# Cloning and expression of Rift Valley fever virus nucleocapsid (N) protein and evaluation of a N-protein based indirect ELISA for the detection of specific IgG and IgM antibodies in domestic ruminants

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## Abstract

Serodiagnosis of Rift Valley fever (RVF) currently relies on the use of live or inactivated whole virus as antigens. The recombinant nucleocapsid (N) protein of RVF virus was tested for diagnostic applicability in an indirect enzyme-linked immunosorbent assay (I-ELISA), using sera from experimentally infected sheep ($n$ = 128), vaccinated sheep ($n$ = 240), and field-collected sera from sheep ($n$ = 251), goats ($n$ = 362) and cattle ($n$ = 100). The N-protein based I-ELISA performed at least as good as VN and HI tests.

In goat the diagnostic sensitivity (D-Sn) and specificity (D-Sp) of the I-ELISA was 100% when using the anti-species IgG conjugate. Using protein G as a detection system, the D-Sn and D-Sp in goats were 99.4% and 99.5%, in sheep field sera both 100%, in cattle 100% and 98.3%, respectively. The I-ELISA based on recombinant N-protein has the potential to complement the traditional assays for serodiagnosis of RVF. Advantages of the N-protein are its safety, stability and cost-effectiveness in use and production.

# Article Outline

# 1. Introduction

Rift Valley fever (RVF) is an arthropod-borne viral disease, which results in severe economic losses due to high mortality particularly in new-borne lambs and kids and abortion in adult sheep, goats and cattle. It causes mild to moderate febrile illness in humans but severe complications such as ocular sequelae, encephalitis or fatal haemorrhagic fever, may occur in a low proportion of infected individuals (Swanepoel and Coetzer, 1994). The most recent and first confirmed outbreaks of RVF among humans and livestock outside Africa, occurred in the Arabian Peninsula (Shoemaker et al., 2002), implying spread of infection into non-endemic areas most likely due to the fact that the causative agent, RVF virus (RVFV), is capable of utilizing a wide range of mosquito vectors (Turrel et al., 1998).

Considerable efforts have been made recently to develop nucleic acid techniques for rapid detection and identification of RVFV (Ibrahim et al., 1997 and Garcia et al., 2001). However, traditional and molecular procedures for diagnosis of RVF may be beyond the resources and capabilities of many laboratories, particularly in developing countries. Accurate diagnosis of RVF can be achieved when serological tests are used in combination with clinical observation and epidemiological history and/or when seroconversion to the virus is demonstrated.

The three most relevant methods for the detection of antibodies to RVFV are haemagglutination-inhibition (HI), virus neutralization (VN) tests and the enzyme-linked immunosorbent assay (ELISA). Although regarded as a gold standard, the VN test is laborious, expensive, and requires 5–7 days for completion. It can be performed only when a standardized stock of live virus and tissue culture facilities are available. Consequently, it is rarely used, and if so only in highly specialized laboratories worldwide. The HI test is sensitive but laborious and cross-reactive with other phleboviruses (Scott et al., 1986 and Swanepoel et al., 1986a). In addition, disadvantages of the classical diagnostic methods include health risk to laboratory personnel (Kitchen, 1934 and Smithburn et al., 1949), restrictions to their use outside RVF endemic areas (Scherer et al., 1980 and Barnard and Gerdes, 2000), their inability to distinguish between different classes of immunoglobulins, lack of standardization and unsuitability for screening large numbers of sera (Wright et al., 1993).

A highly sensitive and specific ELISA based on β-propiolactone inactivated and/or gamma-irradiated, sucrose–acetone-extracted RVFV antigen derived from tissue cultures or mouse brain has recently been developed and validated (Paweska et al., 1995, Paweska et al., 2003a and Paweska et al., 2003b). However, this type of RVFV antigen preparation has to be produced in a containment facility and it poorly binds to the ELISA plates. To overcome this problem a sandwich ELISA that utilizes immobilized antibodies to trap crude or semi-purified preparations of antigen can be used (Paweska et al., 2003a and Meegan et al., 1987).

Recombinant nucleocapsid proteins have successfully been used for the detection of antibodies to other viruses in the family *Bunyaviridae* (Kallio-Kokko et al., 1993, Marriott et al., 1994, Elgh et al., 1997 and Ciufolini et al., 1999). This paper describes the cloning, expression and purification of nucleocapsid protein of RVFV and its evaluation as an antigen in indirect ELISA (I-ELISA) for the detection of RVFV specific IgM and IgG antibodies in ruminant sera.

