Development and characterization of monoclonal antibodies against Rift Valley fever virus nucleocapsid protein generated by DNA immunization

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Key words: RVF, nucleoprotein, competitive ELISA, RVFV lineages

Introduction

Rift Valley fever virus (RVFV) is a mosquito-borne bunyavirus affecting several species of ruminants and man. This virus causes severe epizootics/epidemics in sub-Saharan African countries, and has been traditionally associated with heavy rainfall seasons, favoring infected floodwater mosquito populations to expand and disseminate the virus to livestock and secondary transmission vectors.1 Since RVF has been reported outside subtropical African countries, its possible spread and emergence in northern countries, including those of the Mediterranean basin is now a cause for concern. This circumstance is aggravated since RVFV replication competent mosquito vectors can be found in many European countries, perhaps favored by novel climatic conditions.2,3 Implementation of surveillance systems based on routine diagnostic methods might contribute in avoiding epizootic spread of the disease in susceptible areas.

The virus is transmitted to livestock and humans by mosquito bites or aerosol spills, i.e., when tissues or blood from infected animals are manipulated. The disease caused by RVFV infection in livestock is characterized by acute hepatitis and massive abortion and mortality rates in young animals.4,5 In man, the disease may not be diagnosed since symptoms are usually related with a self-limited febrile illness, but severe complications may occur including retinal degeneration, encephalitis or even hemorrhagic fever.6,7 The virus replicates efficiently in many vector species as well as in a number of different vertebrate species, including wildlife and other experimentally infected animals, whose role in the epidemiology of the disease remains obscure.8 Therefore specific immunodiagnostic assays to test the presence of anti-RVFV antibodies in non-livestock species may be difficult and laborious, relying on neutralization assays.9 Recently, alternative methods for detection of antibodies against RVFV in several species, including wild ruminants, have been developed based on inhibition/competition ELISA assay.10,11

RVFV has a single-stranded, tripartite RNA genome composed of a large (L), medium (M) and small (S) segments. While the L segment encodes an RNA-dependent RNA polymerase, the M segment encodes four proteins, two mature envelope glycoproteins (G1/Gc and G2/Gn), involved in virus-cell interaction, along with two non structural proteins of 14 and 78 kDa. The S segment of RVFV uses an ambisense strategy to code for the nucleocapsid protein, N, in the antigenomic sense, and for a non structural protein, NSs, in the genomic sense.12 The RVFV N protein, as in the case of many other nucleoproteins of negative strand RNA viruses, acts as a scaffold for the packaging of viral genomic, RNA(+) segments. An essential requirement for the controlled RNA packaging in mature particles is the oligomerization of N monomers to provide the adequate template for RNA binding.13 As is the case with other viral examples,14,15 the RVFV nucleocapsid protein is highly immunogenic. Antibodies against N are readily detected early after infection and in convalescent individuals, providing robust basis for diagnostic detection of the disease.

This paper describes the generation of monoclonal antibodies directed to immunogenic nucleoprotein N epitopes of Rift Valley fever virus (RVFV), and their application in diagnostics, both for antibody detection in competitive ELISA and for antigen capture in a sandwich ELISA. Monoclonal antibodies (mAbs) were generated after DNA immunization of Balb/c mice and characterized by western blot, ELISA and cell immunostaining assays. At least three different immunorelevant epitopes were defined by mAb competition assays. Interestingly, two of the mAbs generated were able to distinguish between RVFV strains from Egyptian or South African lineages. These monoclonal antibodies constitute useful tools for diagnosis, especially for the detection of serum anti-RVFV antibodies from a broad range of species by means of competitive ELISA.
As described here, we generated a set of monoclonal antibodies (mAbs) directed against immunogenic N epitopes by DNA immunization in order to mimic the host’s immune response to RVFV antigens and ensuring the generation of a repertoire of antibodies similar to that of serum from convalescent animals. The mAbs were characterized and, particularly, their ability to bind to the immunogenic epitopes on the nucleoprotein N was tested in competition assays. The use of this mAb-based approach may improve the current techniques for the assessment of the immune status of many different animal populations at risk of RVFV infection.

