

## Application of antigen-detection enzyme immunoassay for the diagnosis of porcine *Trypanosoma brucei* infection

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OGUNSANMI, A., V. TAIWO, G. OHORE: Application of antigen-detection enzyme immunoassay for the diagnosis of porcine *Trypanosoma brucei* infection. Vet. arhiv 70, 231-238, 2000.

### ABSTRACT

The prevalence rate of *Trypanosoma brucei* infection in pigs was appraised by a monoclonal antibody-based antigen-detection enzyme immunoassay (antigen-ELISA). Blood samples were collected in the abattoir from pigs reared in the rain forest and derived savannah region of Nigeria under the traditional extensive management system. Blood samples were also collected from 50 exotic pigs reared on a commercial farm with fly-proof pens. These blood samples were analyzed for presence of trypanosomes and antigens in peripheral blood. Of 189 porcine blood samples 51 (27.0%) were positive for circulating antigens, whereas only 4 (2.1%) had demonstrable trypanosomes as revealed by the haematocrit centrifugation/buffy-coat technique. When the 51 blood samples collected in EDTA tube corresponding to those sera that were positive for *T. brucei* antigens were subinoculated into mice, 46 (90.1%), the mice became infected. Demonstration of trypanosomes in the infected mice is supportive proof that the parasites were residing in the infected hosts. Samples collected from 50 exotic pigs in fly-proof pens were all antigen-ELISA negative. In addition, none of the corresponding 50 control blood samples had demonstrable trypanosomes by the buffy-coat method, nor do they show detectable parasites after subinoculation into mice. Thus, antigen-ELISA appeared to be a better and more useful tool for mass sero-epidemiological survey of porcine *T. brucei* infection when compared to the buffy-coat technique.

**Key words:** antigen-detection ELISA, porcine, parasitaemia, antigenaemia, diagnosis

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### Introduction

Nigeria is the largest pig producing country in Africa and pork accounts for about 4.45% of total meat supply in the country (ADEBANJO,

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1983). However, one of the major constraints to pig production in Nigeria is trypanosomiasis, as some species of tsetse have a special predilection for pigs (MADUBUNYI, 1988).

Unlike *Trypanosoma simiae*, which is considered as the most dreaded protozoan of pigs, *T. brucei*, although a common parasite of domestic pigs, is often regarded as non-pathogenic or at best a cause of mild chronic disease (ILEMOBADE and BALOGUN, 1981). Epidemiological reports have implicated *T. brucei* as a cause of several serious natural outbreaks of fatal porcine trypanosomiasis (AGU and BAJEH, 1986, 1987; ONAH and UZOUKWU, 1991). This unfolding importance of *T. brucei* prevalence in pigs has called for regular monitoring of the pig stock in order to avoid huge economic losses and to enhance timely drug intervention (ONAH and UZOUKWU, 1991). To achieve this, an appropriate reliable *T. brucei* diagnostic tool must be put in place. The standard laboratory method for diagnosis of *T. brucei* infections is to identify the parasites in the blood of infected hosts by a variety of techniques that include direct microscopy, concentration methods, and animal inoculation (reviewed by NANTULYA, 1990). The sensitivity of these techniques, however, is quite low, as direct microscopy, for example, may miss the diagnosis in up to 50% of the infections (BARNETT, 1947).

The other alternative laboratory approach to diagnosis of trypanosomiasis is the demonstration of trypanosomal antibodies in the serum of the infected animal by several techniques (NANTULYA, 1990). These techniques, too, have some major limitations. First, antibody detection can provide only a presumptive diagnosis, as it will not differentiate between current and past infections because antibodies may persist for a long time following treatment (LUCKINS et al., 1997) or spontaneous recovery (NANTULYA et al., 1986). Antibody detection tests are thus more useful as epidemiological tools rather than as diagnostic procedures (MASAKE and NANTULYA, 1991).

