Full Length Research Paper

A preliminary characterization of an invariant 52kDa surface antigen in *Trypanosoma evansi* that serve as a diagnostic marker

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The study was carried out to examine the ways that *Trypanosoma evansi* components interact with the host, by investigating the components of the parasite which acted as antigens during infection, and to study the dynamic of some of these antigens during infection. Such approach could help in identifying new diagnostic reagents, a better understanding of existing diagnostic methods and target antigen for production of vaccines. The antigenic components of intact and trypsin-treated *T. evansi* were identified using sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) and immunoblotting against sera from infected rabbits and rabbits immunized with a range of soluble parasite materials. A trypsin sensitive component of ~52 kDa which was cleaved from the parasite by the process of trypsinisation and produced a potent reaction to infection and immunization sera was selected for further study. This antigenic component was purified by electro-elution from acrylamide gels and monospecific antiserum was produced and used in an antigen-capture enzyme linked immunosorbent assay (ELISA). This antigen was found to be preserved between different variants and populations of *T. evansi* and reached a detectable level in the circulation of infected animals before the remission of the first parasitemia and was cleared from the circulation following successful chemotherapy. The antigen represents a good candidate for the diagnosis of infection with *T. evansi* and for developing a serologically-based test for monitoring the effectiveness of chemotherapy.

Key words: *Trypanosoma evansi*, invariant antigens, immunoblotting, dynamic, diagnosis.

INTRODUCTION

Most of the parasites in the sub-genus *Trypanozoon* are of veterinary and medical importance, causing wide-spread disease throughout many tropical and sub-tropical countries. *Trypanosoma evansi*, the causative agent of the disease most commonly known as surra, was the first pathogenic trypanosome described. Infection with this parasite has an extremely wide geographical range in

countries with hot and warm-temperate climates (Hoare, 1972). It infects a range of economically important live-stock including equids, camels, cattle, buffaloes, goats, sheep and pigs (Luckins, 1988), but the principal affected hosts are equines and dromedaries (Hoare, 1972).

Scientific endeavour over the past 100 years has yielded large amounts of information on many aspects of

the parasitic trypanosomes including morphology, infectivity, behavior of intact parasites and host/parasite interaction, and has been the subject of extensive reviews (Hoare, 1972; Antoine-Moussiaux et al., 2009; Morrison et al., 2010). Our knowledge on many aspects of host/parasite interaction and immunology in particular, is mainly gleaned from studies based only on whole parasite extracts. Such extracts consist of a complex mixture of molecules each potentially capable of interacting with the host in a particular way. A full understanding of the way in which trypanosomes interact with the host must take into account the nature and behavior of individual parasite components and the way in which they interact with the host. Such information could help in identifying new diagnostic reagents, provide better insight into host/parasite interaction and provide indicators of infection, progress of the disease, effectiveness of chemotherapy and target antigens for production of vaccines.

Although the variant antigens of Salivarian trypanosomes constitute the major part of the surface, invariant surface antigens have also been reported. Infected mammalian hosts produce antibodies against minor and invariant surface antigens (Burgess and Jerrells, 1985; Radwanska et al., 2000). These invariant molecules are suggested to be immunogenic and may be present as circulating invariant antigens during infection (Burgess and Jerrells, 1985). These invariant surface antigens, reported mainly in *Trypanosoma brucei* are glycoproteins and referred to as invariant surface glycoproteins (ISGs); their abundance is said to be > 100-fold lower than the variant surface glycoprotein (VSG) (Overath et al., 1994). These antigens are considered to be arranged in between the VSG molecules on the surface of the parasite. These invariant surface proteins are necessary for nutrient uptake signaling and structural integrity, and are protected from the adaptive immune system of the mammalian host by the VSG in an unknown way (Schwede and Carrington, 2010).

The 145 and 140 kDa molecules are invariant surface glycoproteins which act as receptors for low-density lipoprotein (LDL) which is required for trypanosome growth (Hide et al., 1989; Coppens et al., 1988; Rolin et al., 1990). Other ISGs function as glucose transporters (Parsons and Nielson, 1990; Bringuad and Baltz, 1992). ISG100 is a polytopic protein partly resident within the endosomal system (Nolan et al., 1997; Pal et al., 2002). ISGs with as yet unknown function have been identified and include a 77 kDa protein isolated from coated endocytotic vesicles of *T. brucei* (Webster and Shapiro, 1990). Two invariant surface glycoproteins of 65 and 75 kDa have been identified by Ziegelbauer and Overath (1992) in the bloodstream stage of *T. brucei*. These particular ISGs having large extracellular domains similar in size to the VSGs and are distributed over the entire surface of the trypanosome (Ziegelbauer and Overath, 1993), were initially thought to be the most abundant

surface proteins with the exception of VSG (Overath et al., 1994). However, with the exception of a weak reaction with anti-ISG 75 antibodies, in live trypanosomes, these ISGs were not considered to be accessible to antibodies (Ziegelbauer et al., 1992) and are not involved in protection against reinfection. However, Lanca et al. (2011) were able to partially protect Balb/C mice against a lethal dose of *T. brucei* by intramuscular immunization with a single dose of a DNA plasmid encoding ISG 65. Although ISG65 is expressed on the trypanosome cell surface, an intracellular pool of this protein was also detected (Chung et al., 2004).

Definitive diagnosis of *T. evansi* infection is still based on microscopic demonstration of the parasite, an insensitive method which depends mainly on the level of parasitemia. A sensitive parasite detection method, mini-anion-exchange centrifugation method, is available but is rarely used for routine diagnosis (Lanham and Godfrey, 1970; Gutierrez et al., 2004). Currently, tests for indirect diagnosis of *T. evansi* infection through detection of specific antibodies in mammalian hosts are widely used. All these antibody detection tests can not differentiate between current and previous infections. Many of these tests are based on the native VSG of the predominant variable antigen type (VAT) RoTat 1.2 of *T. evansi*. These include a card agglutination test for trypanosomiasis for *T. evansi* (CATT/*T. evansi*) (Bajana and Hamers, 1988), an immune trypanolysis (TL) assay (Van Meirvenne et al., 1995), a latex agglutination test for *T. evansi* (Verloo et al., 1998) and an enzyme-linked immunosorbent assay for *T. evansi* (ELISA/*T. evansi*) (Verloo et al., 2000). Antibody-ELISA based on crude *T. evansi* extract was also used to detect anti-*T. evansi* equine antibodies (Reyna-Bello et al., 1998). Recently an antibody detection ELISA independent of VSG and based on recombinant invariant surface glycoprotein 75 (rISG75) derived from *T. b. gambiense* LiTat 1.3 has been developed for diagnosis of *T. evansi* infection (Tran et al., 2009). This test showed a sensitivity of 94.6% and a 100% specificity, and demonstrated an almost perfect agreement with the previous tests.

