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Recombinant RoTat 1.2 variable surface glycoprotein as antigen for diagnosis of *Trypanosoma evansi* in dromedary camels

V. Lejon[a,*], F. Claes[a,b], D. Verloo[a,1], M. Maina[c], T. Urakawa[c,2], P.A.O. Majiwa[c], P. Büscher[a]

[a]*Department of Parasitology, Institute of Tropical Medicine, Nationalestraat 155, B-2000 Antwerpen, Belgium*
[b]*Department of Animal Science, Faculty of Agriculture and Applied Biological Sciences, KU Leuven, Kasteelpark Arenberg 30, 3000 Leuven, Belgium*
[c]*International Livestock Research Institute, ILRI, P.O. Box 30709, Nairobi, Kenya*

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Abstract

The transcript encoding a predominant *Trypanosoma evansi* variable surface glycoprotein RoTat 1.2 was cloned and expressed as a recombinant protein in *Spodoptera frugiperda* and *Trichoplusia ni* (insect) cells. Its potential as an antigen for specific detection of antibody in serum of dromedary camels affected by surra, was evaluated. In ELISA, the reactivity of the recombinant RoTat 1.2 VSG was similar to that of native RoTat 1.2 VSG. An indirect agglutination reagent was therefore prepared by coupling the recombinant RoTat 1.2 VSG onto latex particles. The performance of the latex agglutination test was evaluated on camel sera, and compared with the performance of CATT/*T. evansi* and LATEX/*T. evansi* tests, using the immune trypanolysis assay with *T. evansi* RoTat 1.2 as a reference test. The relative sensitivity and specificity of the latex coated with recombinant RoTat 1.2 VSG, using a 1:4 serum dilution, were respectively, 89.3 and 99.1%. No differences were observed between the performance of latex coated with recombinant RoTat 1.2 VSG and LATEX/*T. evansi* or CATT/*T. evansi*. Here, we describe the successful use of the recombinant RoTat 1.2 VSG for detection of specific antibodies induced by *T. evansi* infections.

Keywords: Diagnosis; *Trypanosoma evansi*; RoTat 1.2; Variable surface glycoprotein; Latex agglutination; Antibody detection; Surra

1. Introduction

Surra is caused by *Trypanosoma evansi* and occurs in Africa, Latin America, the Middle East and South East Asia. Domestic animals such as buffaloes, camels, cattle, horses, small ruminants and dogs are affected by the disease. Wild animals, including capybara and deer, act as reservoir hosts. The trypanosome is mechanically transmitted, mainly by bloodsucking flies (*Tabanidae*) (Stephen, 1986). Although few data are available, economic losses resulting from surra are believed to be large, particularly during epidemic outbreaks of the disease (Luckins, 1988). For example, the estimated total cost of *T. evansi* to the Brazilian Pantanal region's cattle ranchers is about US$ 2.4 million/year (Seidl et al., 1998).

Control of the disease is mainly based on livestock keepers recognising infected animals by clinical signs and treating them individually or on herd basis. This is inefficient and inaccurate since many infected animals may remain undiagnosed and act as reservoirs of the parasite. Since clinical signs are non-specific, latent infection can only be confirmed by the detection of the parasite in the blood of an infected animal (Luckins, 2000). Yet, a majority of parasite detection methods in current use are not sensitive enough to reveal infections when parasitaemia is low. During the past few years, sensitive and specific antibody detection tests have been developed for serodiagnosis of surra, including ELISA, immunofluorescence, direct and indirect agglutination (Dia et al., 1997;

* Corresponding author. Tel.: +32 3 247 6369; fax: +32 3 247 6373.
E-mail address: vlejon@itg.be (V. Lejon).
[1] Present address: Veterinary and Agrochemical Research Centre, Groeselenberg 99, 1180 Ukkel, Belgium.
[2] Present address: Nippori Kako Co. Ltd, 2-38-1 Kyohouen, Yao City, Osaka 581-0817, Japan.