**Results**

**Expression of RVFV nucleoprotein N in mammalian and bacterial cells.** A recombinant Trx-N fusion protein from RVFV was detected using SDS-PAGE in the form of two major polypeptides of 29 and 51 kDa as judged by Coomassie staining (Fig. 1A and columns 1, 2) of pTrx-N transformed bacterial cell extracts. Detected Trx-N protein was further purified using a nickel-NTA purification column. Immunoblotting using an anti-histidine tag mAb detected two polypeptides of around 51 kDa and 29 kDa, respectively (not shown). However, only the 51 KDa polypeptide was detected by western blotting when a rabbit anti-RVFV was used (Fig. 1A and columns 3, 4), indicating that the 29 KDa polypeptide lacked the epitope(s) of the larger protein recognized by the antiserum. This suggests that the smaller polypeptide may be an incomplete or degraded protein product. Identical result was obtained by western blot, when a sheep anti-RVFV was used instead (Fig. 1A). Expression of the RVFV nucleoprotein gene coded by pCMV-N plasmid was detected at 24 hours post-transfection by immunocytochemistry of cell extracts using a rabbit polyclonal anti-RVFV serum. A strong cytoplasmic immunostaining was detected in transfected cells but not in negative controls transfected with pCMV-GFP (not shown). To analyze the size of the expressed polypeptide, an immunoprecipitation assay was conducted, using either infected or transfected cell extracts. A similar sized polypeptide was immunoprecipitated from pCMV-N transfected and RVFV MP12 infected cell extracts, indicating the proper translation and processing of the cloned nucleocapsid gene. Interestingly, higher amounts of nucleoprotein N were detected in the supernatant of transfected cells compared to those found in the supernatant of MP12 infected cells (Fig. 1B).

**Figure 1.** Expression and detection of nucleoprotein N. (A) Expression of recombinant Trx (1) and Trx-N fusion protein (2) in E. coli cells as detected by Coomassie staining of Ni-NTA column purified bacterial extracts, or by anti-RVFV rabbit (3) or sheep (4) hyperimmune sera. (B) Immunoprecipitation of BHK21 cell supernatants (S) and cell lysates (CL) with a rabbit anti-RVFV polyclonal serum. (1) MP12 infected cells, (2) mock infected cells, (3) pCMV transfected cells and (4) pCMV-N transfected cell extracts. (C) Immunoprecipitation of supernatant (S) and cell lysates (CL) of MP12 infected BHK21 cells using serum from mice immunized with pCMV-N (2 and 3) or sheep anti-RVFV hyperimmune serum (1). Mr: relative molecular mass in kilodaltons.

**Production and characterization of anti-N mAbs.** Once expression of nucleoprotein N in mammalian cells was confirmed, the pCMV-N construct was used to immunize Balb/c mice in order to generate mAbs against the RVFV nucleocapsid protein. The mouse sera were able to immunoprecipitate a polypeptide of the expected size from RVFV infected cell extracts, indicating the proper translation and processing of the cloned nucleocapsid gene. Interestingly, higher amounts of nucleoprotein N were detected in the supernatant of transfected cells compared to those found in the supernatant of MP12 infected cells (Fig. 1B). Undiluted hybridoma cell supernatants were screened for anti-RVFV-N specific antibodies by antigen capture or indirect recombinant Trx-N ELISA. After subsequent cloning and further expansion and stabilization of the positive hybridomas, six different hybridoma cultures were
lower or null reactivity. This differential reactivity is suggestive of a differential epitope exposure on the N-protein depending on the assay performed.

The differential epitope recognition by these two mAbs was also illustrated by western blot assays (Fig. 2A). While most selected on the basis of their growth kinetics and stability, and their supernatants titrated using both antigen capture and indirect recombinant Trx-N ELISA assays (Table 1). All six mAbs showed reactivity in antigen capture ELISA, but when using indirect recombinant Trx-N ELISA, mAbs D7E8 and D7D8 showed

![Table 1. Characterization of mAbs anti-RVFV nucleoprotein](https://example.com/table1)

| Immunogen       | Name   | Rec ELISA | Capture ELISA | WB  | ICC | IIF | Ig isotype |
|-----------------|--------|-----------|---------------|-----|-----|-----|------------|
| DNA only        | D7D8   | ≤3        | 27            | -   | +   | +   | IgG2a/K    |
| DNA only        | D9D11  | ≥2187     | ≥2187         | +   | +   | +   | IgG2a/K    |
| DNA only        | D7E8   | -         | 27            | -   | +   | +   | IgG2a/K    |
| DNA only        | A9F12  | 2187      | ≥2187         | +   | +   | +   | IgG2a/K    |
| DNA + protein   | F1D11  | 27        | 81            | +   | +   | +   | IgG2a/K    |
| DNA + protein   | F1B10  | 243       | 729           | +   | +   | +   | IgG2a/K    |