Soluble trypanosome antigens circulating in body fluids of infected animals have been detected using enzyme-linked immunosorbent assay antigen-ELISA. The test is based on a polyclonal antiserum raised against crude trypanosomal antigens or monoclonal antibodies to membrane antigens of procyclic trypanosomes of *T. brucei* or a cell membrane antigen of *T. evansi* (Nantulya et al., 1989; Olaho-Mukani et al., 1993; Rayulu et al., 2007). These antigen capture assays are capable of de-tecting trypanosome antigens released into bloodstream by dying parasites during infection and are very sensitive compared to parasitological methods.

However, it has also been reported that the Ag-ELISA may produce false negative results, possibly due to insuf-ficient destruction of trypanosomes during multiplication phase to produce detectable levels of antigens in the blood

antigens in the blood of infected animals (Waitumbi and Nantulya, 1993). A test that can detect an invariant surface antigen accessible to antibodies during infection might overcome this phenomenon. Molecular methods for specific detection of *T. evansi* infection have also been used. Claes et al. (2004) used polymerase chain reaction based on sequenced gene coding for *T. evansi* RoTat 1.2 VSG which was present in all *T. evansi* strains (Urakawa et al., 2001).

The dynamic aspects and the fate of individual trypanosome antigens during infection are not well understood. Such information might provide better insight into host/parasite interaction and could help in identifying new diagnostic reagents. Nantulya and Lindqvist (1989) in developing antigen ELISA for diagnosis of bovine trypanosomiosis using monoclonal antibodies to membrane antigens of procyclic trypanosomes detected differences in the dynamics of circulating trypanosome antigens. *Trypanosoma vivax* and *Trypanosoma congolense* antigens were detectable between 10 to 12 days following tsetse challenge, while *T. brucei* antigens were detectable between 8 to 14 days. Following treatment of the infected cattle with Berenil, *T. vivax* and *T. congolense* antigens were cleared from the circulation within two weeks but the rate of clearance of *T. brucei* antigens was slower compared to the other two species. The host species has also been found to influence the dynamics of trypanosome antigens. El Amin et al. (1993) during an evaluation of antigen-ELISA for the diagnosis of *T. evansi* infection using the anti-brucei group-specific antigen detected the antigen in camels 2 to 4 days after infection, while in goats the parasite antigens were detected after 7 days of infection.

Thammasart et al. (2001) using antigen-detection ELISA were able to detected *T. evansi* antigens 10 to 14 days in most of the cattle experimentally infected with this parasite. The antigens disappeared from the circulation 3 days after treatment. While in some cattle, Ag-ELISA was not able to detect the parasite antigens despite positive hematocrit (HCT) results and they attributed these findings to variation in the pathogenicity of the parasite strains and in the balance of parasitemia and antibody levels in the circulation. The antigenic components of *T. evansi* were investigated in this study and the dynamic of some of these antigens was studied in search for an invariant surface antigen accessible to antibodies during infection to overcome the false negative results encountered in Ag-ELISA.

MATERIALS AND METHODS

Trypanosome

Two stocks of *T. evansi* were originally isolated from naturally infected camels in Al-Ahsa area, Saudi Arabia, propagated in mice and cryopreserved until used in this study. They include one population designated (TR 2257) and two variants of another population (TR 2165 & TR 2222).

Experimental animals

Mice, rabbits and rats were used as experimental models to expand the trypanosomes, to produce infection serum and polyclonal antibodies against *T. evansi* materials and to investigate the dynamics of the 52 kDa *T. evansi* antigen during infection, respectively.

Separation of trypanosome from mice blood

Trypanosomes expanded in mice were separated from infected blood by ion-exchange chromatography on Diethylaminoethyl (DEAE) cellulose (DE52, Whatman Biochemical, UK) as described by Lanham and Godfrey (1970). The separated trypanosomes were washed three times in phosphate saline glucose (PSG, pH 8.0) by centrifugation at 2650 g for 20 min at 4°C.

Preparation of trypanosome lysate

Crude soluble extract was prepared by re-suspending the pellet of 2.2×10^9 DEAE column separated trypanosomes in an equal volume of PSG and then subjected to three cycles of freezing to -80°C for 10 min, followed by thawing to room temperature for 10 min. The lysate was then centrifuged at 10,000 g for 45 min at 4°C, supernatant removed, its protein concentration determined and the lysate aliquoted into 100 µl volumes and stored at -20°C. Whole trypanosome extract and trypsinised trypanosomes extract were prepared through detergent lysis (Anderson and Blobel, 1983) using sodium dodecyl sulphate (SDS) sample buffer. 2.2×10^9 DEAE column separated trypanosomes were diluted with an equal volume of SDS sample buffer and heated to 100°C for 5 min. After cooling to room temperature and centrifugation at 10,000 g for 5 min, they were aliquoted into 200 µl volumes and stored at -20°C. Trypsinisation of trypanosomes was carried out by incubating the trypanosomes with trypsin (20 µl/ml at a concentration of 5 mg/ml) for 75 min at 37°C and the trypsin neutralized by incubation with trypsin inhibitor (20 µl/ml at a concentration of 5 mg/ml in distilled water) for further 5 min at 37°C. The trypanosomes were then washed by centrifugation at 5,000 g for 5 min in PSG.

Electron microscopy of intact and trypsinised trypanosomes

Electron microscopy was carried out according to the method of Garnett et al. (1978), with minor modification. After post-fixation in 1% osmium tetroxide and washing, the trypanosomes were dehydrated in ascending series of acetone, then infiltrated in araldite mix: analar acetone and infiltrated in araldite mix and accelerator and embedded in araldite mix: accelerator. Sixty nm thick sections of embedded trypanosomes were cut and mounted on 200 mesh copper grids and stained with uranyl acetate and lead citrate before being viewed and photographed using a Philip 400 transmission electron microscope at 100 kV.