Of late, another diagnostic approach has been introduced that is based on the detection of trypanosome antigens in the circulating blood of infected people (NANTULYA, 1989), cattle (NANTULYA and LINDQVIST, 1989) and camels (NANTULYA et al., 1989a, 1989b). The test is an enzyme-immunoassay (antigen-ELISA) based on species-specific monoclonal antibodies (MoAbs) raised against conserved, invariant trypanosome antigens released upon disintegration of the parasites (NANTULYA et al., 1987). The antigen-ELISA is capable of detecting infection in more than 90% of clinical *Trypanosoma brucei rhodesiense* sleeping sickness patients (NANTULYA, 1989). The sensitivity of the assay in the diagnosis of animal trypanosomiasis, however, has not been

adequately tested in pigs. This study was carried out to investigate the possibility and sensitivity of *T. brucei* antigen-detection immunoassay in slaughtered pigs raised by free-range management within the tropical rain forest and derived savannah regions in Nigeria. In comparison, commercial pigs raised in a privately managed, fly-proof environment were also used.

## Materials and methods

One hundred and eighty-nine local pigs brought to the Bodija abattoir for slaughter from different areas of Oyo State, Nigeria, within the tropical rain forest and derived savannah zones between November 1994 to January 1995 were used. The pigs were reared under traditional extensive husbandry systems. Fifty local pigs reared on a private commercial pig farm under a modern intensive, fly-proof husbandry system located in a peri-urban area of Ibadan in rain forest vegetation were also used. One hundred and twenty albino mice obtained from the Experimental Unit of the Department of Veterinary and Pharmacology, University of Ibadan, Ibadan, Nigeria, were also used. They were fed with commercial mouse pellets *ad libitum*.

### *Sample collection, detection of antigenaemia and parasitaemia*

Blood was collected by jugular venipuncture into plain vacutainer tubes to obtain serum and the other into tubes containing ethylenediamine tetracetic acid (EDTA) as anticoagulant. The samples were transported to the laboratory within 40 minutes of collection for sera separation by allowing them to stand at room temperature for 2 hours, followed by centrifugation at 3,000 rpm for 10 minutes using a bench centrifuge (Centromix, UK).

The sera samples were transferred into sterile storage tubes for immediate trypanosome antigen-ELISA screening test. The circulating antigens were detected in sera by a sandwich enzyme-linked immunosorbent assay (ELISA) following the procedure described by NANTULYA et al. (1989b). Briefly, flat-bottomed micro-titer plates (micro-ELISA plates, Dynatech, Alexandria) were coated with an IgM fraction of *T. brucei* monoclonal antibody (Moab) TR7/47.34.16 (NANTULYA et al., 1987) at a concentration of 1  $\mu\text{g}/\text{well}$  in carbonate buffer, pH 9.6, and kept at 4 °C for at least 18 hours prior to use. The plates were washed 3 times at 10-minute intervals with washing buffer (0.15M phosphate-buffered saline, pH 7.4, containing 0.5% Tween 80). Excess buffer was drained off the plates and undiluted porcine sera in volumes of 100

$\mu\text{l/well}$ , were subsequently added in duplicates to the coated wells and mixed using a Dynatech microtiter mixer. The control sera used were standard positive and negative controls (NANTULYA, 1989). The plates were incubated for 30 minutes at room temperature, washed as before, the fluid drained off and replaced with horseradish peroxides labelled homologous monoclonal antibody. The conjugate was diluted 1:1000 in a buffer solution consisting of 0.01 M phosphate buffer containing 0.15 M NaCl, 0.2% Tween 80 and 1% normal mouse serum 100  $\mu\text{l/well}$ . The plates were incubated and washed as before, after which chromogen (88  $\mu\text{g/ml}$  of 2,2' azinobis (3-ethyl)-benzthiazoline-6-sulfonic acid diammonium salt (ABTS, Sigma, St. Louis, USA) and the substrate (0.003% hydrogen peroxide in 50 mM citric acid buffer pH 4.0), were added (100  $\mu\text{l}$ ). Sixty minutes after incubation at room temperature, absorbance (O.D.) of the reactants was read at 414 nm wavelength using a Titertek Multiskan micro-ELISA auto-reader. O.D. readings of 0.050 and above were regarded as positive, while O.D. below 0.050 was regarded as negative.