*a* mean reciprocal dilution of three sample replicates which generates an OD_{450 nm} reading of 1.5. *c* cells infected with RVFV MP12 strain. WB, western blot; ICC, immunocytochemistry; IIF, indirect immunofluorescence.

**Figure 2.** Detection of nucleoprotein N in infected cells. (A) Western blot of MP12 infected cellular extracts and immunoblotting with six different mAb supernatants. C+: mouse hyperimmune anti-RVFV serum; C-: preimmune mouse serum. Relative molecular mass (M_r) is given in kilodaltons. (B) Immunofluorescence of MP12 and South African RVFV strain AR20368 infected Vero cells using the six mAb supernatants tested in (A). (C) Immunoprecipitation of nucleoprotein N expressed in BHK-21 cells infected with ZH548-MP12 or with South African virulent isolates, using mAbs D7D8 (1) and D7E8 (2). A polyclonal mouse anti-RVFV serum (3) was used as positive control. Detection of the immunoprecipitated antigen was performed by western blot using the purified D9D11 mAb conjugated to peroxidase.
two mAbs were still able to label cells infected with the Egyptian origin MP12 strain by indirect immunofluorescence (IIF) (Fig. 2B, lower). The staining pattern was consistently similar in all mAbs tested, characterized by a homogeneous distribution across the cytoplasm of the infected cell.

Interestingly, by immunofluorescence assay (IFA), both D7E8 and D7D8 mAbs failed to react with BHK-21 cells infected with the AR20368 RVFV isolate from South African origin (Fig. 2B, upper). Identical results were obtained with the other three South African isolates (not shown). Similar results were obtained when the antigen capture ELISA was set up using the South African isolates instead of the MP12 strain as the source of viral antigen, confirming that D7D8 and D7E8 mAbs were specific only for the MP12 attenuated strain (data not shown). Moreover, immunoprecipitation and subsequent western blot assay confirmed the lack of binding of both D7D8 and D7E8 mAbs to the RVFV N

**Table 2.** Percent of binding inhibition of labelled antibodies in competitive ELISA

| Competitor mAb | A9F12-HRP | F1D11-HRP | D9D11-HRP |
|----------------|-----------|-----------|-----------|
| A9F12          | 92%       | 73%       | 89%       |
| F1D11          | 88%       | 95%       | 95%       |
| D9D11          | 91%       | 71%       | 89%       |
| D7D8           | 43%       | 1%        | 10%       |
| D7E8           | 49%       | 0%        | 8%        |
| F1B10          | 86%       | 75%       | 91%       |

Figure 3. (A) Conservation plot of the 1–60 (upper) and 121–180 (lower) amino acid regions of the RVFV nucleoprotein N from several Egyptian lineage isolates and the South African strains used in this study. Only the regions of the primary sequence in which amino acid changes were found are showed. (B) Immunofluorescence of transfected BHK-21 cells with pCMV-N<sub>AR20368</sub> and pCMV-N<sub>MP12</sub> constructs. Expression of N protein was detected with the indicated mAbs.

anti-N mAbs were able to react to nucleoprotein N in western blotting, mAbs D7E8 and D7D8 failed to recognise the denatured nucleoprotein, thus suggesting that their corresponding epitopes might be conformation dependent. In contrast, these
protein expressed upon infection of Vero cells with South African isolates, but not when infected with the Egyptian RVFV strain (Fig. 2C). To identify amino acid positions potentially involved in the differential binding of these mAbs, the N ORFs from both the MP12 and the four South African strains were sequenced. Noticeably, the MP12 strain carried G159, while the rest of South African isolates sequenced bore E159 (Fig. 3A).

