Short communication report

EVALUATION OF INDIRECT ENZYME-LINKED IMMUNOSORBENT ASSAY (ELISA) SYSTEMS FOR THE SERODIAGNOSIS OF BOVINE TRYPANOSOMOSIS IN DISEASE ENDEMIC AREAS OF KENYA

*OUMA, J. O.; MWANGI, J. M,; MDACHI, R. E. & MURILLA, G. A

Kenya Agricultural Research Institute
Trypanosomiasis Research Centre (KARI-TRC)
P.O. Box 362 - 00902, Kikuyu, KENYA.
**(Corresponding author)*
joouma@gmail.com or jouma@kari-trc.org

African animal trypanosomosis caused by *Trypanosoma brucei* Plimmer & Bradford 1899*, T. congolense* Broaden 1904 and *T. vivax* Ziemann 1905 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, diagnosis of trypanosomosis cannot be based on clinical signs alone (Nantulya 1990). Reliable diagnostic methods are a prelude to understanding the epidemiology of the disease and an important consideration when assessing the success of trypanosomosis control programmes. Conventional parasitological methods have a limited sensitivity and may lead to under-estimation of the prevalence of the disease (Paris *et al.* 1982). More sensitive diagnostic tests, including those for the detection of *Trypanosoma* specific antibodies (Luckins 1977) and antigens (Rae and Luckins, 1984; Nantulya *et al.* 1992) have therefore been developed. Recently, four indirect ELISAs have been developed, and their robustness and diagnostic performance evaluated (Rebeski *et al.* 2000 a, b). One of the problems that has characterised users of the IAEA ELISA kits for bovine trypanosomosis has been that of maintaining the stability of the antigen during shipment to counterpart laboratories. This study utilised antigen pre-coated plates as a way to try and circumvent the problem. This paper reports on the validation of four indirect ELISA systems using antigen pre-coated polystyrene microplates. The usefulness of the current version of indirect ELISA as an epidemiological tool for studies in bovine trypanosomosis is discussed.

Sample collection and description: Cattle populations kept in two different trypanosomosis endemic areas of coastal Kenya, namely; Galana, and Ukunda and on Mfangano island in Nyanza Province were used in this study. Trypanosomosis control programmes were on-going in Galana and Ukunda, but not on Mfangano. All the animals were monitored once a week for trypanosomosis using the buffy coat technique (BCT) and packed cell volume (PCV%). Blood for serum preparation was collected once a month and all field sera classified as either positives or negatives on the basis of whether or not parasites could be demonstrated in the blood by microscopy. For the purpose of validating the current version of indirect ELISA, a pool of 500 known positive sera obtained from any of the above locations was used. It is therefore worth noting that even though the samples were collected sequentially over a period of time, the diagnostic sensitivities reported in this paper were calculated from BCT positive samples only. However, for each of the assay systems a set of samples from the disease endemic, but BCT negative was included in the analysis. Known negative serum samples used to determine the cut-off percent positivity (PP%) value were obtained from cattle belonging to the Kenya Agricultural Research Institute (KARI) and the Kenya Trypanosomiasis Research Institute (KETRI) dairy herds. Both the KETRI and KARI animals are kept in a non-trypanosomosis endemic area in Muguga, near Nairobi. It was against this background that these animals were classified as known negative population.

Validation of *Trypanosoma congolense* and *Trypanosoma vivax* indirect ELISAs: Serum samples collected from cattle of known infection status in trypanosomosis endemic areas (described above) and known uninfected cattle were tested using the indirect *Tc*AGd, *Tc*AGn, *Tv*AGd and *Tv*AGn ELISA systems to determine the diagnostic sensitivity (D-SN) and diagnostic specificity (D-SP) of the test. The reagents used for the assay were supplied by the International Atomic Energy Agency (IAEA) and included internal quality control reference sera of C++, C+ and C-, conjugate and antigen pre-coated polystyrene microplates.

ELISA Protocol: The indirect *T. congolense* and *T. vivax* ELISAs were used to test both the negative and positive sera following the bench protocol version iTAB 1.0, provided by FAO/IAEA (1998). Briefly, antigen-precoated Immulon 1® microplates were reconstituted by adding ice-cold PBS (100 µl/well) and incubating at 37 °C for 1 hour while shaking. The microplates were then washed four times with PBS containing Tween 20 (PBS-T, pH 7.4). Test and control sera diluted 1/400 (*Tv*AGd and *Tv*AGn), 1/100 (*Tc*AGd) and 1/200 (*Tc*AGn) in sample diluent buffer (PBS-T with 5% w/v skimmed milk) were then dispensed in duplicate (100 µl/well) and incubated at 37 °C for 1 hr with rotation. Anti-bovine IgG conjugate diluted 1/20, 000 was added and incubated for another 1 hour. After four cycles of washing, hydrogen peroxide and TMB (substrate/chromogen) were mixed at a ratio of 1:1 and dispensed (100 µl/well) then incubated at 37 °C for 15 min after which 1M orthophosphoric acid was added (100 µl/well) to stop the reaction. The absorption was measured at 450nm wavelength using a multichannel spectrophotometer (Multiskan Plus MKII, Labsystems, Finland).

