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# Recombinant expression of trypanosome surface glycoproteins in *Pichia pastoris* for the diagnosis of *Trypanosoma evansi* infection

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ABSTRACT

Serodiagnosis of surra, which causes vast economic losses in livestock, is still based on native antigens purified from bloodstream form *Trypanosoma (T.) evansi* grown in rodents. To avoid the use of laboratory rodents in antigen preparation we expressed fragments of the invariant surface glycoprotein (ISG) 75, cloned from *T. brucei gambiense* cDNA, and the variant surface glycoprotein (VSG) RoTat 1.2, cloned from *T. evansi* gDNA, recombinantly in *Pichia (P.) pastoris*. The M5 strain of this yeast has an engineered N-glycosylation pathway resulting in homogenous $Man_5GlcNAc_2$ N-glycosylation which resembles the predominant $Man_{9\text{-}5}GlcNAc_2$ oligomannose structures in *T. brucei*. The secreted recombinant antigens were affinity purified with yields of up to 10 mg and 20 mg per liter cell culture of rISG $75_{29\text{-}465\text{-}E}$ and rRoTat $1.2_{23\text{-}385\text{-}H}$ respectively. In ELISA, both recombinant proteins discriminated between pre-immune and immune serum samples of 25 goats experimentally infected with *T. evansi*. The diagnostic potential of rRoTat $1.2_{23\text{-}385\text{-}H}$ but not of rISG $75_{29\text{-}465\text{-}E}$ was confirmed with sera of naturally infected and control dromedary camels. The results suggest that rRoTat $1.2_{23\text{-}385\text{-}H}$ expressed in *P. pastoris* requires further evaluation before it could replace native RoTat 1.2 VSG for serodiagnosis of surra, thus eliminating the use of laboratory animals for antigen production.



## 1. Introduction

Surra is an infectious disease in domestic (buffaloes, cattle, horses, camels etc.) and pet animals (dogs and cats) caused by the protozoan parasite *Trypanosoma (T.) evansi*. It occurs in Africa, the Middle East, Asia and South and Central America with sporadic outbreaks in Europe (Desquesnes

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et al., 2008; Gutierrez et al., 2010; Tamarit et al., 2010). The parasite is transmitted mechanically by bloodsucking flies such as *Tabanidae* and *Stomoxys* species and occasionally by vampire bats in Latin America (Stephen, 1986). It causes severe anemia, edema, immunosuppression and various neurological disorders resulting eventually into the death of the affected animals. Hence, surra leads to serious economic losses to the farmers in terms of morbidity, mortality, abortion, infertility, reduced milk yield and costs for trypanocides (Dobson et al., 2009).

The surface of trypanosomes is covered with a densely packed layer of about $5 \times 10^6$ dimers of one type of variant surface glycoprotein (VSG). This VSG is a strong immunogen, but the parasite avoids eradication by the host immune system by antigenic variation, i.e. changing the variant antigen type (VAT) of its VSG coat. Each parasite genome contains a large repertoire of different VSG genes, supplemented by recombination and gene conversion events, but only one is predominantly expressed at a time (Berriman et al., 2005; McCulloch and Horn, 2009). Switching the expression of one VSG gene to another results in a trypanosome bearing a different VAT that may escape immune destruction as long as the infected host has no antibodies against this particular VAT (Barry and McCulloch, 2001). VSG RoTat 1.2 is found to be predominantly expressed in *T. evansi* except in *T. evansi* type B strains circulating in Kenyan and in Ethiopian camels (Bajyana Songa and Hamers, 1988; Hagos et al., 2009; Ngaira et al., 2005; Payne et al., 1991; Verloo et al., 2000, 1998). In addition, other *Trypanosoma* sp. do not express this particular VAT (Claes et al., 2004). Current diagnostic tests for surra are therefore based on antibody detection against *T. evansi* RoTat 1.2. The card agglutination test for trypanosomiasis CATT/*T. evansi* is one of the OIE reference tests for antibody detection in surra diagnosis (Manual of Diagnostic Tests and Vaccine for Terrestrial Animals (OIE, 2012)).

Buried within the VSG layer, some receptors and invariant surface glycoproteins (ISGs) such as the ISG 75 are expressed in all *Trypanozoon* species with an amino acid homology of 92% and 90% between group I and II ISG 75 sequences respectively (Tran et al., 2006). The trypanosome surface contains $5 \times 10^4$ copies of ISG 75 that are believed to be evenly distributed over the surface (Jackson et al., 1993; Ziegelbauer and Overath, 1992). Since ISG 75 is strongly immunogenic and invariant, it is a good candidate antigen for diagnosis of *Trypanozoon* infections (Büscher and Lejon, 2004; Hutchinson et al., 2004; Tran et al., 2008).

