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Short Communication

ISOLATION OF A HUMAN SERUM-RESISTANT *TRYPANOSOMA BRUCEI* FROM A NATURALLY INFECTED PIG IN THE NSUKKA AREA OF ENUGU STATE

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SUMMARY

A strain of trypanosome was isolated from one of the trade pigs held at the Orie Orba market lairage in Udenu Local Government Area of Enugu State. It was identified by its motility in wet blood film and morphological characteristics in Giemsa-stained thin blood film as *Trypanosoma brucei.* To further characterize the parasite and identify to which of the *brucei*-type sub-species it belonged, it was subjected to the blood incubation infectivity test. Trypanosomes were incubated in phosphate-buffered saline glucose (PSG), normal pig serum or normal human serum for 4 hours at 37° C before aliquots of 200 µl containing 10^6 trypanosomes were used to infect each mouse in three groups of 10 mice per group. The animals were monitored daily for parasitaemia for a period of 30 days using routine parasitological techniques. All mice infected with parasites incubated in PSG and pig serum became parasitaemic with a mean pre-patent period of 3 and 8 days respectively. In contrast, only one of the 10 mice infected with trypanosomes incubated in human serum did not become parasitaemic, indicating that the strain is resistant to human serum and potentially infective to humans. It was concluded that this isolate is possibly *Trypanosoma brucei gambiense* and that this study supports the epidemiological claim that domestic pigs may serve as reservoir hosts for human sleeping sickness due to *T. b. gambiense.*

KEY WORDS: *Trypanosoma brucei*, Human serum resistance, Pigs, Reservoir host, *T. b. gambiense*

INTRODUCTION

Tsetse-transmitted *Trypanosoma brucei* exists in three morphologically indistinguishable sub-species: *Trypanosoma bru-cei brucei*, the causative agent of animal trypanosomosis in a number of livestock and pet animals; *T. b. gambiense*, the causative agent of West African or Gambian sleeping sickness in man and *T. b. rhodesiense*, responsible for human East Afri-can or Rhodesian sleeping sickness. This morphological similarity is important in the epidemiology of the human pathogens as it makes it impossible to specifically identify the subspecies especially when the *brucei*-type parasite is isolated from animal hosts. In such cases, it is usually taken for granted that the isolates must be *T. b. brucei*. Man is refractory to *T. b. brucei* which infects a wide range of animal hosts including equines, camels, dogs and laboratory animals in which it is highly pathogenic and cattle, sheep, goats and pigs in which it produces a chronic infection (Stephens, 1986). In contrast, *T. b. gambiense* infects and produces disease only in man but may infect a number of wild and domestic animals, which are considered potential reservoirs of infection for the human disease it

causes (Smith *et al.*, 1998). *T. b. rhodesiense* is a true and well established zoonotic disease on epidemiological grounds, infecting man and a wide range of game and domestic animals (Stephens, 1986; Kiminyo and Lucey, 2001). The problem that therefore arises in the understanding of the epidemiology of Gambian trypanosomosis as regards the identification of animal reservoirs of the disease include how to determine whether a *brucei*-type trypanosome isolated from animals is human infective or not.

In the past, because pleomorphic trypanosomes were found in the blood of man suffering from sleeping sickness, investigators regarded similar trypanosomes whether isolated from man, domes-tic or wild animals as having similar host spectrum. It was not until various isolates from animals were inoculated into human volunteers that it was realized that man was refractory to most of the organisms isolated from animals (Stephens, 1986). Thus, the only ways of distinguishing between the three pleomorphic subspecies became the hosts in which they cause disease, geographic location of the disease they produce in case of the human parasites and by the inoculation of human volunteers for infectivity in order to distin-guish the human from the animal infective sub-species. Ideally, although the inoculation of human volunteers is the most useful means of proving that a *brucei*-type trypanosome isolated from an animal is infective to man and therefore that the animal from which it was isolated is a reservoir host, ethical considerations preclude the use of this ultimate step as a routine tool in the study of the epidemiology of the disease. In 1970, Rickman and Robson developed a simple test, the blood incubation infectivity test (BIIT), by which *T. brucei* (*sensu stricto*) may be differentiated from *T. rhodesiense* without recourse to human volunteers (Rickman and Robson, 1970a; b). Using this techni-que, a number of animal-derived *Trypanozoon* stocks (from dog, pig and sheep) have been identified as *T. b. gambiense* (Zillmann, *et al.*, 1984). Numerous epide-miological studies in the past showed that pig populations constitute the main reservoir of *Trypanozoon* in the West African sub-region, whether human-infective or not (Killick-Kendrick and Godfrey, 1963; Mehlitz *et al.*, 1982; Zillmann, *et al.*, 1984; Onah, 1991, Omeke, 1994). In this study therefore, we decided to re-evaluate the role of the domestic pig as a reservoir of *T. b. gambiense.* We carried out a survey of natural trypanosome infections in domestic trade pigs in defined geographic locations in Nsukka zone and subject-ed trypanosome isolates to BIIT.

