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A PRELIMINARY CHARACTERISATION OF A POSSIBLE NON-SURFACE VARIANT SPECIFIC 42kDA ANTIGEN IN *Trypanosoma evansi*

E.M. El Hassan

Department of Microbiology and Parasitology, College of Veterinary Medicine,
King Faisal University, P.O. Box 400, Al-Ahsa 31982, Kingdom of Saudi Arabia

ABSTRACT

The objective of this study was to investigate the antigenic components of *Trypanosoma evansi* (*T. evansi*) during infection. The antigenic components of intact and trypsin-treated *T. evansi* were identified using a combination of SDS-PAGE and Western immunoblotting against sera from infected rabbits and rabbits immunised with a soluble extract of the parasite. These sera recognised 14 components ranging from ~ 172kDa to ~21.5kDa. A non-trypsin-sensitive component of ~ 42kDa, recognised strongly by sera from infected rabbits and rabbits immunised with the parasite soluble extract was, selected for further studies. This antigen purified by electro elution from acrylamide gels and mono-specific serum produced and used in both Western immunoblotting and enzyme linked immunosorbent assay (ELISA). Serum raised against this antigen recognised only antigenic materials in the homologous *T. evansi* population by both Western immunoblotting and ELISA. Being a non-trypsin-sensitive antigen that was not, cleaved from the parasite by the process of trypsinisation, possibly indicate a non-surface association, yet a variant-specific antigen. The presence of such antigens in *T. evansi* parasites and their role in the process of antigenic variation is discussed. Conclusions were drawn and recommendations were suggested.

Key words: Antigen immunoblotting, Antigen variants, *Trypanosoma evansi*, western immunoblotting

Trypanosomes contain a complex mixture of components many of which recognised as antigens by the host during the course of infection. Some of these antigens are located on the surface of the parasite (Vickerman and Luckins, 1969; Overath *et al,* 1994) or as a part of other morphological structures of the parasites (Muller *et al,* 1992; Yadav *et al,* 2013). The major surface-antigen of the trypanosomes is a variant antigen specific to each particular population (Cross, 1990). The other major trypanosome antigens are invariant and common between stocks of the same species and between different species of trypanosomes. Some of these invariant antigens are located in the surface of the parasite (Radwanska *et al,* 2000; Schwede and Carrington, 2010; El Hassan, 2014), while the majority are non-surface antigens (Sullivan *et al,* 2013). Non-surface antigens studied so far, were, found to be invariant and common between stocks (Laha and Sasmal, 2008) and species of trypanosomes (Eisler *et al,* 1998). The way in which the host deals with the release of internal antigens after VSG-specific lysis is not fully understood, but could form an important part of the response to infection. Once the host has initiated immune-mediated parasite destruction, a range of non-surface parasite components will be, exposed to the host. It is possible that, the type of host response to such individual components might be important in influencing the final outcome of an infection.

To date, a variant-specific antigen, apart from the VSG and to some extent the expression site-associated genes (ESAGs) has not been identified. Antigenic variation of the VSG molecules in Salivarian trypanosomes is the most important mechanism by which the trypanosomes evade the host immune responses. When antibody production against a particular VSG has occurred, trypanosomes bearing this molecule are lysed, but a small proportion of the population switches, expresses a new VSG and eventually become the predominant population in the vascular system of the host (Turner and Barry, 1989).

The objective of this study was to investigate the antigenic components of *Trypanosoma evansi* (*T. evansi*) during infection.

Materials and Methods

Trypanosomes

T. evansi stocks used in this study originally isolated from naturally infected camels in Al-Ahsa

SEND REPRINT REQUEST TO E.M. EL HASSAN email: umbashi2@yahoo.com

area, Saudi Arabia. They include one population designated (TR 2257) and 2 variants of another population (TR 2165 and TR 2222). Variants were obtained through several passages of 3 days interval in mice.

Purification of parasites

Trypanosomes expanded in mice were separated from infected blood by anion-exchange chromatography on diethylaminoethyl (DEAE) cellulose column (DE52, Whatman Biochemical, UK) as described by Lanham and Godfrey (1970). The trypanosomes then washed 3 times by centrifugation at 2650g for 20 minutes at 4°C in phosphate saline glucose (PSG, pH 8.0).

Preparation of trypanosome lysate

The parasite crude soluble extract was prepared by re-suspending $2.2x10^9$ DEAE column separated trypanosomes in an equal volume of PSG then freezed to – 80°C for 10 minutes and thawed to room temperature for another 10 minutes. The process repeated 3 times and the lysate was then centrifuged at 10000g for 45 minutes at 4°C. The supernatant removed, its protein concentration determined and aliquoted into 100 µl volumes and stored at -20°C. Detergent lysis (Anderson and Blobel, 1983) using sodium dodecyl sulfate (SDS) sample buffer was used to prepare extracts of whole trypanosome and trypsinised trypanosomes. In this process $2.2x10^9$ DEAE column separated trypanosomes, were diluted with an equal volume of SDS sample buffer, heated to 100°C for 5 minutes, then cooled to room temperature, centrifuged at 10000 g for 5 minutes, aliquoted into 200 µl volumes and stored at -20°C.