# 2. Materials and methods

### 2.1. Virus

Inactivated RVFV strain FVR 1976 was kindly supplied by Dr. J. Groen, Institute of Virology, The University Hospital Rotterdam, The Netherlands.

### 2.2. Construction of plasmids

Vector pET43b (Novagen) enables purification of fusion proteins by immobilised metal affinity chromatography. The vector was adapted to GateWay cloning (Invitrogen) and a second purification tag (a chitine binding domain—CBD) was included to enable additional purification of the recombinant protein if desired, after purification with immobilized metal ($Ni^{2+}$) affinity chromatography (IMAC). Using pTYB11 (New England Biolabs) as a template, the CBD gene was amplified by polymerase chain reaction and suitable restriction sites were introduced using the forward primer 5′-CGC GTC TAG *ACT AGT* GGT GCT AGC ACA AAT CC-3′ and reverse primer 5′-CGA CAT ATG ACC ACC ATG GCC ACC TTG-3′. The PCR product was digested with *Xba*I and *Nde*I (underlined in primer sequences) and ligated into the Gateway cassette of

pDEST17 (Invitrogen), digested with the same enzymes. The resulting plasmid was purified from carbenicillin and choramphenicol resistant colonies obtained after transformation of *E. coli* DB3.1 (Invitrogen) that is resistant to the toxic protein encoded by the *ccdB* gene present on the GateWay cassette of pDEST17. The purified plasmid was digested with *Spe*I (italics in the forward primer sequence) and *Hin*dIII. The fragment containing the CBD and GateWay cloning cassette was ligated into *Spe*I–*Hin*dIII digested pET43b. After transformation of DB3.1 cells, plasmid DNA was purified from selected colonies and sequenced to confirm that the correct plasmid, designated pET43GW, had been obtained. The plasmid was digested with *Spe*I, blunted with T4 DNA polymerase and, subsequently, digested with *Hin*dIII. The smaller fragment, containing the CBD sequence and GateWay cassette, was cloned into *Sma*I–*Hin*dIII digested pET43b to yield pETH2GW. Genes cloned into this vector are expressed with an additional hexa-histidine tag.

**2.3. Cloning and expression of the N-protein**

The RNA of RVFV was extracted using the RNeasy kit (Qiagen) and subjected to RT-PCR. Based on published sequences of the S segment (Gene Bank #9632365) two primers, the forward primer RVF-F2 (5′-GCC GCG CGG GAG CAT GGA CAA CTA TCA AGA CGT TGC GAT CC-3) and the reverse primer RVF-R (5′-GCG GCG GCG GGC CCC CTG GGC AGC CAC-3′) were designed to amplify the RVFV gene encoding the nucleocapsid (N) protein. The underlined sequences were not encoded by the virus, but were included as annealing sites for a second PCR performed with the forward primer GW2-F (5′-GGG GAC AAG TTT GTA CAA AAA AGC AGG CTT GGT GCC GCG CGG GAG C-3′) and the reverse primer GW-R (5′-GGG GAC CAC TTT GTA CAA GAA AGC TGG GTG CGG CGG CGG G-3′). This second PCR introduced the *attB1* and *attB2* sites, which enabled subsequent GateWay cloning. The PCR product was harvested from the low melting point agarose gel and inserted into the vector pDONR201 (Invitrogen) by the BP Gateway reaction performed according to the instructions of the manufacturer. After transformation of *E. coli* strain DH5α, plasmid DNA was purified from selected colonies and sequenced to check the cloned fragment. Subsequently, the fragment was subcloned into the expression vector pETH2GW by the LR Gateway

reaction. Carbenicillin resistant colonies obtained after transformation of *E. coli* DH5α were checked by PCR, using primers GW2-F and GW-R. Plasmid DNA of the resulting vector, designated pETH2-NP was purified from a culture grown from a PCR positive colony.