Raising antisera

Infection serum to intact and trypsinised trypanosomes, polyclonal anti-*T. evansi* crude soluble extract antibodies and polyclonal anti-52 kDa monospecific antibodies were raised in rabbits. Rabbit inoculated with trypsinised trypanosomes received 3 intravenous injections of 1×10^7 freshly prepared trypanosomes in 1 ml PSG on days 1, 28 and 57. On each occasion, two mice were also inoculated with 1×10^7 trypsinised trypanosomes in 0.5 ml PSG to serve as infection controls. Parasitemia in both rabbits and mice was

monitored daily. The immunization regime for production of polyclonal antibodies was adapted from Harlow and Lane (1988). Anti-52 kDa IgG fraction was prepared using the commercially available ImmunoPure[R] IgG kit (Pierce Chemical Company, USA) utilizing immobilized protein A columns (Protein A affinatyPak[TM] columns).

Electrophoresis and Western blotting

Fifty microliter of freeze-thawed soluble extract containing 45 μg protein, 50 μl of each of SDS-solubilized whole trypanosome extract and trypsinised trypanosomes each equivalent to 1.1×10^8 trypanosomes were subjected separately to SDS-PAGE (Laemmli, 1970). 7 to 20% gradient poly-acrylamide gels were used and protein banding pattern visualized by Coomassie Blue stain. Western blots (Towbin et al., 1979) were prepared by electro-blotting separated antigens in unstained gels onto nitrocellulose paper. Antigens were detected by Western blotting against infection serum to intact and trypsinised trypanosomes and antisera raised against soluble extract.

Monitoring the specificity and reactivity of anti-52 kDa antibodies

Specificity and reactivity by antibody-ELISA

Serum collected from rabbits immunized with 52 kDa antigen, purified by electro-elution from stained gels, was tested by antibody-ELISA against freeze-thawed soluble extracts of homologous (TR 2165) and heterologous (TR 2222 and 2257) T. evansi populations coated at a dilution of 1/80. Each trypanosome extract was coated into 30 wells of the ELISA plate. Pre-immunization serum and PBS/Tween were also included in the plate as negative controls. All sera were tested at a dilution of 1/1000. The test serum, pre-immunization serum and PBS were each added to 10 wells of the three trypanosome extracts. Mean optical density (OD) values of test samples in each trypanosome extract was compared with the mean OD values of pre-immunization serum and PBS. Sample with an OD value of more than two standard deviations (2SD) above the mean was considered positive.

Specificity and reactivity by Western blotting

The antiserum raised to the 52 kDa antigen was tested by immunoblotting against whole trypanosome extract from homologous (TR 2165) and heterologous (TR 2222 and 2257) T. evansi populations. The serum samples were diluted to 1/50 in blocking buffer, while the conjugate was used at a dilution of 1/500.

Labeling of anti-52kDa IgG with horseradish peroxidase (HRP)

IgG fraction was conjugated to HRP using the modified periodate method as described by Wilson and Nakane (1978). The conjugate reactivity was tested against the freeze-thawed antigens of the homologous T. evansi population at a dilution of 1/80 using direct ELISA. The conjugated antibody was tested at 2-fold dilutions starting from 1/50 to 1/3200 and the reaction was visualized using TMB substrate.

ELISA development for T. evansi antigens

Sensitivity of conjugated anti-52kDa IgG for antigen detection

The sensitivity of the labeled IgG preparation was tested against titrated T. evansi soluble extract (1.8 mg/ml) along with titrated normal rabbit serum (NRS) as control over a 2-fold dilution range from 1/50 to 1/6400 in PBS/Tween. Each dilution of trypanosome extract and NRS was run 6× on the plate. The labeled IgG preparation (5 mg/ml) was used at a dilution of 1/200 in PBS/Tween containing 1% (v/v) NRS. Results were expressed as an antigen titer equivalent to the last antigen dilution that showed an absorbance value greater than 2SD above the mean of the NRS at the same dilution.

Standardization of the ELISA test

Un-labeled IgG preparation to the 52 kDa antigen diluted from 1/50 to 1/6400 was tested against freeze-thawed T. evansi extract or normal rabbit serum (NRS). The trypanosome extract and NRS were tested in duplicates at a dilution of 1/50. The remaining area of the plate was incubated with PBS/Tween. The anti-52 kDa IgG preparation labeled with HRP diluted 1/200 in PBS/Tween containing 1% (v/v) NRS was used as a conjugate. Mean OD values of trypanosome extract greater than 2SD above the means of corresponding NRS and PBS were considered positive.

Evaluation of the developed assay

After the establishment of the ELISA assay under in vitro conditions the assay was subsequently evaluated for its ability to detect the antigen in vivo in a rabbit inoculated intravenously with 10 mg of freeze-thawed T. evansi soluble extract. Serum samples as a source of antigen were collected from the rabbit one day before inoculation, at 0.5, 1, 2, 4, and 24 h post-inoculation, and then daily until the end of the experiment 6 days later. Antigen-ELISA was performed by coating ELISA microtiter plate with un-labeled anti-52 kDa antibodies at a dilution of 1/200 and then the antibody was reacted with a duplicate of undiluted serum of each of the serum samples. HRP-labeled anti-52 kDa antibody was used as a conjugate at a dilution of 1/200. The cut-off point was calculated according to the formula: Cut-off point = (mean + 3s) + 10% (mean = mean of the mean values of all low color wells; S = standard deviation of these mean values).

The dynamics of the 52 kDa T. evansi antigen during infection

Ten male rats were used in this experiment, 4 of which served as un-infected controls. The remaining 6 rats were infected intraperitoneally with 1×10^4 T. evansi (TR 2165) trypanosomes per rat. Parasitemia was monitored daily until the end of the experiment on day 17 post-infection using the microhaematocrit centrifugation technique. Tail blood was collected daily from the infected and control rats as a source of serum. All infected rats were injected intraperitoneally with a single therapeutic dose of 3.5 mg/kg Berenil (Al-Mohammed, 2008) on day 8 post-infection to release the antigen from killed trypanosomes for monitoring its dynamic within the host. Effectiveness of chemotherapy was measured using both parasitemia and antigenemia. The developed antigen-ELISA was used to monitor the level of the 52 kDa antigen in the blood of the infected rats. Pooled serum collected daily from the each infected or un-infected rats was diluted to 1/3 before use in the assay. All serum samples were run in duplicates. The cut-off point was calculated according to the formula:

Cut-off point = (mean + 3s) + 10% (mean = mean of the mean values of all negative control sera;