Trypsinisation of trypanosomes was performed by adding trypsin (20µl/ml at a concentration of 5mg/ml) to trypanosomes and incubation for 75 minutes at 37°C, under these conditions, the trypanosomes were still intact, motile but their infectivity to mice was completely, abolished, as trypanosomes were not detected by 40 days after inoculation. The trypsin then neutralised by incubation with trypsin inhibitor (20µl/ml at a concentration of 5mg/ml in distilled water) for further 5 minutes at 37°C and the trypanosomes then washed by centrifugation at 5000 g for 5 minutes in PSG.

Electron microscopy of trypanosomes

This was performed following the method of Garnett *et al* (1978). Sixty nm thick sections of trypanosomes embedded in araldite mix: accelerator 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.

Preparation of T. evansi antisera

Infection serum was, obtained from a rabbit injected intravenously with $1x10^5$ trypanosomes (TR 2165) in 1ml PSG. The rabbit bled for serum prior to infection and on day 2, 5, 9, 14 and 21 post infection. The experiment terminated on day 21 by treating the rabbit with a single intramuscular injection of diaminazine aceturate (BerenilR, Hoechst Lab., Germany) at a dose rate of 7mg/Kg body weight.

Hyper-immune sera to *T. evansi* (TR 2165) crude soluble extract was raised in a rabbit. The immunisation regime was adapted from Harlow and Lane (1988). The rabbit immunised with the crude soluble extract received a subcutaneous injection of 200 µg soluble extract emulsified in Freund's complete adjuvant (FCA) on day one, then boosted by s/c injection with a further 100 µg of the same materials in Freund's incomplete adjuvant (FIA) at 28 and 56 days post-immunisation. A final boost of 100 µg soluble extract in PSG was administered intravenously at 68 days after the first immunisation. The rabbit was, bled for serum on days -1, 7, 10, 21, 34, 39, 49, 61, 67 and 75.

Electrophoresis and Western blotting of T. evansi lysates

T. evansi soluble extract, whole trypanosome extract and trypsinised trypanosomes were subjected separately to SDS-PAGE (Laemmli, 1970) on 7-20% gradient poly-acrylamide gels. Coomassie Blue stain was, used to visualise the protein-banding pattern in each extract. Unstained parasitic components were, electro blotted onto nitrocellulose membranes and detected by Western blotting (Towbin *et al,* 1979) against infection and hyper-immune serum. The serum samples collected from each rabbit pooled equally and then diluted to 1:50 in blocking buffer before used in the assay.

Purification of the 42 kilo Dalton (kDa) antigen

Electro elution from acrylamide gels

Ten SDS-PAGE gels used to provide materials for immunisation. *T. evansi* (TR 2165) soluble extracts separated on 7-20% gradient SDS-PAGE gels stained by Coomassie blue stain and the 42kDa protein located in each gel by reference to a molecular weight standard lane and excised from the gel. The protein then electrophoretically eluted from the excised gel

portion using an electro elution device (Model 422 Electro eluter, Bio Rad. USA) set up according to the manufacturer's instructions. The homogeneity and absence of contaminants in the eluted protein was, assessed by SDS-PAGE using 10% homogeneous acrylamide mini-gel (Mini-ProteinR II, Bio-Rad, USA) prepared according to manufacturer's instructions. Proteins were, visualised by sliver staining. All electro eluates pooled, transferred to dialysis tube (Molecular weight "M.W." cut-off 12000 Daltons, Sigma chemical Co. St. Louis, USA) and dialysed overnight against phosphate-buffered saline (PBS). The total protein concentration of the dialysed protein was measured using a BCA^R test kit (Pierce, USA) and then concentrated ~ 6-fold using a Centrifugal Ultrafiltration System (Sartorius Ltd., Germany) and stored at – 20°C until needed.

High performance liquid chromatography (HPLC)

A $3x10^8$ column separated and washed *T. evansi* (TR 2165) trypanosomes were suspended in 0.015M phosphate buffer, pH 8.0 containing N-CBZ-L-Phenylalanine Chloromethyl Ketone (ZPCK) and n-Octyl β-D-Glucopyranoside (OGP) and centrifuged at 20000 g at 10°C. The supernatant was subjected to fractionation by HPLC. One ml of the detergent solubilised trypanosome materials was injected onto a Spherogel TSK DEAE-5 PW column (Beckman, USA) in 20mM tris (hydroxymethyl) aminomethane (Tris buffer), pH 7.0. Proteins were eluted from the column using a continuous gradient at a flow rate of 1ml/minute over a period of 30 minutes. The absorbance of the column eluate was, monitored continuously at 280 nm and the eluate collected at one minute, intervals. Five fractions representing the major ion-exchange elution peak collected during the first 3-7 minutes were analysed using 8-25% gradient Phast gel (Pharmacia, USA) SDS-PAGE system and the separated proteins were, visualised by sliver staining.