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Payne et al., 1991; Bajyana Songa and Hamers, 1988; Verloo et al., 1998, 2000). Among these antibody detection tests, the CATT/*T. evansi* (Bajyana Songa and Hamers, 1988), ELISA/*T. evansi* and LATEX/*T. evansi* (Verloo et al., 2000) make use of native variable surface glycoprotein (VSG) from the predominant *T. evansi* variable antigen type RoTat 1.2 (Verloo et al., 2001). Production of these tests requires mass production of cloned *T. evansi* homogeneously expressing RoTat 1.2 VSG, normally grown in mice and rats.

In order to facilitate and standardise the production of the RoTat 1.2 VSG, its coding gene was cloned and expressed as recombinant antigen in *Spodoptera frugiperda* (insect) cells (Urakawa et al., 2001). Preliminary data from ELISA, conducted using this recombinant protein as an antigen, indicated its diagnostic potential and now we have developed an indirect agglutination reagent by coupling the recombinant RoTat 1.2 VSG onto latex particles. The resulting latex agglutination test was evaluated on camel sera, and compared with CATT/*T. evansi* and LATEX/*T. evansi*, using the immune trypanolysis assay with *T. evansi* RoTat 1.2 as a reference test for presence of antibody.

2. Materials and methods

2.1. Expression of recombinant RoTat 1.2

The cloning and expression of recombinant RoTat 1.2 diagnostic antigen in *S. frugiperda* cells has been described (Urakawa et al., 2001). However, in order to obtain immunogenic, soluble RoTat 1.2 VSG in large amounts, the coding region of the RoTat 1.2 VSG cDNA was truncated at the C-terminal end by approximately 20% and was ligated into a transfer vector for recombinant baculovirus expression. In addition, for convenient purification of the recombinant protein, a His6x-tag was introduced at the carboxyl terminus. Briefly, a 28-mer forward primer (RoTat1.2-PstATG: 5′-AAACTGCAGTATGCAAACCAA GGCGCTC-3′) and a 39-mer reverse primer (AcRo-Tat1.2Δ3′His-tag: 5′-CGAATTCTAGTGATGGTGATGG TGATGTGTGTAAGCGGC-3′) were used for the amplification of a truncated RoTat 1.2 VSG by PCR. The PCR product was cut with *Pst* I and *Eco* RI, and then ligated into the *Pst* I/*Eco* RI insertion site of a transfer vector, pVL1392 plasmid. The nucleotide sequence of the truncated RoTat 1.2 VSG gene in the transfer plasmid was confirmed by re-sequencing to ascertain that no artificial mutations occurred during the molecular manipulations. Subsequently, a recombinant baculovirus was generated as described previously (Urakawa et al., 2001).

2.2. Purification of recombinant RoTat 1.2

The high-titre seed recombinant virus was prepared by the infection in *S. frugiperda* insect cells. The combination of *Trichoplusia ni* (insect) cells (High Five cells, BTI-TN-5B1-4, Invitrogen, USA) and EX-CELL 401 serum-free culture medium (JRH Biosciences, USA) was used for maximum protein expression. The recombinant RoTat1.2 VSG, which was expressed intracellularly by baculovirus-infected insect cells and secreted into the culture medium in a soluble form, was purified from both the medium and the disrupted cells using the QIAexpressionist kit (Qiagen, UK). Briefly, the cell culture was centrifuged at 1500×*g* at 22 °C (Heraeus Megafuge 2.0R). The supernatant culture medium and the sedimented cells were recovered separately.

To achieve complete lysis of the cells, three volumes of lysis buffer (composition specified by Qiagen (UK), containing 0.1% Tween 20, 100 mM phenyl methyl sulfonyl fluoride, 0.5 mM EDTA, pH 8.0) were added. The mixture was vortexed gently, left on ice for 30 min and centrifuged for 30 min at 10,000×*g* at 4 °C (Tomy minifuge, Japan). The supernatant recovered after lysis was passed through the Ni-NTA agarose column, following the manufacturer's instructions (Qiagen, UK).

The supernatant cell culture medium was concentrated to approximately 20 ml using either polyethylene glycol 6000 (or 8000) or centrifugation through an Amicon-30 (Millipore) at 2500 revolutions per min in a Heraeus Megafuge 2.0R (1000×*g*). The concentrated cell culture medium was dialysed twice against 1L of PBS at 4 °C. After removal of possible precipitation by centrifugation, it was passed through the Ni-NTA agarose column. To recover the bound protein from the column, 500 µl of elution buffer was added four times and eluates were collected in separate tubes.