### 2.4. Expression and purification of the his6-tagged NusA-N-protein

The expression strain *E. coli* BL 21DE3 (RIL) Codon Plus (Stratagene) was transformed with pETH2-NP using carbenicillin and chloramphenicol resistance for selection. Twenty-five milliliter cultures of carbenicillin (25 μg/ml) and chloramphenicol (34 μg/ml) containing LB were incubated shaking overnight at 37 °C. Then the culture was diluted 1:20 in 500 ml LB containing the same antibiotics and incubated at 37 °C, with shaking, till the optical density at 600 nm (OD600) reached 0.6–0.9. At that time, expression of the NusA-N-fusion protein was induced by the addition of isopropyl-β-d-thiogalactopyranoside (IPTG) to a final concentration of 1 mM/l. The culture was incubated for an additional 4 h at 37 °C with vigorous shaking. Cells were harvested by centrifugation for 15 min at 5000 × *g*. The pellet was resuspended in 25 ml buffer B (20 mM Tris/HCl (pH 8.0), 500 mM NaCl) containing 0.1 mg/ml lysozym, and the suspension incubated for 30 min at room temperature. Subsequently 2.5 ml of a solution containing 50 mM EDTA, 10 mM 2-mercaptoethanol and 10% Triton-X100, was added. After three freeze-thaw cycles, 1.5 ml 0.5 M $MgCl_2$ and 3 μl benzonase (Novagen) were added to the lysate to break down the released DNA. Once the lysate was no longer viscous (approximately 15 min at room temperature) it was centrifuged at 5000 × *g* for 15 min. The supernatant was collected and the pellet (inclusion bodies) was washed once with buffer B containing 1% Triton-X100 and subsequently dissolved in buffer B containing 8 M urea and 20 mM imidazole. After incubation at room temperature for 25 min the solution was centrifuged at 5000 × *g* for 15 min at room temperature to remove insoluble material. The clear supernatant was purified on a column packed with $Ni^{2+}$—charged chelating Sepharose (Amersham). After washing the column with the starting buffer (20 mM Tris–HCl pH 8.0, 500 mM NaCl, 20 mM imidazole and 8 M urea), the sample was eluted with Buffer B containing 8 M urea and 50 mM EDTA and dialysed overnight at 4 °C against PBS to allow refolding of the protein. Any material

precipitated during the buffer exchange was removed by spinning at 5000 × *g* at 4 °C for 20 min. The resulting supernatant was used as antigen in ELISA. The protein concentration of the supernatant was 0.34 mg/ml as determined by ND-1000 Spectrophotometer (NanoDrop Technologies, Inc.). Samples taken during the purification process were subjected to SDS-PAGE analysis.

**2.5. Sera**

All test sera were obtained from the Special Pathogens Unit, National Institute for Communicable Diseases (SPU-NICD), South Africa.

Sera (*n* = 128) from eight sheep inoculated subcutaneously with 1 ml of cell culture supernatant fluid containing $10^{6.5}$ $TCID_{50}$/ml of the AR 20368 strain of RVFV isolated in 1981 from *Culex zombaensis* in South Africa (Paweska et al., 2003a), taken on days 0, 1–14, 21, 70 and 77 after inoculation and sera (*n* = 240) from 10 sheep vaccinated subcutaneously with 1 ml of tissue-culture preparation of modified live vaccine of the Smithburn strain of RVFV, taken on days 0, 1–18, and 20 after vaccination, were used to study the kinetics of antibody production and to compare the analytical sensitivity and specificity of the ELISA with those of VN and HI tests under control conditions.

A total of 713 field-collected sera from goats (*n* = 362), sheep (*n* = 251), and cattle (*n* = 100) was used. The sera were collected in East Africa (Kenya and Somalia) to monitor the 1997–1998 outbreak of Rift Valley fever in this region (Woods et al., 2002). ELISA results in field sera that were categorized as positive and negative in the VN test were used for the selection of cut-off values and determination of diagnostic accuracy.

2.6. Virus neutralisation and haemagglutination-inhibition tests

The virus microneutralization and haemagglutination-inhibition tests were conducted as described previously (Paweska et al., 2003b).

2.7. ELISA internal controls

Freeze-dried, gamma-irradiated serum controls were obtained from the SPU-NICD, SA. Selection, inactivation, and characterization of high, low and negative controls followed recommended guidelines for the preparation of reference standards for an ELISA (Wright et al., 1993 and Wright et al., 1997) and was described elsewhere (Paweska et al., 2003a).