MATERIALS AND METHODS

Experimental animals

Male out-bred mice aged between 8-10 weeks were purchased from the laboratory animal unit of the Faculty of Veterinary Medicine, University of Nigeria Nsukka. They were acclimatized in our Departmental fly-proof laboratory animal facility for a week before the commencement of the experiments. Animals were kept in cages in groups of 10 mice/cage and given feed and water *ad libitum.*

Trypanosome isolates

Blood samples were collected from trade pigs kept at the Ibagwa, Nsukka and Orba market lairages over a period of four months (September–December, 2001). Each samples was subjected to routine parasitological techniques for the detection of trypanosomes, including animal inoculation, and was declared negative only after inoculated animals remained negative for trypanosomes after 21 days observation. Positive cases were identified by motility in wet blood smears and morphological characteristics in Giemsa-stained thin blood preparations. Isolates were

multiplied by passage in Wister rats, which were then used as donor animals for the BIIT experiments.

Pig and human serum samples

10 ml of blood for serum was collected in plastic universal bottles from all the pigs sampled. Blood clot was removed from each of the samples, which were then centrifuged for 10 min at 3000 rotations per minute (r.p.m.). 5 ml serum was taken from each sample into plastic bijou bottles and stored at –20° C until used. Only serum samples from pigs that proved negative for trypanosomes (normal pig serum) were used in the BIIT technique. Similarly, 10 ml of human blood was collected from each student volunteer from the Faculty of Veterinary Medicine, University of Nigeria, Nsukka. The samples were processed for serum collection and storage as was described for pig serum above.

Blood incubation infectivity tests

Each trypanosome isolate was inoculated into donor rats, which were monitored until parasitaemia was assessed to be log 8/ml (Herbert and Lumsden, 1976). They were then exsanguinated by cardiac puncture under ether anaesthesia and the blood collected in heparinised-vacutainer bottles. The blood samples were used to carry out the BIIT as described by Rickman and Robson (1970a) and Hawkings (1976) with slight modification. Briefly, 50µl of the rat blood containing approximately 5 x 10^6 trypanosomes was incubated in 1 ml human serum for 4 h at 37° C. The same amounts were similarly incubated in normal pig serum and phosphate-buffered saline glucose (PSG) as positive controls. Preincubation, two- and four-hour post-incubation wet films were prepared from each of the samples and examined for motility and morphological characteristics of the trypanosomes in each of the incubation medium. At the end of the incubation, 200µl each, containing approximately 10^6 trypanosomes, was taken from each of the properly mixed samples and injected into three mice/sample intraperitoneally. They were then monitored for parasitaemia over a period of 21 days. Any iso-late incubated in human serum which resulted in parasitaemia in any or all of the three mice was subjected to BIIT again, using 10 mice each for the test and control samples and monitoring them for parasitaemia over a period of 30 instead of 21 days in order to confirm human serum resistance.

RESULTS

Trypanosome isolates and prevalence

A total of 19 isolates, code named Trypanosome Research Isolate, University of Nigeria (TRIUN) 01-19, were made during the period. By morphological characteristics, 4 (21.05%) of these isolates: TRIUN 02, TRIUN 07, TRIUN 11 and TRIUN 12 were identified as mixed infections of *T. brucei* and *T. congolense* while 15 (78.96%) TRIUN 01, TRIUN 03-06, TRIUN 08-10 and TRIUN 13-19 were identified as single infections due to *T. brucei*. Twelve (63.16%) of the 19 isolates (TRIUN 01-12) were obtained from pigs sampled at Orba, 5 (26.32%, TRIUN 13-17) were obtained from Ibagwa while 2 (10.53%, TRIUN 18-19) came from pigs sampled at Nsukka.