Up to now, the available antibody tests for surra are all still based on native proteins purified from bloodstream form trypanosomes grown in rodents. To eliminate the use of laboratory rodents, VSG RoTat 1.2 has been recombinantly expressed in *Spodoptera frugiperda* with clear diagnostic potential but poor expression reproducibility (Lejon et al., 2005; Urakawa et al., 2001). Later, the diagnostic value of ISG 75 recombinantly expressed in *Escherichia coli* was demonstrated in camels and goats (Tran et al., 2008, 2009). To avoid the difficult and tedious purification of intracellular, recombinant proteins, we aspired a model for secreted expression also taking into account that glycosylation of a protein has a profound effect on its folding, thus on its potential to react with antibodies elicited by its native counterpart. Therefore, in the present study, we expressed VSG RoTat 1.2 and ISG 75 recombinantly in *Pichia pastoris*, a yeast that has proven to be successful for the recombinant expression of several trypanosomal proteins such as acid α-mannosidase and trans-sialidase from *T. cruzi*, rhodesain from *T. brucei rhodesiense* and congopain from *T. congolense* (Caffrey et al., 2001; Huson et al., 2009; Laroy and Contreras, 2002; Vandersall-Nairn et al., 1998). Glycoproteins produced in *P. pastoris* contain high mannose glycan structures. Those structures can hamper downstream processing, might be immunogenic and can cause rapid clearance from the circulation. To prevent hypermannosylation, our proteins are expressed in the M5 strain of *P. pastoris*. This strain has an engineered N-glycosylation pathway resulting in homogenous $Man_5GlcNAc_2$ N-glycosylation which resembles the predominant $Man_{9\text{-}5}GlcNAc_2$ oligomannose structures in *Trypanosoma brucei* (Acosta-Serrano et al., 2004; Jones et al., 2004; Mehlert et al., 1998; Vervecken et al., 2004; Zamze et al., 1991, 1990).

The expressed and secreted recombinant proteins are affinity purified and tested for their diagnostic potential against experimentally *T. evansi* infected goat sera and against uninfected and naturally infected dromedary camel sera.

## 2. Materials and methods

### 2.1. Yeast strain

For recombinant expression of the trypanosome proteins, the *P. pastoris* GS115 M5 strain was used in view of homogenous $Man_5GlcNAc_2$ N-glycosylation of the secreted proteins (Vervecken et al., 2004).

### 2.2. Construct engineering

The ISG 75 sequence was amplified from cDNA collected from *T. brucei gambiense* LiTat 1.3 (clone cb4; GenBank accession no. DQ200254) with a primer set starting from the first residue of the mature polypeptide (amino acid 29) and ending immediately upstream of the transmembrane domain (at amino acid 465 of a total of 523 amino acids). This clone exhibits a sequence and protein identity of >99% with an ISG 75 clone from *T. evansi* RoTat 1.2 (clone g21; GenBank accession no. DQ200175). A *PvuII* site was incorporated in the forward primer (ISG 75_FP). An E tag coding sequence (GE Healthcare), a stop codon and an *AvrII* site were added to the reverse primer (ISG 75_RP) (Table 1). The RoTat 1.2 VSG sequence was amplified from *T. evansi* RoTat 1.2 gDNA (GenBank accession no. AF317914) (Urakawa et al., 2001) by PCR. A *XhoI* site was incorporated in the forward primer (RoTat 1.2_FP) followed by 18 nucleotides reconstituting the α-mating factor signal sequence necessary for secreted expression in yeast (Daly and Hearn, 2005). Since the first amino acid codon of RoTat 1.2 showed self-complementarity with the reconstituted signal sequence, we started translation at the second residue of the ORF (amino acid 23). In order to obtain soluble RoTat 1.2 VSG, the coding region of the RoTat 1.2 VSG cDNA was truncated at the C-terminal end

**Table 1**
Primers developed for the recombinant expression of ISG 75 and RoTat 1.2 constructs with incorporation of a C-terminal E and His tag respectively.

| | | | | | |
|---|---|---|---|---|---|
| ISG 75_FP | ATA | GTACAG | CTGGAGGAGCTCTCTGTTGCGCAAAACAG | | |
| | Red | *PvuII* | ISG 75 (AA 29) | | |
| ISG 75_RP | ATA | CCTAGG | TTA | ACGCGGTTCCAGCGGATCCGG-ATACGGCACCGCGCGCACC | CCCAATCCAGCCACTCTTGGC |
| | Red | *AvrII* | Stop | E tag | ISG 75 (AA 465) |
| RoTat 1.2_FP | CTCGAG | AAAAGAGAGGCTGAAGCT | | AATGTAGCTCTTAAACGCAA | |
| | *XhoI* | Signal peptide | | RoTat 1.2 (AA 23) | |
| RoTat 1.2_RP | GAATT | CTA | GTGATGGTGATGGTGATG | TGTGTAAGCGGCTCGC | |
| | *EcoRI* | Stop | His tag | RoTat 1.2 (AA 385) | |

Red: redundant nucleotides.

by approximately 20% resulting in an expressed protein sequence of 363 amino acids of a total of 487. A His tag coding sequence, stop codon and *EcoRI*-site were added to the reverse primer (RoTat 1.2_RP) (Table 1). The PCR-fragments were subcloned into the pCR®2.1-TOPO® vector (TOPO TA Cloning®, Invitrogen™). The resulting constructs (rRoTat $1.2_{23\text{-}385\text{-}H}$ and rISG $75_{29\text{-}465\text{-}E}$) were transformed into TOP10 *E. coli* (Invitrogen). Subsequently, plasmid DNA was purified and double digested with *PvuII*/*AvrII* for ISG 75 and *XhoI*/*EcoRI* for RoTat 1.2 VSG respectively. The resulting fragments were ligated into the pPIC9 vector (Invitrogen, *Pichia* Expression kit), previously digested with *SnaBI*/*AvrII* for ISG 75 and *XhoI*/*EcoRI* for RoTat 1.2. The nucleotide sequences of the truncated genes in the transfer plasmid were confirmed by sequencing.

