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A pilot sero-survey for surra in livestock in Karnataka by ELISA using flagellar antigen of *Trypanosoma evansi*

Ligi Jose, Sengupta P. P*, Rudramurthy G. R., Rahman H.
National Institute of Veterinary Epidemiology & Disease Informatics (NIVEDI), Ramagondanahalli, Yelahanka, P.B.no. 6450, Bengaluru- 560064, Karnataka, India
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Abstract

Background: *Trypanosoma evansi* causes trypanosomosis or 'surra' in domestic and wild animals and is transmitted by haematophagus tabanid fly. For successful control of the disease, it is important to detect the carrier animals followed by successive treatment. For this purpose a reliable serological test is useful for mass screening of the animals. **Methodology:** In the present study, emphasis was given to address the need for the development of a mass screening serological test by using purified flagellar antigen of *T. evansi* for the detection of antibody against surra. Also a pilot sero-epidemiological study for trypanosomosis in different species using purified flagellar (FLA) antigen based indirect enzyme linked immunosorbant assay (I-ELISA) was conducted and compared with standard CATT/*T. evansi* test. The diagnostic potentiality of purified FLA antigen based I-ELISA (FLA-I-ELISA) was evaluated using 197 sera samples from field animals including cattle, buffaloes, horses and donkeys. **Results:** The test showed an overall 14.72% sero positivity (S.P). Among cattle, S.P was found to be 18.03%, in buffalo 25%, in horse 6.25% and in donkeys 5.5%. **Conclusion:** Thus, epidemiological survey were done with purified FLA antigen based I-ELISA test in Karnataka reveals that, buffalo samples showed the maximum sero positivity for trypanosomosis.

Keywords*: Trypanosoma evansi;* surra; purified flagellar (FLA) antigen; I- ELISA; sero-epidemiology.

1. Introduction

Trypanosoma evansi (*T.evansi*) is an extracellular haemoflagellate protozoan parasite of livestock that causes a disease termed as 'surra' or trypanosomosis. *T.evansi* has the widest geographical distribution among all the pathogenic trypanosome species[1]. Cattle, buffaloes, camels and horses are the most susceptible hosts of surra in south-east Asia[2,3]. The disease is generally characterized by fever, anemia, cachexia, immunosupression, oedema, muscular weakness, loss of appetite, abortion with a morbidity and mortality loss of 50-70%. In the sub- clinical stage of infection, it can cause significant decrease in milk production also in lactating cows[4]. Even after recovery, the animals serve as carriers for the disease by exhibiting low levels of parasitaemia for years. Therefore, it is very essential to detect and diagnose the carrier animals for subsequent treatment and for successfully stamping out the disease, as they may act as a source of infection to other healthy animals. Thus, in such scenario, there is an urgent need for the development of sensitive serological tests for the detection of carrier status of animals and its mass screening in the field which can lead to effective control of the disease with better production. For the detection of carrier status of the infection, many diagnostic tools have been developed such as nucleic acid detection by PCR[5,6] .Several serological tests such as indirect fluorescent antibody test (IFA),enzyme linked immunosorbent assay (ELISA), card agglutination test (CATT/*T. evansi*), etc and parasitological diagnostic tests such as thick or wet blood smear examination tests have also been developed for the detection of trypanosomosis. However, by parasitological test, only clinical stages of infection can be diagnosed satisfactorily and not latent or chronic infection[7]. Thus, serological test such as, ELISA qualifies as a universal test as it is not strain specific[8]. The parasites are attached to the host surfaces by flagellum[9] and several flagellar pocket- associated

Corresponding author
Full Address :
National Institute of Veterinary Epidemiology & Disease Informatics (NIVEDI), Yelahanka, Bengaluru- 560064, Karnataka , India
Phone no. +91 080 23093100
E-mail: pinakiprasad_s@rediffmail.com

proteins have been identified and found to contribute in trafficking and virulence[10]. The flagellar pocket region can be considered as a privileged site for immunological intervention[11]. Moreover, it has been reported that paraflagellar rod protein may be the critical organelle mediating attachment to vector cell surface[12].Thus, in the present study, purified flagellar (FLA) based indirect ELISA (I-ELISA) was developed for detailed epidemiological study for the diagnosis of antibody of surra in cattle, buffaloes, horses and donkey in Karnataka state. As per literature search, such endeavor was not found available

2. Materials and methods

2.1. *T. evansi* stabilate preparation and experimental animals

Trypanosoma evansi (*T. evansi*) buffalo isolates, isolated from Karnataka (India) which were maintained in Parasitology Laboratory, NIVEDI, Bangalore, India were used for purified flagellar (FLA)[5,6,13-15] .Four wistar albino rats were used for propagation of *T.evansi* stabilates and hyperimmune serum preparation procedures.