To exclude the possibility that the observed lack of reactivity was due to lower expression levels or other factors influencing the accessibility of N protein epitopes expressed by the South African strains, the complete N ORF from both the AR20368 and MP12, differing only in the amino acid residue 159 were expressed in BHK-21 cells upon transfection with plasmids pCMV-N<sub>AR20368</sub> and pCMV-N<sub>MP12</sub>, respectively. Immunofluorescence data confirmed the previous results, and excluded the lack of reactivity against N<sub>AR20368</sub> as a consequence of lower expression levels upon infection with the South African isolates (Fig. 3B). These data suggest that the amino acid residue 159 might influence the binding of mAbs D7E8 and D7D8. Furthermore, a comparison of all RVFV nucleoprotein sequences available at GenBank showed that only Egyptian strains maintained this non-conserved substitution (Fig. 3A). These data would support the value of these mAbs as a diagnostic tool to discriminate strains from the Egyptian lineage.

A further epitope mapping of these antibodies was performed by competitive assay among the mAbs. As shown in Table 2, these mAbs define at least three antigenic sites on the nucleoprotein N based on the percent of inhibition, ranging from high (≥70%), low (20–50%) or no competition at all (≤10%). Following this criteria, a first antigenic region may be defined by mAb A9F12. As expected, mAbs D7D8 and D7E8 defined a third antigenic region in nucleoprotein N. Interestingly, these two mAbs could be further differentiated on the basis of their competition with mAb A9F12, suggesting a close relationship among these two antigenic regions. Unfortunately, the specificity of mAbs D7D8, D7E8 and F1B10, was affected when subjected to purification procedures based on acid pH elution, precluding further peroxidase labelling.

**Analysis of mAbs in a competitive binding ELISA assay.** Since all six antibodies demonstrated binding specificity to the RVFV nucleoprotein N in antigen capture ELISA, we then analyzed whether their binding epitopes were also recognized by convalescent sheep serum, in order to design and develop a serological assay based on these mAbs. A preliminary study with different positive sera, including reference sheep field sera, experimental vaccine sera or a hyperimmune rabbit polyclonal serum was carried out. Sera were set to compete for the binding of each mAb to the viral antigen captured in ELISA. Four-fold dilutions of these competitive sera were assayed, ranging from 1/10 to 1/160. Figure 4 shows the mean percentage of binding inhibition of each mAb after incubation with different anti-RVFV sera. All serum dilutions assayed were able to inhibit, with varying degrees, the binding of all the mAbs assayed. In all cases, a clear difference in the percent inhibition values could be established for the negative serum with respect to the positive sera tested. These results indicate that the epitopes recognised by our mAbs are represented in the antibody repertoire generated following immunization or natural/experimental infections.

To further estimate a cut off value for discrimination of positive and negative samples, RVFV positive (n = 12) and negative (n = 41) sera were assayed in competition with all six mAbs. The distribution of the inhibition percentages observed for positive

*www.landesbioscience.com mAbs*
The epidemic potential of Rift Valley fever virus is a matter of international concern for livestock trade and human health. Currently, the risk of unexpected RVF outbreaks in non-endemic areas may be increased by changing climate conditions, including warming oceanic-derived rainfall and increased temperatures, allowing for the establishment of competent vector species in naïve or virgin areas. Therefore reliable, fast and versatile diagnostic assays are needed. The RVFV induces a potent antibody response against the nucleoprotein N, but to a much lesser extent to both mature structural glycoproteins. Since the nucleocapsid protein is highly conserved among all sequenced RVFV isolates, this antigen was an obvious choice for newer developments in diagnostic procedures. A recombinant nucleoprotein N-based ELISA test has been recently reported, showing more sensitivity than classical neutralization and haemagglutination-inhibition tests in detecting antibody responses against RVFV infection. This method avoids the use of live virus production for subsequent use as ELISA antigen, which is of interest in disease-free areas; however, the use of recombinant antigens may not ensure the proper folding of the protein, thus compromising the sensitivity of an ELISA assay. Two mAbs (D7D8 and D7E8) and negative samples indicates that the percentage of inhibition of positive sera is ≥60% whereas inhibition by negative sera is ≤40%. Therefore, a cut off value in the 40–60% range interval could be assigned for any of the mAbs tested in the competition assay (Fig. 5). Two mAbs, A9F12 and F1D11, were also selected for further characterization due to the high yield of immunoglobulin production by their corresponding hybridoma cells. These mAbs were also evaluated for their ability to concentrate viral antigen from non-purified samples. Figure 6A shows that both F1D11 (upper) and A9F12 (middle) were able to capture viral antigen in a similar way to the rabbit anti-RVFV hyperimmune serum (lower) as judged by the specific binding of hyperimmune sheep or rabbit anti-RVFV sera.