### 2.8. Anti-ruminant immunoglobulin and recombinant protein G horseradish peroxidase (HRPO) conjugates

Using a checkerboard titration and standard direct ELISA procedures (Crowther, 1995), commercially available anti-species-Ig-HRPO conjugates were tested for their capacity to detect antibody bound to the recombinant N-protein of RVFV. Rabbit antiserum to the μ chain of sheep IgM (ICN Pharmaceuticals. Inc., Aurora, Ohio, USA) was selected for detection of IgM. For the detection of IgG two different anti-IgG-HRPO conjugates were used: rabbit anti-sheep IgG(H + L) (Zymed Laboratories, Inc., San Francisco, CA, USA) to detect sheep and goat IgG, and goat anti-bovine IgG(H + L) (Kirkegaard & Perry Laboratories, Inc., Gaithersburg, MD, USA) to detect cattle IgG. In addition, peroxidase-conjugated protein G (HRPO-(rec)Protein G, Zymed, USA) was used. This conjugate can be applied to detect IgG antibody of different species (Bjorck and Blomberg, 1987, Abdu-Zeid, 2002, Paweska et al., 2003b and Saegerman et al., 2004).

### 2.9. Procedures for the indirect ELISA (I-ELISA)

Immunoplates (Maxisorb, Nunc, Denmark) were coated directly with 100 μl stock recombinant N-protein diluted 1:200 for the IgM and 1:100 for the IgG I-ELISA in PBS pH 7.4 and incubated overnight at 4 °C. After washing three times with PBS containing 0.1% Tween 20 (washing buffer) the plates were blocked with 200 μl of 10% fat-free milk powder (“Elite”, Clover SA, Pty Ltd.) in PBS, then incubated in a moist chamber for 1 h at 37 °C and washed as described before. Control and test sera were diluted 1:400 in PBS containing 2% milk powder (diluting buffer) and 100 μl were added to the plates. Each test serum was tested in duplicate and the control sera in quadruplicate. After incubation in a moist chamber for 1 h at 37 °C, plates were washed 6 times with the washing buffer and 100 μl of the peroxidase conjugated anti-IgM (diluted 1:500) or anti-IgG (diluted 1:3000) or Protein G (diluted 1:4000) were added. Plates were incubated at 37 °C for 1 h and after washing six times, 100 μl 2,2′-azino di-ethyl-benzothiazoline-sulfonic acid (ABTS) substrate was added to each well. Subsequently plates were incubated in the dark at room temperature for 30 min. The reactions were stopped by the addition of 100 μl 1% sodium dodecyl sulfate (SDS), and the optical densities (OD) determined at 405 nm. The results were expressed as percentages of the high-positive

control serum (PP) using the formula: (mean OD of the test serum/mean OD of the high positive control) × 100.

Sera from experimentally infected and vaccinated sheep were tested for the presence of antibodies to RVFV using rabbit anti-sheep IgM and rabbit anti-sheep IgG, and sera from vaccinated sheep were additionally tested using protein G. The field serum panel was tested using protein G, in addition goat sera were tested using rabbit anti-sheep IgG.

2.10. Selection of cut-offs and estimation of diagnostic accuracy

ELISA cut-off values at 95% accuracy level were selected using the two-graph receiver operating characteristics analysis (TG-ROC) (Greiner, 1995 and Greiner et al., 1995). The following statistical approaches (Greiner and Gardner, 2000) were used to estimate: sensitivity (D-Se) = [Tp/(Tp + Fn)] × 100 and specificity (D-Sp) = [Tn/(Tn + Fp)] × 100; where Tp represents the true-positive sera Fn the false-negative sera, Tn the true-negative sera and Fp is the false positive sera.

# 3. Results

### 3.1. Cloning, expression and purification of the N-protein

Amplification of the RVFV gene encoding the N-protein by RT-PCR yielded a fragment of the expected size of approximately 800 bp. After addition of the GateWay cloning sites *attB1* and *attB2* by a second PCR round, the fragment was inserted into pDONR201 and subsequently transferred to the expression plasmid pETH2GW, yielding plasmid pETH2-NP. Sequence analysis demonstrated 100% homology with the published gene for the N-protein of RVFV (results not shown). Analysis by SDS-PAGE demonstrated that *E. coli* BL 21DE3 (RIL) Codon Plus cells transformed with pETH2-NP, expressed, considerable amounts of a 97 kDa protein (Fig. 1: lanes 2 and 3) after IPTG induction. The majority of the fusion protein was in the insoluble fraction (inclusion bodies) after cell lysis (Fig. 1: lanes 4 and 5). It was dissolved in 8 M urea and purified on $Ni^{2+}$ chelating sepharose, employing its two his6-tags. Most of the fusion protein bound to the column (Fig. 1: lanes 6 and 7) and was successfully eluted by the addition of EDTA (Fig. 1: lane 8). Once purified, the fusion protein remained soluble after urea removal by extensive dialysis against PBS (Fig. 1: lane 9).