Size exclusion chromatography

The 5 fractions collected from the HPLC ion-exchange column were pooled together and re-fractionated according to molecular size by size exclusion HPLC (Welling and Welling-Wester, 1989) using a Biosep-SEC-S column (Phenomenex, UK). Samples were eluted from the column over a period of 30 minutes using a flow rate of 0.65ml/minute. The absorbance of the column eluate was monitored continuously at 280 nm and the eluate fractions collected at 1 minute, intervals. Nine fractions were collected between 12 and 20 minutes of the chromatography run representing the major elution peak. These fractions were, analysed by SDS-PAGE using a homogenous 15%, acrylamide mini-gel (Mini-ProteinR II, Bio-Rad, USA) prepared and run according to the manufacturer's instructions and proteins were visualised by sliver staining.

Lectin-affinity chromatography

The pool of 5 fractions eluted from the HPLC ion-exchange column was subjected to lectin affinity chromatography using Concanavalin A sepharose 4B (Con A sepharose, Sigma Ltd. UK). Prior to chromatography, 4 ml of the Con A sepharose gel was equilibrated with 10 ml Con A binding buffer pH 6.0. Three millilitres of the sample were mixed with 7 ml of binding buffer and allowed to pass through the column. The column eluate was, passed back through the column a total, of 5 times to wash unbound materials. Material bounds to the column was then eluted by passing 7 ml elution buffer and the eluate was collected as 7x1ml fractions. All fractions were analysed by SDS-PAGE using the Phast system.

Preparation of anti-42kDa antibodies

Two rabbits were, used to produce antibodies to the 42kDa antigen purified by electro elution from stained gels. Stained gels were, used to localise the target antigen band to avoid the risk of contamination by highly immunodominant VSG since the 2 molecules have a large molecular range. Each rabbit received I.M. injection with 100µg antigen emulsified in FCA and this was, repeated 14 days later. The rabbits were, then boosted 3 times by s/c injection with 50µg antigen emulsified in FIA on days 49 and 77 and again with 25 µg of antigen in FIA on day 196. The rabbits were, bled for serum on days -1, 24, 56, 84, 203 and 210.

Monitoring antibody production

An antibody-ELISA was, used to measure the amount of antibody in the sera from the rabbits on days 24, 56, 84 and 203, post primary injection of the antigen. The assay was, also used to determine the titer of the antibodies in the final serum collected on day 210 post-injection. Each serum was tested against a 1/80 dilution of a freeze-thawed soluble extract of the homologous *T. evansi* population as antigen. Sera were, evaluated over a 2-fold dilution range from 1/250-1/8000 for the sera collected 24-203 days and over a 10-fold dilution range from $1/10^2$-$1/10^6$ for the final serum collected at 210 days. In each case, pre-immunisation serum at a similar dilution range was included as a negative control. All serum samples were, tested in duplicates and the antibody

titer of the final serum was, taken as the last dilution that continued to show an OD value of more than two standard deviations (2SD) above the mean of the negative control.

Specificity of anti-42kDa antibodies in day 210 sera

Specificity by antibody-ELISA

Serum collected on day 210 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 soluble trypanosome extract was, coated into 30 wells of the ELISA plate. Pre-immunisation 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-immunisation serum and PBS were each added to 10 wells of the 3, soluble trypanosome extracts. Mean O.D values of test samples in each trypanosome extract was compared with the mean O.D values of pre-immunisation serum and PBS. Sample with an O.D value of more than two standard deviations (2SD) above the mean of pre-immunisation serum was considered positive.

Specificity by Western blotting

The antiserum was tested by immunoblotting against whole trypanosome extract from homologous (TR 2165) and heterologous (TR 2222 and TR 2257) *T. evansi* populations. The serum samples were diluted to 1/50 in blocking buffer, while the peroxidase-labelled donkey anti-rabbit IgG conjugate was used at a dilution of 1/500.

Specificity by indirect fluorescent antibody test (IFAT)

IFAT (Nadeem *et al,* 2011; OIE, 2012) was used to provide information on variant specificity and location of the 42kDa antigen within *T. evansi* by testing the antisera against homologous (TR 2165) and 2 heterologous (TR 2222 and TR 2257) *T. evansi* populations. Parasitemic whole blood obtained from mice infected with the appropriate *T. evansi* population was fixed either as smears in acetone or in suspension using formalin and was, used as antigen. In both cases, reaction zones were marked on the microscopic slides bearing the fixed trypanosomes. The antiserum was, diluted to 1/50 in PBS before added to the reaction zones. Serum collected from the rabbit infected with *T. evansi* (TR 2165) in section 2.5, pre-immunisation serum and PBS were included on each slide as positive and negative controls. FITC-labelled donkey anti-rabbit IgG at a dilution of 1/40 was, used as a conjugate and the test was performed as described by the above authors.

Specificity by agglutination test

Variant-specificity and agglutinating property of anti-42kDa antigen antibodies was examined by agglutination testing against homologous (TR 2165) and heterologous (TR 2222 and TR 2257) *T. evansi* populations. The serum was tested over a 2-fold dilution range from 1/2 – 1/1024 in a micro-well plate.