2.3. ELISA

Microplates (Maxisorp, Nunc) were coated overnight at 4 °C with 150 µl/well of purified variable surface glycoprotein of *T. evansi* RoTat 1.2 (Büscher et al., 1999) or recombinant RoTat 1.2 VSG at a concentration of 2 µg/ml in PBS (0.01 M phosphate, 0.14 M NaCl, pH 7.4). Antigen free control wells received 150 µl/well of PBS. Plates were blocked for 1 h at ambient temperature with 350 µl/well of PBS-Blotto (0.01 M phosphate, pH 7.15, 0.2 M NaCl, 0.05% w/v NaN_3, 1% w/v skimmed milk powder Régilait). For testing, camel serum was diluted 1:100 in PBS-Blotto. Fifty microlitre of serum dilution was added in duplicate to antigen containing and antigen free wells and the plates were shaken for 30 min at ambient temperature on a plate shaker (AM69 microshaker, Cooke microtiter system, UK). Using an automated plate washer (Elx50 washer, BIO-TEK Instruments), microplates were washed 3×1 s with 350 µl/well of PBS-Tween (0.01 M phosphate, 0.14 M NaCl, 0.05% v/v Tween 20, pH 7.4). Protein A peroxidase (Sigma) was diluted to 0.2 µg/ml in PBS-Tween and incubated for 1 h (150 µl/well). After five washes, wells were incubated for 1 h at ambient temperature with 150 µl ABTS substrate-chromogen solution. The latter was prepared from 50 mg ABTS

(2,2′-azinobis(3-ethylbenzothiazoline)-6-sulfonic acid, Boehringer, Germany) dissolved in 100 ml of ABTS-buffer (phosphate-citrate-sodium perborate solution, pH 4.6, Boehringer, Germany). The plate was shaken for 10 s and the OD read at 415 nm (Multiskan RC Version 6.0, Labsystems). Corrected OD values were obtained by subtracting the mean OD of the antigen-free control wells from the mean OD of the corresponding antigen containing wells.

2.4. Coating of latex reagent with recombinant RoTat 1.2 VSG

The recombinant RoTat 1.2 VSG was dialysed in phosphate buffer (0.01 M, pH 8 supplemented with 1 mM $CaCl_2$ and 1 mM $MgCl_2$) and concentrated to 1 mg/ml on an Ultrafree-PF 10.000 NHWL polysulfone membrane (Millipore). Coupling onto the latex particles was performed according to Büscher et al. (1999). Briefly, 100 mg of carboxyl modified polystyrene latex (Estapor K1.08, 10%, 0.857 μm, 9 μeq COOH/g) was mixed with 1 mg of recombinant RoTat 1.2 VSG for 15 min. 250 mg of 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide·HCl (Pierce), freshly dissolved in 1 ml of H_2O was added and the suspension was mixed for 1 h. The reagent was washed three times by centrifugation (470×*g*, 1 h, 4 °C) with 8 ml of TBSA (Tris buffered saline 0.02 M pH 7.4 complemented with 1% BSA). The final sediment was resuspended in TBSA supplemented with 10% w/v sucrose, to obtain a 1.6% latex suspension. The latex reagent was sonicated on ice (Vibra-cell, amplitude 80, 1 min, pulse 3 s, 9 W output) and aliquots of 0.5 ml were frozen at −20 °C.

2.5. LATEX test protocol

To obtain a final latex concentration of 0.8%, lyophilised LATEX/*T. evansi* (Verloo et al., 1998) was resuspended in 1 ml of PBS (containing NaN_3 1 g/l; pH 7.2). Similarly, the latex coated with recombinant RoTat 1.2 VSG was thawed and to it was added 0.5 ml of PBS. Two-fold dilution series of serum were prepared in PBS. Twenty microlitre of LATEX/*T. evansi* or of latex coated with recombinant RoTat 1.2 VSG, and 20 μl of serum dilution were mixed onto the black reaction zone (15 mm diameter) of a test card. The test card was rocked for 5 min at 70 revolutions per min on a horizontal rotator (eccentric deviation 12 mm). The reaction was scored positive when macroscopic agglutination was visible. The end-titre was defined as the highest dilution of test serum still showing a positive result. A serum sample with an end-titre ≥ 8, i.e. agglutination occurred at serum dilution 1:8 or higher, was considered positive in LATEX/*T. evansi* (Verloo et al., 1998).

