cooling system and LED bulbs to illuminate the cart during night. A 12 V/7 AH battery is also used to store solar power during the day and to run the system during night or on a cloudy day when solar radiation is not adequate.

The solar-powered vending cart was tested for its performance during summer season. The minimum and maximum drop in temperature ranged between 8.1°C and 11.2°C and increase in RH was observed to be up to 15% and 25% in June (Figure 4). The requirement of water ranged between 16.5 and 20.0 litre/day. There was considerable effect on physiological loss in weight of different vegetables kept either inside or outside the mobile chamber (Table 1). When the produce loses up to 10% of its fresh weight, it begins to wilt and soon becomes unusable. Methi (leafy vegetable) became unusable on the first day itself when kept outside, but was usable when kept inside the chamber. Tomato exhibited minimum moisture loss at the end of storage of up to 10 days, but due to decay it was unusable beyond the tenth day of storage. There was very low moisture loss from cabbage as well, which was usable even on the tenth day when kept inside; cauliflower and carrot could be stored up to 7 days. Therefore, the shelf life of vegetables significantly increased by storing them in the solar-powered evaporatively cooled chamber of the vending cart.

Thus, this system is an excellent alternative for short duration storage of fresh fruits and vegetables at a low cost. It not only reduces the storage temperature but also increases RH which is essential for maintaining the freshness of the commodities. In the solar-powered vending cart, fruits and vegetables can be stored safely for a longer period of time at the retail vendor's level.

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Development of ELISA exploring recombinant variable surface glycoprotein for diagnosis of surra in animals

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In the present study, the variable surface glycoprotein (VSG) gene of *Trypanosoma evansi* was cloned and expressed in *Pichia pastoris* (X-33). The diagnostic potential of recombinant VSG (*r*VSG) in ELISA has been determined using 1818 field sera samples collected from different species across different states of India. The developed test was compared with the standard reference test such as, CATT/*T. evansi*; moreover, the new assay was also compared in ELISA using VSG RoTat 1.2 antigen. The diagnostic sensitivity and specificity of recombinant protein were found to be 95.4% and 93.8% respectively, with Cohen's kappa value of 0.86. The epidemiological study revealed varied prevalence of surra in different species and across different geographical regions of India. Cattle experienced higher prevalence of surra with 42.2% seropositivity from eastern region of India, whereas camel showed 19.9% seropositivity from Rajasthan. Hence, the present study is useful as an effective tool in sero-diagnosis as well as surveillance.

Keywords: Diagnosis, ELISA, *r*VSG, surra, *Trypanosoma evansi*.

TRYPANOSOMA EVANSI, which is considered as a petite mutant of *Trypanosoma brucei*[1], is a causative agent of a disease called surra which results in significant economic loss in the agricultural industry. A wide range of animals such as horses, mules, donkeys, camels, cattle and buffaloes are susceptible to *T. evansi* infection. Cattle, buffaloes and horses are the most likely hosts for surra in the South East Asian region[2]. The clinical symptoms of surra include recurrent fever, anaemia, muscular weakness, oedema, loss of appetite and abortion, with 50–70% morbidity and mortality. The animals which exhibit low levels of fluctuating parasites even after recovery/cure serve as carriers of the disease for years. Hence, detection of carrier animals is the key factor in controlling the disease. Conventional parasitological techniques can satisfactorily detect acute or sub-acute infections. However, chronic/latent infection (where parasitic load is less) diagnosis is difficult by conventional method. The development of new diagnostics such as parasitic DNA detection and/or immunodiagnostics would help in the detection of carrier