Results

Electron microscopy of trypanosomes

Electron microscope sections prepared from untreated trypanosomes were, seen to possess a thick, compact and dense surface coat overlying the plasma membrane. 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 (Fig 1).

Electrophoresis and Western blotting of T. evansi lysates

The protein profile of *T. evansi* lysates resolved by Coomassie blue staining showed a low number of protein bands in the trypsinised trypanosome extract compared to the other 2 extracts (Fig 2, lane 4). Only few proteins including the 42kDa component showed a similar staining intensity to that of the other 2 extracts.

Infection and hyperimmune sera when tested against the 3 trypanosome, extracts using immunoblotting (Fig 3, lanes 1 and 2), recognised 14 components ranging from ~ 172kDa to ~21.5kDa. The majority of the parasite components which acted as antigens during the first 21 days of infection were soluble proteins. Some of these antigens were trypsin-sensitive and were absent from the trypsinised trypanosomes extract. Of the immuno-dominant components of *T. evansi* recognised by the infection serum, the 42kDa component was recognised strongly by this serum in the whole trypanosome extract and to a lesser extent in the other two preparations (Fig 3, lane 1). This component however, was recognised strongly in the 3 preparations by the hyper-immune serum raised against the soluble extract (Fig 3, lane 2).

Electro elution of the 42kDa antigen

The eluate of the gel slices from the 42kDa region in the gel showed a single band of the

appropriate molecular size when subsequently analysed by SDS-PAGE. The amount of protein

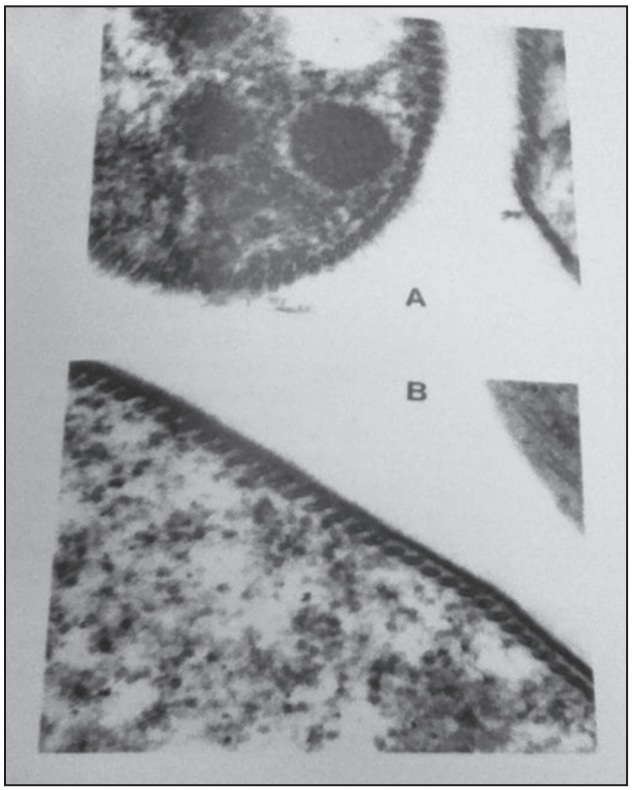

Fig 1. Transmission electron micrograph of section through surface body of *T. evansi* (TR 2165). 1mm ≡10nm. A-Trypsinised trypanosomes lacking a surface coat. B-Intact non-treated trypanosomes possessing a surface coat.

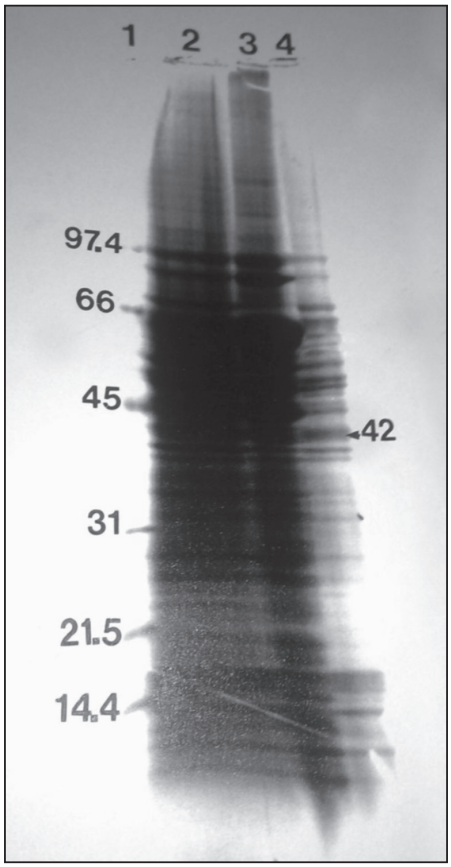

Fig 2. Protein profile of Coomassie stained TR 2165 *T. evansi* 1. Molecular weight marker. 2. Whole trypanosome extract. 3. Soluble extract. 4. Trypsinised trypanosome extract.

obtained following electro elution and ultrafiltration was 671 µg/ml.