S = standard deviation of these mean values). Serum sample showing an absorbance value above the cut-off point was considered positive for the antigen.
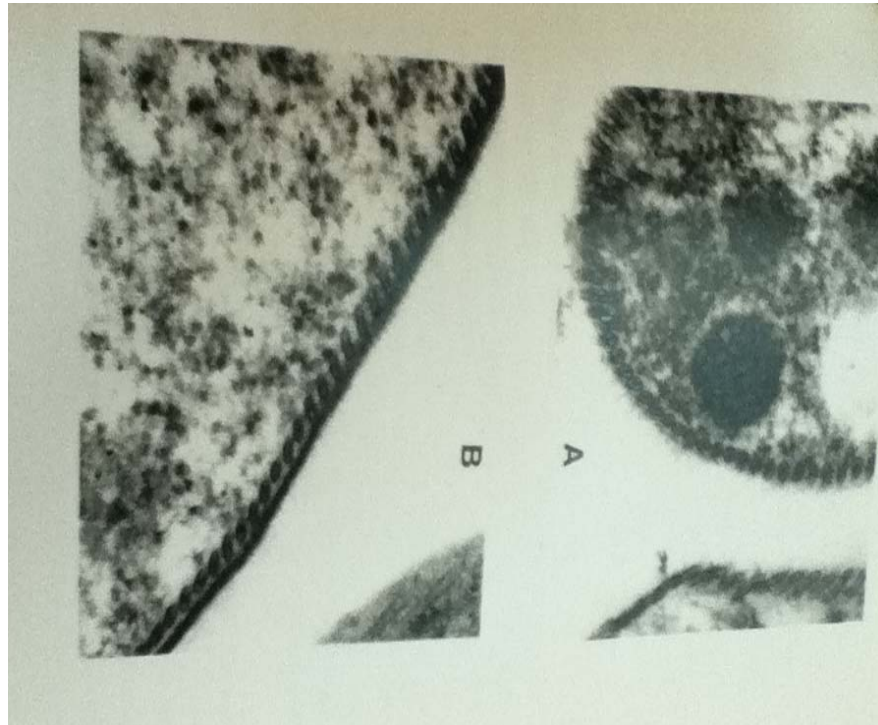
Figure 1. Transmission electron micrograph of section through surface of body of *T. evansi* (TR 2165). 1 mm ≡ 10 nm.
(A) 75 min trypsin-treated trypanosomes lacking a surface coat and (B) Intact non-treated trypanosomes possessing a surface coat.

RESULTS

Trypsinisation of *T. evansi*

Electron microscope sections prepared from untreated trypanosomes were seen to possess a thick (20 nm), compact and dense surface coat overlying the plasma membrane (Figure 1B). In the sections prepared from trypsinised trypanosomes, the surface coat was removed, leaving behind the plasma membrane which was sometimes difficult to resolve but the microtubules appeared normal (Figure 1A).
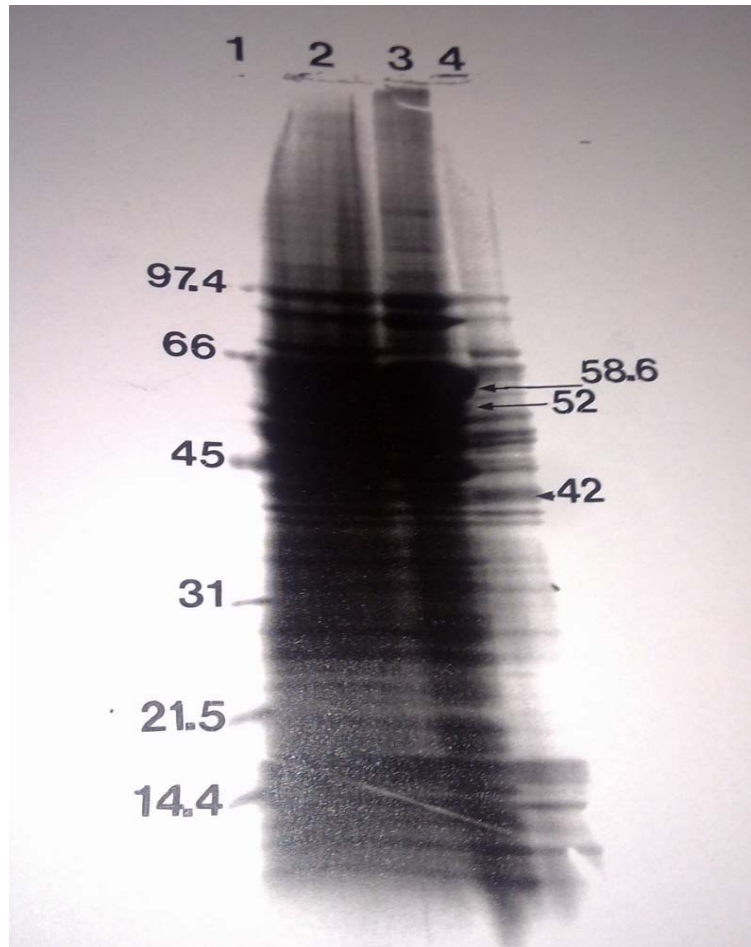
Protein profile of *T. evansi* materials

Slight difference was seen in the number of the protein bands resolved by Coomassie blue stain of the whole trypanosome extract and the soluble extract. The highest number of individual proteins were seen in the whole trypanosome extract, with a total of 39 individual protein bands with molecular weight ranging from 158.6 kDa to about 9.1 kDa (Figure 2, lane 2). Despite the equal amount of trypanosome proteins in the three extracts, two of these protein bands with molecular weight of 146 and 142 kDa were absent from the soluble extract which

contained 37 proteins (Figure 2, lane 3). Otherwise both extracts had similar banding pattern with high molecular weight components in the range of 158.6 to 125.1 kDa, and showed the lowest staining intensities. Of the 39 bands in the whole extract, only 32 polypeptide bands were visible by Coomassie blue staining of the trypsinised trypanosomes extract in the size range of 96 to 9.1 kDa (Figure 2, lane 4). Seven of the protein bands present in the whole trypanosome extract were absent from the trypsinsed trypanosome extract. Five of these bands were in the high molecular weight part of the gel (158.6 to 125 kDa), with the other two at 58.6 and 52 kDa. Furthermore only few proteins dominated by a 42 kDa component showed a similar staining intensity to that of the other two extracts. The staining intensity of the other proteins in the trypsinised trypanosomes extract appeared to be lower than that of the other two extracts despite equivalent amounts of trypanosomes.