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animals. Many different genes have been employed in the development of diagnostics for trypanosomosis, such as VSG[3], invariant surface glycoprotein (ISG)[4], kinetoplastid/nuclear DNA and isoenzyme analysis[5–10]. The antibody response against *T. evansi* in the host is primarily elicited by VSG. Several potential antibody detection techniques have been developed for *T. evansi*, based on RoTat 1.2 (ref. 11), such as, CATT/*T. evansi*[11,12], LATEX/*T. evansi*[11,13] and ELISA/*T. evansi*[11]. VSG has been expressed by several groups in different host cells including insect cell line[14], yeast[15] and *E. coli*[16,17]. Furthermore, several other genes from different species of trypanosomes have also been successfully expressed in *Pichia pastoris*, for instance, acid α-mannosidase and trans-sialidase from *T. cruzi*[18,19], rhodesain from *T. brucei rhodesiense*[20] and congopain from *T. congolense*[21]. In the present study, the VSG gene of *T. evansi* has been expressed in *P. pastoris* and assessed its immunoreactivity. Further, the recombinant protein has been characterized for its potential in the serodiagnosis of surra in ELISA.

Different isolates of *T. evansi* such as buffalo, lion, dog and leopard (available in the Parasitology Laboratory, NIVEDI, Bengaluru) were used in the present study. The isolates of *T. evansi* were propagated in rats[3] and purified[22]. The whole cell lysate (WCL) from different isolates were prepared[16] and the protein concentration of the supernatants was estimated[23] and kept in aliquots at –80°C till further use.

The VSG RoTat 1.2 antigen was procured from the Koning Leopold Institute of Tropical Medicine, Antwerp, Belgium (OIE reference laboratory of surra). The antigen was reconstituted following the manufacturer's instruction to use in ELISA (600 ng/well). Further, the results obtained were used in the comparative immunoreactivity study with expressed protein.

The hyper-immune/immune sera raised against different isolates[16] and available in the laboratory were used in the present study. However, the hyper immune serum against recombinant VSG (*r*VSG) was produced in New Zealand white adult rabbits following the protocol mentioned earlier[16]. The experimental animals were dealt following standard animal ethics and feed and drinking water were given *ad libitum.*

The field sera samples were collected from Karnataka (cattle $n = 195$, buffalo $n = 172$, donkey $n = 87$, horse $n = 98$), Odisha (cattle $n = 42$), West Bengal (cattle $n = 272$) and Rajasthan (camel $n = 952$) from the field. The horse sera ($n = 98$) samples were collected from an organized herd from Karnataka, India. After sampling, the serum was separated and preserved at –80°C for further use. The serum samples were screened in duplicate for surra by different serological tests including, CATT/*T. evansi* and indirect ELISA using *r*VSG and VSG RoTat 1.2 antigens.

A set of expression primer TEVSG-F (5′-CAT*GAATTC* CAAGGCGCTCGTTGG-3′) corresponding to 10–24 bp and TEVSG-R (5′-GCCTGT*AAGCTT*TTTGTTTTTTG CATCTGATTC-3′) corresponding to 1216–1196 bp were designed from our published sequence EF495337. The *Eco*RI and *Hin*dIII restriction sites were introduced respectively in the forward and reverse primers. Both forward and reverse primers were also introduced with histidine (His) sequences (for purification of recombinant protein). RT-PCR followed by PCR was carried out to amplify the VSG gene of *T. evansi*. The total RNA isolated from the purified trypanosomes (buffalo isolate) and random nanomer were used in RT-PCR to synthesize VSG specific cDNA[3]. Further, the cDNA was amplified by PCR using VSG-specific primers (TEVSG-F/R). The 25 µl PCR reaction mixture contained *Taq* buffer, dNTPS (10 mM), TEVSG-F/R primers (20 pmol each) and *Taq* DNA polymerase (3 units – MBI Fermentas). PCR reaction was carried out with an initial denaturation at 94°C for 3 min, followed by 35 cycles of 1 min, denaturation at 94°C for 1 min, primer annealing at 55°C for 1 min and extension at 72°C for 1 min 20 sec with a final extension at 72°C for 10 min. The amplified product was then cloned into pGEMT/A cloning vector following the standard protocol[17].