To check the efficacy of a capture assay in a sandwich ELISA or in competitive assay, purified A9F12 was used to capture virus antigen from MP12 infected cell culture. For detection, A9F12 was peroxidase labeled. In Figure 6B, the ability of A9F12 mAb to discriminate positive sera in a competitive ELISA is shown. The results obtained were very similar to those obtained when rabbit polyclonal sera was used as a capture antibody, indicating the reliability of this format. In particular, lower inhibition values for negative serum are shown when the mAb is used for capture.

Discussion

The epidemic potential of Rift Valley fever virus is a matter of international concern for livestock trade and human health. Currently, the risk of unexpected RVF outbreaks in non-endemic areas may be increased by changing climate conditions, including warming oceanic-derived rainfall and increased temperatures, allowing for the establishment of competent vector species in naïve or virgin areas. Therefore reliable, fast and versatile diagnostic assays are needed. The RVFV induces a potent antibody response against the nucleoprotein N, but to a much lesser extent to both mature structural glycoproteins. Since the nucleocapsid protein is highly conserved among all sequenced RVFV isolates, this antigen was an obvious choice for newer developments in diagnostic procedures. A recombinant nucleoprotein N-based ELISA test has been recently reported, showing more sensitivity than classical neutralization and haemagglutination-inhibition tests in detecting antibody responses against RVFV infection. This method avoids the use of live virus production for subsequent use as ELISA antigen, which is of interest in disease-free areas; however, the use of recombinant antigens may not ensure the proper folding of the protein, thus compromising the sensitivity of an ELISA assay. Two mAbs (D7D8 and D7E8)
validated for sheep, goats, cattle and humans, but their usefulness for antibody detection in other susceptible ruminant species, such as camels or wild ruminants should be carefully addressed. The availability of serological assays in which samples can be tested irrespective of their origin, such as the competitive ELISA designed here, may facilitate the determination of the role of other non-livestock species in the RVF enzootic cycle.

In our study, we show the ability of anti-N mAbs to compete with the binding of immune serum to nucleoprotein epitopes exposed by antigen capture ELISA. The ability of the described mAbs to compete is remarkable and may be indicative of the presence of immunogenic epitope(s) located in the nucleocapsid protein. The DNA based immunization strategy used for the generation of mAbs may have contributed to the proper exposure of surrogate determinant(s) mimicking the natural antigens. Studies are in progress to determine the exact location of the corresponding epitope(s). Preliminary data indicate that these epitopes map

![Figure 6. (A) Performance of mAb F1D11 and A9F12 as capture antibodies in ELISA. Positive (experimentally vaccinated sheep, shaded bars and rabbit anti-RVFV hyperimmune serum, open bar) and negative sheep sera (1/120 dilution) were tested in ELISA for their ability to bind to mAb captured viral antigen from infected cell culture supernatants. An anti-sheep HRP-conjugated (diluted to 1/6,000) was used for detection of the immunocomplexes. (B) Competitive ELISA using either purified A9F12 mAb or rabbit anti-RVFV serum as capture antibodies. Experimental sera from vaccinated sheep were used to compete the binding of A9F12-HRP labelled antibody.](image-url)
at the second half of the N polypeptide, when expressed in a baculovirus system (H. Boshra, et al. unpublished observations). Previous reports indicated the generation of monoclonal antibodies against structural elements of the virus particles.19-25 Thus, antibodies against glycoproteins were generated, along with nucleoprotein specific mAbs.25 These antibodies were generated after immunization with inactivated virus preparations, therefore multiple mAb specificities were found. Our immunization scheme ensured a directed antibody response towards nucleoprotein N.

The two highest producing hybridomas (A9F12 and F1D11) were selected for further downstream applications. In particular, these mAbs could efficiently capture virus antigen in a similar manner as the polyclonal rabbit serum used in this study as well as for detection of virus in tissues from experimentally infected animals (R. Martín-Folgar, et al. unpublished observations). The use of mAbs in diagnostic tests offers obvious advantages over methods based on the use of polyclonal serum either as capture or detecting antibodies. Therefore, these mAbs may constitute useful tools for improving current tests for identification of RVFV in biological samples and for studies on virus pathogenesis and/or virus cycle functions.