2.6. CATT/T. evansi test protocol

CATT/*T. evansi* was reconstituted with 2.5 ml of PBS and two-fold serum dilutions in PBS were prepared. Twenty-five microlitre of serum dilution were mixed with 1 drop of reagent onto the white reaction zone (1.8 mm) of a test card, and rocked for 5 min at 70 revolutions per min. An end-titre ≥ 8, i.e. agglutination occurred at serum dilution 1:8 or higher, was considered positive in CATT/*T. evansi* (Verloo et al., 1998).

2.7. Trypanolysis

Immune trypanolysis was performed as previously described (Van Meirvenne et al., 1995) with *T. evansi* variable antigen type RoTat 1.2. Sera were tested at a 1:4 dilution. Live trypanosomes were incubated for 60 min with test serum in the presence of guinea pig serum as the source of complement. When variant specific antibodies are present in the serum, lysis of the RoTat 1.2 trypanosomes occurs. The sample was considered positive for the presence of anti-RoTat 1.2 antibodies when 50% or more of the trypanosomes were lysed.

2.8. Serum samples

Samples were collected from dromedary camels between February 1995 and September 1995 in the Tahoua, Abalak and Tchin Tabaraden districts in Niger.

3. Results

3.1. Expression of recombinant RoTat 1.2 VSG

The recombinant RoTat 1.2 VSG was efficiently expressed in High Five insect cells (Fig. 1). More than 95% of the expressed protein was secreted, yielding about 45 mg of RoTat 1.2 VSG per litre of supernatant recovered from the cell culture medium. The protein had a deduced total amino acid number of 391, with 21 residues of signal peptide, 364 residues of RoTat 1.2 VSG, and six additional histidines. The absence of a potential N-glycosilation site was supported by the fact that tunicamycin treatment of the cells during infection did not change the migration pattern of the expressed protein in SDS-PAGE analysis (data not shown).

3.2. ELISA

The reactivity of the recombinant RoTat 1.2 VSG, compared with native RoTat 1.2 VSG was tested in ELISA using 12 trypanolysis-negative and 17 trypanolysis-positive camel sera. There was a good correlation between the OD obtained with the native RoTat 1.2 VSG and recombinant RoTat 1.2 VSG (Fig. 2). The ELISA cut-off value for

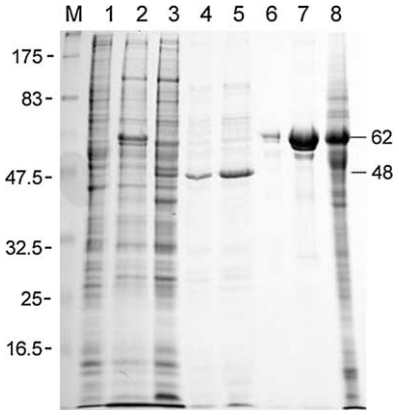

Fig. 1. Expression of recombinant RoTat 1.2 VSG in High Five cells. Shown is a photograph of an SDS-PAGE, containing in lane (1) Lysate of mock infected High Five cells; (2) Lysate of AcRoTat1.2 full length protein infected High Five cells; (3) Lysate of AcRoTat1.2Δ3′-His infected High Five cells; (4) Supernatant culture medium of cells loaded in lane 3; (5) 4× concentrated sample loaded in lane 4; (6 and 7) 0.5 and 5 μg purified RoTat 1.2 VSG; (8) Lysate *Trypanosoma evansi* RoTat 1.2; M. New England Biolabs protein marker. Molecular masses are in kDa.

positive was calculated as the mean OD plus three standard deviations of the trypanolysis-negative camel serum, and was 0.078 for native RoTat 1.2 VSG and 0.111 for recombinant RoTat 1.2 VSG. For both antigens the OD of all trypanolysis-positive camel sera was higher than this cut-off, while all trypanolysis-negative camel sera had an OD below the cut-off. This confirmed the antigenic potential of both proteins.