The cloned VSG was released from recombinant pGEMT/A cloning vector by digesting with *Eco*RI and *Hin*dIII and ligated into *Eco*RI and *Hin*dIII digested eukaryotic expression vector pPICZα(A). The recombinant plasmid pPICZα(A)VSG was transformed into *E. coli* (top 10) competent cells by following the standard protocol[24]. The transformed cells were plated on LB agar medium containing zeocin (100 µg/ml) and incubated at 37°C overnight. The colonies that appeared on the plate were screened by colony lysis, colony touch PCR and restriction enzyme (*Eco*RI and *Hin*dIII) digestion. Furthermore, recombinant plasmid was isolated from the positive clone (Invitrogen) and sequenced using vector specific (AOXF/R) primers and insert specific (TEVSG-F/R) primers to determine and confirm the orientation of the insert.

The recombinant plasmid pPICZα(A) VSG, isolated from the positive clone was linearized using *Pme*I and then electroporated (Gene pulser, Xcell; Biorad) into eukaryotic expression host *P. pastoris* (X-33) competent cells (Invitrogen) at 25 µF capacitance, 1492 V (voltage) for 3.4 ms. After electroporation, the cells were plated on yeast extract, peptone, dextrose and sorbitol (YPDS) agar plate containing different concentrations of zeocin (100, 200 and 300 µg/ml) and incubated at 30°C for 5 days. The translucent colonies which appeared on each plate were further streaked on to YPD agar plates containing zeocin (300 µg/ml) and incubated at 30°C for 48 h. The DNA from the colonies was isolated (Qiagen, USA) and subjected to PCR using vector-specific (AOX F/R) and gene-specific (TEVSG-F/R) primers to select positive recombinant clones for the expression of protein.

Two recombinant *P. pastoris* (X-33) positive clones were analysed for the expression of VSG. To induce the

gene expression, the inoculum was prepared by inoculating a single positive colony into 10 ml buffered glycerol complex medium (BMGY, Invitrogen) in 100 ml baffled flask and incubated in a shaker (300 rpm) incubator at 30°C for 18 h (OD_{600} = 4). The cells were pelleted out at 3000 *g* for 5 min at room temperature and then resuspended in 50 ml buffered methanol complex medium (BMMY, Invitrogen) to an OD_{600} of 1.0. The culture was then placed in 500 ml baffled flask and returned to the incubator for growth. The induction of gene expression was maintained by adding 100% methanol to give a final concentration of 0.5% at every 24 h. During the induction process, 1 ml of culture was collected at every 24 h interval (0–120 h) and subjected to SDS-PAGE analysis to determine optimum post-induction time (PIT). After this, the protein was expressed in bulk, followed by the purification of *r*VSG (His tag VSG) by NiNTA agarose column (Qiagen, USA) according to the manufacturer's instructions. The control clones such as, induced X-33, X-33 with pPICZα(A) and uninduced X-33 with pPICZα(A) VSG were run simultaneously and the protein from each control clone was purified and used as control proteins/antigens. The purified proteins were dialysed against phosphate buffered saline (PBS) pH 7.2 and after estimating the protein concentration[23], stored in aliquots at –20°C till further use.

The supernatant (300 µg/well) and purified *r*VSG (60 µg/well) and control proteins were loaded into polyacrylamide gel and electrophoresed. After the completion of electrophoresis, the gel was stained with PAGE blue staining solution (Invitrogen) following the manufacturer's instructions. The purity of the histidine tag *r*VSG was authenticated using anti-histidine-tag antibodies (Bethyl Laboratories, USA). In brief, the electrophoresed proteins were transferred onto nitrocellulose membrane following the standard protocol and the membrane was then treated with anti-histidine tag antibodies (1 : 1000 dilution). Finally the immunoblot was developed by adding the substrate solution containing diamino benzidine tetrahydrochloride. Further, the immuno-reactivity of *r*VSG was determined using hyper-immune/immune sera (1 : 50 dilution) raised in rabbits/bovine/buffalo and respective secondary antibody conjugated with horse radish peroxidase.