The unclotted blood samples obtained with EDTA as the anticoagulant from both the abattoir and private pig farm were examined for trypanosomes using the microhaematocrit centrifugation technique (WOO, 1971) as modified by MURRAY et al. (1977) and PARIS et al. (1982). The blood samples corresponding to sera samples that were trypanosome antigen-ELISA positive were thereafter sub-inoculated into appropriately tagged albino mice within 24 hours of collection (EKWURUKE, 1985; OTESILE, 1990). The mice were examined for parasitaemia (MURRAY et al., 1977; PARIS et al., 1982) for up to 30 days before disposal.

## Results

Only 6 (3.2%) of the 189 pig samples had demonstrable (*T. brucei*) trypanosomes as determined by the microhaematocrit centrifugation technique (Table 1). In contrast, circulating antigens of *T. brucei* were detected in 51 (27%) of the 189 serum samples. Of the 51 pig blood in

Table 1. Trypanosome parasitaemia, antigenaemia and mice sub-inoculation of porcine blood and sera

Source	Number of samples	Number positive		Mice sub-inoculation*	
		Parasitaemia	Antigenaemia	Positive	Negative
Abattoir	189	6 (3.2%)	51 (27%)	46 (90.2%)	5 (9.8%)
Private pig farm	50	0 (0%)	0 (0%)	0 (0%)	0 (0%)

\*This was carried out in 51 antigenaemic blood samples from the abattoir and in all 50 from the private farm

EDTA tubes corresponding to sera samples with demonstrable *T. brucei* circulating antigens that were sub-inoculated into mice, 46 (90.2%) mice became parasitaemic. Five (9.8%) mice failed to develop parasitaemia even after 30 days of sub-inoculation.

All the 50 sera from pigs under private management were trypanosome negative, both by the parasitaemia and antigen-ELISA techniques. None (0%) of the mice inoculated with blood samples from the private commercial pig farm became infected up to 30 days of examination.

## Discussion

The results of this study showed that the microhaematocrit centrifugation technique detected *T. brucei* infection in 3.2% of pig blood samples, compared to the 27.0% prevalence of *T. brucei* exhibited by the antigen-ELISA technique. The low sensitivity of the microhaematocrit method was apparent because it demonstrated the gross deficiency of this method under field conditions (NANTULYA, 1989). Previous studies on the sensitivity of this test placed emphasis on defining the lowest number of parasites that can be detected in a millilitre of blood (PARIS et al., 1982), which was obviously not the correct definition of the sensitivity of the test (VECCHIO, 1966).

The antigen-ELISA demonstrated a higher sensitivity that revealed a high *T. brucei* prevalence rate (27.0%) among the test pigs. The reason for the pronounced difference between the two techniques is that they measure different values. The microhaematocrit centrifugation technique detects only trypanosomes present in the peripheral blood circulation. The number of trypanosomes in peripheral circulation is often too small in chronic infections for detection by this technique, despite the fact that there may be many trypanosomes present. The second technique, on the other hand, detects soluble antigens released by living and dying trypanosomes into the serum. Thus, the sensitivity of this test does not depend upon parasite numbers in peripheral circulation, hence the high sensitivity even when there may be no detectable parasitaemia, especially in the tissue-invasive brucei-group (LOSOS and IKEDE, 1972). The minimum amount of circulating antigen detected by ELISA is around 250 ng of protein (NANTULYA and LINDQVIST, 1989).