2.2. Purification of whole trypanosomes and preparation of purified flagellar (FLA) antigen of *T. evansi*

The trypanosomes were isolated from whole blood through standard protocol using diethyl amino ethyl (DEAE) cellulose Column chromatography[13]. *T. evansi* purified FLA antigen was prepared from whole parasites[16-18]. Presence of purified flagella (FLA) of *T. evansi* was confirmed by Giemsa stained smear. The protein concentration of FLA antigens were estimated[19] and preserved in aliquots at -80°C till further use.

2.4. Card agglutination test for Trypanosomosis (CATT/*T.evansi*)

The CATT/*T.evansi* kit uses the freeze dried trypanosomes of *T.evansi* VSG RoTat1.2[20-21] and was obtained from the Koning Leopold Institute of Tropical Medicine, Antwerp, Belgium (OIE reference Laboratory of Surra) and used as per manufacturer's instruction.

2.5. Hyperimmune sera preparations in animal

In the present study, hyperimmune sera against purified FLA antigen was raised in rat by following the standard protocol[13].The experimental animals were dealt as per the standards of animal ethics; feed and drinking water were given *ad libitum*.

2.6. Field sera collections

Field sera samples (197) were collected randomly between the period 2012-2014 from different districts of Karnataka state including Bijapur, Gulbarga, Bangalore urban, Chickballapur, Gadag from different species including cattle (61), buffalo (52), horse (48) and donkey (36). After sampling, the sera samples were separated and preserved at -80°C for further use. The field sera samples were subjected to FLA based indirect ELISA (FLA-I-ELISA) and CATT/*T. evansi* in duplicates for the screening of trypanosomosis. Also the bovine samples infected with *Theileria annulata* and *Babesia bigemina* were also incorporated in the test.

2.7. Characterization purified FLA antigen of *T. evansi*

2.7.1. SDS PAGE and immunoblot analysis of purified FLA antigen of T. evansi

SDS PAGE was carried out by loading purified FLA antigen (50µl) into 10% polyacrylamide gel and subjected to electrophoresis to detect purified FLA antigen as described by following the standard protocol[22]. Prior to loading, the protein sample was boiled in electrophoresis sample buffer (50 mMTris pH 6.8, 10% glycerol, 5% β- mercaptoethanol, 2% SDS and 0.1% bromophenol blue) for 5 minutes. The electrophoresed gel was stained with PAGE blue staining solution (Invitrogen, USA) followed by destaining. For immunoblot analysis, the electrophoresis protein was transferred on to the nitrocellulose membrane following the standard protocol and then the immunoblot was developed by treating the membrane initially with primary antibody using hyperimmune sera (1:100 dilution) raised in rat and later, the membrane was treated with secondary antibody conjugated with horse raddish peroxidase (1:1500 dilution). Finally, the membrane was treated with solution containing substrate diamino benzidine tetrahydrochloride (DAB) to develop the blot.

2.7.2. Indirect -Enzyme linked immunosorbant assay (I-ELISA)

The immunoreactivity of the purified FLA antigen was determined using I-ELISA. The antigen concentration, sera dilution and conjugate dilution were optimized by checker board titration for I-ELISA. Thus for I-ELISA, the concentration of purified FLA antigen at 200ng/well, dilution of hyperimmune/field sera samples at 1:100 and conjugate was chosen at 1:1500 dilutions respectively. The microtitre plates (maxisorp®, nunc) were coated overnight at 4°C with 100µl well of purified FLA antigen (200 ng/well) in PBS (pH 7.2). The microtitre plates after overnight incubation were washed four times with washing buffer [0.25% (v/v) Tween- 20 in PBS, pH 7.2] and blocked with 150µl/well blocking buffer (3% skimmed milk powder (SMP) and 0.05% Tween- 20 in PBS) for one hour at 37ºC and washed. The rat hyperimmune sera samples were diluted (1:100) with half strength of blocking buffer. The plates were incubated for one hour at 37ºC and washed as described as above. Later, the respective secondary antibody such

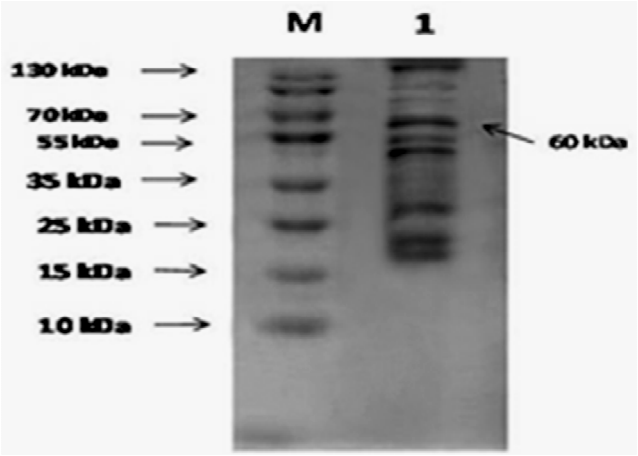

Figure I: SDS-PAGE analysis of purified flagellar antigen (FLA) Lane 1 = Purified FLA antigen, Lane M= Prestained protein ladder

