Immunodiagnosis of bovine trypanosomiasis in Anambra and Imo states, Nigeria, using enzyme-linked immunosorbent assay: zoonotic implications to human health

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Abstract

Background & objectives: The prevalence of trypanosomiasis was studied in cattle, being a major source of animal protein in Nigeria, thus, a very likely means of spread of Human African Trypanosomosis (HAT).

Methods: Enzyme-linked immunosorbent assay (ELISA) was used to diagnose bovine trypanosomiasis in 264 samples collected from adult cattle of mixed breeds, age and sex, in Anambra and Imo states, Nigeria.

Results: Out of 264 samples analysed, 21 (7.96\%) were seropositive for \textit{Trypanosoma congolense} while 20 (7.58\%) were seropositive for \textit{T. vivax} and 8 (3.03\%) were seropositive for \textit{T. brucei} infections in both the states.

Interpretation & conclusion: The predominant species was found to be \textit{T. congolense}. Mixed infection of three species, \textit{T. vivax}, \textit{T. congolense} and \textit{T. brucei} was found to dominate other mixed infections in both the states. ELISA detected the infection of the three species of trypanosomes in the same group of animals. The usefulness of antigen capture ELISA in the diagnosis of human or animal trypanosomiasis was established, and the possibility of the spread of HAT caused by \textit{T. brucei gambiense} and \textit{T.b. rhodesiense} through cattle was expressed.

Key words Bovine – diagnosis – human – implications – trypanosomiasis – zoonosis

Introduction

African animal trypanosomiasis caused by \textit{Trypanosoma brucei}, \textit{T. congolense} and \textit{T. vivax} remains one of the major constraints to health and productivity of cattle and other domestic animals in the tsetse infested areas of tropical Africa owing to its varied clinical manifestations\textsuperscript{1}.

In Nigeria, it has continued to be a menace in the livestock industry despite the age-long attempts to control the disease\textsuperscript{2}. In the same vein, Human African Trypanosomosis (HAT) arising from \textit{T. brucei gambiense} and \textit{T.b. rhodesiense}, constitute a major problem arising from the controversial role of animal reservoir hosts. Although campaigns against HAT in the 1960s brought the disease below epidemic pro-
portions, there is presently a dramatic resurgence in both forms of HAT which has been blamed on wide spread of civil disturbances and wars, declining economies, reduced health financing, dismantling of disease control programmes and animal reservoir hosts.

The HAT, sleeping sickness is a complex and debilitating disease of man. The disease still ravages in several parts of sub-Saharan Africa despite decades of efforts aimed at control. The HAT poses as an emerging public health crisis in several countries including Nigeria, and a major health risk to tourists coming to tropical Africa. Human animal trypanosomiasis is caused by the parasitic protozoa of the Genus Trypanosoma. World Health Organization (WHO), had commented on the complex nature of the epidemiology of the human disease arising from two species of the infecting trypanosome in various parts of Africa.

The fact that many animals had T. brucei infection might be of zoonotic importance, since domestic animals are known reservoirs of T. b. gambiense in west Africa. A recent sero-prevalence study employing the standard direct sandwich technique of ELISA showed that of 320 sheep and goats, blood samples assayed in Ondo and Ekiti states, Nigeria, 47.8, 46.6 and 47.18% have had previous contact with T. brucei, T. congolense and T. vivax respectively. Trypanosomiasis seems to be remerging as an important livestock disease in Nigeria, assuming major clinical importance in small ruminants and extending to previously designated tsetse free zones. The reasons for this need are to be investigated in order to avoid animal human transmission.

**Material & Methods**

A total of 264 adult cattle of mixed breeds and sexes (male 220, female 44) from cattle markets in Awka and Orlu in Anambra and Imo states respectively were used for this study.

**Serum sample collection:** Appropriately labelled plain 10 ml vacutainer tubes with monoject needles were used to collect 5–8 ml of blood from the cattle via the jugular veins. The tubes with blood were kept in ice-packs in slanting position and transported to the laboratory, where the tubes were stored at 4°C overnight to allow for adequate retraction and clotting. The samples were centrifuged at 3000 rpm for 10 min and sera dispensed in duplicate into appropriately labelled sample tubes and stored at −20°C until used analysed.

