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# Bead-based suspension array for simultaneous detection of antibodies against the Rift Valley fever virus nucleocapsid and Gn glycoprotein

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A multiplex bead-based suspension array was developed that can be used for the simultaneous detection of antibodies against the surface glycoprotein Gn and the nucleocapsid protein N of Rift Valley fever virus (RVFV) in various animal species. The N protein and the purified ectodomain of the Gn protein were covalently linked to paramagnetic Luminex beads. The performance of the resulting multiplex immunoassay was evaluated by testing a comprehensive and well-characterized panel of sera from sheep, cattle and humans. The suitability of this multiplex immunoassay to differentiate infected from vaccinated animals (DIVA) was investigated by testing sera from lambs vaccinated with a paramyxovirus vaccine vector expressing the RVFV surface glycoproteins Gn and Gc. The results suggest that the bead-based suspension array can be used as a DIVA assay to accompany several recently developed experimental vaccines that are based on RVFV glycoproteins, and are devoid of the N protein.



## 1. Introduction

Rift Valley fever virus (RVFV) is a mosquito borne virus that belongs to the Phlebovirus genus of the *Bunyaviridae* family (Elliott, 1996). The virus was first isolated in 1930 (Daubney et al., 1931) near Lake Naivasha in Kenya and has since caused devastating outbreaks throughout Africa (Bird et al., 2009; Gerdes, 2004).

The RVFV RNA genome comprises a small (S), medium (M) and large (L) segment. The S genome segment encodes the nucleocapsid (N) protein in genomic-sense orientation and a non-structural protein (NSs) in the antigenomic-sense orientation (Elliott, 1996). The NSs protein suppresses host innate immune responses and was shown to be the major virulence determinant of the virus (Billecocq et al., 2004; Bouloy et al., 2001; Habjan et al., 2009; Ikegami et al., 2009). The M segment encodes the two structural glycoproteins Gn and Gc and two non-structural proteins, a 78-kDa protein and the NSm protein, of which the latter was shown to have an anti-apoptotic function (Won et al., 2007). The L segment encodes the RNA-dependent RNA polymerase protein.

The first commercially available ELISAs used inactivated whole virus as the antigen (Paweska et al., 2003, 2005a, 2005b). Although these ELISAs in general perform very well, the use of recombinant proteins can provide advantages with respect to safety, stability and cost-effectiveness. Several ELISAs based on recombinant antigen have been developed and commercialized, but all these assays detect antibodies against the N protein (Jansen van Vuren and Paweska, 2009; Jansen van Vuren et al., 2007; Paweska et al., 2007, 2008). Preferably, a serological confirmation test should detect antibodies against other immunogenic proteins of the virus. The virus neutralization test is based on the detection of antibodies against the structural glycoproteins, and can therefore be used for such serological confirmation (Gerdes, 2008). However, the classical virus neutralization test requires live virus to be handled under appropriate biosafety containment facilities, and takes up to seven

*Abbreviations:* DIVA, differentiate infected from vaccinated animals; GneS3, secreted Gn ectodomain with three Strep-tags; Trx, thioredoxin protein; PE, phycoerythrin; MFI, median fluorescence intensity; P/N, positive/negative.

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days for completion. A less laborious, glycoprotein-based serological assay would therefore be very advantageous.

Recently, an efficient method to produce milligram amounts of the Gn ectodomain in a highly pure, soluble form was developed (de Boer et al., 2010). In the work described in the present paper, the production of this protein was improved, and both the recombinant Gn ectodomain and purified N protein were used to develop a Luminex assay for the simultaneous detection of antibodies against both proteins.

Luminex technology uses variously coloured polystyrene beads, the surface of which is carboxylated to allow covalent coupling of antigens. Conjugated beads can be incubated with serum to capture specific antibodies, after which a fluorescent secondary antibody is added to bind to the captured serum antibodies. The Luminex system uses a red laser to determine the colour of the bead and a green laser to detect bound secondary antibodies (Krishhan et al., 2009). With this system, multiple serological components can be tested simultaneously with a single sample (Perkins et al., 2006). The work described in this paper demonstrated that a newly developed Luminex assay can detect antibodies against both the RVFV Gn and N proteins simultaneously. Moreover, the results suggest that this assay may be used to differentiate infected from vaccinated animals (DIVA) when combined with new experimental vaccines based on RVFV glycoproteins.

## 2. Materials and methods

### 2.1. Production of the N protein

Production of recombinant RVFV nucleoprotein N has been described previously (Martin-Folgar et al., 2010). Briefly summarized, the gene encoding the N protein of RVFV strain MP-12 (GenBank accession number DQ380154.1, protein ID ABD38739.1), flanked by *Sal*I and *Xho*I sites, was cloned into plasmid pET32a (Novagen, Merck KGaA, Darmstadt, Germany) to generate a fusion protein with the 109 amino acid thioredoxin protein (Trx) containing a 6× His-tag for purification purposes. The resulting plasmid, pTrx-N, was transformed into *Escherichia coli* BL-21 cells. Protein expression was induced by addition of 1 mM isopropyl-β-thiogalactoside (Fermentas, Madrid, Spain) to the culture medium with constant shaking for 4 hours. As a negative control, expression of thioredoxin was induced in BL21 cells transformed with 'empty' plasmid pET32a. Bacterial cells were collected by centrifugation and lysed in phosphate-buffered saline (PBS) containing 2 mg/ml lysozyme. Supernatants were incubated with Ni-NTA resin (Qiagen, Hilden, Germany). After extensive washing steps bound protein was eluted with 500 mM imidazole in PBS.