3.3. Determination of cut-off titre in latex coated with recombinant RoTat 1.2 VSG, and comparison with LATEX/T. evansi and CATT/T. evansi

In order to find the optimum serum screening dilution for the latex coated with recombinant RoTat 1.2 VSG, the end titre of 112 trypanolysis-positive and 116 trypanolysis-negative camel serum samples was determined. Sera were tested starting from a 1:4 dilution, since preliminary experiments revealed a prozone effect, which is the absence of agglutination due to high antibody concentrations, at lower dilutions. The highest relative sensitivity (89.3%, 95% exact binomial confidence interval CI: 82.0 to 94.3%) and relative specificity (99.1%, 95% CI: 95.3 to 100%) occurred at a cut-off titre of 1:4, i.e. if all samples with end titre 1:4 or higher were considered positive (Fig. 3). A prozone effect was still observed in one trypanolysis-positive serum, which was negative at 1:4 and gave agglutination at 1:8 to 1:64 dilutions. This prozone was taken into account for calculation of relative sensitivity and for construction of the receiver operator characteristics (ROC) curve.

The relative sensitivities of CATT/*T. evansi* and LATEX/*T. evansi* on the same samples were 92.9%, (95% CI: 86.4 to 96.9%), the relative specificities were respectively, 100% (95% CI: 97.5 to 100%) and 99.1% (95% CI: 95.3 to 100%). No significant difference was observed between the latex coated with recombinant RoTat 1.2 VSG at screening dilution 1:4, and LATEX/*T. evansi* (McNemar Chi Square with Yates correction $P=0.34$), or CATT/*T. evansi* (McNemar Chi Square with Yates correction $P=0.55$).

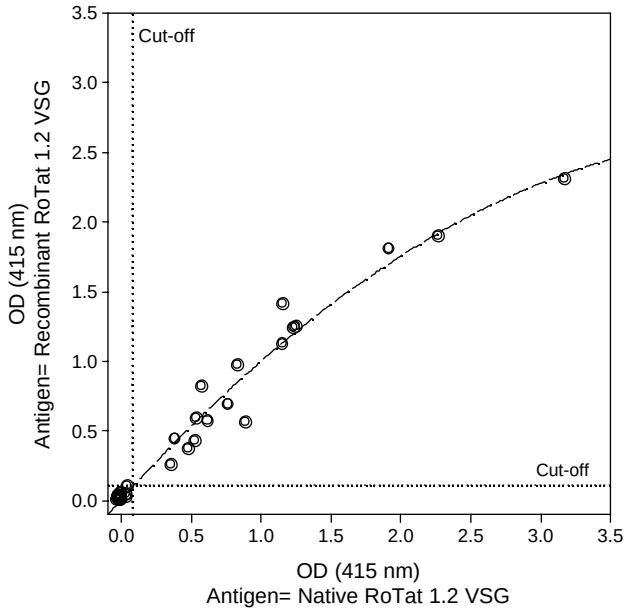

Fig. 2. Antigenic capacity of recombinant RoTat 1.2 VSG compared with that of native RoTat 1.2 VSG for detection of *T. evansi* specific antibodies in 29 camel sera by ELISA. (○, OD of individual sample; —, non-parametric smoothed line).