Chromatographic purification of the 42kDa antigen

The 5 fractions representing the major elution peak of the HPLC ion-exchange column showed multiple protein bands when analysed by SDS-PAGE Phast gel (Fig 4). Three to nine protein bands per fraction were detected by electrophoresis with molecular weights ranging from approximately 14kDa to 94kDa. The first 4 fractions included the 42kDa target protein (Fig 4, lanes 2-5).

After fractionation of the pooled ionex, fractions 3-7 by size exclusion chromatography 9 fractions were, obtained that constituted the major elution peak. After analysis by SDS-PAGE 1 to 3 protein bands per fraction were identified (Fig 5, lanes 4-10) with a molecular size of approximately 66, 87 and 94kDa.

When the major ion-exchange peak (pooled fractions 3-7) was subjected to lectin affinity chromatography using a Con A sepharose column, the first 6 fractions eluted from the column all contained bands when analysed by SDS-PAGE using Phast system (Fig 6, lanes 2-7). Five protein bands were, identified in each fraction with molecular weights of approximately 15, 30, 62, 66 and 94kDa. The 7th eluate fraction from this column (Fig 6, lane 8) did not contain any protein material resolvable by SDS-PAGE.

Antibody response in the immunised rabbits

Serum from test bleeds collected from the immunised rabbits 10 days following each injection of the antigen showed a progressive increase in absorbance values when tested by ELISA against a soluble extract of the homologous population (Table 1). At a serum dilution of 1/250, almost a 3-fold increase in the absorbance value from day 24 to day 203 post-primary injection was, observed.

The antibody titer of the final serum collected on day 210 post-immunisation was 1/100000 as

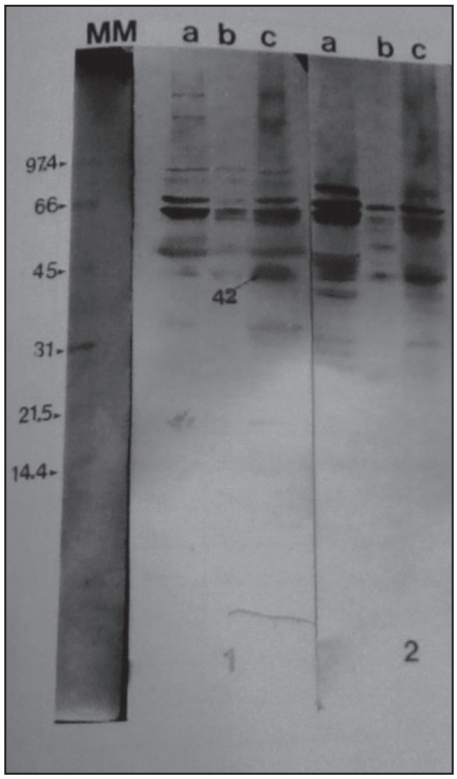

Fig 3. Immuno-dominant components of TR 2165 *T. evansi* **MM.** Molecular weight marker. **1.** Infection serum to intact trypanosomes. **2.** Serum raised against soluble extract. **a.** Soluble extract. **b.** trypsin-treated trypanosomes. **c.** Whole cell extract.

defined by antibody-ELISA against the homologous soluble extract (Table 2).

Characterisation of anti-42kDa antibodies in day 210 sera

Specificity by antibody-ELISA

Serum collected on day 210 from rabbits immunised with the 42kDa antigen when tested for specificity by antibody-ELISA against soluble extracts of the homologous and heterologous *T. evansi* populations reacted only with the homologous population giving an absorbance value of more than 8x that of the pre-immunisation serum (Table 3). In the case of the heterologous populations TR 2222 and TR 2257 absorbance values were less than 2SD above the mean of the negative control (Table 3).

Specificity by Western blotting

Serum collected on day 210 from rabbits immunised with the 42kDa antigen when tested for specificity by western immunoblotting against the whole trypanosome extracts recognised 4 faintly stained protein bands of molecular weight of approximately 65, 35, 29 and 26kDa and a strong

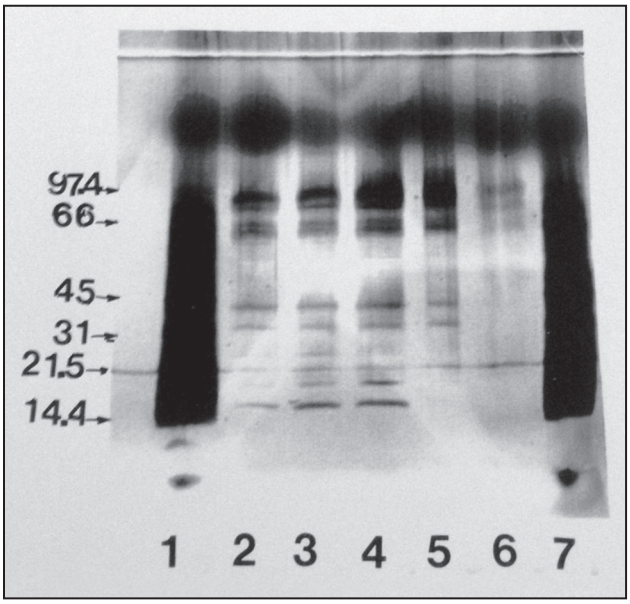

Fig 4. SDS-PAGE analysis of the HPLC ion-exchange chromatography fractions.