The development of these mAbs might allow for the identification of virus from different geographical origins. In particular, two of the mAbs generated can readily recognise the Egyptian derived mutagenized strain MP12, but not four isolates from South African origin. Results obtained with these mAbs (D7D8, D7E8) in transfection assays with pCMV-N2036827 would confirm their capability to distinguish between Egyptian and South African strains of RVFV. Due to the high degree of conservation displayed by N sequences, this was completely unexpected. Although more viral strains should be tested to confirm this result, it is reasonable to suggest that the location of the epitope(s) defined by D7E8 and D7D8 might be related by residue 159. Interestingly, this is not a conserved amino acid substitution since transition from N159H to D7D8 might be related by residue 159. This change in the function is not a conserved amino acid substitution since transition from N159H to D7D8 might be related by residue 159. Interestingly, this is not a conserved amino acid substitution since transition from glycine to glutamic acid implies a significant increase in polarity and charge as well. The relevance of this change in the function of N should be further explored.

The data presented here show that a competitive, mAb-based ELISA (C-ELISA) assay can be designed for detection of RVFV antiserum using mAbs directed against the immunogenic nucleocapsid protein. Further validation of this C-ELISA is required, using a larger number of positive and negative serum samples from animal or human origin.

Material and Methods

Cells and virus. BHK-21 and Vero cells were maintained in Dulbecco’s modified minimum essential medium (DMEM) (Invitrogen) containing 10% fetal calf serum (FCS). Penicillin (100 U/ml), streptomycin (100 µg/ml) (Invitrogen) and non-essential amino acids were added to the media. The myeloma cell line SP2/0-Ag14 was grown in RPMI medium supplemented with 10% FCS (Sigma). The mutagenized RVFV vaccine strain ZH-548 MP12,26 and 4 virulent South African strains of RVFV, designed AR 20368, AN 1830, 252/75 and 56/74 were propagated in BHK-21 cells at a multiplicity of infection (m.o.i.) of 0.2. When cytopathic effect (cpe) was extensive, cell monolayers were harvested and supernatant fluids were clarified at 3,000 xg for 30 minutes.

Plasmid construction and expression of recombinant antigens. A DNA SalI/XhoI fragment encoding the nucleoprotein N ORF was excised from a previously generated pXL-TOPO-N construct,27 digested, and subsequently cloned into pET32a (Novagen) to generate a thioredoxin (Trx) fused N ORF (pTrx-N), using the following forward and reverse primers: 5’-ACG CTG CGT CAT GGA CAA CTA TCA AGA GCT TGC GAT CCA-3’ and 5’-CCG CTC GAG GGC TGC TGT CTT GTT GTA AGC CTG AGC GCC TGC TG-3’. The generation of the pCMV-N construct has been described previously.27 The identity and proper insertion of DNA fragments was confirmed by automated DNA sequencing on both strands. For bacterial expression analysis the plasmid was transformed in BL21 cells and expression was induced by addition of 1 mM isopropyl-β-thiogalactoside (IPTG) to the culture medium with constant shaking for 4 hours. Bacterial cultures were then centrifuged, lysed with lysozyme (2 mg/ml) in phosphate buffered saline (PBS) and purified using Ni-NTA resin (Qiagen). Samples were analyzed by SDS-PAGE. Purified proteins from bacterial extracts were visualized after Coomassie staining and their identity confirmed by western blotting using both polyclonal sera against RVFV, or anti-histidine tag mAbs. Monolayers of BHK-21 cells were transfected with the pCMV-N construct using Lipofectamine reagent (Invitrogen) using manufacturer’s instructions. Expression of transfected cells was confirmed by immunocytochemistry analysis using a rabbit polyclonal anti-RVFV serum. A pCMV vector expressing the green fluorescent protein (pCMV-GFP) was used as a negative expression control to assess transfection efficiencies.