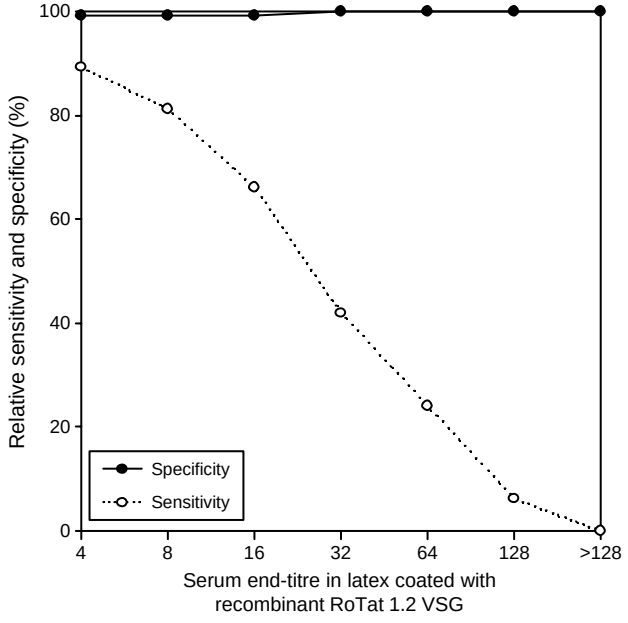

Fig. 3. Modified receiver operator characteristics curve (Jacobson, 1998) of relative sensitivity and specificity obtained with a latex coated with recombinant RoTat 1.2 VSG, in function of serum end-titre. Immune trypanolysis was used as a reference test for presence of antibodies.

4. Discussion

RoTat 1.2 is a predominant VSG expressed early during infection of susceptible animals, with the majority of *T. evansi* strains (Verloo et al., 2001; Ngaira et al., 2004). Consequently, RoTat 1.2 VSG has been used as an antigen in antibody detection tests for diagnosis of *T. evansi* infections in camels (Atarhouch et al., 2003; Dia et al., 1997; Diall et al., 1994; Gutierrez et al., 2000; Verloo et al., 1998). The CATT/*T. evansi* reagent consists of whole, fixed, stained and freeze-dried RoTat 1.2 trypanosomes, while LATEX/*T. evansi* and ELISA/*T. evansi* require purification of native soluble RoTat 1.2 VSG from trypanosomes grown in laboratory rodents. For the production of these tests, mass cultivation of cloned trypanosomes is needed which is laborious and requires much experience. These drawbacks can be eliminated by expression of RoTat 1.2 VSG as a recombinant protein, which may facilitate and standardise the production of antibody detection tests (Urakawa et al., 2001).

Recombinant RoTat 1.2 VSG was therefore expressed in 'High Five' insect cells and could be purified from the supernatant. A first confirmation of its antigenic potential for detection of RoTat 1.2 specific antibodies was obtained in ELISA. Optical densities with recombinant RoTat 1.2 VSG were comparable to those obtained with native RoTat 1.2 VSG (Fig. 2), and the test showed 100% relative sensitivity and specificity on a limited series of camel sera. In a next step it was therefore decided to replace the native RoTat 1.2 VSG in LATEX/*T. evansi* by the recombinant VSG. Priority was given to further development of rapid and simple agglutination tests, since such 'point-of-care' tests can be used on the spot for diagnosis of surra and do not require the use of a laboratory. The highest combination of relative sensitivity and specificity of the latex coated with recombinant RoTat 1.2 VSG was observed at a camel serum dilution 1:4. At this serum dilution, no significant difference could be observed between latex coated with recombinant VSG and CATT/*T.evansi* or LATEX/*T.evansi* agglutination tests based on native RoTat 1.2 VSG.

Due to the lack of serum samples of camels infected with other trypanosome species, strict specificity of the test based on recombinant RoTat 1.2 VSG for *T. evansi* infection could not be evaluated. In theory, some trypanolysis-negative samples may have originated from animals infected with trypanosome species other than *T. evansi*. However, *T. evansi* is the most abundant trypanosome in camels, and infections with *Trypanosoma brucei*, *Trypanosoma congolense* or *Trypanosoma vivax* are sporadic (Hornby, 1952; Wilson et al., 1983; Dirie et al., 1989). Lack of specificity and cross reactivity of the test might be advantageous for diagnostic purposes, since in practice, treatment of these other trypanosome infections in camels is based on the same drugs as for *T. evansi*.

Although our results are encouraging for replacement of native RoTat 1.2 VSG by recombinant RoTat 1.2 VSG in agglutination tests, further evaluation of the antigen in a phase 3 diagnostic trial remains necessary. In such a phase 3 trial, the test sensitivity and specificity should be validated prospectively in an endemic area on a target population of more than 1000 camels. This requires standardised mass production of recombinant RoTat 1.2 VSG, which can be performed in any laboratory with experience in insect cell culture. Furthermore, the potential of the reagent to detect *T. evansi* specific antibodies in other species affected by surra such as water buffaloes, dogs etc. should be evaluated. On these host species, possible cross reactivity with *T. congolense*, *T. vivax* and *T. brucei* infections should be studied.

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