Immunological characterization of *T. evansi* proteins

Pooled infection serum collected from the rabbit infected with whole trypanosomes extract for up to 21 days after infection recognized a total of 17 antigenic components ranging from 172 to 21.2 kDa (Figure 3, lane 1) in the three trypanosomal preparations used in this study. Of

Figure 2. Total protein profile of *T. evansi* (TR 2165) fractionated on SDS-PAGE and stained with Coomassie Blue.
(1) Molecular weight markers; (2) whole cells extract; (3) soluble extract;
(4) trypsinised trypanosomes extract.

the 39 protein components present in the whole trypanosome extract, 16 reacted with the infection serum. Twelve of these antigens were also recognized by this serum in the soluble extract. Five antigens of 65.5, 58.6, 31.5, 30 and 21.2 kDa present in the whole extract were absent from the soluble extract, with one 44 kDa component present only in the soluble extract. The infection serum recognized 7 components in the trypsinised extract, with corresponding antigens in both whole and soluble extracts while a 65.5 kDa component was common to the trypsinised and whole extracts. Hyperimmune serum raised to soluble extract recognized a total of 20 components in the three trypanosomal preparations with a molecular weight ranging from 166.1 to 26.6 kDa (Figure 3, lane 2). Ten antigens recognized during 21 days of infection were also recognized by the hyperimmune serum. The majority of the antigens recognized by the hyperimmune serum were present in the soluble extract with 16 components recognized by the

serum. Seven of these 16 antigens were also present in the whole cell extract and one 58.6 kDa component was present only in the whole extract. The hyperimmune serum recognized 4 of the 16 components in the trypsinsed trypanosome extract which also contained two additional components of 54.7 and 26.6 kDa. Serum raised to trypsinised trypanosomes did not recognize any protein component on any of the three trypanosomal extracts (Figure 3, lane 3).

Specificity and reactivity of anti-52kDa antibodies

Antibodies raised to the 52 kDa antigen when tested by ELISA reacted with both the homologous (TR 2165) and heterologous (TR 2222 and 2257) populations of *T. evansi* (Table 1). As shown in the table each of the soluble extracts showed absorbance levels greater than 2SD above the mean of the negative control serum and
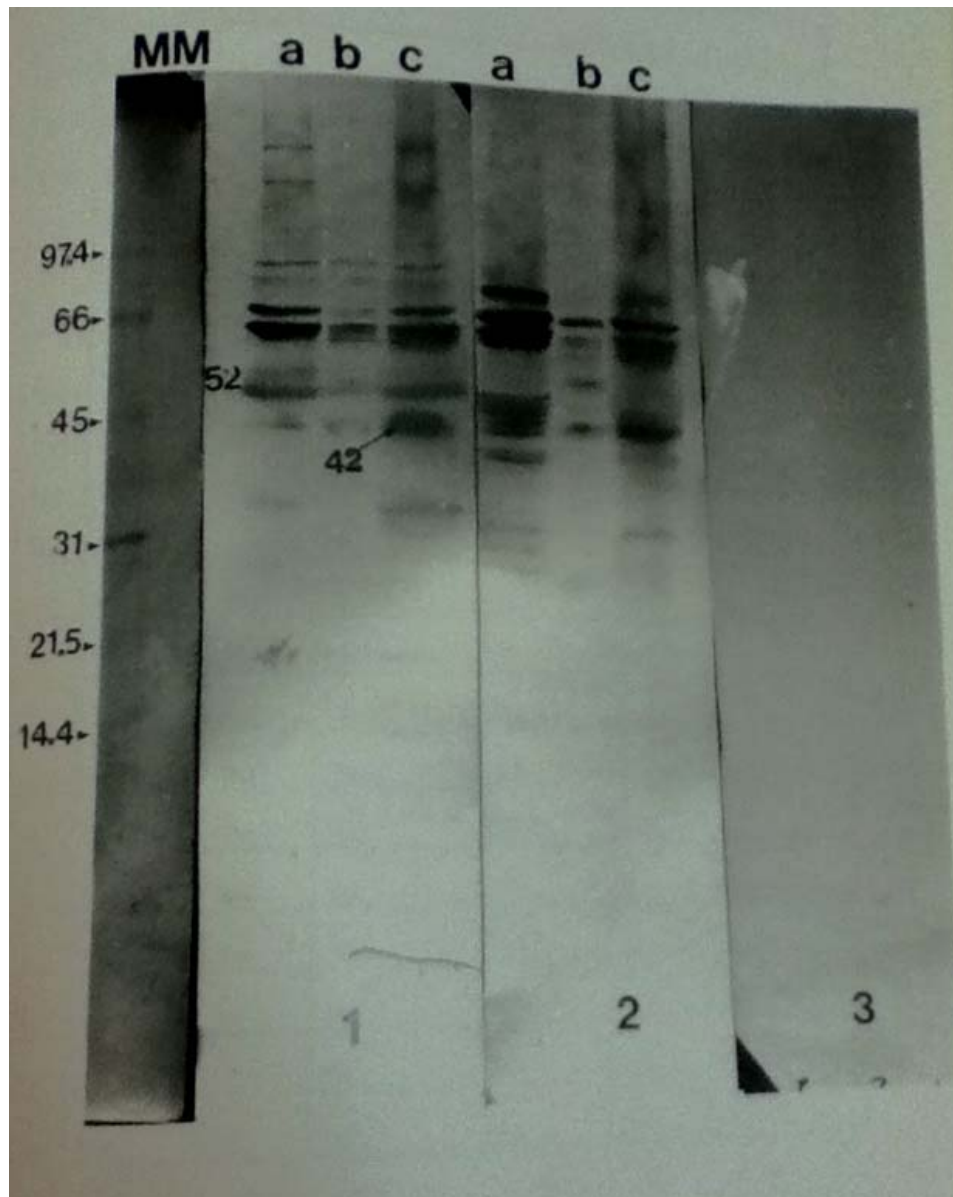
Figure 3. *T. evansi* (TR 2165) proteins identified by infection serum from rabbits infected with intact living trypanosomes and trypsin-treated trypanosomes and polyclonal serum to soluble extract. MM = molecular weight marker.
(1) Infection serum to intact trypanosomes; (2) serum raised against soluble extract; (3) infection serum against trypsin-treated trypanosomes; (a) soluble extract; (b) trypsin-treated trypanosomes and (c) whole cell extract.

PBS. The *T. evansi* heterologous population TR 2222 showed the highest absorbance (0.717) followed by TR 2257 (0.668) while the homologous population of *T. evansi* (TR 2165) showed an absorbance value of 0.630. In immunoblots, the antibodies recognized a single protein band of molecular weight ~52 kDa in the homologous population of *T. evansi* (TR 2165) (Figure 4, lane 2). While in the heterologous population of *T. evansi* (TR 2222 and 2257), two components were recognized: a major   component of ~52 kDa and a   minor 33 kDa

component (Figure 5, lanes A and B).