The antigen concentration, serum dilution and conjugate dilution for ELISA were optimized by checker board titration. Microtiter plates (Maxisorp®, Nunc) were coated overnight at 4°C with 100 µl/well of purified *r*VSG (4 µg/well)/VSG RoTat 1.2 (600 ng/well) antigens in PBS (pH 7.2). After coating, the microtiter plates were washed 4 times with washing buffer (0.25% (v/v) Tween-20 in PBS pH 7.2) and blocked with 150 µl/well blocking buffer (3% skimmed milk powder (SMP) and 0.05% Tween-20 in PBS) for 1 h at 37°C and washed. The hyperimmune/immune sera and field/herd serum samples (1 : 100 dilutions) were added (100 µl/well), followed by incubation for 1 h at 37°C. After washing, the respective secondary antibodies, anti rabbit IgG (for rabbit sera), anti bovine IgG (for bovine sera), anti horse IgG (for horse sera) and protein G (for camel and donkey sera) conjugated with horseradish peroxidase (Sigma) (diluted as per manufacturer's instruction) was added (100 µl/well) and incubated for 1 h at 37°C. The microtiter plates were then washed and 100 µl/well enzyme substrate (chromogenic) solution containing 5 mg *o*-phenylene diamine dihydrochloride (Sigma) and 0.03% H_2O_2 was added to develop the colour. The reaction was stopped by adding 1 M H_2SO_4 (100 µl/well) and the OD was read at 492 nm in an ELISA reader (Bench mark microplate reader, Biorad). Except antigen coating step, in all the other steps the microtiter plates were incubated on ELISA shaker (Heidoltch titramax 101). The field/herd serum samples were tested in ELISA (in duplicate) using *r*VSG and VSG RoTat 1.2 antigens.

The optimum combination of diagnostic specificity, sensitivity and cut-off OD value of *r*VSG and VSG RoTat 1.2 in ELISA were determined by receiver operating characteristic (ROC) analysis. ROC was analysed using data obtained from field sera sample by keeping CATT/*T. evansi* as a gold standard test. ROC curves and area under curves (AUC) were generated using the software MedCalc (Version 12.7.2, USA). Cohen's kappa values[25] were determined to correlate between different diagnostic tests. The specificity of *r*VSG was evaluated with cattle serum samples clinically infected with *Theileria annulata* and *Babesia bigemina*.

The epidemiological study of surra with *r*VSG was compared with two standard antigens/test (VSG RoTat 1.2 in ELISA and CATT/*T. evansi*). The epidemiological data obtained with all the three tests were subjected to Chi square (χ^2) analysis (species and state-wise) to find out the significance of the tests.

The CATT/*T. evansi* kit developed with freeze-dried trypanosomes of *T. evansi* VAT, RoTat 1.2 (refs 12, 26) was procured from the Koning Leopold Institute of Tropical Medicine, Antwerp, Belgium (OIE, reference laboratory of surra) and used as per manufacturer's instruction. The hyper-immune serum samples were diluted two-fold (1 : 4 to 1 : 64) in CATT buffer and tested. However, bovine/camel/horse serum samples were diluted to 1 : 4 and tested in duplicate for comparative study/evaluation.

The VSG sequence cloned in pPICZα(A) is of 1206 nucleotides (nt) and encodes a polypeptide with an apparent molecular weight of 42.7 kDa (402 amino acids). The sequencing results showed that cloned VSG in pPICZα(A) is in correct orientation with respect to promoter sequence and also in frame with the ATG codon. The recombinant *P. pastoris* (X-33) clones, identified and confirmed by PCR (figure not shown) were preserved in –80°C till further use.

