number of days before relapse occurred ($p < 0.001$), relapse occurred in all animals irrespective of the dose of berenil or whether treatment was given at the early stage (day 4) or late stage (day 12) of the infection.

TABLE I: Mean number of days taken prior to a relapse in mice infected with *T. brucei* and treated early (day 4 p.i.) or late (day 12 p.i.) with either 7-mg/kg or 14-mg/kg body weight of diminazene aceturate

Group	Dose	Day Treated	No. Treated	No. Relapsed	No. relapse ± SD	Days before
1	7 mg/kg	4 p.i.	10	10	17 ± 3.5	p.t.
2	7 mg/kg	12 p.i.	10	10	11 ± 1.8	p.t.
3	14 mg/kg	4 p.i.	10	10	42 ± 2.5	p.t.
4	14 mg/kg	12 p.i.	10	10	25 ± 1.6	p.t.

NB: p.i. = post infection; p.t. = post treatment

DISCUSSION

It has been reported that in most African countries the prevalence and impact of trypanocidal drug resistance is largely unknown and requires investigation (Holmes, 1997). The case study presented in this paper has shown that trypanosome strains resistant to diminazene aceturate occur naturally in the Nsukka area of Enugu State, Nigeria. This situation constitutes a problem to successful treatment and management of not only canine trypanosomosis but possibly other livestock trypanosomosis in the area. In 2003, trypanosomosis was diagnosed in a horse and a sheep at the University of Nigeria Veterinary Farm and both animals were treated with berenil. Eventually they relapsed and repeated treatment with berenil was given. Despite the treatments, the horse rapidly lost weight, became recumbent and was salvaged while the sheep remained chronically infected without serious clinical manifestations until it was also disposed of (unpublished personal observation).

This situation is worrisome given that berenil is reputed to be the only drug to which trypanosomes do not easily develop resistance (Aliu *et al.*, 1984). However, it is easy to conceive why the drug has become almost ineffective in the treatment of trypanosomosis in the Nsukka area. Resistance of trypanosomes to trypanocides can be induced in a number of ways in the field. These include: **a)** long term and extensive use of a particular drug in an area (Elrayah and Kaminsky, 1991); **b)** unsupervised use by untrained persons resulting in frequent under-dosage and use of expired drugs; and **c)** the preponderance and use of adulterated and fake drugs with little or no therapeutic activity, which has become a growing international menace in Africa where it was estimated that 60% of available drugs were fake (Broussard, 1996; Holmes, 1997). It is possible that all three reasons may subsist in the Nsukka area. It is on records that berenil is the only trypanocide routinely in use for the treatment of trypanosomosis cases in the Nsukka environ since 1970 (unpublished UNVTH records). Also, State-funded veterinary services in the area and Nigeria in general have suffered neglect over the years and have been replaced by an upsurge of private veterinary practices run mostly by young and inexperienced graduates and a lot of illegal para-veterinary practices. The result is a combination of drug abuse, under-dosage and use of fake and adulterated drugs.

Expired trypanocides are also sold to farmers and used without regards to the serious implications. Moreover, a recent quality analysis of commercially available diminazene aceturate products in Nigeria showed that none contains 100% of the claimed active ingredient, with one containing as low as 89.8% (g/g) of diminazene aceturate (Anene *et al.*, 2006). It is therefore not surprising that for some time now strains of trypanosomes shown to be resistant to berenil and other drugs in experimental studies have continued to be isolated from naturally infected animals in the Nsukka area (Anika *et al.*, 1987; Onah and Uzoukwu, 1991; Anene, 1997; Anene *et al.*, 1999a; 1999b;).