**Generation of monoclonal antibodies:** This was carried out as per the procedure described by Milstein and Köhler, with little modifications. Succinctly, monoclonal antibodies were raised by immunising Balb/c mice with the T. congolense, T. vivax and T. brucei antigens lysate in Freud’s adjuvant. The spleens were removed from the mice and the individual cells fused with constantly dividing (immortal) B-tumour cells in polyethylene glycol (PEG). The B-tumour cells were selected for a purine enzyme deficiency and often for their inability to secrete immunoglobulin. The resulting cells were distributed into micro-well plates in hypoxanthine aminopterin thymidine medium which killed off the fusion partners, in that each well was left with one hybridoma cell. The immunoglobulins were prepared from the ascetic fluid purified and tested by ELISA against different trypanosome species-specific antigens, Anaplasma marginale antigen, Babesia bijemina antigen Theileria parva antigen, to establish their specificity.

**Generation of conjugate:** Briefly, 10 mg horseradish peroxidase (HRP) type VI was dissolved in 0.2 ml of 1.25% glutaraldehyde in phosphate buffered saline (PBS) (pH 7.2) and left at room temperature overnight. About 1.0 ml of 0.1 M carbonate buffer (pH 9.2) was added and dialysed changing twice in 4 h. To this 5 mg of purified monoclonal antibodies raised against different bovine trypanosome species were added in 0.25 carbonate buffer and incubated at room
temperature overnight and 0.1 ml of 0.2M lysine was added followed by the addition of equal volume of glycerol and kept at −20°C. A chequer board titration was used to determine the dilution of the conjugates.

Sandwich antigen capture ELISA: The monoclonal antibodies raised against *T. brucei*, *T. congolense* and *T. vivax*, separately were used at a dilution of 1 : 500 in coating buffer to coat each well of the flat bottom micro ELISA plates (Dynateck, Virginia, U.S.A.), and kept at 4°C overnight. The monoclonal antibody IgM raised specifically against *T. brucei* and IgG raised specifically against *T. congolense* and *T. vivax* were used11. The plates were flipped empty and rinsed once with washing buffer and the excess buffer drained off the plates by gentle tapping of the inverted plate on a thick towel and 100 μl of diluent buffer (washing buffer) was added in each well, then sera to be tested were added in duplicate in the wells using micro-pipettes with tips as follows 5 μl in *T. brucei* plates and 10 μl in the *T. vivax* and *T. congolense* plates. The wells in row A column 1 and 2 and wells in row B column 1 and 2 contain no sera and were left as reagent controls, while wells in row G column 11 and 12 contain positive controls and row H column 11 and 12 contain negative controls. The plates were incubated at 37°C for 15 min, then flipped empty and rinsed once with washing buffer. About 100 μl of antis species monoclonal antibodies (IgM for *T. brucei* and IgG for *T. congolense* and *T. vivax*), separately raised against the species and conjugated to horseradish peroxidase and diluted at 1: 1000 in conjugate diluting buffer (phosphate buffer, 0.5% tween 80, 1% BSA) were added in each case. The plates were incubated at 37°C in dark and the change in colour was observed and absorbance was read after 30 min at 405 nm wave length using a micro ELISA reader SFC 400 (Biotek Instruments, Inc, Vermont, U.S.A.). The wells with colour change were recorded. The quantitative value of absorbance of the colour intensity was extrapolated from the standard plot, indicating the quantity of antigen in the test serum.

Results

All 264 different serum samples were collected from selected local governments in Imo and Anambra states. The animals were adults and of mixed breeds and sexes and were transported to the states from northern parts of Nigeria. From each state, 132 samples were collected. Out of the 264 a total of 110 (41.66%) different serum samples were positive for *T. congolense* while 108 (40.91%) were positive for *T. vivax* and 86 (32.58%) were positive for *T. brucei* in both the states.