Out of the two recombinant *P. pastoris* (X-33) clones, one clone showed maximum expression level. The

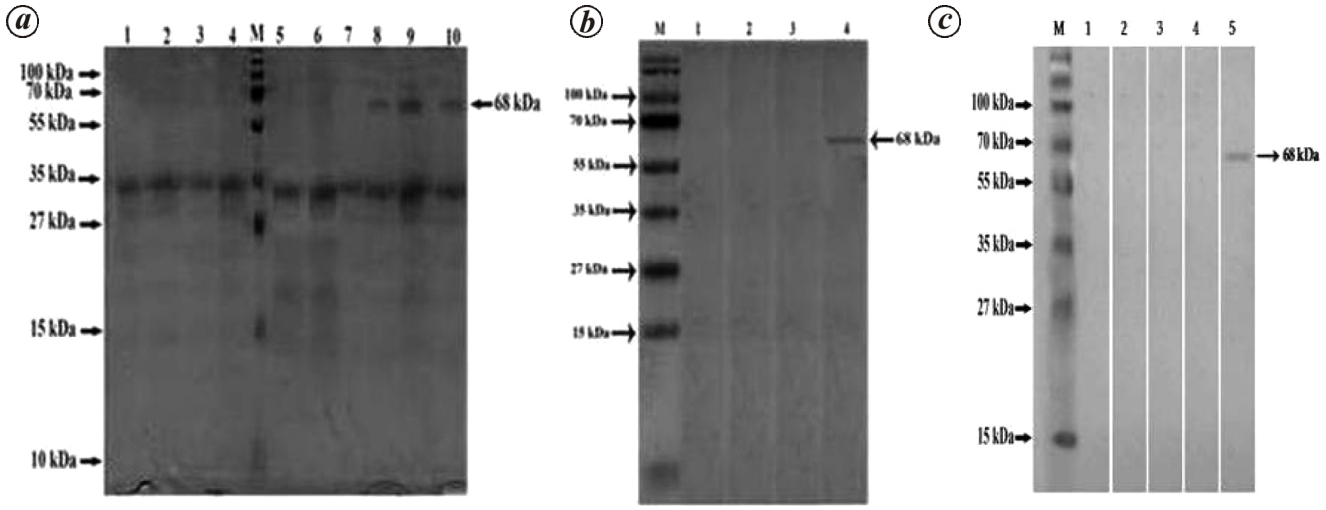

Figure 1. Characterization of *r*VSG. ***a***, SDS PAGE analysis of non-purified protein samples. Lanes 1 and 2: 72 h and 96 h induced X-33 clones respectively; lanes 3 and 4: 72 and 96 h induced X-33 with pPICZα(A) clones respectively; lanes 5–10: 0, 24, 48, 72, 96 and 120 h induced X-33 with pPICZα(A) + VSG clones respectively. ***b***, SDS PAGE analysis of purified protein samples. Lanes 1, 2 and 4: purified protein samples from induced X-33, X-33 with pPICZα(A) and X-33 with pPICZα(A) + VSG respectively; lane 3: uninduced X-33 with pPICZα(A) + VSG. ***c***, Immunoblot analysis. Lanes 1–3: purified protein samples from, induced X-33, X-33 with pPICZα(A) and uninduced X-33 with pPICZα(A) + SG respectively; lanes 4 and 5: purified protein samples from induced X-33 with pPICZα(A) +VSG; lanes 1, 2, 3 and 5 treated with different hyper immune/immune sera/anti His tag antibody; lane 4 treated with control/healthy serum; lane M: prestained protein ladder.