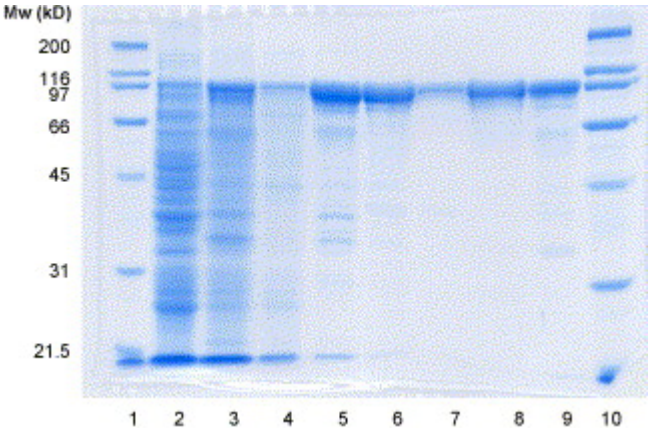


Fig. 1. SDS-PAGE analysis of the N-protein of RVFV. Lines: 1. Mw-marker; 2. not induced bacteria; 3. induced bacteria (total lysate); 4. soluble fraction; 5. insoluble fraction (inclusion bodies); 6. inclusion bodies solved in 8 M urea; 7. flow-through $Ni^{2+}$ column; 8. protein eluted from $Ni^{2+}$ column; 9. soluble protein after dialysis against PBS 10. Mw-marker.

### 3.2. Analytical evaluation of the N-protein based indirect ELISA for detection of IgM and IgG anti-RVFV

Comparison of the HI, VN and I-ELISA (using anti-species IgM and IgG conjugates) in RVF-experimentally infected sheep is shown in Fig. 2A–C, respectively. The ELISA and the VN test detected antibodies earlier than the HI. The dynamics of IgM and IgG responses in experimentally infected and vaccinated sheep using the recombinant N-protein I-ELISA are shown in Fig. 3 and Fig. 4, respectively. In experimentally infected sheep the IgM PP values started to rise from day 3 onwards and reached the maximum value at day 9. At day 77 the IgM PP values had returned to values recorded before inoculation. The IgG PP values started to rise at day 5 and were still high at day 77 (Fig. 3). In vaccinated sheep the IgM levels started to rise from day 4, reached the highest PP values at day 10 followed by their gradual decrease. The IgG PP values rose from day 6 onwards and were still high at day 34 after vaccination (Fig. 4).