Out of 110 (41.66%) sera positive samples for *T. congolense*, 48 (18.18%) were positive in Anambra state whereas 62 (23.48%) were positive samples in Imo state. Out of 108 (40.91%) positive for *T. vivax* in both the states, 50 (18.94%) were detected in Anambra and 58 (21.97%) in Imo state. Out of the 86 (32.58%) sera samples positive for *T. brucei* in both the states, 33 (12.5%) were detected in Anambra and 53 (20.08%) were detected in Imo state (Table 1).

Single infections were detected for these three species in both the states as 21 (7.95%) for *T. congolense*, 20 (7.58%) for *T. vivax* and 8 (3.03%) for *T. brucei*. In Anambra state alone, single infections were 11 (4.17%) for *T. congolense*, 10 (3.79%) for *T. vivax* and 5 (1.89%) for *T. brucei*. In Imo state, single infections were 10 each for *T. congolense* and *T. vivax* and 3 (1.14%) for *T. brucei* (Table 2).

Mixed infections were also detected. This was ob-
served during the plate reading. Since the three organisms were tested for in each serum sample, in separate plates, a serum appearing positive in the three plates is an indication of mixed infection of the three organisms. Positive in only two plates indicates mixed infection of the two organisms while positive in only one plate indicates single infection. The most predominant, involved three species—*T. congolense*, *T. vivax* and *T. brucei* and this occurred in 35 (13.26%) animals in Imo and 18 (6.81%) animals in Anambra, with a total of 53 (20.08%) in the two states. This is followed by the mixture of *T. vivax* and *T. congolense* which was detected in 14 (5.30%) animals in Anambra state and 6 (2.27%) in Imo state with a total of 20 (7.58%) in both the states. Mixed infections of *T. vivax* and *T. brucei* were detected in 8 (3.03%) cattles in Anambra and 5 (1.89%) cattles in Imo states making a total of 13 (4.92%) in two states. *T. congolense* and *T. brucei* mixed infections were also detected in 3 (1.14%) animals in Anambra and 10 (3.79%) animals in Imo states making a total of 13 (4.92%) in both the states (Table 2).

The absorbance values which determine the quantitative value of each trypanosome antigen used in each state were shown in Table 3. The readings that fall between 0.05 and 0.1 showed a very high quantity of antigen, hence depicting an active infection. *T. vivax* is found to have the highest number of high absorbance value with 14, followed by *T. congolense* and *T. brucei* having four each (Table 3).

**Discussion**

Indications from the results show that mixed infections caused by *T. congolense*, *T. vivax* and *T. brucei*...
in Anambra and Imo states, were the prevalent infections in the two states. The findings depict a contrary view to that found by Dipeolu\textsuperscript{12}, that the prevalence of mixed infection caused by \textit{T. congolense} and \textit{T. vivax} is the most significant. It also differs from the observations of Akinboade\textsuperscript{13} who reported that a mixed infection due to \textit{T. congolense} and \textit{T. brucei} was most prevalent in cattle. However, the contrary observations could be attributed to differences in diagnostic methods used. Dipeolu\textsuperscript{12} worked on “survey of blood parasites in domestic animals” using parasitological methods. The sensitivity of these methods especially in low parasitaemic conditions is unreliable compared to the reliable and highly sensitive ELISA technique used in this study. Supportably Losos and Ikede\textsuperscript{14} reported a mixed infection of \textit{T. congolense}, \textit{T. vivax} and \textit{T. brucei} in one cattle using DNA hybridization technique\textsuperscript{14}. This technique is also sensitive on its own although hybridisation signals failed to be obtained when samples that are to be used were kept at 4°C for up to four days where by the DNA gets degraded because of micro-condensation.

The failure to detect mixed infection of the three species by the parasitological method (which is based on the mortality and the morphology of the trypanosomes), is not unusual because of the fluctuating parasitaemic behaviour of blood stream trypanosomes (\textit{T. vivax} and \textit{T. congolense}). This means that the trypanosome species with highest proportion are likely to be diagnosed and infection attributed to these parasites only, whereas as the species which are low in numbers might not be identified microscopically.