### 2.3. Transformation into P. pastoris

The constructs were linearized with *StuI* and purified (QIAquick, QIAGEN). The concentration was measured with the NanoDrop® ND-1000 Spectrophotometer (ISOGEN Life Science) and 5 μg linearized plasmid DNA in maximum 10 μl were electroporated (25 μF, 2000 V; Gene*Pulser* XCell™, Bio-Rad) into the *P. pastoris* M5 strain. Selection of transformed colonies was performed on SDC-His agar plates (synthetic dextrose complete histidine-deficient medium (BIO 101)).

### 2.4. Screening for expression of recombinant ISG 75 and RoTat 1.2

Positive transformants were selected by inoculating individual colonies in 10 ml Buffered Glycerol-complex Medium (BMGY, Invitrogen) in 50 ml falcon tubes. After 24 h growth (29 °C, 250 rpm; Innova 44R, New Brunswick Scientific), the cells were collected by centrifugation for 5 min at 3220 × *g*. The supernatant was discarded and the cells resuspended in 10 ml Buffered Methanol-complex medium supplemented with 2% (w/v) casamino acids (BMMY-2%CA, Becton Dickinson) to increase protein accumulation. The cells were again collected by centrifugation and the supernatant was discarded to remove all traces of glycerol as carbon source. After resuspension of the cells in 10 ml BMMY-2%CA, expression was allowed for 24 h at 250 rpm and, to minimize protein degradation, at 15 °C (Shi et al., 2003). The supernatant was collected by centrifuging the resulting cell culture for 15 min at 3220 × *g*. The proteins of 1 ml of the supernatant were precipitated with trichloroacetic acid (TCA) and resuspended in 100 μl of SDS-PAGE reducing loading buffer (0.125 M Tris–HCl (pH 6.8), 4% SDS, 20% sucrose, 0.04% bromophenol blue, 0.2 M dithiothreitol). The secreted proteins were separated on a 12% polyacrylamide gel (50 min at 200 V; Mini-PROTEAN Tetra Cell Electrophoresis System, Bio-Rad) and transferred to a nitrocellulose membrane by Western blotting (30 min at 100 V; Criterion Blotter, Bio-Rad). The nitrocellulose membrane was blocked overnight with TBS-Blotto (0.02 M Tris–HCl (pH 7.5), 0.5 M NaCl, 0.004% $NaN_3$, 5% skimmed milk powder (Fluka)) The proteins were visualized by immunostaining with polyclonal goat anti-E tag antibody (1:10,000; Bethyl

Laboratories), rabbit anti-ISG 75 antiserum (1:500), mouse anti-His tag antibody (1:4000; AbD Serotec) or rabbit anti-RoTat 1.2 antiserum (1:250) as primary antibodies followed by alkaline phosphatase conjugated donkey anti-goat IgG (1:10,000; Jackson ImmunoResearch), goat anti-rabbit IgG (1:20,000; Jackson ImmunoResearch) or rabbit anti-mouse IgG (1:15,000; Sigma), and nitro blue tetrazolium (NBT) and 5-bromo-4-chloro-3-indolyl phosphate (BCIP).

### 2.5. Bulk protein expression and purification

The selected protein secreting colonies were inoculated in 500 ml BMGY with addition of 0.2 g/l chloramphenicol and 0.1% P2000 antifoam (Sigma) (Routledge and Bill, 2012). The cultures were grown in Fernbach shake flasks for 24–66 h at 29 °C and 250 rpm to create biomass. Afterwards the cells were collected (5 min at 4417 × *g*) and resuspended in an equal volume of BMMY-2%CA containing 1% methanol. After measurement of the optical density at 600 nm the culture was further diluted with BMMY-2%CA to an $OD_{600\,nm}$ of 5.0, transferred to an Ultra Yield flask with an AirOtop seal (BioSilta) and 0.1% P2000 antifoam was added. Protein expression was induced for 24 h up to 96 h at 15 °C and 250 rpm with addition of 0.5% methanol every 8 h. The supernatant was collected by centrifugation for 30 min at 17,670 × *g* (no brake).