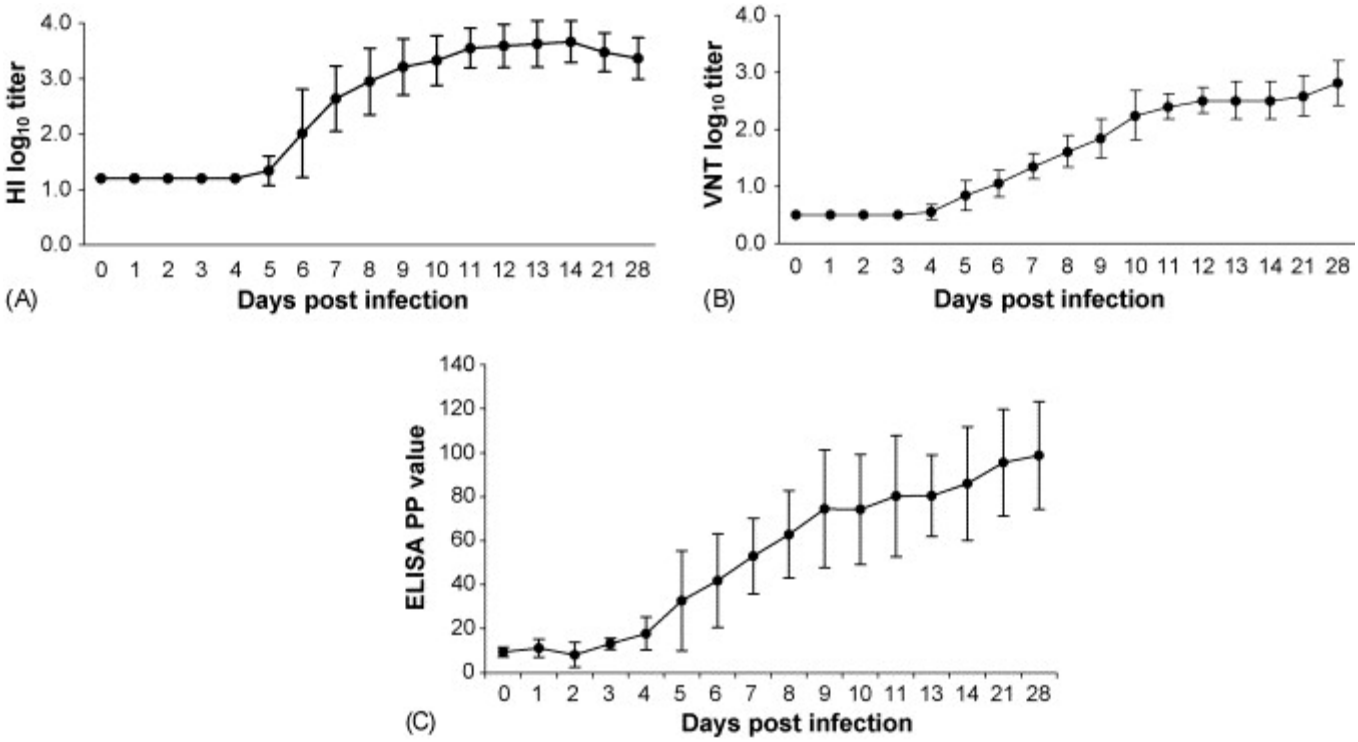


Fig. 2. (A) Mean ± 1S.D. Immune response of eight sheep experimentally infected with wild type of RVFV as measured by HI. (B) Mean ± 1S.D. Immune response of eight sheep experimentally infected with wild type of RVFV as measured by VN. (C) Mean ± 1S.D. Immune response of eight sheep experimentally infected with wild type of RVFV as measured by and recombinant N-based I-ELISA.

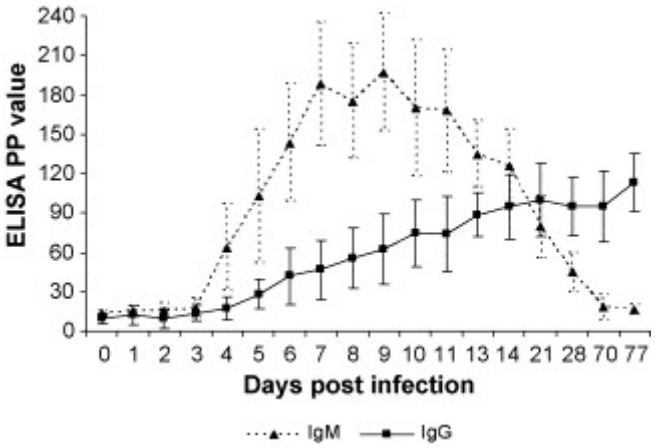


Fig. 3. Mean ± 1S.D. IgM and IgG responses in sheep ($n$ = 8) infected with wild type AR 20368 strain of RVFV using the recombinant N-protein I-ELISA.

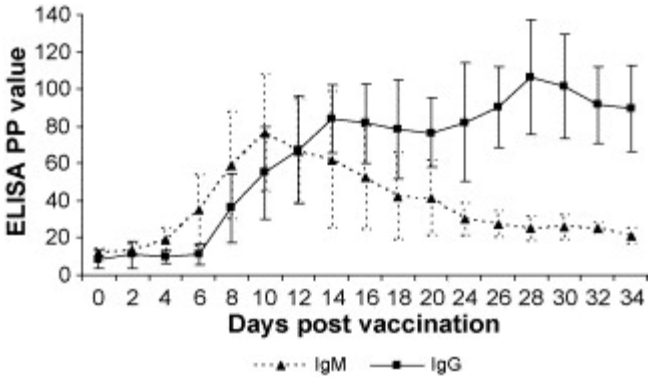


Fig. 4. Mean ± 1S.D. IgM and IgG responses in sheep ($n$ = 10) vaccinated with live-attenuated Smithburn strain of RVFV using the recombinant N-protein I-ELISA.

In the vaccinated sheep IgG responses were also measured by the N-protein based I-ELISA using protein G as a detection system, which produced higher PP values than the assay run with anti-species IgG (Fig. 5). However, the correlation coefficient between the two data sets was high (0.823).