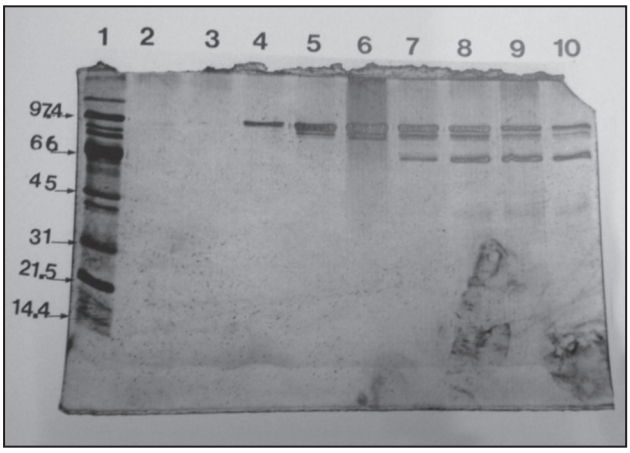

Fig 5. SDS-PAGE analysis of the size exclusion, chromatography fractions.

42kDa band in the homologous TR 2165 population (Fig 7, lane 2). The serum did not recognise any antigenic component in the heterologous populations TR 2222 and TR 2257 (Fig 8, lane 1A and 1B, respectively).

Specificity by indirect fluorescent antibody test (IFAT)

Although, photographs are not shown, fluorescent trypanosomes were detected in both formalin and acetone-fixed preparations of the homologous population (TR 2165) when tested against this serum by IFAT. A strong green fluorescence was observed over the entire outline of both formalin and acetone-fixed trypanosomes. No fluorescent trypanosomes were, detected with the heterologous populations TR 2222 and TR 2257.

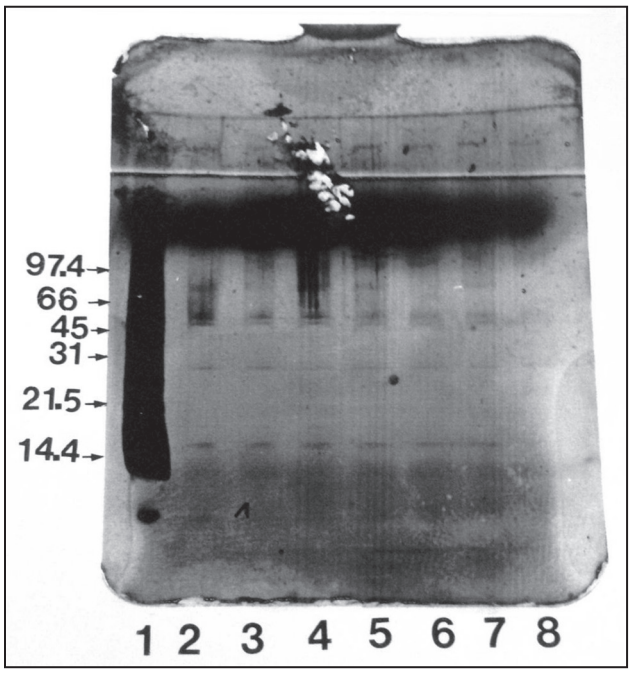

Fig 6. Lectin affinity chromatography fractions analysed by SDS-PAGE

Specificity by agglutination test

The serum did not agglutinate either homologous or heterologous *T. evansi* populations.

Discussion

In prescent study trypsinised trypanosome extract contained the lowest number of *T. evansi* proteins, compared to soluble and whole trypanosome extracts when fractionated by SDS-PAGE and stained with Coomassie stain. Although, trypsin digestion is reputed to cleave mainly the VSG from the parasite surface (Cross, 1975), other proteins were reported to be cleaved during trypsinisation (Frommel *et al,* 1988). Most of the antigens recognised during the course of infection were also immunogens when presented as a soluble extract as they reacted with both infection serum and serum raised against the parasite soluble extract. Differences were however, detected in the antigenicity of the parasite components released during infection and by physical disruption. Some of the parasite components which were not antigenic during the course of infection acted as antigens when presented to the host in soluble extract. This difference in the antigenicity of these parasite materials could be accounted for by difference in the way they were released from the parasite (Barriga, 1981) and the way in which they were presented to the host e.g. immunogenicity of those incorporated with adjuvant will be increased due to their presentation in an aggregated form (Harlow and Lane, 1988).