#### 2.5.1. Affinity purification of rISG $75_{29\text{-}465\text{-}E}$

The supernatant was filtered over a 0.22 μm filter (Corning) and affinity purified over a 5 ml pre-packed anti-E tag column (RPAS Purification Module, GE Healthcare) at a flow rate of 1–2 ml/min at 4 °C. The fluid was sent through a UV detector (Spectra/Chrom® UV Monitor; Spectrum Chromatography) measuring absorbance at 280 nm. The column was washed with binding buffer (0.2 M sodium phosphate buffer, 0.05% $NaN_3$, pH 7.0) until the baseline (i.e. absorbance of the binding buffer) was reached. The rISG $75_{29\text{-}465\text{-}E}$ was eluted with elution buffer (1 M glycine, pH 3.0). Elution fractions of 2 ml were collected in tubes containing 400 μl of neutralizing buffer (1 M Tris base, 0.05% $NaN_3$, pH 8.2).

#### 2.5.2. Affinity purification of rRoTat $1.2_{23\text{-}385\text{-}H}$

rRoTat $1.2_{23\text{-}385\text{-}H}$ was purified over Ni beads (HisPur™ Ni-NTA Resin; Pierce). The collected culture supernatant was first 1:2 diluted in 50 mM $Na_2HPO_4$, 600 mM NaCl and 20 mM imidazole (pH 9.0), added to the Ni beads (50 ml per 1 ml beads in a 50 ml falcon) and mixed during 1 h on a roller at room temperature. After 1 h the beads were collected by 5 min centrifugation at 700 × *g*. The beads were resuspended in 1 ml of wash buffer and decanted into a 20 ml Econo column (Bio-Rad). All further purification steps were performed at 4 °C. After the columns were packed, the unbound proteins were washed away with wash buffer (25 mM imidazole) until the baseline absorption value at 280 nm was reached. The bound proteins were eluted with elution buffer (250 mM imidazole).

### 2.6. Protein concentration

The purified proteins were desalted over a HiPrep 26/10 Desalting column (GE Healthcare) with phosphate buffered saline (50 mM sodium phosphate, 0.15 M NaCl, pH 7.0) at 4 °C according to the manufacturer's instructions. The concentration was measured by the BCA™ Protein Assay Kit (Thermo Scientific).

### 2.7. Mass spectrometry and protein sequencing

The sequence of the purified proteins was analyzed by mass spectrometry (4800 Plus MALDI-TOF/TOF; AbSciex). Coomassie SDS-PAGE bands were excised and destained by means of alternating incubation and washing in 50 mM ammonium bicarbonate (ABC) and 80% acetonitrile (ACN). Cysteine disulfide bonds were reduced by 5 mM dithiothreitol (DTT) and alkylated by 55 mM iodoacetamide (IAM) to form stable S-carboxyamidomethylcysteine. Both solutions contained 50 mM ABC and 5% ACN. The modification was performed at ambient temperature. Peptides were extracted from the gel bands after digestion with trypsin (Sequencing Grade Modified Trypsin, Promega) and samples were desalted using C18 cleanup tips (ZipTip, Millipore). The in-gel digestion protocol is based on Rosenfeld et al. (1992). The resulting peptides were co-crystallized on a MALDI-target using α-cyano-4-hydroxycinnamic acid (ACHA) spiked with three internal standards. Peptides were selected for fragmentation in the collision cell (CID) and the resulting mass spectra were submitted to MASCOT (MASCOT 2.4, Matrix Science) for peptide sequence identification against all eukaryotic sequences in the NCBInr protein database.

Edman degradation was performed on PVDF electroblotted protein bands from SDS-PAGE using an Applied Biosystems 494 Procise protein sequencer.

### 2.8. Reactivity of the purified proteins in ELISA with goat and camel sera

Microplates (Maxisorp, Nunc™) were coated overnight at 4 °C with 200 μl/well of purified rISG $75_{29\text{-}465\text{-}E}$ and/or rRoTat $1.2_{23\text{-}385\text{-}H}$ and native RoTat 1.2 at 2 μg/ml in PBS. Antigen negative control wells were left empty. Further manipulations were done at ambient temperature. The protocol was based on the procedure according to Lejon et al. (2005, 2003). To correct for aspecific reactions, caused by contaminating yeast proteins which were not eliminated from the protein mixture by the one-step affinity purification, a culture of untransformed *P. pastoris* M5 strain was grown for 47 h in BMMY-2%CA (after a pre-culture in BMGY). This culture was induced with methanol in exactly the same way as the transformed M5 strains (i.e. addition of 0.5% methanol every 8 h). Previous to the ELISA the sera were desorbed by diluting them in PBS-Blotto (0.01 M sodium phosphate, 0.2 M sodium chloride, 0.05% $NaN_3$, 1% skimmed milk powder, pH 7.4) supplemented with 10% (v/v) untransformed M5 medium for 1 h at room temperature. The sera were centrifuged for 5 min at 3000 × *g* before use (desorption). Twenty five sera of goats before infection and 30