HRP conjugate reactivity

HRP conjugate reacted with the homologous *T. evansi* antigens when tested by a direct ELISA which titrated out with increasing conjugate dilution (Figure 6). The absorbance values ranged from 1.713 at a dilution of 1/50 to 0.105 at a dilution of 1/3200. The conjugate showed an
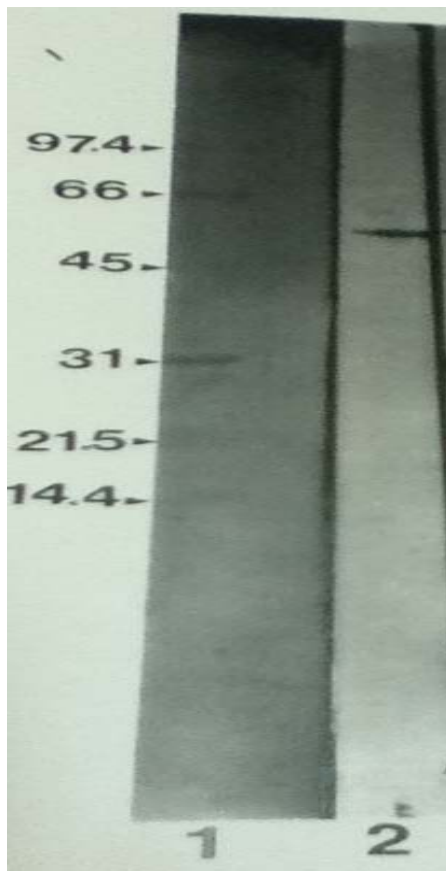
Figure 4. Western immunoblotting of homologous *T. evansi* population (TR 2165) against serum to the 52kDa antigen. (1) Molecular weight markers and (2) antiserum to 52 kDa antigen.
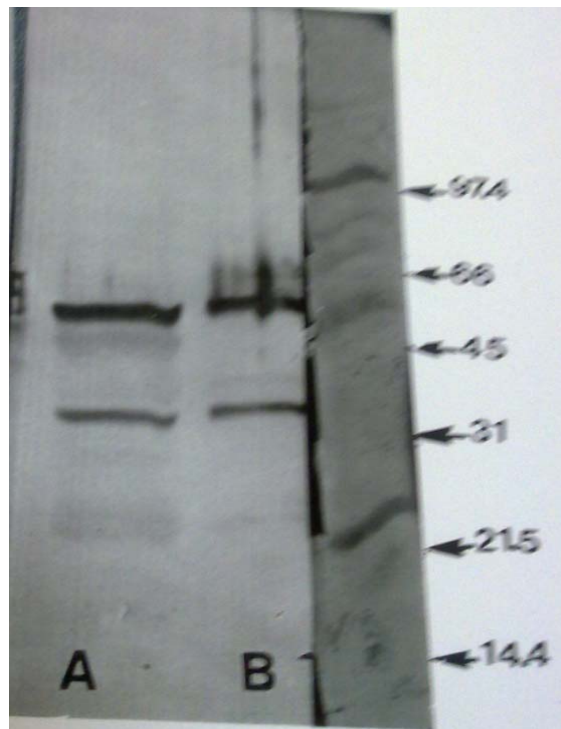
Figure 5. Western immunoblotting of heterologous *T. evansi* populations (TR 2222 and 2257) against serum to the 52 kDa antigen. (A) *T. evansi* TREU 2222 and (B) *T. evansi* TREU 2257.

estimated titer of 1/200 at an absorbance value of 1.0.

Sensitivity of conjugated anti-52 kDa IgG for antigen detection

The peroxidase labeled anti-52k Da antibodies produced an antigen end-point titer of 1/400 along with an antigen binding ratio of 2.2 (Table 2).

Standardization of the ELISA test

The assay utilizing 1% NRS in PBS/Tween as a blocking agent in the buffer used to dilute HRP-conjugate was able to differentiate between the antigen and the NRS. Results for the antigen and NRS at 1/50 dilution is shown in Table 3.

Evaluation of the developed assay

The anti-52 kDa antibodies detected antigens in the

blood of the inoculated rabbit by 2 h after inoculation. Thereafter, there was a gradual decline in the antigen titer which reached pre-inoculation level at 72 h post-inoculation (Figure 7).

**The dynamics of the 52 kDa *T. evansi* antigen during infection**

Parasitemia

The parasites were first detected in the tail blood of the rats at 4 days after infection. Thereafter, their number increased reaching a peak on day 8 post-infection. Following treatment with Berenil on day 8, the parasites disappeared from the circulation the next day and were not detected up to the time that the experiment was terminated at 17 days post-infection (Table 4).