The 42kDa component of *T. evansi* was not cleaved from the parasite by the process of trypsinisation as was present in the Coomassie stained trypsinised trypanosome extract. This would possibly indicate that it does not possess trypsin sensitive sites, arginine and lysine (Stryer, 1988) and not surface-associated as electron microscope sections prepared in this study from trypsin-treated trypanosomes revealed the removal of the surface coat, leaving behind intact plasma membrane. The 42kDa components was found to be antigenic during the course of infection and immunogenic when administered as a soluble extract as indicated by its reaction with both infection serum and serum raised to the parasite soluble extract. This antigen is also

Table 1. Monitoring antibody response in rabbits immunised with 42kDa antigen tested by Ab-ELISA against homologous *T. evansi* population.

	NRS	Days after primary injection			
		24	56	84	203
Absorbance at 1/250 dilution	0.193 ±0.007	0.773 ±0.002	1.125 ±0.001	1.305 ±0.002	2.028 ±0.001

Table 2. The antibody titre of the final serum collected on day 210 post-immunisation, measured by Ab-ELISA.

Dilution	1/100	1/1000	1/10000	1/100000	1/1000000
Anti-42kDa antibodies	2.36±0.02	1.5±0.02	0.4±0.01	0.1±0.01	0.05±0.002
Pre-immunisation serum	0.2±0.02	0.1±0.01	0.09±0.01	0.02±0.002	0.05±0.002

Table 3. Specificity by antibody-ELISA of serum collected on day 210 from rabbits immunised with the 42kDa antigen.

Trypanosome population	TR2165	TR2222	TR2257
Anti-42kDa antibodies	1.5±0.019	0.181±0.005	0.191±0.02
Pre-immunisation serum	0.181±0.005	0.179±0.004	0.182±0.005
PBS/Tween	0.15±0.005	0.151±0.010	0.153±0.005
2SDEV+mean(Pre-immunisation serum)	0.191	0.187	0.192

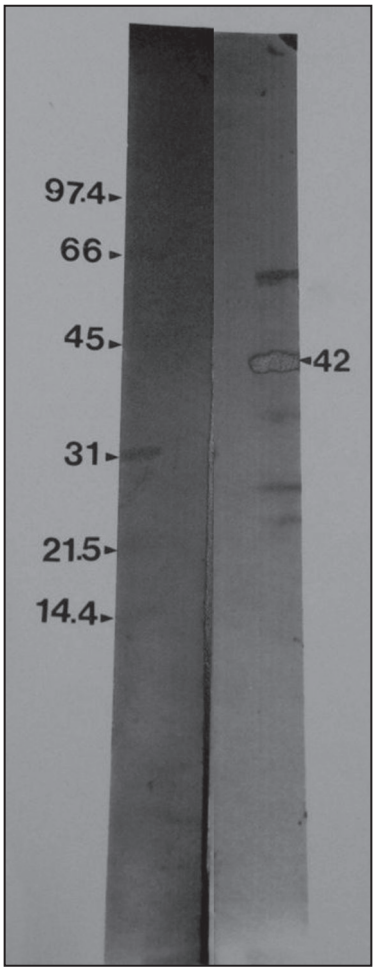

Fig 7. Reactivity of anti-42kDa antibodies tested by Western blotting against the homologous population.
Lane 1: Molecular weight markers. Lane 2: Anti-42kDa antibodies.

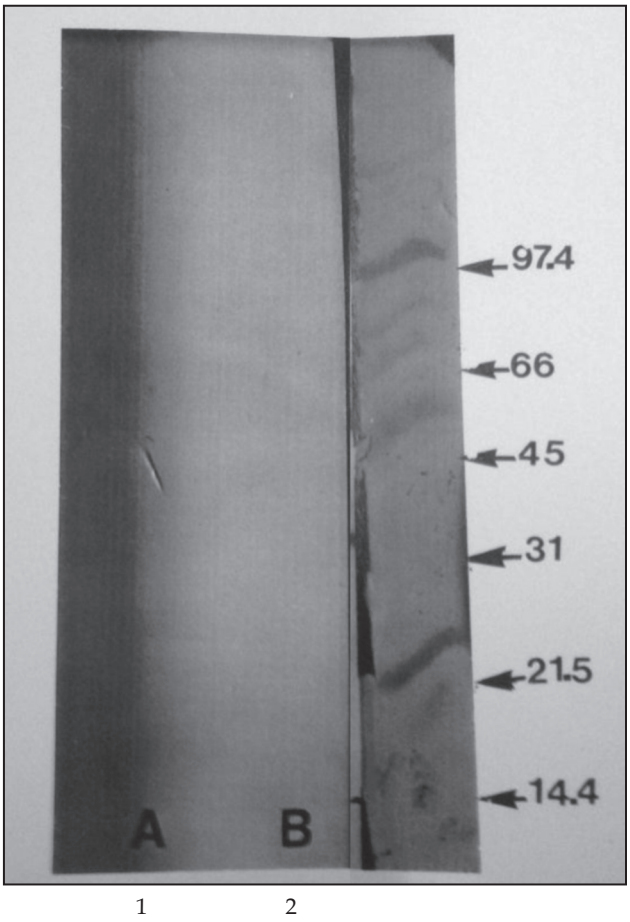

Fig 8. Specificity of anti-42kDa antibodies tested by Western blotting against heterologous *T. evansi* populations.
Lane 1: Anti-42kDa antibodies. Lane 2: Molecular weight markers. A: TR 2222. B: TR 2257.

one of the immuno-dominant antigens of *T. evansi* as indicated by its strong reaction with both types of sera and its presence in the 3 trypanosomal extracts. Immuno-dominant antigens in the range of 41-43kDa were reported in *T. evansi* (Yadav *et al,* 2013).