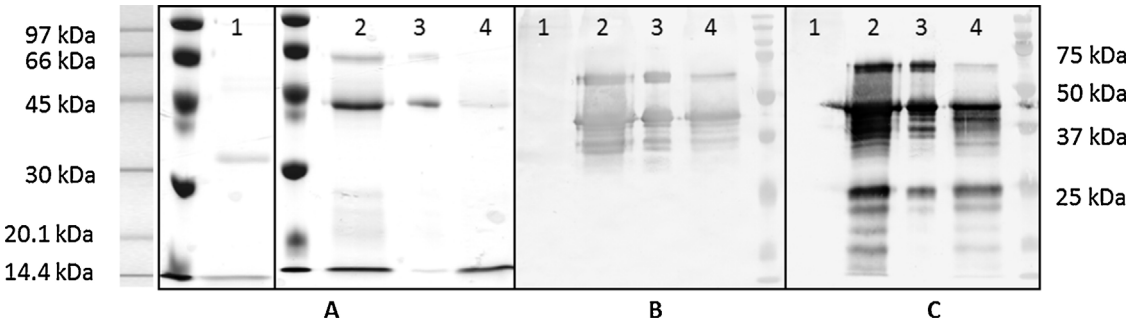


**Fig. 1.** Expression of rISG $75_{29\text{-}465\text{-}E}$ by *Pichia pastoris*. (A) Coomassie stained SDS-PAGE gel; (B) Western blot with polyclonal goat anti-E tag antibody; (C) Western blot with rabbit anti-ISG 75 antiserum; protein markers: LMW-SDS protein marker (GE Healthcare) and Dual Color Precision Plus prestained protein standard (Bio-Rad); lane 1: 20× concentrated supernatant of non-transfected *Pichia pastoris* M5 after 47 h induction; lane 2: 20× concentrated supernatant of transfected *Pichia pastoris* M5 after 46 h induction; lane 3: E tag purified recombinant ISG 75; lane 4: 20× concentrated flow-through of E tag purified supernatant.

days post-infection with *T. evansi* were tested at 1:1000 dilution (250 μl/well). Antibody binding was visualized with rabbit anti-goat IgG conjugated with horseradish peroxidase (1:10,000; Sigma) and the chromogen ABTS (2,2′-azinobis[3-ethylbenzothiazonline-6-sulfonic acid]-diammonium salt; Roche) both at 250 μl/well. The optical densities (ODs) were read at 414 nm (Multiskan RC Version 6.0; Labsystems). The tested dromedary camel sera originated from Niger, collected between February 1995 and September 1995 in the Tahoua, Abalak and Tchin-Tabaraden districts. A total of 185 sera were used in this study, of which 93 from camels that were positive in trypanolysis (i.e. containing anti-RoTat 1.2 antibodies) (Verloo et al., 2001, 1998), including 47 parasitologically confirmed animals, and 92 from trypanolysis negative control animals without clinical, parasitological and serological evidence of infection. The camel sera were diluted 1:100 in PBS-Blotto + 10% M5 medium (desorption) as described above. Protein A-peroxidase (1:10,000; Sigma) and ABTS were used as conjugate and chromogen respectively. The ODs were read at 414 nm.

## 3. Results

### 3.1. Expression and purification of rISG $75_{29\text{-}465\text{-}E}$ and rRoTat $1.2_{23\text{-}385\text{-}H}$

After induction of the transfected *P. pastoris* M5 cell cultures, recombinant ISG 75 and RoTat 1.2 were secreted in the supernatant. After 46 h, the culture supernatants were harvested for affinity purification of the recombinant proteins and analysis by SDS-PAGE followed by Coomassie staining or Western blot.

In the Coomassie stained gel rISG $75_{29\text{-}465\text{-}E}$ is visible as two predominant protein bands of approximately 40–45 and 60–65 kDa (Fig. 1). In Western blot, several shorter, putative degradation products that react with anti-E tag and anti-ISG75 antibodies become visible. A considerable fraction of the recombinant proteins was not retained on the E tag affinity column and was found back in the flow through. Attempts to optimize the purification conditions were unsuccessful. Typically, 1 mg rISG $75_{29\text{-}465\text{-}E}$ could be purified from 100 ml yeast culture.

After Coomassie staining rRoTat $1.2_{23\text{-}385\text{-}H}$ is visible as one band at approximately 45 kDa (Fig. 2). In Western blot, the anti-His tag serum detected several putative degradation products in the culture supernatant that were not visible in the nickel purified fraction. These shorter proteins were not detected with the anti-RoTat 1.2 antiserum. A small fraction of the recombinant protein was not retained by the affinity column. Typically, a yield of 2 mg rRoTat $1.2_{23\text{-}385\text{-}H}$ could be purified from 100 ml yeast culture.

The N-terminal amino acid sequence of the purified recombinants was determined by Edman degradation. No sequence was obtained for the 60–65 kDa band of rISG 75, while the N-terminal sequence of the 40–45 kDa band is DYYYEH(S)XLD, corresponding with amino acids 146–155 of the native ISG 75 without its signal peptide (where X is arginine) thus with amino acids 148–157 of the recombinantly expressed ISG 75. For the rRoTat 1.2 45 kDa protein, the N-terminal sequence obtained was EAEANVALKG. The first 4 amino acids (EAEA) are remnants of to the KEX2 protease cleavage site incorporated after the α-mating factor signal sequence and should have been cleaved off by dipeptidyl aminopeptidase (STE13 gene product). In this case however, cleavage of the Glu-Ala repeats did not occur, probably due to insufficient yeast dipeptidyl aminopeptidase to process the large amounts of secreted recombinants (Brake et al., 1984). The sequence NVALKG corresponds with amino acids 23–28 of the native RoTat 1.2. Tandem mass spectrometry of tryptic peptides from the purified proteins revealed several peptide sequences from native ISG 75 and RoTat 1.2 with protein scores of 218 and 181 (identity threshold: 78; $p < 0.05$). The mass spectrometry analyses (CeProMa, UA) were repeated and confirmed independently at the Laboratory for Protein Biochemistry and Protein Engineering (UGent) (data not shown).