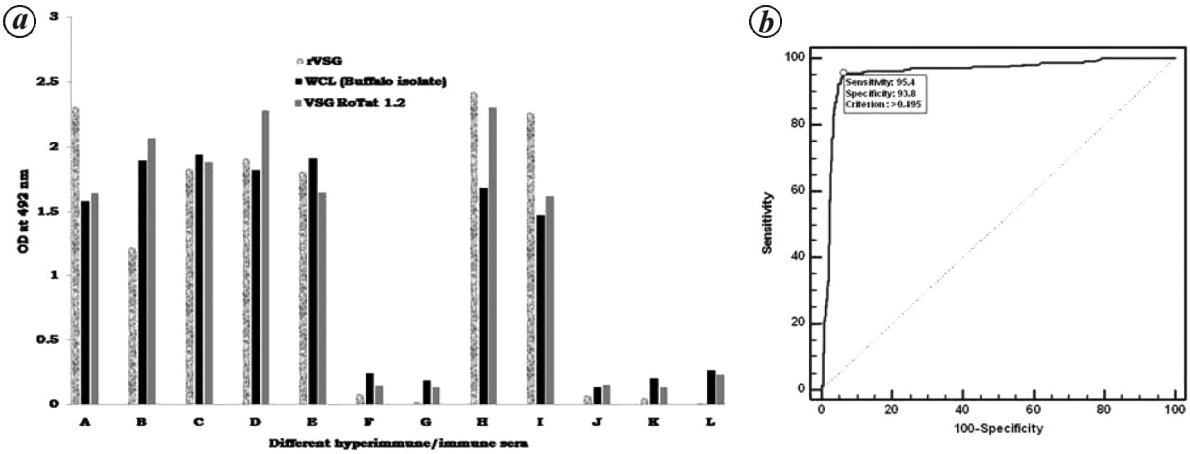

Figure 2. Diagnostic potential of *r*VSG. ***a***, Comparative immunoreactivity of different antigen in ELISA. A–E, hyper immune serum raised against *r*VSG, *T. evansi* buffalo, dog, lion and leopard isolates respectively; F and G, rabbit healthy serum and anti-rabbit conjugate control respectively; H, hyper immune serum (against *T. evansi* buffalo isolate WCL) from bovine; I, immune serum (against *T. evansi* buffalo isolate) from buffalo; J, antibovine conjugate control; K and L, healthy serum from bovine and buffalo respectively. ***b***, ROC curve depicting diagnostic sensitivity and specificity of *r*VSG in ELISA.

optimum PIT was found to be 120 h (Figure 1 *a*); the concentration of recombinant protein remained the same even after 120 h PIT. The yield of the purified *r*VSG ranged from 30 to 40 mg/l of culture. The SDS-PAGE analysis of the induced supernatant and purified products revealed the presence of *r*VSG (~68 kDa). However, the protein band corresponding to ~68 kDa was not found in control antigens (Figure 1 *b*). The increase in molecular weight of the expressed protein might be due to glycosylation of the expressed protein and addition of 11 kDa polypeptide from the vector pPICZα(A) to the insert as a fusion (*r*VSG (42.7 kDa) + vector fusion (11 kDa) + glycosylation = ~68 kDa). The increase in molecular weight of the protein (glucoamylase) expressed in *P. pastoris* was observed in the previous study and this could be attributed to glycosylation[27]. The *P. pastoris* is advantageous over prokaryotic system due to the existence post-translational modification systems such as, glycosylation and disulphide bond formation[28]. Moreover, earlier studies reported the presence of three potential N-glycosylation sites on VSG at the amino terminal region[29].

Immunoblot analysis showed that the expressed *r*VSG is highly immunogenic. The *r*VSG and control antigens remained respectively, reactive and non-reactive with

Table 1. Prevalence of surra in India: a comparative analysis using different serological tests

Test →		Indirect ELISA with *r*VSG		Indirect ELISA with VSG RoTat 1.2		CATT/*T. evansi*			
State	Species	P	N	P	N	P	N	χ^2 value	Significance *P* value
Karnataka	Cattle	68	127	70	125	71	124	0.10	0.949
	Buffalo	52	120	51	121	54	118	0.12	0.937
	Horse	5	93	5	93	5	93	0.00	1.000
	Donkey	6	81	6	81	6	81	0.00	1.000
Odisha	Cattle	13	29	11	31	8	34	0.15	0.451
West Bengal	Cattle	115	157	115	157	102	170	0.17	0.423
Rajasthan	Camel	190	762	196	756	178	774	0.11	0.573