as anti-rat IgG (for rat sera), anti bovine IgG (for buffalo and cattle sera) and anti horse IgG (for horse and donkey sera) conjugate with horse peroxidase (diluted as per manufacturer's instruction (sigma)) was added (100µl/ well) and incubated for one hour at 37ºC. The microtitre plates were then washed and 100µl/well enzyme substrate (chromogenic) solution (5 mg of O- phenyl diamine dihydrochloride (sigma) and 0.03% (v/v) H_2O_2) was added to develop the colour. After few minutes, the reaction in the plates were stopped by adding 1M H_2SO_4 (100 µl/well) and O.D was recorded at 492 nm in an ELISA reader (Bench mark microplate reader, Bio-rad). The specificity of the purified FLA antigen was determined with serum samples of cattle clinically infected with *Theileria annulata* and *Babesia bigemina* diagnosed by blood smear examination.Thus for the epidemiological study for the detection of antibody against *T. evansi,* 197 field/herd sera samples of different species in Karnataka, were subjected to I-ELISA in duplicate using purified FLA antigen and was compared against CATT/*T. evansi.*

2.8. Statistical Analysis

An agreement of purified FLA-I-ELISA test in relation to CATT/*T. evansi* using cattle, buffalo, horse and donkey sera samples were determined[23] .All degrees of significance were determined by chi-square test using winepiscope software. A group of field serum samples collected from Karnataka from different species like cattle, buffaloes, horse, and donkey were subjected to FLA- I-ELISA and CATT/*T.evansi* for the various statistical analysis of the test.

3. Results and discussion

SDS-PAGE analysis showed the presence of nine bands ranging from 18 to 130 KDa at 18, 20, 30, 50, 55, 60, 75, 90 and 130 KDa for purified flagellar portion of *T.evansi* (**Figure I**). It was showed in the earlier studies that even in some geographical regions, 4 to 12 polypeptide bands found in the range of 17.6 to 80.2 kDa in flagellar antigen in different isolates[17]. For I-ELISA, the purified FLA antigen and control healthy sera remained respectively reactive and non-reactive. The purified FLA antigen showed high reactivity of O.D value of > 1.2 with hyperimmune sera and control healthy rat sera showed lesser O.D value of <0.2 respectively by I-ELISA. Also purified FLA antigen remained non reactive with serum samples clinically infected with *Theileria annulata* and *Babesia bigemina* from bovines (O.D value of <0.2) which concluded that purified FLA antigen is highly specific to trypanosomes. In ELISA and SDS analysis, the purified FLA antigen of *T. evansi* showed immunoreactivity with the panel of sera samples consisting of experimentally produced field samples including different host species namely cattle, buffalo, horse and donkey. The degree of agreement was determined by chi-square test using winepiscope software. Out of 197 field sera samples, 29 sera samples were found to be positive by I-ELISA with purified FLA antigen, while approximately 31 sera samples were found to be positive by CATT/*T. evansi* test. The overall seroprevalence (S.P) of trypanosomosis in various species in Karnataka was found to be 14.72 % and 15.73 % respectively by I-ELISA and CATT/*T. evansi*. Moreover, species wise sero-prevalence survey by I-ELISA in Karnataka state in India showed 18.03 % S.P in cattle, 25 % in buffalo, 6.25% in horse and 5.5 % in donkey. Overall species wise sero-prevalence survey in Karnataka showed the dominant statistics with buffalo of 25 % S.P, followed by cattle with 18.03 % S.P, Donkey with 5.5% S.P and Horse with 6.25% S.P (**Table I**).

Table I: Comparison of I-ELISA and CATT/*T. evansi* test

Test →		FLA-I- ELISA			CATT/*T. evansi*		
State	**Species**	**P**	**N**	**% Positive**	**P**	**N**	**% Positive**
Karnataka	**Cattle**	11	50	18.03	12	49	19.67
	Buffalo	13	39	25	14	38	26.9
	Horse	3	45	6.25	3	45	6.25
	Donkey	2	34	5.5	2	34	5.5
Total		29	168	14.72	31	166	15.73

Earlier among bovines 29.3% S.P was observed involving six states[14] whereas earlier from eastern India, 12.74% S.P was observed[24]. In Karnataka, no significant variations were observed between sero-positivity and negativity among two tests (χ^2=0.079, df=1, p>0.05). It was also found that in Karnataka, among cattle, no significant difference were observed in both the tests (χ^2=0.054, df=1, p>0.05). And when cattle and buffalo sera samples were compared, no significant variations were observed (χ^2= 0.815, df=1, p>0.05. The developed ELISA showed high agreement in screening of samples from animals when compared with CATT/*T. evansi* test (Cohen's kappa value of 0.73). However a larger panel of sera samples is required to determine the exact sensitivity and specificity of the test.

4. Conclusion

Thus, according to the diagnostic performance of purified FLA antigen based I-ELISA, it was found that purified FLA-I-ELISA for surra for the epidemiological study among different species in Karnataka in India and the authors expect that the developed test in the present study can be employed for mass screening for effective control regime by detecting the carrier animals with trypanosomosis.

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Conflict of interest

The author's declares none.

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