The detection of mixed infection comprising \textit{T. congolense}, \textit{T. vivax} and \textit{T. brucei} in the same group of animals and the high prevalence rate of trypanosomes in this study in spite of the increased use of trypanocides have highlighted the following—the stable occurrence of drug resistant strains of trypanosomes; the utmost need for more effort towards the control of the disease; and the distribution of animal reservoir and insect vectors. All these factors act either individually or in concert to maintain the infection in domestic animals and as reservoir for HAT.

In this study, the prevalent species among the three species of trypanosomes is \textit{T. congolense} in both the states though the difference is not significant from \textit{T. vivax} infection in between the states. In Anambra alone, the prevalent specie is \textit{T. congolense} while equity is the case in Imo between \textit{T. congolense} and \textit{T. vivax}. One can significantly say that infections due to \textit{T. congolense} and \textit{T. vivax} are the main constraint on

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<th>O.D. range</th>
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<td>Anambra</td>
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<td>Anambra</td>
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<td>001–002</td>
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Table 3. Optical density range, showing prevalence of trypanosome antigen quantity among the species used in this study
livestock development in the two states and in Nigeria as a whole considering the fact that preponderant majority of the livestock raised for consumption in Nigeria are raised in the northern part of the country and transported to the southern part. However, in Africa, T. congolense infection usually manifests as a chronic form of the disease and milder than that caused by T. vivax. The importance of this work should not be over emphasised considering lack of current research on trypanosomiasis in Nigeria. Some of the factors that affect the prevalence of trypanosomiasis in Nigeria include animal breed, type of management, season of the year and the type of vegetation. It is also known that nomadism tends to expose animals to high tsetse challenge and hence trypanosome infection.

In Nigeria, cattle are considered as one of the principal livestock, and their survival and development are necessary to ameliorate the worsening situation regarding the supply of animal protein. Cattle production in the country has been restricted to the northern part because of the erroneous belief that the southern part (forest zone, including Anambra and Imo states) was highly infected by tsetse flies which transmit trypanosomiasis. Afterwards the cattle are transported to the south for marketing.

Consequently, the prevalence of these parasites in cattle and other domestic animals gives room for concern, as indications have occurred that T.b. gambiense and T.b. rhodesiense, to which man is the natural host, have been detected in several animal reservoirs, using molecular techniques. HAT described as Rhodesian sleeping sickness is caused by T.b. rhodesiense, resulting to acute disease course leading to death of infected persons within few weeks or months. In west and central Africa, HAT has a wider geographical spread than Rhodesian disease with about 77.8% of HAT endemic countries suffering from T.b. gambiense. In Nigeria, although the exact sleeping sickness situation is not well known, there is increase in number of volunteer cases presented for treatment each year. Apart from the old Gboko endemic focus remaining active, outbreak of sleeping sickness in the new Abraka focus presently constitute a major health risk, resulting to several deaths. During an outbreak, out of 3,583 volunteers from 24 communities scattered around this focus, 359 were seropositive and 104 parasitologically positive for T.b. gambiense.

Identification of serum-resistance-association (SRA) gene in eight Trypanozoon isolates resistant to human serum has been reported while such genes were absent in isolates sensitive to human serum. Although this technique identified human-infective trypanosomes in cattle as reservoirs in the sleeping sickness endemic foci in Uganda arising from T.b. rhodesiense, it may similarly be used for identification of animal reservoir hosts in T.b. gambiense endemic.
areas\textsuperscript{27} using PCR methods reported 8\% $T. b. gambiae$ infection rate in wild animals in the Bipindi sleeping sickness focus of Cameroon which was believed to be responsible for resurgence and perpetuation of the disease in the area. High prevalence of $T. b. gambiae$ was observed in rodents ($Atherurus africanus$ and $Cricetomys gambianus$), monkeys ($Cercopithecus$ and $Cercocebus$) and ungulates ($Cephalophus$ spp). Two small carnivores ($Genetta servalina$ and $Nandinia binotata$) also harboured trypanosomes infective to man.