Immunisation of rabbits with the *T. evansi* 42kDa antigen eluted from Coomassie stained polyacrylamide gels in the present study elicited strong antibody response to the antigen by immunoblotting and ELISA. This result indicates that the antigen retained its immunogenicity and confirms the usefulness of the SDS-PAGE and electro elution for the purification of antigens for immunisation as reported by previous workers (Harlow and Lane, 1988; Kurien and Scofield, 2012).

None of the HPLC chromatographic procedures produced the 42kDa antigen from *T. evansi* lysates in sufficient purity for further study. However, despite the overall failure of this system to separate the antigen, it did provide additional information on the characteristic of this antigen. The isoelectric point (pI) of the target antigen is probably close to 7.0 as it was, eluted during the early stages of ion-exchange chromatography indicating that it was not tightly bound to the column at pH 7.0. The smallest component resolved by size exclusion chromatography was 66kDa. This would be in keeping with the failure to isolate the 42kDa antigen by this method under these conditions. The antigen does not possess any of the sugar residues that are, known to bind Con A such as α-linked D-mannosyl, D-glucosyl and N-acetyl-D-glucosaminoyl (Frommel and Balber, 1987; Chmielewski *et al,* 2014) as it did not bind to Concanavalin A.

The present study has therefore confirmed the usefulness of SDS-PAGE separated proteins as a mean of producing highly specific, high titered antisera to individual trypanosome proteins and highlighted the

problems associated with chromatographic separation of complex mixtures of proteins with similar physico-chemical properties.

The *T. evansi* 42kDa antigen appeared to be a population-specific antigen as serum raised against it only recognised antigenic materials in the homologous population TR 2165 by immunoblotting, ELISA and IFAT. Results from immunoblotting, however, showed that the serum recognised 5 protein bands in the homologous population of the parasite although dominated by the 42kDa protein. Such multiple recognition could be due to contamination of the original gel slice or breakdown products arising from protease activity during preparation of extracts. Contamination from the nearby bands during excision of the antigen from gels is unlikely since the contaminants have a large molecular size range and the target band was excised from a stained gel. The 65kDa contaminant in the present study is clearly not a breakdown product of the 42kDa. Although, the 42kDa component might be a breakdown product of the 65kDa component in the extract used for SDS-PAGE. However, the 42kDa component was present in Coomassie blue stained whole trypanosome extract and trypsinised trypanosome extract that were solubilised by detergent lysis, which is known to hinder protease activity (Sumathi and Dasgupta, 2006) indicating that this component is not a breakdown product of 65kDa. The other components could be breakdown products of either the 65 or 42kDa components.

One of the heterologous populations of *T. evansi* used in the present study was derived from the same stock as the homologous population and known to be antigenically different from homologous population. This suggest that the 42kDa antigen complex is variant-specific. Although, the molecular weight of all but the 65kDa component is lower than the 65-67kDa range reported for *T. evansi* VSG (Uche, 1989). Although, there is evidence that the 42kDa antigen is surface-associated from the overall green fluorescence seen by IFAT, the antiserum did not agglutinate the homologous population of the parasite. This suggest that if surface-associated, the 42kDa is not present in enough sites to cross-link for agglutination formation. It is possible however, that drying and fixation of the trypanosomes during antigen preparation could have exposed the antigen to the serum in IFAT making it appear to be surface-associated.

Results (not presented) from densitometric measurements on Coomassie Blue-stained SDS-PAGE gels indicate that the 42kDa antigen is present in a large amount in the parasite materials and this might also explain the reaction of antiserum in IFAT. Another explanation of the reaction of the anti-42kDa serum in IFAT is that the antigen is possibly sharing an identical epitope with the surface coat antigen. This hypothesis will also explain the importance of the variant-specificity, of the 42kDa antigen. As after switching from one VSG to another the previous VSG will only be gradually diluted during succeeding divisions (Overath *et al,* 1994) and the presence of the 42kDa will possibly lower the chances of VSG-specific antibodies to bind to the surface of the trypanosomes, thereby, allowing the switching process to succeed and the infection to continue.

The *T. evansi* 42kDa antigen reported in the present study may represent, a possible evidence of the presence of non-surface variant specific antigens in this parasite. Further studies, however, are needed to identify the amino acid sequence of this protein and to check whether it corresponds with the VSG sequence. The study also confirmed the usefulness of SDS-PAGE separated proteins as a mean of producing highly specific, high titered antisera to individual trypanosome proteins and highlighted the problems associated with chromatographic separation of complex mixtures of proteins with similar physico-chemical properties.

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