Data expression: The optical density (OD) values were expressed as percent positivity (PP) values. PP values for internal quality control (IQC) samples were calculated as part of the quality assurance and used as a basis of accepting the test sera results. The PP values for each plate were calculated based on the OD value of the respective C++ as follows;

$$\frac{\text{Replicate OD values of each control}}{\text{Median OD value of C++ control}} \times 100 = \text{PP values for quality control}$$

$$\frac{\text{Replicate OD value (test serum)}}{\text{Median OD value of C++ control}} \times 100 = \text{PP values for acceptance of test sera}$$

Determination of cut-off PP values: A number of methods are in existence for the determination of seropositive/ seronegative threshold (cut-off) values. However, for this study, cut-off selection was done according to the visual inspection method described by Jacobson (1998). Based on the visual inspection of frequency distributions of test results from known infected and uninfected animals, the cut-off was placed at the intersection of the two distributions. The cut-off was also determined by a modified receiver-operator characteristic (ROC) analysis (Greiner *et al.* 1995). In both cases, a PP value of 30% was taken as the cut-off value. Any value from the negative population above 30% was considered as false positive, while those from the positive population falling below this value were false negatives.

Determination of diagnostic sensitivity and specificity: The diagnostic sensitivity (D-SN) and diagnostic specificity (D-SP) were calculated as using the table below and as described by Jacobson (1998).

TABLE 1: CALCULATION OF DIAGNOSTIC SENSITIVITY (D-SN) AND DIAGNOSTIC SPECIFICITY (D-SP)

	Animals of known infection status		TOTAL
	Infected	Uninfected	
AB-ELISA +VE	True positive (TP)	False positive (FP)	TP+FP
AB-ELISA –VE	False negative (FN)	True negative (TN)	FN+TN
	D-SN= TP/TP+FN	D-SP= TN/FP+TN	

Diagnostic sensitivity (D-SN) of the test systems: Table 1 and 2 show the diagnostic sensitivities and specificities achieved by the four assay systems.

TABLE 2: SENSITIVITY OF THE FOUR ASSAY SYSTEMS CALCULATED FROM BCT POSITIVE SAMPLES

Assay System	Total no. tested by Ab-ELISA (BCT -ve from endemic areas)	BCT positive (a)	Ab-ELISA positive (b)	Diagnostic Sensitivity (%) (b/a x 100)
*Tc*AGd	920 (420)	500	476	95
*Tc*AGn	880 (380)	500	455	91
*Tv*AGd	920 (420)	500	485	97
*Tv*AGn	720 (220)	500	430	86

The four indirect ELISA systems validated in the current study showed diagnostic sensitivity of between 86% and 97% and specificity of 82% to 100%. Similar levels of diagnostic sensitivity and specificity were reported in goats infected with infected with *T. congolense* and *T. vivax* (Lejon *et al.* 2003) although the number of animals sampled was much lower than used in the current study. The highest diagnostic sensitivity (97%) and specificity (100%) were achieved using *Tv*AGd. Magona *et al.* (2002) demonstrated that *Tc*AGd had lower diagnostic sensitivity (63.7%) and specificity (57.5%) than *Tv*AGd (sensitivity = 81.3%; specificity = 81.3%).

TABLE 3: DIAGNOSTIC SPECIFICITY OF THE ASSAY SYSTEMS AS DETERMINED BY SERA FROM A NON-TRYPANOSOMOSIS ENDEMIC AREA

Assay system	No. of known -ve samples tested	-ve by ELISA (TN)	+ve by ELISA (FP)	Diagnostic specificity (%) (TN/TN+FP)x 100
*Tc*AGd	80	76	4	95
*Tc*AGn	120	98	22	82
*Tv*AGd	80	80	0	100
*Tv*AGn	160	151	9	94

The reason for the relatively higher sensitivity and specificity obtained using *Tv*AGd when compared with *Tc*AGd assay system is unclear. However, it is known that *T. congolense* has four different genetically distinct types (savannah, forest, Tsavo and Kilifi-types). The effect of the different genotypes on indirect-ELISA test sensitivity and specificity is a subject for further investigation.

This study also showed that assay systems employing denatured antigen preparations had slightly higher diagnostic sensitivities and specificities than those using non-denatured (native) antigens. Past experience (Ouma *et al.* unpublished data; Rebeski, Personal communication) has shown that use of native antigen for coating ELISA plates results in general decrease in the optical density (OD) signals. It is possible that by treating the antigen prior to coating of micro-plates, certain factors that either deteriorate or cause deactivation of the antigen are stabilised. Although the OD values for both the native antigen-coated plates exhibited more variation over time than denatured antigen-coated plates, the differences were not significant. OD values of the quality control sera (C++, C+ and C-) also remained within the expected lower and upper control limits throughout the study. Given that antigen instability has been one of the major setbacks that has characterised IAEA test kits before, it appears the pre-coating of ELISA plates offers a solution to this problem. As a way of enhancing assay proficiency, it would be important to consider the production of lyophilised reagents.

One of the major aims of this study was to incorporate the indirect ELISA in monitoring trypanosomosis control programmes in Kenya. A major obstacle to achieving this objective is the fact that antibodies are known to persist in circulation up to 10 months after successful therapy or self-cure (Authie *et al.* 1993), hence introducing the phenomenon of false positives in a herd. Interpretation of results gets even more complicated when dealing with serum samples from animals kept in tsetse infested (disease endemic) areas. In such areas it is difficult to discriminate between past and current infection. In the present study, some of the animals from disease endemic areas that were BCT negative were positive by Ab-ELISA. This phenomenon implies that the antibody detection assays cannot be used as a basis to institute treatment. However, Ab-ELISA is considered an important epidemiological tool for monitoring sero-prevalence of the disease and not for diagnosis of

individual animals (Hopkins 1997). Combining Ab-ELISA with BCT gives more reliable results than using Ab-ELISA alone.

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