Similarly dogs, pigs, wild animals and bovids were identified as reservoir hosts in Liberia, Cote d’Ivoire and Burkina Faso based on isoenzyme electrophoresis, resistance to human serum and DNA analysis. The capacity of $T. b. gambiae$ to proliferate and persist in alternative hosts in the absence of symptoms with prolonged maintenance of infectivity to the vectors makes it maintain a wide range of reservoir hosts which may differ from one endemic area to another. Adaptation of PCR technique for identification of $T. b. gambiae$ and blood meals of both animal and human origins in tsetse flies\textsuperscript{5}, has paved way for determination of the roles of animal reservoir hosts in epidemics of Gambian sleeping sickness. The reports have shown involvement of $Glossina palpalis$ in cyclical transmission of sleeping sickness from pigs to man in Cote d’Ivoire\textsuperscript{28}. Pig-tsetse-human, cattle-tsetse-human and sheep-tsetse-human transmission cycles have similarly been reported in three endemic foci in south-eastern Uganda\textsuperscript{5}.

Even though the role of animal reservoir hosts in the transmission of $T. b. gambiae$ have been controversial current biotechnologies that differentiate between $T. b. brucei$, $T. b. gambiae$ and $T. b. rhodesiense$ in the tsetse vector and animals confirm the true zoonosis of Gambian trypanosomosis. Going by such findings maintenance of animal-tsetse-animal $T. b. gambiae$ transmission cycle is not unlikely. The zoonotic nature of the disease seems to be further enhanced by the feeding pattern of $G. palpalis$ group which apart from feeding on man, feeds on domestic pigs, wild ruminants and lizards\textsuperscript{29}. Animal reservoir hosts for $T. b. gambiae$ has been identified as one of the principal factors associated with the persistence of Gambian trypanosomosis in endemic areas in spite of chemotherapeutic campaigns\textsuperscript{27}. For example, the presence of pigs in Mbini focus of Equatorial Guinea is believed to be responsible for the persistence of infection despite several years of treatment\textsuperscript{30}. This is supported by the ability of $G. palpalis$ group to cyclically transmit $T. b. gambiae$ from unlimited number of animal reservoir hosts, to man\textsuperscript{28,5} and the difficulty associated with the control of trypanosomosis in game animals. Based on observations using human serum resistance, isoenzyme electrophoresis and DNA-test for molecular characterisation of trypano-zaans leading to more definitive identification of $T. b. gambiae$ in pigs and other domestic and wild animals, the role of animals as reservoir hosts for Gambian sleeping sickness and resurgence of the disease can not be questioned any longer. Going by the feeding habit of $G. palpalis$ group on unlimited range of animal hosts, and the ability of $T. b. gambiae$ to perpetuate in such hosts without clinical symptoms supports the fact that animal reservoirs indeed constitute important complications militating against eradication of Gambian sleeping sickness in sub-Saharan Africa. An integrated approach to the control of human and animal trypanosomosis is essential in the control of the current upsurge in human trypanosomosis and in limiting the present economic impact on Africa and tourism potentials of sleeping sickness endemic countries.

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Immunodiagnosis of bovine trypanosomiasis in Anambra and Imo states, Nigeria, using enzyme-linked immunosorbent assay: Zoonotic implications to human health. Journal Vector Borne Dis., 45:292-300. Ezebuiro, O. G. C., Abenga, J. N., Ekejindu G. O. C. 2009. An Outbreak of Bovine Trypanosomiasis in the Blue Nile State, Sudan. Short report. Parasites and Vectors, 4:74. In the rabies field the enzyme-linked immunosorbent assay (ELISA) was initially developed for the titration of rabies virus-neutralizing antibodies. The technique was applied to the quantification of rabies antigen by Atanasiu et al using fluorescein-labelled IgG to the purified nucleocapsid. Subsequently, Perrin et al developed an ELISA called rapid rabies enzyme immunodiagnosis (RREID), which was based upon the detection of rabies virus nucleocapsid antigen in brain tissue. In this test, microplates are coated with purified IgG and an IgG-peroxidase conjugate is used to react with immunocapt