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Identification of immuno-dominant antigens of *Trypanosoma evansi* for detection of chronic trypanosomosis using experimentally infected equines

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ABSTRACT

Trypanosoma evansi is the most extensively distributed trypanosome responsible for disease called surra in livestock in many countries including frequent outbreaks in India. The prevalence of this disease is most commonly reported by standard parasitological detection methods (SPDM); however, antibody ELISA is being in practice by locally produced whole cell lysate (WCL) antigens in many countries. In the present investigation, we attempted to identify and purify immuno dominant, infection specific trypanosome antigens from *T. evansi* proteome using experimentally infected equine serum by immuno blot. Three immuno dominant clusters of proteins i.e. 62–66 kDa, 52–55 kDa and 41–43 kDa were identified based on their consistent reactivity with donkey sequential serum experimentally infected *T. evansi* up to 280 days post infection (dpi). The protein cluster of 62–66 kDa was purified in bulk in native form and comparatively evaluated with whole cell lysate antigen (WCL). ELISA and immuno blot showed that polypeptide of this cluster is 100% sensitive in detection of early and chronic infection. Further, this protein cluster was also found immuno reactive against hyper immune serum raised against predominantly 66 kDa exo antigen, revealed that this is a common immunodominant moieties in proteome and secretome of *T. evansi*.

1. Introduction

Trypanosoma evansi an extracellular haemo flagellate causes trypanosomosis, (commonly known as surra) in a wide range of animals (OIE, 2010) and has been documented in equines by Payne et al. (1991), Monzon (1993), Lun et al. (1993), Claes et al. (2005), Laha and Sasmal (2008), Tamarit et al. (2010) and Mavadia et al. (2010). The disease is transmitted mechanically by the Tabanids flies (*Tabanus*, *Haematopota*, *Lyperosia*), from one host to another therefore, the magnitude of enzootic trypanosomosis caused by non tsetse borne *T. evansi* is about three times greater than due to tsetse borne trypanosomes (Woo, 1977). Traditionally *T. evansi* infection has been observed in domestic and wild animals in acute, sub-acute and in chronic forms. This disease affects most severely in equines particularly horses and mules. The disease in donkeys is not fatal and persists in chronic form for years together, however, in horses disease has a short duration of up to two weeks with fever, weakness or it may stretch up to four months, characterized by anemia, emaciation, with neurologic signs, as the parasite is reported to reach blood brain barrier and showing marked ataxia, blindness, circling, hyper-excitability, depression and gradual onset of paralysis of hind quarters (Rodrigues et al., 2009).The clinical signs of trypanosomosis in general are indicative but are not sufficiently patho-gnomonic and diagnosis must be confirmed by laboratory methods. This study entails efforts for search on infection specific antigens, which can be reproduced in complete homogenity and purity and can be applied universally for sero epidemiology. Therefore, in the present investigation, an attempt has been made to identify and purify antigenic components from *T. evansi,* proteome to detect the antibodies at early and chronic stages of infection.

2. Materials and methods

2.1. Source and maintenance of T. evansi

T. evansi was isolated from an organized equine farm of National Research Centre on Equines in Hisar, Haryana during 2009 and the isolate was maintained *in vivo* by inoculating infected

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blood in Swiss albino mice. Briefly, whole blood was collected in ethylenediaminetetraacetic acid (EDTA) from parasitologically positive horse and was subsequently, injected intra-peritoneally (i/p) in mice. The peripheral blood from tail of mice was examined microscopically daily for scoring the degree of parasitaemia. On peak parasitaemia the *in vivo* passaging was done at 5–6 days interval by inoculating 10^4 parasites by i/p route in naive mice, so as to maintain the isolate of *T. evansi* in the laboratory. The parasites are also maintained by cryopreservation in liquid nitrogen and were being used as when required.

2.2. Purification of parasites

The purification of *T. evansi* from mice blood (first parasitaemic peak) was accomplished by DEAE-cellulose chromatography, followed by centrifugation (Lanham and Godfrey, 1970). Briefly, 5–10 g of pre-swollen DEAE-cellulose (DE52, Whatman, India) was suspended in phosphate buffered saline (PBS, pH 8) and the column was packed with slurry and equilibrated with PBS pH 8.0. The column was charged with PBS-G (57 mmol/L Na_2HPO_4, 3 mmol/L NaH_2PO_4, 43.8 mmol/L NaCl, pH 8.0 with 1% Glucose) without disturbing the surface. Then, 2–3 ml infected mice blood, collected aseptically from mice heart in heparin (10 IU/ml), was poured onto the equilibrated column and the live parasites were eluted using PBS-G (pH 8.0) containing 1% glucose. The parasites thus collected were washed twice with PBS, pH 7.2 and stored at −40 °C until used.