The fact that 46 (90.2%) of the 51 mice inoculated with corresponding antigen-ELISA positive blood became paraistaemic lends credence to the superiority and high sensitivity of antigen-ELISA technique. This also showed that the test can play a useful role, and is

therefore a promising tool, for the screening and monitoring herds of pigs in trypanosome endemic areas, especially in animals with very low and undetectable parasitaemia. The 5 (9.8%) sub-inoculated antigenaemic blood samples that failed to develop parasitaemia in mice up to 30 days following sub-inoculation raise some points. This observation supports previous observations that the parasite may not be in circulating peripheral blood but still reside in other body organs such as the spleen, liver, and lymph nodes, since *T. brucei* is a tissue-invading parasite (LOSOS and IKEDE, 1972). The five pigs that were antigenaemic, but whose blood could not be infective to mice, indicate that either the pigs that had previous exposure to the parasite had self-cured (MORRISON et al., 1981) or that the parasites were tissue-resident at the time of blood collection.

However, judging by the results of this study, it is concluded that there is a high prevalence of porcine trypanosomiasis in the rain forest and derived savannah regions of Nigeria. Pig farmers should protect their animals with trypanocidal drugs. As observed in the privately managed fly-proof piggery, it is possible to maintain a trypanosome-free herd by preventing exposure to tsetse fly and other biting flies that may mechanically transmit trypanosomes (DAVIES, 1977). The antigen-ELISA technique proved to be far more sensitive than the parasitological test for the detection of porcine trypanosomiasis. The full automation of procedures, as well as making facilities available at a reasonable cost, could make it a valuable and indispensable tool for mass screening. In some situations, it may be necessary to combine antigen-ELISA with other parasitological tests, especially when morphological demonstration of the parasites becomes necessary.

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Received: 30 November 1998  
Accepted: 11 September 2000

**OGUNSANMI, A., V. TAIWO, G. OHORE: Primjena imunoenzimnog testa za dijagnosticiranje zaraze svinja praživotinjom *Trypanosoma brucei*. Vet. arhiv 70, 231-238, 2000.**

**SAŽETAK**

Proširenost zaraze praživotinjom *Trypanosoma brucei* u svinja pretraživana je imunoenzimnim testom za dokaz antigena (antigen-ELISA). Uzorci krvi su prikupljeni u klaonici, od svinja uzgojenih u uvjetima tradicionalnog ekstenzivnog gospodarenja u području džungle i u području savane u Nigeriji. Uzorci su uzeti i od 50 egzotičnih svinja uzgojenih na komercijalnim farmama sa svinjcima zaštićenim od muha. Krvni uzorci su pregledani na prisutnost tripanosoma i na antigene u perifernoj krvi. Od 189 uzoraka krvi svinja, 51 (27,0%) je bio pozitivan na antigene u krvi, dok su samo u 4 (2,1%) otkrivene praživotinje tripanosome pomoću hematokrita. Kada su uzorci krvi od 51 svinje čiji su serumi bili pozitivni na praživotinju *Trypanosoma brucei* inokulirani miševima, 46 (90,1%) ih je postalo zaraženo. Nalaz tripanosoma u zaraženih miševa je dokaz da su paraziti živjeli u tim zaraženim svinjama. Uzorci od 50 egzotičnih svinja iz svinjaca bez muha su svi bili antigen-ELISA negativni. Uz to, niti jedan od tih 50 kontrolnih uzoraka nije pokazao prisutnost tripanosoma u sloju bijelih krvnih stanica, niti su pronađeni nametnici nakon inokulacije miševima. Tako se pokazalo da je antigen-ELISA bolja i korisnija metoda za masovne sero-epidemiološke pretrage svinja na zarazu s praživotinjom *T. brucei*, u usporedbi s dokazom tripanosoma u bijelim krvnim stanicama.

**Ključne riječi:** dijagnostika, antigen-ELISA, svinja, *Trypanosoma brucei*, parazitemija, Nigerija

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