A total of 85 pigs: 50 (58.82%) at Orba, 20 (23.53%) at Nsukka and 15 (17.65%) at Ibagwa were sampled during the period. For the three locations, a total of 45, 21, 14 and 5 pigs were sampled during September, October, November and December respectively. The overall pre-valence of trypanosomosis for the area during the period was 22.35% representing 19 positive cases out

of the 85 pigs sampled. The pooled monthly prevalence and the individual location prevalence for the areas during the period are shown in Figs. 1 and 2 respectively.

Pre- and post-incubation characteris-tics of the isolates

Pre-incubation wet film preparations made of the trypanosome isolates suspended in PSG, normal pig serum and human serum showed that they were all alive and moving actively.

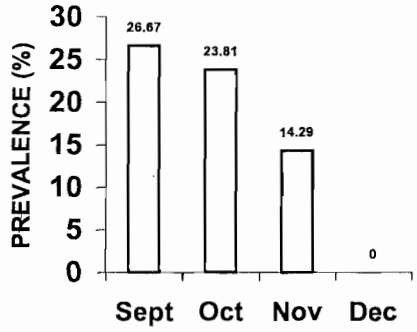

Fig. 1: Pooled monthly prevalence of trypano-somiasis in trade pigs at Ibagwa, Nsukka and Orba.

After two and four hours of incubation, isolates incubated in PSG moved actively in singles while those incubated in pig serum moved actively but many were in clusters. In contrast, only one isolate, (TRIUN 08) incubated in human serum showed considerable degree of activity after four hours incubation, the rest being populated by clumps of disintegrated and misshapen parasites with little or no movement. TRIUN 08 had single parasites moving actively although many moved in aggregated clumps.

Infectivity test in mice

Three mice each were infected with 10^6 trypanosomes incubated either in the PSG, normal pig or human serum and monitored daily for parasitaemia for a period of 21 days. All the mice inoculated with trypanosome isolates incubated either in the PSG or normal pig serum became infected by day 7 post infection. In contrast, none of the isolates incubated in human serum was infective for

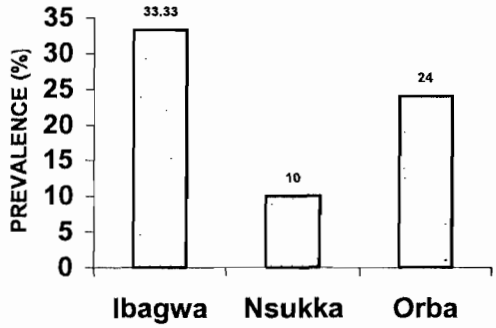

Fig. 2: Overall prevalence of trypanosomiasis at Ibagwa, Nsukka and Orba during a 4-month survey in trade pigs.

the mice except TRIUN 08 that showed patent infection in 2 of the three inoculated mice by day 14 post-infection. This suggested that the isolate was resistant to human serum. To confirm this, the isolate was used to repeat BIIT by infecting each of three groups of 10 mice/ group with 10^6 trypanosomes incubated either in the PSG, pig or human serum and monitoring their parasitaemia daily for a period of 30 days. At the end all control mice were infected. Mice infected with parasites incubated in PSG had an average pre-patent period of 3 days, those infected with parasites incubated in pig serum had an average pre-patent period of 8 days while all but one of the 10 mice infected with trypanosomes incubated in human serum showed patent infection with an average prepatent period of 15 days (Table I).

TABLE I: Prepatent periods (Days) of a human serum-resistant *T. brucei* in mice infected after a 4-hour incubation either in PSG, normal pig serum and human serum

	Mice										
	i	ii	iii	iv	v	vi	vii	viii	ix	x	
Medium	**Pre-patent period (Days)**										**Mean ± SD**
PSG	3	3	2	2	4	4	3	2	4	3	**3 ± 0.8**
PS	7	7	8	11	7	8	7	9	8	8	**8 ± 1.2**
HS	14	15	14	15	15	16	15	14	15	-	**14.8 ± 0.7**