P, Positive, N, Negative.

hyper-immune/immune sera/anti-His tag antibodies in immunoblot (Figure 1 *c*) and ELISA. Moreover, hyper-immune serum raised against *r*VSG showed immuno-reactivity with homologous/WCL and VSG RoTat 1.2 antigens. However, *r*VSG remained non-reactive with healthy/control serum. The comparative performance of *r*VSG, CATT/*T. evansi* and VSG RoTat 1.2 with different sera combination in ELISA has been demonstrated (Figure 2 *a*).

The optimum combination of diagnostic sensitivity and specificity of *r*VSG was found to be 95.4% (95% confidence interval (CI), 92.9 to 97.9) and 93.8% (95% CI, 92.0 to 95.6) respectively, at >0.495 OD value (Figure 2 *b*). The sensitivity and specificity of VSG RoTat 1.2 were found to be 95.0% (95% CI, 92.4 to 97.7) and 94.4% (95% CI, 92.6 to 96.1) respectively, at >0.508 OD value. AUC indicates that *r*VSG has classification accuracy up to 95.6%.

The seroprevalence study of surra showed that the disease is more prevalent in cattle compared to other species; the seropositivity (SP) in cattle is up to 42.2% ($\chi^2 = 0.17$, $df = 2$, $P > 0.05$) in West Bengal (eastern India), followed by Karnataka (SP = 34.8%, $\chi^2 = 0.10$, $df = 2$, $P > 0.05$) and Odisha (SP = 30.9%, $\chi^2 = 0.15$, $df = 2$, $P > 0.05$). However, SP in buffalo, horse and donkey is 30.2% ($\chi^2 = 0.12$, $df = 2$, $P > 0.05$), 5.1% ($\chi^2 = 0.0$, $df = 2$, $P > 0.05$) and 6.8% ($\chi^2 = 0.0$, $df = 2$, $P > 0.05$) respectively, from Karnataka. The SP of surra in camels was found to be 19.9% ($\chi^2 = 0.11$, $df = 2$, $P > 0.05$) from Rajasthan. When compared with other parts of India, the eastern region revealed high SP of surra especially in cattle. Earlier studies[17,30,31] also reported up to 40% seroprevalence of surra in cattle from eastern region of India. Moreover, χ^2 analysis revealed no significant difference among the three tests. The comparative analysis of seroprevalence by different tests is shown in Table 1.

T. evansi is an etiological agent of surra in wild herbivores and carnivores in the subtropics. Moreover, *T. evansi* infection in humans has been reported from Asian countries such as India and Sri Lanka[32,33]. VSG is expressed at early, middle and later stages of infection[26]. Moreover, the VSG antigenicity elicits sufficient level of antibody production in the host against the parasite[34]. In living trypanosomes, the surface epitopes are conformationally labile[35]. The above findings and molecular epidemiological studies on trypanosomosis suggest that VSG can act as a potent antigen in the diagnosis of *T. evansi* infection. Moreover, the number of genes of trypanosomes has been expressed in different host systems for several purposes. Earlier studies also report the significance of VSGs in the diagnosis of sleeping sickness and surra[26,36]. The whole cell lysates of *T. evansi* lead to strong cross-reactions with *T. vivax*, *T. congolense* and even *T. cruzi*[37]. Earlier reports suggest that VSG expressed in heterologous system can be used as a potent antigen in the diagnosis of *T. evansi* infection[12,17,38].

In conclusion, the recombinant protein explored in the present study does not depend on the use of laboratory rodents for production. Hence, the developed test exploring recombinant variable surface glycoprotein of *T. evansi* can be exploited as a potential, reliable and promising perspective tool for future application in the serodiagnosis of surra in different species.

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