### 3.2. Diagnostic potential assessed with experimentally infected goats

An assessment of the diagnostic potential of rISG $75_{29\text{-}465\text{-}E}$ and rRoTat $1.2_{23\text{-}385\text{-}H}$ was performed in ELISA with sera from 25 goats, before and after infection with *T. evansi*. The results were compared to those obtained with native RoTat 1.2 (Fig. 3). The recombinant proteins

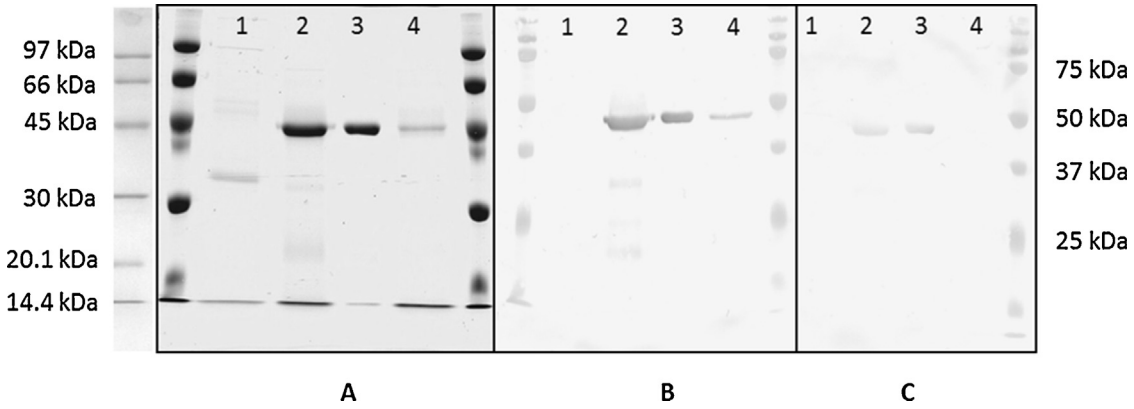


**Fig. 2.** Expression of rRoTat $1.2_{23\text{-}385\text{-}H}$ by *Pichia pastoris*. (A) Coomassie stained SDS-PAGE gel; (B) Western blot with anti-His tag antibody; (C) Western blot with rabbit anti-RoTat 1.2 antiserum; protein markers: LMW-SDS protein marker (GE Healthcare) and Dual Color Precision Plus prestained protein standard (Bio-Rad); lane 1: 20× concentrated supernatant of non-transfected *Pichia pastoris* M5 after 47 h induction; lane 2: 20× concentrated supernatant of transfected *Pichia pastoris* M5 after 46 h induction; lane 3: His tag purified recombinant RoTat 1.2; lane 4: 20× concentrated flow-through of His tag purified supernatant.

were recognized by antibodies in sera collected 30 days post-infection (median OD 0.676 for rRoTat $1.2_{2\text{-}363\text{-}H}$ and 0.653 for rISG $75_{29\text{-}465\text{-}E}$) as was the case with native RoTat 1.2 (median OD 0.890). The combination of both recombinant antigens showed an additive effect for the detection of infected goats (median OD 0.922). The reactions with the pre-infection sera were much lower (median OD 0.080 for rRoTat $1.2_{23\text{-}385\text{-}H}$, 0.161 for rISG $75_{29\text{-}465\text{-}E}$ and 0.048 for native RoTat 1.2). Neither recombinant antigen was able to fully discriminate between pre-infection and post-infection samples. With rRoTat $1.2_{2\text{-}363\text{-}H}$ and rISG $75_{29\text{-}465\text{-}E}$, respectively six and three post-infection sera yielded ODs lower than the maximum OD of the pre-infection sera (respectively 0.324 and 0.375). With the mixed recombinant proteins, the lowest OD obtained with the post-infection sera was equal to the highest OD obtained with the pre-infection sera.

### 3.3. Diagnostic potential assessed with camel sera

The diagnostic performance of both recombinant antigens was further evaluated with a panel of 185 camel sera and compared to native RoTat 1.2 in ELISA (Fig. 4). All trypanolysis negative samples, except one outlier, showed minimal reactivity with all the antigen preparations. Median OD values were 0.059 with native RoTat 1.2, 0.042 with rRoTat $1.2_{23\text{-}385\text{-}H}$, 0.045 with rISG $75_{29\text{-}465\text{-}E}$ and 0.055 with the mixture of the two recombinant fragments. Median OD values obtained with the trypanolysis positive samples were high with native RoTat 1.2 (2.322), somewhat lower with rRoTat $1.2_{23\text{-}385\text{-}H}$ (0.953) but very low with rISG $75_{29\text{-}465\text{-}E}$ (0.066). The lack of reactivity of the rISG $75_{29\text{-}465\text{-}E}$ with the positive samples is also reflected in the median OD obtained with the mixed recombinant antigens (0.646). For the native RoTat 1.2, only 2 of 93 positive samples showed an OD lower than the maximum OD (0.704) observed with the trypanolysis negative samples confirming the discriminative power of the native antigen. For rRoTat $1.2_{23\text{-}385\text{-}H}$, the discriminative power is less since 17 of 93 trypanolysis positive samples showed an OD lower than the maximum OD (0.474) of the negative samples with this recombinant.