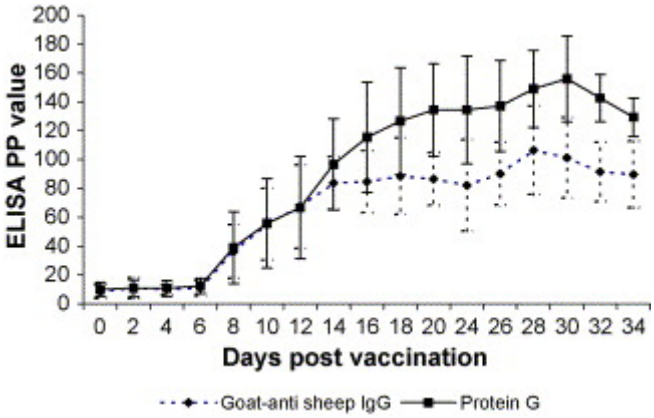


Fig. 5. Mean ± 1S.D. IgG responses in sheep ($n$ = 10) vaccinated with live-attenuated Smithburn strain of RVFV as measured by the N-protein based RVFV IgG I-ELISA using anti-sheep IgG and protein G HRPO conjugates.

### 3.3. Determination of cut-off values

Cut-off values derived from the TG-ROC analysis are given in Table 1. Optimisation of cut-off values using the TG-ROC analysis was based on the non-parametric programme option (Greiner, 1995) due to departure from a normal distribution of data sets analysed.

The optimal cut-off PP values were different for the distinct subpopulations of species tested. Example of the graphic presentation of the TG-ROC analysis is shown in Fig. 6.

Table 1.

Diagnostic accuracy of the Rift Valley fever recombinant N-based I-ELISA

| **Measure** | **Cattle** | **Goat** | **Sheep** |
|---|---|---|---|
| Cut-off[a] | 35.3 PP | 73.5 PP; **41.4PP**[b] | 48.3 PP |
| Sensitivity (%) | 100 (VNT+[c] = 42) | 99.4; **100** (VNT+ = 162; **VNT+ = 197**) | 100 (VNT+ = 51) |
| Specificity (%) | 98.3 (VNT−[d] = 58) | 99.5; **100** (VNT− = 200; **VNT− = 116**) | 100 (VNT− = 200) |

[a] Cut-off values expressed as a percentage positivity (PP) of an internal high positive serum control was optimized at 95% accuracy level by the TG-ROC analysis.

[b] Non-bold data: ELISA run with protein G; bold data: ELISA run with anti-sheep IgG.

[c] Number of sera tested positive in the virus neutralisation test.

[d] Number of sera tested negative in the virus neutralisation test.

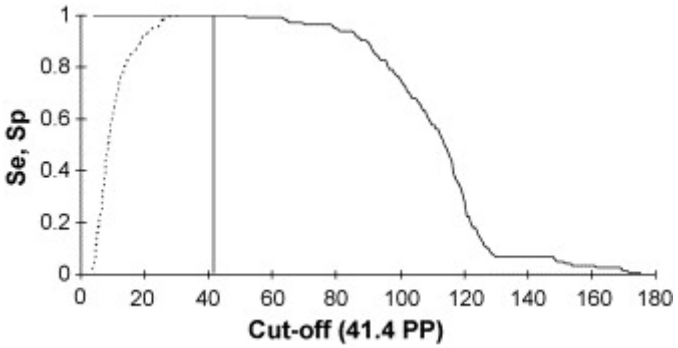


Fig. 6. Selection of cut-off for N-protein based RVFV IgG I-ELISA in goats by TG-ROC analysis. The insertion point of the sensitivity (Se, smooth line) and specificity (Sp, dashed line) graphs represents a cut-off PP value (41.4) at which the highest and equivalent test parameters are achieved at 95% accuracy level.

### 3.4. Diagnostic accuracy

Very high estimates of D-Sn and D-Sp were derived from the study data sets both when using anti-species IgG and protein G conjugates for each of the species (Table 1).

# 4. Discussion

Many countries where RVF is endemic, and where disease outbreaks occur in both ruminants and humans, do not have adequate containment facilities required to work with RVFV. For this reason and the fact that the virus has recently been shown to spread beyond its traditional endemic areas (Shoemaker et al., 2002) there is an urgent need for the development of diagnostic methods allowing for safe and cost effective production and application of RVF immunoreagents. The N-protein of RVFV was chosen as an antigen because it was shown to be the immunodominant protein in other members of the *Bunyaviridae* family (Swanepoel et al., 1986b, Vapalahti et al., 1995 and Schwarz et al., 1996).

