



A native whole cell lysate antigen (WCLA) based ELISA for the sero-detection of surra in Indian cattle

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The DNA based molecular detection techniques are though reliable, yet their application in large throughput epidemiological studies is not cost effective, especially for developing nations (Sudan *et al.* 2014). The present communication deals with the use of native whole cell lysate based ELISA for the sero-diagnosis of trypanosomosis in cattle.

In vivo propagation of *Trypanosoma evansi* in mice: Inbred Swiss albino mice of either sex of 6–12 weeks of age, weighing about 25–30 g, were maintained under standard rearing. Cryopreserved *T. evansi* cattle stablilate, maintained in the Protozoology laboratory, were revived and passaged through Swiss Albino mice by intraperitoneal inoculation. At teeming level of parasitemia, *T. evansi* infected blood was collected from mice by cardiac puncture under chloroform anaesthesia using heparinised syringe. Freshly collected blood was mixed with equal volume of trypanosome dilution buffer containing glycerol (TBDG-20), avoiding frothing before chromatographic separation.

Preparation of *T. evansi* whole cell lysate antigen (WCLA): Blood stage trypomastigotes in heart blood of experimentally infected mouse were purified by standard DEAE cellulose chromatography (OIE 2004) The trypanosome suspension was subjected to rapid freezing and thawing for 5- times in presence of phenyl methyl sulphonyl fluoride (PMSF; added to a final concentration of 0.1mM). The lysate was subjected to ultrasonication. Sonication was done on ice for 3 cycles at 15 amplitudes for 30 sec, with an interval of 1 min between the cycles. The sonicate was centrifuged at 13,000 rpm at 4°C for 30 min. The supernatant was carefully aspirated and the protein concentration was estimated (Lowry *et al.* 1951).

Serum samples

Reference sera: Prior approval from the animal ethics

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committee was obtained for animal experimentation. A group of 4 crossbred bovine calves was experimentally infected with *T. evansi* (5×10^6) twice at fortnight intervals. Reference positive sera samples were separated from blood collected from experimental calves on day 21 post infection. Reference negative sera were obtained from naïve (confirmed by PCR and mice inoculation tests) bovine calves. All the infected calves were treated with diminazine aceturate @10 mg/kg body weight at the end of the experiment.

Test sera: Sera samples (320) were separated from blood collected randomly from indigenous cattle reared under field conditions from Western Uttar Pradesh. Blood samples were collected aseptically from the jugular vein for separation of sera and preparation of blood smears on spot.

Whole cell lysate antigen-based enzyme-linked immunosorbent assay (WCLA-ELISA): ELISA was performed following the protocol originally described by Luckins *et al.* (1977) with some minor modification. Initially the WCLA-ELISA protocol was laboratory standardized for optimum reactivity by checker-board titrations of antigen, serum and conjugate. Flat bottom polystyrene ELISA plates were coated overnight with 100 µl of WCLA (concentration 15 µg/ml) in carbonate-bicarbonate buffer (pH 9.6) at 4°C. Excess unbound antigen was removed from the wells by washing 3-times with PBS-Tween 20 (PBS-T) and blocked with 3% fat-free milk powder dissolved in PBS at 37°C for 2 h. The plates were washed 3-times with PBS-T and the sera samples diluted 1:100 in PBS containing 1% non fat milk powder were loaded in duplicate wells and incubated for 1 h at 37°C. Following stringent washing with PBS-T, rabbit anti bovine IgG HRP conjugated antibody was added at 1:15,000 dilution. Known reference positive and negative sera were used alongside as controls. The plates were incubated further at 37°C for 60 min, washed and developed with freshly prepared O-phenylenediamine substrate. The plates were read at 492 nm in an ELISA reader after stopping the reaction with 50 µl of 3M HCl.

Statistical analysis: The cut-off value was determined by adding the 4 standard deviations (SD) value to the mean OD₄₉₂ value of 6 negative controls. The sensitivity and

specificity of ELISA were calculated and compared with that of blood smear examination, as a gold standard, using online software (<http://graphpad.com/quickcalcs/kappal.cfm>). The kappa value, hence calculated, was compared and results were formulated.

Observations on infectivity of cryopreserved *T. evansi*: Patent infection was established in mice following intraperitoneal infection by days 4 to 6 post inoculation (PI) and a teeming level of parasitemia observed microscopically in wet mice blood smears. The trypanosomes were separated from the heart blood of infected mice at this level for preparation of whole cell lysate antigen (WCLA). The protein concentration of the WCLA was determined as 4.1 mg/ml.

Whole cell lysate antigen -based enzyme-linked immunosorbent assay (WCLA-ELISA): The ELISA test was standardized in the laboratory using experimentally infected bovine calves and further used for screening of *Trypanosoma* specific antibodies present in the sera samples procured from field conditions. An overall prevalence of surra was recorded at 19.38% with WCLA ELISA

Microscopical examination revealed 23 blood smears positive for *T. evansi*. The ELISA detected *T. evansi* specific antibodies in significantly higher number of samples than direct blood smear examination (Table 1). WCLA ELISA yielded high sensitivity and specificity compared to direct microscopy, with adequate positive and negative predictive values (Table 1).