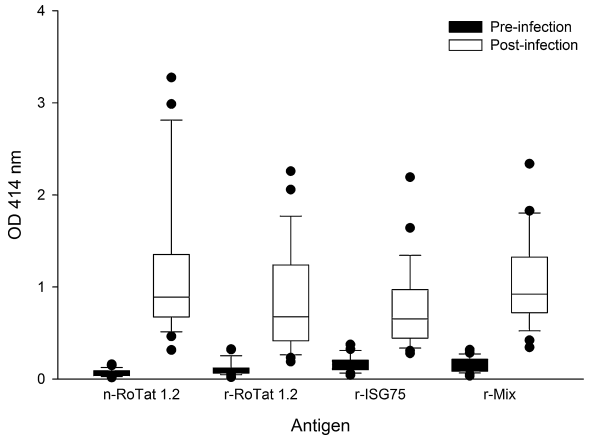


**Fig. 3.** Box plots of the optical density value (OD) obtained in ELISA with native RoTat 1.2 (2 μg/ml), rRoTat $1.2_{23\text{-}385\text{-}H}$ (2 μg/ml), rISG $75_{29\text{-}465\text{-}E}$ (2 μg/ml) and a mix of both recombinant antigens (rMix, 1 μg/ml each) against 25 pre-immune and 25 immune sera of goats experimentally infected with *T. evansi*.

## 4. Discussion

Aiming at the eventual replacement of native antigens in serodiagnostic tests for surra, we successfully expressed the *T. brucei* ISG 75 and *T. evansi* VSG RoTat 1.2 in the yeast *P. pastoris*. The results demonstrate that *P. pastoris* not only represents a good expression system for trypanosomal enzymes, such as trans-sialidase from *T. cruzi* (Laroy and Contreras, 2002), rhodesain (Cys-protease) from *T.b. rhodesiense* (Caffrey et al., 2001), acid α-mannosidase from *T. cruzi* (Vandersall-Nairn et al., 1998) and cathepsin B-like proteases and congopain from *T. congolense* (Huson et al., 2009; Mendoza-Palomares et al., 2008), but also for invariant and variant surface glycoproteins. The inclusion of the α-mating factor signal sequence for secreted expression and of an E tag (rISG $75_{29\text{-}465\text{-}E}$) or His tag

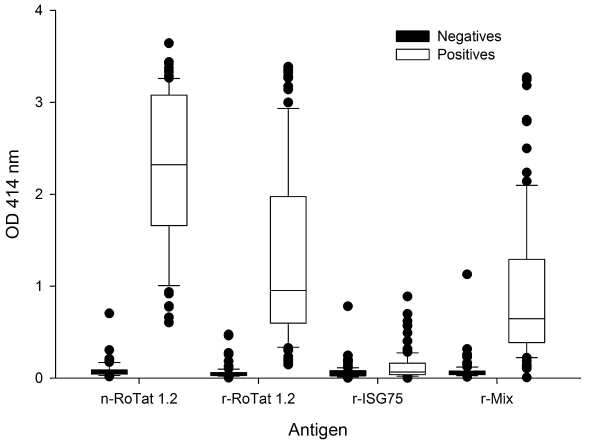


**Fig. 4.** Box blots of the optical density value (OD) obtained in ELISA with native RoTat 1.2 (2 μg/ml), rRoTat $1.2_{23\text{-}385\text{-}H}$ (2 μg/ml), rISG $75_{29\text{-}465\text{-}E}$ (2 μg/ml) and a mix of both recombinant antigens (rMix, 1 μg/ml each) against sera from 92 non-infected and 93 *T. evansi* infected camels.

(rRoTat $1.2_{23\text{-}385\text{-}H}$) for affinity purification simplifies the purification of the expressed proteins. Yields of up to 10 mg and 20 mg recombinant protein per liter cell culture were obtained for respectively rISG $75_{29\text{-}465\text{-}E}$ and rRoTat $1.2_{23\text{-}385\text{-}H}$. Moreover, the production in *P. pastoris* was highly reproducible, in contrast with VSG RoTat 1.2 recombinantly expressed in *S. frugiperda* (Lejon et al., 2005).