The use of unpurified whole virus preparations in the I-ELISA (Forghani and Schmidt, 1979) gave rise to high background absorbance and purification of the virus is laborious and expensive. Recombinant antigens lack infectivity and have shown to be very stable (Maree and Paweska, 2005) which makes them suitable for wide distribution in ELISA kit format. In the present study the I-ELISA using HRPO-conjugated anti-species IgM, IgG and protein G were used to assess the recombinant N-protein as potential diagnostic immunoreagent for serodiagnosis of RVF.

In the pETH2-NP vector the gene for the N-protein, confirmed by sequence analysis, was cloned in-frame with gene segments encoding the NusA protein, the Chitin binding domain (CBD) and two hexa-histidine-tags.

Good solubility as previously shown for proteins expressed in *E. coli* as a fusion with the NusA protein (De Marco et al., 2004) could not be shown in the present approach. However, the CBD and his6-tags allowed purification of the fusion protein dissolved in 8 M urea on columns of immobilised $Ni^{2+}$ ions that resulted in a soluble fraction that could be used in ELISA. The molecular mass of the 97 kDa recombinant protein produced by *E. coli* BL 21 DE3 (RIL) Codon Plus cells transformed with pETH2-NP (Fig. 1: lanes 2 and 3) corresponded well to the calculated mass of NusA-NP fusion

protein. Results of the N-protein I-ELISA indicated that the purity of the recombinant protein purified by IMAC only was sufficient to obtain very high sensitivity and specificity, thus additional purification was not necessary.

Results obtained with sera from experimentally infected and vaccinated sheep show high analytical sensitivity of the N-protein I-ELISA in detection of RVFV specific antibodies. The I-ELISA detected RVFV specific IgG earlier than the HI test, and its sensitivity was comparable to the VN test. Results in this study were similar to those obtained by Paweska et al. (2003b) using an indirect or a sandwich ELISA (Paweska et al., 2003a) based on inactivated sucrose–acetone extracted antigen.

The diagnostic accuracy of the N-protein based I-ELISA to correctly classify animals as infected or uninfected was assessed by testing field-collected goat, sheep and cattle sera known to be positive or negative in the VN test. Very high estimates of D-Sn and D-Sp of the assay were obtained when using both anti-species IgG and protein G conjugates. In goat the D-Sn and D-Sp was 100% when using the anti-species IgG conjugate. Using protein G as a detection system, the D-Sn and D-Sp in goats were 99.4% and 99.5%, in sheep field sera both 100%, in cattle 100% and 98.3%, respectively. There was a good degree of correlation (0.823) between results obtained with the N-protein based I-ELISA using anti-species IgG and protein G in vaccinated sheep the latter showing lower diagnostic accuracy. However, the use of protein G in I-ELISA has the advantage of detecting anti-RVFV antibodies in different ruminant species with one set of reagents. This would provide a very practical diagnostic tool, especially during outbreaks of RVF involving different domestic and wild (ruminant) species and humans.

Central to any serological assay is determination of the diagnostic threshold or cut-off, for which appropriateness of data used is important as this impacts on D-Sn and D-Sp (Jacobson, 1998). The TG-ROC analysis provides a simple graphical means of evaluating sensitivity and specificity and allows selection of cut-off values to obtain the desired level of accuracy (Greiner, 1995). In the present study, the sera used for this evaluation were from animals with unknown vaccination or infection status. Therefore, the virus neutralisation test (current gold standard) was used to categorize individuals according to their RVF infection status. It is important to note that infection with RVFV induces life long virus neutralising immunity in animals (Barnard, 1979), and that there is no

evidence of serological subgroups or major antigenic variation between virus isolates of disparate temporal or geographic origins (Swanepoel and Coetzer, 1994). Antigenic cross-reactivity studies in sheep (Swanepoel et al., 1986b) and field studies in cattle (Swanepoel, 1976) failed to provide evidence that other African phleboviruses could hamper the serodiagnosis of RVF.

The estimates of diagnostic accuracies of the N-protein based I-ELISA are similar to those reported previously (Paweska et al., 2003b) using the sucrose–acetone extracted antigen for ELISA. Hence, the results presented here confirm the potential value of the recombinant N-protein as an antigen in I-ELISA for detection of specific anti-RVFV antibodies. Additional advantages of the N-protein are its safety, stability and cost-effectiveness in use and production.

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