2.3. Preparation of whole cell lysate (WCL) antigen

The sonicated whole cell lysate antigen of *T. evansi* was prepared following the method (Yadav et al., 2011). Briefly, cell-free trypanosomes obtained by anion exchange chromatography were collected and washed in PBS (pH 7.2) and submitted to ultrasonication. The disruption of cells was performed using ultrasonic cell disruptor (Branson Sonifier 450, Branson, USA) at 20 kHz using 5 mm tapered tip with four disruptions of 15 s each with 30 s interval in ice-bath at 20% duty cycle. The sonicated whole cell lysate was centrifuged at 10,000*g* for 15 min at 4 °C and supernatant collected, aliquoted and stored at −40 °C till further use. The protein concentration of antigen was determined following protocol described by Bradford (1976).

2.4. Experimental infection in equines

Eight, healthy donkeys aged 2–3 years free from piroplasmosis were screened for presence for *T. evansi* infection using standard parasitological methods as well as by antibody ELISA for 10–15 days prior to procurement from the local contractor. All the animals were also examined for presence of helminth ova/oocyst and treated with an anthelmintic albendazol@10 mg/kg body wt. prior to experiment. These animals were stall-fed on balanced diet housed in fly proof stable and maintained throughout the experiment under intensive system of management. The diet consisted of water and green fodder *ad libitum* and 1 kg general purpose horse feed mix and bran were given twice daily. The blood from infected mice was collected and six female donkeys were experimentally infected sub-cutaneously (s/c) each with 2×10^6 trypanosomes. Two animals were kept as control throughout the experiment. Blood and serum was collected weekly/fortnightly/monthly up to 280 days for parasitological and serological examination. Serum was retrieved and stored at −40 °C until further use. The serum samples of all the six infected donkeys were also pooled sequentially and tested by ELISA and immunoblot.

The animal experimentation was carried out according to rules and regulations set forth by Committee for the Purpose of Control and Supervision of Experiments on Animals (CPCSEA) Animal Welfare Division, Ministry of Environment, Government of India, The research protocol for experimentation was duely approved by the Institute Animal Ethics Committee of the National Research Centre on Equines, Sirsa Road Hisar Haryana.

2.5. Sodium dodecyl sulphate–polyacrylamide gel electrophoresis (SDS–PAGE)

SDS–PAGE of antigens (whole cell lysate and semi purified antigen) was carried out under reducing conditions, as per the method of (Laemmli, 1970), employing mini gel electrophoresis apparatus (Atto Corporation, Japan) and using prestained/unstained protein molecular weight (MW) markers (Fermentas USA). The antigen (60–80 μg/well) was electrophoresed at 50 V through stacking gel and subsequently at 100 V through resolving gel until the tracking dye reached the bottom. The gel was stained with Coomassie brilliant blue R-250 stain, and de-staining was performed using isopropanol:acetic acid:water (10:10:80, v/v/v).

2.6. Immunoblotting

Immunoblotting was done as described by Towbin et al. (1979) with slight modifications. Briefly, *T. evansi* WCL antigens of equine isolate was electrophoresed on 10% SDS–PAGE and trans-blotted on PVDF membrane by employing semi-dry transfer apparatus (Atto Corporation, Japan) using tris–glycine–methanol transfer buffer (25 mM Tris, 192 mM glycine and 20% methanol) at constant current of 0.8 mA/cm^2 for 90 min. After transfer, the membrane was blocked overnight by incubating at 37 °C in PBS-Tween-20 buffer (pH 7.2) containing 5% (w/v) skimmed milk (SM-PBST). The membrane was then incubated with serum samples of equines at 1:100 dilutions in SM-PBST for 1 h at 37 °C. The membrane was washed five times with PBS-T (wash buffer) and then incubated for 1 h with anti-horse IgG conjugated to alkaline phosphatase (Sigma) as secondary antibody (1:4000 dilution in SM-PBST). The immunoreactive bands were visualized using nitro-blue tetrazolium (NBT) and 5 bromo-4-chloro-3-indolyl phosphate (BCIP) substrate (Fermentas USA) in alkaline phosphatase buffer (pH 9.5). The substrate reaction was stopped by washing the membranes with distilled water.

2.7. Purification of immuno dominant antigen

The immuno dominant proteins were purified from preparative poly acrylamide gels following protocol described by Retamal et al. (1999), with slight modification. The WCL antigen was loaded in equal amount in different preparatory batches in mini gels. Out of three immuno reactive proteins, a cluster of 62–66 kDa protein designated as semi purified antigen was purified by cutting out with a razor blade and washed (three times for 5 min) with 2 ml of 250 mM Tris buffer/2 mM EDTA, pH 7.4, in a Falcon tube (25 ml), followed by three rinses of 5 min with distilled water. The gel slices were chopped finely or homogenized with a spatula (in pieces of 2–5 mm). Then two volume of 20 mM Tris buffer, pH 7.4, containing 0.1% (v/v) SDS was added. The samples were sonicated for 3 min in an ice bath (five or six cycles of 30 s) with a 3.0 mm probe sonicator.

2.8. Raising of hyper immune serum

The hyper immune serum (HIS) against whole cell lysate/semi purified antigen were raised in New Zealand White (NZW) rabbits by inoculating 100 μg antigen subcutaneously, emulsified in an equal volume of complete Freund's adjuvant, followed by two inoculations with 100 μg protein each, emulsified in incomplete