Seroprevalence of *T. evansi* antibodies in cattle from different parts of India has been reported using native glycoprotein based ELISA (Kundu *et al.* 2013). The use of defined antigens in the diagnosis of cattle surra in the Indian subcontinent is rare. Glycoprotein antigen based competitive ELISA and double antibody sandwich ELISA has been reported earlier for sero-detection of *T. evansi* in camels. The lack of adequate information on the prevalence of surra in cattle, especially from the northern parts of India, prompted us to develop and evaluate a user friendly low cost *T. evansi* native antigen based serological assay.

Bovids often suffer from a chronic form of infection with very low level of parasitaemia in the peripheral blood which often goes undetected by routine microscopy. Therefore, standardization of high throughput serological assays are considered vital for effective large scale detection and

control of the infection. *T. evansi* whole cell lysate antigen (WCLA) based ELISA has been conventionally used for sero-diagnosis of surra (Luckins 1977) as many of the diagnostically relevant proteins are often useful for detection of infection in polyclonal serum isolated from the host thereby increasing the diagnostic sensitivity of the test. In this study, we found that sero-diagnosis using native whole cell lysate antigen yielded diagnostic sensitivity in detecting chronic surra of cattle.

Crude surface antigen based ELISA for seroprevalence study of *T. evansi* were reported by various workers from different parts of world (Kundu *et al.* 2013) and is in good agreement with other tests in terms of sensitivity and specificity (OIE 2004). The ground reality of the diagnosis of chronic surra in cattle and buffaloes is much more complicated than expected in any other chronic infection. Though trypanosomosis may go undetectable by PCR, stained thin smears or even by mice hemo-transfer assays due to numerous factors, yet cattle may still remain infected particularly in those cases where the parasites sequestered in tissue fluids thereby having only occasional reappearances in the host blood circulation. Serological studies are definitely having an uncut advantage under such circumstances as the animals once infected remain infected for life (Atarhouch *et al.* 2003) and are having sufficient serological antibody titers to be get detected by sophisticated serological tools.

In field conditions with majority of chronically infected animals, WCLA ELISA would definitely offer a better option in terms of good sensitivity, specificity, positive and negative predictive values in comparison to microscopic or molecular tools. Still mere presence of specific anti-*T. evansi* antibodies in sera is definitely not suggestive of an active infection; drug-cured animals may also appear sero-positive for a considerable time and will give false positive tests as trypanosome specific antibodies can be detected up to 83 days (Luckins 1977) and 69–78 days (Monzon *et al.* 2003) post drug treatment in cattle and horses respectively. Still the advantage of only seldom missing a true positive sample usually outweighs this deficiency, more precisely from an epidemiological standpoint of view. WCLA ELISA is definitely of greater value in the herd testing for adopting sufficient quarantine measures and during declaration of surra free status of a population in a

Table 1. Diagnostic performance of WCLA ELISA in respect to blood microscopy for diagnosing bovine trypanosomiosis

Test WCLA ELISA	Blood smear			Sensitivity (95%CI)	specificity (95% CI)	Kappa value
	Positive	Negative	Total			
Positive	23	38	61	95.83%	87.16%	Kappa=0.486
Negative	1	258	259	(78.81% to 99.30%)	(82.81% to 90.75%)	SE of kappa= 0.067 95% confidence interval from 0.355 to 0.616
Total	24	296	320			

*Kappa value > 0.81: almost perfect agreement, 0.61–0.80: substantial agreement, 0.41–0.60: moderate agreement, 0.21–0.40: fair agreement, 0.01–0.20: slight agreement and 0.00: poor agreement.

particular geographical niche. Considering the potentiality of inflicting pathogenesis in a wide range of animals, a stringent implementation of available control measures is required. For that a clear picture on prevalence of the infection in different animal species is imperative. To accomplish this, a wider study involving quite a large number of samples from across the country is required. In this regard, *T. evansi* whole cell lysate antigen ELISAs may prove to be a quite bright option for high through put epidemiological surveys of *T. evansi* infection in field levels.

SUMMARY

Cattle sera samples procured from some selected farms of western Uttar Pradesh were screened by using ELISA employing *Trypanosoma evansi* whole cell lysate antigen. The WCLA ELISA was compared with microscopic examination and a high level of sensitivity and specificity were achieved based on kappa prediction value estimates. A diagnostic ELISA was laboratory standardized with 15 µg/ml *T. evansi* soluble protein. A total of 320 sera samples were tested and 62 samples (19.38%) showed seropositivity. In comparison, 23 samples (7.18%) were found positive for *T. evansi* parasites in peripheral blood smears. *T. evansi* whole cell lysate antigen ELISAs may prove quite bright options for high through put epidemiological surveys of *T. evansi* infection in field levels.

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