Antigenemia

The antibodies to the 52 kDa antigen detected the antigen in the blood of rats by day 6, reaching peak activity on day 8 post-infection. After treatment on day 8 the activity declined, reaching negative control level on day 17 post-infection at the end of the experiment (Table 4).
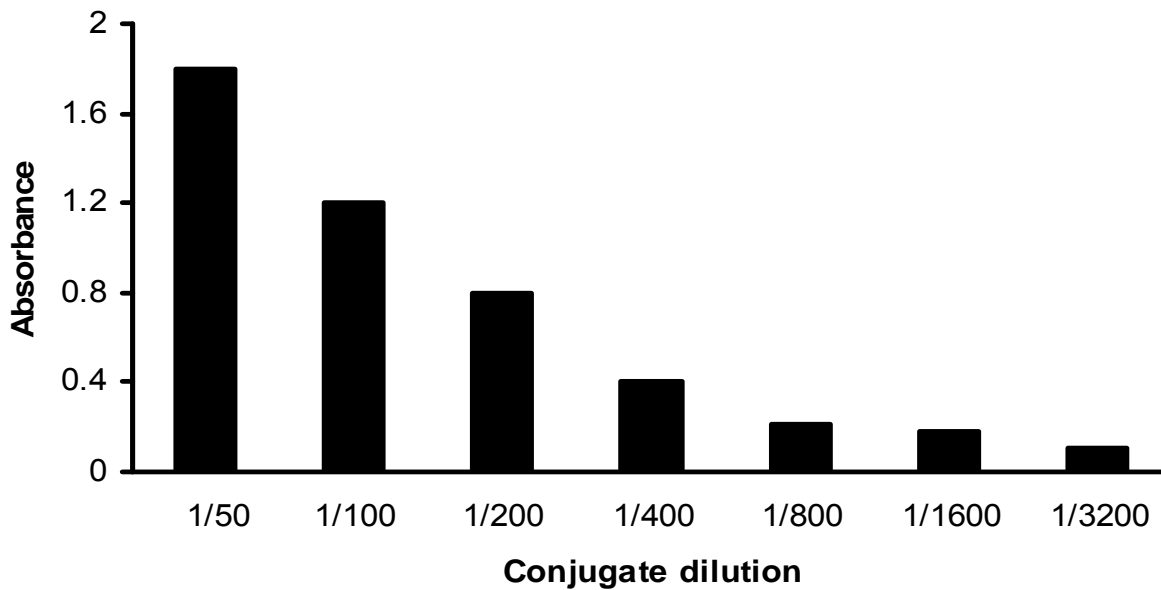
Figure 6. Reaction of labeled anti-52 kDa antibodies to homologous *T. evansi* population by direct ELISA.
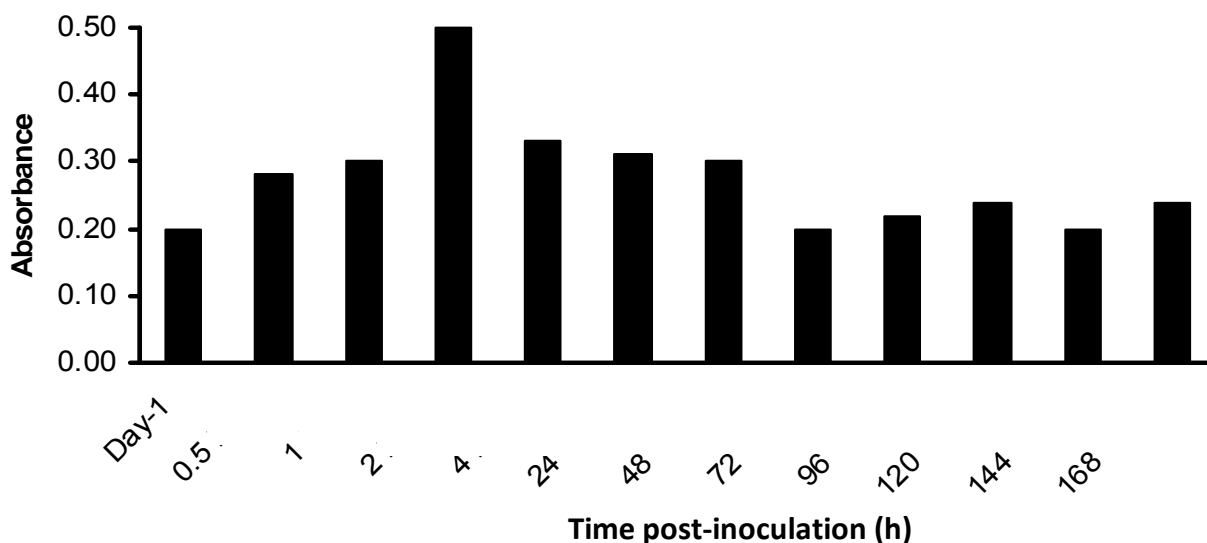
Figure 7. The dynamics of 52 kDa antigen in the circulation of the rabbit inoculated with *T. evansi* crude soluble extract (cut-off point = (mean + 3s) + 10% = 0.443).

DISCUSSION

The whole trypanosomes extract contained the largest number of the parasite proteins compared to the other two trypanosomal preparations used in this study. Two of these proteins with molecular weight of 146 and 142 kDa were not released by aqueous solubilisation as they were absent from the soluble extract, suggesting a possible membrane association as detergent lysis is known to solubilize the membrane proteins by replacement of the lipid bilayer, with a micelle of detergent (Garner et al., 2008). Two molecules with similar molecular weights (145 and 140 kDa) were reported in *T. brucei* (Coppens et al., 1992). The trypsinised trypanosomes extract contained the lowest number of the parasite proteins, with 7 bands absent from this extract compared to the whole extract. Although trypsin digestion is reputed to cleave mainly the VSG from the parasite surface (Mehlert et al., 2002), other proteins were reported to be cleaved during trypsinisation (Frommel et al., 1988). Possibly, in the present

Table 1. Specificity and reactivity of anti-52kDa antibodies tested by enzyme linked immunosorbent assay (ELISA).

Sample	TR 2165	TR 2222	TR 2257
Test serum	0.630±0.004	0.717±0.004	0.668±0.009
NRS	0.179±0.005	0.178±0.006	0.178±0.005
PBS	0.166±0.005	0.166±0.006	0.166±0.006
2SD + mean	0.189	0.187	0.188

Table 2. Sensitivity of conjugated anti-52kDa IgG for antigen detection.

Antigen end point titer	Absorbance at end-point titer		Antigen binding ratio
	Antigen	NRS	
1/400	0.537 ± 0.017	0.246 ± 0.018	2.2

study, these molecules possessed trypsin sensitive sites, arginine and lysine (Brown et al., 2002), and were released from the parasite together with the surface coat. Of these molecules, a 58.6 kDa protein fall within the 53 to 64 kDa range reported for VSG molecules of *T. evansi* (Uzcanga et al., 2004).

Twenty seven components of *T. evansi* were found to be antigenic in the present study depending on the extraction procedure. The molecular weight of these antigens ranged from 172 to 21.2 kDa although the upper molecular weight limit revealed by Coomassie blue stain was 158.6 kDa. This difference is possibly due to the fact that Western immunoblotting is a more sensitive detection system than Coomassie blue. Coomassie blue has a detection limit of 1 µg protein (Harlow and Lane, 1988), while as little as 100 pg of protein can be detected by immunoblotting (Towbin et al., 1979). Nine of the antigens recognized by the infection serum were sensitive to trypsin as they were absent from the trypsinised trypanosomes extract. This finding suggests that these antigens possess trypsin sensitive sites and are present in the surface of the parasite as the trypanosomes were viable and motile after the trypsinisation. Surface antigens other than the VSG have been reported in the trypanosomes and were used in protection experiments and as diagnostic reagents (Lanca et al., 2011; Tran et al., 2009).