### 2.2. Production of the Gn protein

The ectodomain of the Gn protein was produced from the insect expression vector pMT/BiP/V5-HisA (Invitrogen, Carlsbad, CA, USA). The sequence encoding the authentic Gn signal peptide was replaced by the sequence encoding the BiP signal peptide, specifying the junction sequence "GLSLG-**RSLRSL**AEDPH" (BiP, **linker**, Gn ectodomain). In the pMT-GneS3 plasmid, the Gn ectodomain sequence was fused to a sequence encoding a combined FLAG-tag/enterokinase cleavage site for easy detection and purification of the monomeric protein (DYKDDDDK) and three Strep tags (WSHPQFEK) separated by glycine linkers (GGGSGGGSGGGS), resulting in the sequence …YQCHT**DPTG**DYKDDDDK**AGPG**WSHPQFEK and GGGSGGGSGGGSWSHPQFEKGGGSGGGSGGGSWSHPQFEK (sequences resulting from introduced restriction sites are indicated in bold). The enterokinase cleavage site was introduced to allow future removal of the Strep-tag after purification.

The secreted Gn ectodomain (GneS3) was purified from the cell culture supernatant by virtue of three C-terminal Strep-tags using Strep-Tactin Sepharose, according to the manufacturer's recommendations (IBA, Göttingen, Germany). The GneS3 protein was eluted from the washed beads with 4 mM D-desthiobiotin (IBA) and concentrated using an Amicon® Ultra-4 concentrator with a molecular mass cut-off of 30 kDa (Millipore, Billerica, MA, USA).

### 2.3. Coupling of RVFV proteins to carboxylated beads

The purified GneS3, Trx-N and Trx (thioredoxin; control) proteins were dialyzed against PBS using Slide-A-Lyzer MINI dialysis units with a cut-off of 7 kDa (Pierce, Thermo Fischer Scientific, Rockford, IL, USA). Using a generic two-step carbodiimide coupling using sulfo-*N*-hydroxysulfosuccinimide (NHS) and 1-ethyl-3-[3-dimethylaminopropyl] carbodiimide hydrochloride (EDC) (Pierce) according to instructions, 50 μg of each protein was coupled to $2.5 \times 10^6$ carboxylated paramagnetic beads (MagPlex microspheres, Luminex, Oosterhout, the Netherlands).

### 2.4. Multiplex assay

Assays were performed with a flow cytometry-based Luminex 200 as follows. A bead mix was prepared with 1000 beads of each bead set in 50 μl PBS containing 0.05% Tween-20 and 0.867 M NaCl (PBS-T-HS). Aliquots of 50 μl bead mix and 50 μl serum, diluted in PBS-T-HS, were mixed and incubated in a 96-well plate for 30 minutes in the dark at room temperature on a plate shaker (600 rpm). The plate was subsequently placed on a magnet (Dynal MPC-96-S; Invitrogen) for 1 min, after which the beads were washed with 100 μl PBS containing 0.05% Tween-20 (PBS-T). Subsequently, 100 μl phycoerythrin (PE)-conjugated secondary antibody in PBS-T was added, and the beads were incubated for 30 minutes, as described above. When appropriate, beads were incubated instead with biotinylated secondary antibodies, followed by a 30-minute incubation with 1:2000-diluted PE-conjugated streptavidin (25 mg/ml stock, Invitrogen). Following incubation with PE-conjugated secondary antibodies or streptavidin-PE, the beads were resuspended in 80 μl PBS-T. Samples were analysed using xPONENT 3.1 software (Luminex) by measuring the fluorescence of at least 100 beads per bead set per sample, using default settings. Results were expressed as median fluorescence intensity (MFI).

### 2.5. Establishing the optimal serum dilution

To determine the optimal serum dilution to be used in combination with the GneS3 (Gn) and Trx-N (N) beads, two-fold serial dilutions of characterized positive and negative sheep sera were assayed for IgG antibodies in singleplex (individual beads), duplex (Gn and N beads) or triplex (Gn, N and Trx beads) with PE-conjugated anti-sheep IgG (Jackson ImmunoResearch, Suffolk, UK), diluted in PBS-T. The serum dilution that resulted in the highest positive/negative (P/N) ratio was used for all further assays.

### 2.6. Detection of RVFV-specific antibodies

A set of 70 serum samples compiled for a RVFV ELISA ring trial (Kortekaas et al., in preparation) was assayed with the suspension array. This set contained RVFV-positive and RVFV-negative sera of sheep ($n = 57$) and cattle ($n = 13$). An additional set of 31 field sera (kindly provided by Dr. Christiaan Potgieter, Agricultural Research Council-Onderstepoort Veterinary Institute [ARC-OVI],