DISCUSSION

A positive BIIT result is given where both test and control animals develop a persistent parasitaemia; a negative result where the control animals alone do so (Rickman and Robson, 1970a). Negative BIIT results were obtained with 18 out of the 19 isolates, in this study indicating that they were human serum sensitive, presumably non-infective for man and that all the *T. brucei* isolates including those in the mixed *T. brucei/T. congolense* infections were *T. b. brucei* (*sensu stricto).* The resistance of TRIUN 08 to human serum, by establishing persistent parasitaemia in two out of three mice and then in nine out of ten animals after four hours incubation in human serum, indicates that it is presumably infective for man and therefore *T. b. gambiense.* Recently, regular surveillance of at-risk areas has shown dramatic resurgence in West African trypanosomosis in Central and Western Africa but the countries with the highest incidence include the Democratic Republic of Congo, Angola and Sudan (Kiminyo and Lucey, 2001; WHO, 2000). In many countries such as Ghana, Liberia, Nigeria and Sierra Leone there is no surveillance and the situation is poorly understood (WHO, 2000). However, it is said that about 200-300 cases are recorded annually in Cote d'Ivoire, Guinea, Nigeria and Uganda (Kiminyo and Lucey, 2001), which indicates that Gambian sleeping sickness is still a serious problem in these areas. Thus there was the need for the present epidemiological study.

The isolation of a human serum-resistant *T. brucei* from trade pigs in this study suggests that *T. b. gambiense* is present in Nsukka area of Enugu State. The epidemiological significance is that pigs may act as reservoir host for the parasite and that humans in this area are potentially at risk. Most of these trade pigs were purchased from several locations in the middle belt region of Nigeria where there was evidence that black pigs commonly kept by the Tiv tribe (an endemic area of sleeping sickness in Nigeria) were capable of acting as reservoirs of *T. b. gambiense* (Watson, 1962

cited by Stephen, 1986). Moreover, the observation that there is low prevalence of *Trypanozoon* infections in domestic animals in the absence of pigs and that pig populations constitute the main reservoir of *Trypanozoon* (Killick-Kend-rick and Godfrey, 1963; Mehlitz *et al.*, 1982; Zillmann *et al.*, 1984), further suggest that the domestic pig is a potential reservoir of *T. b. gambiense*. Based on the above observations, other studies which used human serum resistance, isoenzyme electrophoresis and DNA-test to character-ize a number of *Trypanozoon* isolates from pigs as *T. b. gambiense* (Mehlitz *et al.*, 1982; Zillmann *et al.*, 1984) and on this study, the role of the domestic pig as a reservoir host for Gambian sleeping sick-ness cannot be questioned any longer.

The fact that 2 out of the 13 test animals used in this study did not become infected does not invalidate the significance of the positive BIIT we obtained and thus the validity of the pig as a reservoir host. Such "equivocal results" have been reported in the past and has been attributed to a number of reasons. These include: 1). Insufficient number of the serum-resistant individuals in the stock, which makes them incapable of establishing an infection, 2). Death of many trypanosomes including the resistant individuals during the *in vitro* incubation period and 3). Other weak-nesses of the technique that may have resulted in the loss of the trypanosome lytic factor such as using blood samples that have been badly handled or stored for too long (Hawking, 1979). Moreover, it is possible to distinguish levels of sensitivity and resistance. For instance, there are: a), fully sensitive isolates in which no individual resists human serum, b). Sub-resistant strains where a few individuals (≈ one /million) resists human serum and survive to produce infection, c). Resistant strains where most individuals are still sensitive but resistant individuals may be up to 10 or 1000/million and d). Highly resistant strains where almost all the individuals are serum resistant (Hawking, 1976; 1977; 1979). It is possible that our isolate in this study may belong to either the sub-resistant or resistant group in which the resistant individuals were able to establish an infection after incubation in most but not all the test animals. However, it has been stated that for practical purposes, if a trypanosome isolate is serum-resistant at all, then it must be ruled that that isolate is potentially infective for man and therefore *T. b. gambiense* or *T. b. rhodesiense* (Hawking, 1979).

Finally, we conclude that the domestic pig continues to be a favoured host for the trypanosomes of the *brucei*-type and therefore a reservoir of *T. b. gambiense*; the isolated human serum-resistant trypanosome is probably *T. b. gambiense* and potentially infective for man and; the Nsukka area should be considered as an at-risk area for human Gambian sleeping sickness. Despite the importance of the findings in this study, the restricted geographic area covered and the limited number of animals sampled, make it necessary that further and expanded epide-miological study be carried out including the surveillance of human infections in the area.

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