Serum raised against trypsinised trypanosomes in the present study did not react with any of the three trypanosomal preparations. The trypsinised trypanosomes, unlike the intact trypanosomes, did not replicate within the host body as indicated by their absence in the circulation of the rabbit inoculated with these trypsinised trypanosomes. This would limit the amount of antigens released from them compared to the continuous stimulation of the immune system in animals infected with the intact trypanosomes (Shapiro and Murray, 1982). This lack of antibody response to trypsinised trypanosomes

suggests that the surface coat antigen might be important in the initiation of the humoral immune response against the non-surface antigens. This might take place through direct activation of B lymphocytes without the involvement of antigen-presenting cells such as macrophages. The role of macrophages in the presentation of trypanosomes antigens has been reported negligible (Paulnock et al., 1988; Namangala et al., 2000).

One of the trypsin sensitive antigens with molecular weight of 52 kDa reacted potently with both infection serum and serum raised to the soluble extract and was also a good immunogen when administered as soluble extract, making it suitable for use in a range of purification methods. In the present study, this antigen was purified from the parasite extract through electro-elution from Coomassie stained polyacrylamide gels and antibodies were raised to it. Immunization of rabbits with this purified antigen elicited strong antibody responses as revealed by immnuoblotting and ELISA. This result indicates that this antigen retained its immunogenicity and confirms the usefulness of the SDS-PAGE for the purification of antigens for immunization as reported by previous workers (Harlow and Lane, 1988).

The 52 kDa antigen was found to be conserved between different populations and stocks of *T. evansi*. Immunoblotting with the serum recognized the target antigen as a single component in the homologous population but as two components of 52 and 33 kDa in the heterologous *T. evansi* populations. This 33 kDa component might be a breakdown product or another component only present in the heterologous population carrying similar epitopes to the 52 kDa. These findings indicate that the 52 kDa represents an invariant antigen. Invariant antigens had been reported from different species and populations of trypanosomes, some of them have been shown to be located on the surface of the parasite (Overath et al., 1994), while others are non-surface associated antigens (Muller et al., 1992). Evidence

Table 3. Labeled anti-52 kDa antibodies at a dilution of 1/200 with PBS/Tween containing 1% NRS.

Coating antibody	1/50	1/100	1/200	1/400	1/800	1/1600	1/3200	1/6400
Antigen	0.994±0.003	0.900±0.003	0.763±0.004	0.652±0.003	0.656±0.003	0.640±0.004	0.459±0.004	0.460±0.001
NRS	0.294±0.003	0.247±0.004	0.178±0.003	0.159±0.001	0.142±0.001	0.134±0.001	0.106±0.003	0.103±0.001
PBS	0.290±0.005	0.288±0.005	0.179±0.005	0.151±0.003	0.126±0.003	0.138±0.004	0.105±0.003	0.117±0.003
2SD + mean OD	0.298	0.326	0.179	0.167	0.156	0.142	0.106	0.130

NRS: normal rabbit serum. SD: standard deviation, OD: optical density.

Table 4. Parasitemia and antigenemia in the experimental rats.

Days after infection	Tryps/20 fields	52 kDa OD	Control serum OD*	Days after infection	Tryps/20 fields	52 kDa OD*	Control serum OD*
1	0	0.257	0.189	10	0	1.159*	0.234
2	0	0.286	0.249	11	0	0.407*	0.239
3	0	0.249	0.220	12	0	0.401*	0.234
4	10	0.286	0.215	13	0	0.432*	0.238
5	22	0.283	0.231	14	0	0.417*	0.212
6	41	0.562*	0.255	15	0	0.421*	0.221
7	85	0.894*	0.259	16	0	0.397*	0.207
8	200	1.346*	0.265	17	0	0.292	0.209
9	0	1.078*	0.212	-	-	-	-

*Positive samples. Mean optical density (OD) of control sera = 0.229 ± 0.02. Cut-off point = 0.229 + 0.06 + 0.0289 = 0.318.

from the present study suggests that the 52 kDa antigen is surface associated as it was cleaved from the parasite by the process of trypsinisation.

The antibodies produced to this antigen were used in the present study to develop an immunoassay for the detection of the antigen in serum of infected animals. The antigen-capture ELISA developed in this study was able to detect *T. evansi* antigens in serum. When soluble extract of the parasite was inoculated in an aqueous form intravenously into a rabbit, the 52 kDa antigen was detected within two hours post-inoculation due to its circulation with blood throughout the animal body. Un-aggregated protein antigens are known to distribute evenly throughout the animal's blood before being cleared by the phagocytic cells (Tizard, 1992). Thereafter the level of the antigen declined, reaching pre-inoculation level within 3 days either as a result of dilution or phagocytic clearance.

The 52 kDa antigen was detected early in the circulation of *T. evansi* infected rats in this study. Such early appearance might be allied with the antigen's surface-association, making it one of the first antigens that would be accessible to antibodies early during infection. The detection of this antigen during low parasitemia levels in the blood of the infected rats would possibly suggests its detection during chronic phase of the disease when parasitemia is low. Although the level of this antigen remained high for two days following treatment, a sharp decline was seen on the second day. The rapid clearance of the antigen from the circulation suggests that it could have been circulating as antigen-antibody complexes. Such complexes are known to be rapidly cleared by phagocytic cells (Tizard, 1992) and elimination of antigens from the circulation by these mononuclear phagocytic cells is inefficient when antibodies have low affinity for the antigen. The peak level of antigenemia generally coincided with the highest levels of parasitemia. It is likely that the

ELISA result reflects the presence of free target antigen in the circulation of the infected rats rather than antigen appearing in the blood sample due to breakdown of parasites after blood sampling, since the antigen persisted in the circulation for eight days after complete disappearance of trypanosomes from rat's blood. Previous studies had shown that trypanosomes at concentrations of approximately 1×10^9 trypanosomes/ml would be needed in the blood sample to reach the absorbance levels obtained with serum from the rats. These levels were never reached during the course of infection in the present study.

The clearance of the 52 kDa antigen from the circulation after successful chemotherapy suggests a possible role in serologically-based assay for measuring the effectiveness of chemotherapy. This antigen, being an invariant antigen accessible to antibodies during infection and can reach a detectable level in the circulation before the remission of the first parasitemic wave, is a possible candidate for the diagnosis of infection at least in areas where *T. evansi* is the only trypanosome species present.

Further inquiry in its presence in other trypanosome species is recommended for investigation of cross reactivity. The surface association and accessibility of this antigen to antibodies during early stages of infection warrants further investigation in its viability in vaccine production. Some of these invariant surface antigens were involved in partial protection against infection (Lanca et al., 2011).

ACKNOWLEDGEMENT

Conflict of Interest

The authors declare that they have no conflict of interests.

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