The mature *rISG* $75_{29\text{-}465\text{-}E}$ consists of 452 amino acids with one putative N-glycosylation site at Asn108 (NetNGlyc; ExPASy). It contains one remaining amino acid of pPIC9 (*SnaBI* site), one amino acid of the *PvuII* site incorporated in the forward primer, 437 residues of the extracellular ISG 75 domain and a 13 residues long C-terminal E tag. The Coomassie stained gel and Western blots showed two bands, with the thickest band around 40–45 kDa. Edman degradation on the highest band was not successful (60–65 kDa) which could be caused by acetylation of the N-terminal threonine. The N-terminal sequencing of the lower band however revealed that it started with amino acid 148 of the recombinant ISG 75. The 147 missing amino acids could account for the 20 kDa difference between the higher and lower band. Thus, the 60–65 kDa band visible in the Western blot and Coomassie stained gel represents the entire rISG $75_{29\text{-}465\text{-}E}$ that is cleaved between amino acids 147 and 148 (R and D), probably by a protease from *P. pastoris*. This protein degradation does not occur with recombinantly expressed ISG 75 in *E. coli* (Tran et al., 2008). Since the addition of protease inhibitors in the expression medium could not prevent the cleavage of rISG $75_{29\text{-}465\text{-}E}$, an alternative way to prevent degradation of the recombinant protein could be to start from a different cDNA clone. ISG 75 is encoded by a multicopy gene family consisting of two main groups, which share 75% and 77% similarities among their cDNA and gDNA sequences respectively (Tran et al., 2006). The rISG $75_{29\text{-}465\text{-}E}$ constructed in this study belongs to group I of the ISG 75 gene family while ISG 75 clones from group II differ around the putative cleavage site (EPNAYKR-DYYYEHSRLD (Group I) versus DPSAYKH-DYHRNFGHDD (Group II)). Therefore it may be worthwhile to prepare rISG 75 from group II. The rRoTat $1.2_{23\text{-}385\text{-}H}$ consists of 373 amino acids, 2 N-terminal Glu-Ala repeats that are not efficiently cleaved off by dipeptidyl aminopeptidase as shown by Edman degradation, 363 residues from RoTat 1.2 and 6 residues of the C-terminal His tag.

rRoTat $1.2_{23\text{-}385\text{-}H}$ has a theoretical mass of 39.6 kDa without potential N-glycosylation sites, whereas the theoretical mass of rISG $75_{29\text{-}465\text{-}E}$ is 50.5 kDa (complete construct) or 34.3 kDa (cleaved construct) (Compute pI/Mw; ExPASy). ConA purification of both rISG $75_{29\text{-}465\text{-}E}$ and rRoTat $1.2_{23\text{-}385\text{-}H}$ proved that they are both glycosylated (data not shown). This glycosylation could explain why in SDS-PAGE, the apparent mass of the recombinants is 5–10 kDa higher than their theoretical mass since glycoproteins generally migrate slower caused by interference of the oligosaccharides with the binding of SDS on the protein backbone (Segrest and Jackson, 1972).

In ELISA, the purified recombinants could mostly distinguish the pre-immune and immune serum samples of 25 goats experimentally infected with *T. evansi*. There is a clear difference between the pre- and post-infection sera, albeit less outspoken than with native RoTat 1.2, the latter being the antigen used in reference tests for *T. evansi* infection such as CATT/*T. evansi* and immune trypanolysis (OIE, 2012; Verloo et al., 1998). The superior reactivity of the sera with native RoTat 1.2 can be explained by the fact that the native antigen is a longer protein bearing more potentially diagnostic epitopes and with the original three dimensional conformation. The tests with naturally infected and control camels confirmed the diagnostic potential of rRoTat $1.2_{23\text{-}385\text{-}H}$ but not of rISG $75_{29\text{-}465\text{-}E}$. This is quite unexpected since Tran and coworkers reported that recombinant ISG $75_{S\text{-}29\text{-}457\text{-}H}$ derived from a *T.b. gambiense* strain and expressed in *E. coli* was able to distinguish infected and non-infected camel sera within the same collection tested here (Tran et al., 2009). It is possible that the ISG 75 specific epitopes best recognized by camels are different from those recognized by goats and that the former are located on the first 147 amino acids cleaved off from the rISG $75_{29\text{-}465\text{-}E}$ expressed in *P. pastoris* and not from rISG $75_{S\text{-}29\text{-}457\text{-}H}$ expressed in *E. coli*. For other hosts (cattle, horses etc.), the diagnostic potential of rISG $75_{29\text{-}465\text{-}E}$ and rRoTat $1.2_{23\text{-}385\text{-}H}$ compared to native RoTat 1.2 remains to be evaluated. Once their diagnostic properties are confirmed, they can be incorporated in diagnostic tests such as ELISA, latex agglutination or lateral flow tests. Since ISG 75 is expressed by all *Trypanozoon* taxa and strains, it also qualifies as a possible antigen for the diagnosis of human African trypanosomiasis or sleeping sickness (Hutchinson et al., 2004).

## 5. Conclusion

The results suggest that rRoTat $1.2_{23\text{-}385\text{-}H}$ expressed in *P. pastoris* requires further evaluation before it could replace native RoTat 1.2 VSG for serodiagnosis of surra, thus eliminating the use of laboratory animals for antigen production. Further research is needed on a recombinant ISG 75 fragment that is less prone to degradation and that is reactive with camel sera.

## Conflict of interest statement

All authors declare no conflict of interest.

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