Relapses in canine trypanosomosis may occur as a result of one or a combination of factors. These include: sequestration of the parasites in the brain or cryptic foci poorly perfused by blood which makes them inaccessible to drugs that do not cross the blood-brain-barrier (Jennings *et al.*, 1980; Waitumbi *et al.*, 1988; Chukwu *et al.*, 1990; Onah and Uzoukwu, 1991; Mamman *et al.*, 1995); low efficacy due to under dosage or poor quality drug or use of fake and expired drugs; and finally, natural emergence (or evolution by mutation) of drug resistant strains in the field. In this study, the relapse could not have been as a result of drug abuse in terms of under dosage or the use of poor quality, fake, expired and/or inappropriately stored drugs in the UNVTH since the drug was obtained from a reliable source. On the other hand, it is possible that the relapse may have resulted from reinvasion of the blood system by trypanosomes sequestered in the brain and other cryptic sites since the infection had lasted

for so long before the dog was brought for treatment.

The persistent corneal opacity supports this. However, since none of the other nervous signs usually associated with cerebral trypanosomosis in the dogs and other animals were seen (Omamegbe *et al.*, 1984; Anene *et al.*, 1989; Onah and Uzoukwu, 1991) it is reasonable to rule out CNS involvement and favour the conclusion that the isolate is truly drug-resistant. This was proven by the infection and treatment studies in the mice. Relapses which occur as a result of the reinvasion of the blood circulation by berenil-susceptible trypanosomes from the brain are usually fully susceptible to the drug if treated early or with a high dose of the drug (Silayo *et al.*, 1992; Biswas and Hunter, 1993). Neither the doubling of the normal dose of berenil nor early treatment of the infected mice with both normal and double dose was effective in curing the mice. Thus, the *T. brucei* isolated in this case is truly berenil-resistant. The work of Anene *et al.* (2006) also confirmed this in albino rats and further showed that the isolate is cross-resistant to pentamidine. However, this cross-resistance could not be demonstrated in dogs experimentally infected with the isolate and treated with either diminazene aceturate or pentamidine (Akpa *et al.*, 2008).

The prognosis of this case was no doubt exacerbated by the concurrent hookworm worm infection, despite the fact that the single dose of ivermectin treatment was effective in curing the helminth infection. However, this study in addition to unpublished hospital records at the UNVTH further confirm that concurrent trypanosome/hookworm infection in dogs is common in Nsukka area as previously reported (Omamegbe *et al.*, 1984; Omamegbe and Uche, 1985). It is therefore necessary that these parasites be considered and investigated in the differential diagnosis of clinical cases in dogs presenting with anaemia and regional oedema in the Nsukka area of Enugu State, Nigeria.

CONCLUSION

The emergence of drug resistant field strains of trypanosomes in the area is attributable to the long (over 30 years) and continuous use of berenil as the only trypanocide for the treatment of the disease in the area. This is compounded by the preponderance of fake and poor quality diminazene products in the market. The problem is bound to get worse unless other trypanocides such as quinapyramine, isometamidium and suramin are reintroduced for 'combination' and 'sanative' treatment and prophylactic management of the disease (Anene *et al.*, 2001). In addition, efforts should be intensified in the search for new, effective and affordable drugs for the treatment of the disease. Heads of government and scientists in African must spearhead this effort. Ethnoveterinary medical research involving investigation of the anti-trypanosomal chemotherapeutic properties and efficacy of the plethora of natural herbs available in African rain forests as well as the study of alternative immunological control such as the interaction between trypanosomes and certain helminths (Onah *et al.*, 2004) must be consideded in this effort. In conclusion, the *T. brucei* isolated in this case is truly berenil-resistant and berenil has become ineffective in the treatment of trypanosomosis in Nsukka area of Nigeria.

ACKNOWLEDGEMENTS

We acknowledge with thanks the excellent technical assistance offered by late Mr. Johnson Nwosu during the course of this investigation and Professor H.P. De Koning (Institute of Biomedical and Life Sciences, Division of Infection and Immunity, University of Glasgow) for confirmatory identification of the subspecies of the trypanosome isolate by PCR. The diminazene aceturate used in the case treatment and murine drug resistance studies was a kind gift from Hoechst AG, Frankfurt am Main, Germany through Professor Derek Wakelin of the University of Nottingham, England, UK.

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