Immunization of Balb-c mice and hybridoma cell production. The pCMV-N construct was used to carry out immunization of Balb-c mice. Two mice were inoculated, by the intramuscular (i.m.) route, with 100 µg of endotoxin free DNA in 100 µl of saline solution. Mice received a total of three doses, every two weeks. Ten days after the last inoculation, mice were bled and the sera were analyzed by immunoprecipitation and/or ELISA. An additional DNA boost was given to one mouse while the other received a subcutaneous boost with an emulsion of recombinant N protein (70 µg) in Freund’s incomplete adjuvant and a last boost, by the intraperitoneal (i.p.) route, of a Percoll-purified MP12 virus preparation (125 µg). Three days later, spleens were aseptically removed for hybridoma preparation following standard procedures.28 The positive wells were initially screened by antigen capture and recombinant ELISA and further cloned. Immunoglobulin isotyping was performed using the Monoclonal Antibody Isotyping Kit (Pierce).

Antibody purification and labelling. The supernatants of hybridoma cultures were collected, clarified by centrifugation (20,000 x g, 20 min, 4°C) and filtered through a 0.22 µm membrane filter (Millipore). NaCl was added to reach 3 M, equilibrated in 50 mM sodium borate/boric acid buffer, pH 8.9. The resulting solution was loaded on an Econo-Pac protein-A affinity column (Bio-Rad,) pre-equilibrated with five column volumes of NaCl 3 M, 50 mM sodium borate/boric acid buffer. Bound
antibodies were eluted stepwise with five column volumes each of PBS and sodium citrate/citric acid buffer 75 mM pH 3.2. Both elutes were collected separately, neutralized to pH 7.2 with HEPES buffer when needed, and analyzed by SDS-PAGE. The antibody containing fractions were pooled and stored for further use.

Labelling of purified Ig was performed as follows. Briefly, horseradish peroxidase (HRP) (Sigma) was dissolved at 50 mg/ml in water. To this solution sodium acetate/acetic acid buffer pH 5.5 (final concentration 15 mM) and sodium periodate (final concentration 3 mM) were added sequentially. The oxidation was allowed to proceed for 7 minutes at 37°C in the dark and was stopped by adding EPPS buffer pH 7.5 (final concentration 25 mM) followed by sodium arsenite (final concentration 13 mM). This solution was mixed with an equal volume of affinity-purified antibody solution (1 mg/ml of immunoglobulin in 75 mM sodium cyanoborohydride, 0.1 M HEPES pH 7.5) and incubated for 12 hours at 37°C. The conjugation reaction was stopped with ethanolamine (final concentration 6 mM), incubating 15 min at room temperature, and then sodium borohydride (final concentration 2 mM). The entire reaction mixture was loaded on a gravity fed Econo-Pac 10 DG Gel filtration column (Bio-Rad), which was eluted with 15 ml of phosphate buffered saline solution. The fraction eluting at the void volume was collected and assayed for peroxidase activity.

**Western blot and immunodetection in infected cells.** Viral proteins (supernatants and cell lysates from BHK-21 cells infected with RVFV-MP12 strain) separated by SDS-PAGE were electrophoretically transferred onto nitrocellulose membranes (Whatman). After overnight blocking with 5% fat-free milk powder in PBS, the membranes were incubated with monocular antibodies from hybridoma culture supernatants for 1 h at 37°C. After being washed 3 times with washing solution (5% milk PBS; 0.1% Tween 20), HRPO-conjugated anti-mouse IgG (Sigma) diluted 1:10,000 was added to the membranes and incubated for 1 h at 37°C, washed 3 times and developed with ECL (Pierce).

For cell immunostaining infected BHK21 cells were fixed in 4% paraformaldehyde and permeabilised with 0.25% Triton X-100. After blocking with 20% Fetal Bovine Serum (FBS) the primary mAb was used at 1/2 prior to adding an HRP-conjugated peroxidase-conjugated mAb. The non-saturating dilution of antibody was then used to incubate the cells at room temperature. The reaction was stopped by adding EPPS buffer pH 7.5, washed 3 times, and 50 µl of soluble tetra methyl benzidine (TMB) substrate (Sigma) was added to each well. Plates were then incubated in the dark at room temperature for a further 5 min. The reactions were stopped by the addition of 50 ul of H₂SO₄ 3 N and the optical density (OD) was determined at 450 nm.

**Competitive binding assays.** Different anti-RVFV sera, either experimentally immunized sheep, rabbit or sheep anti-RVFV reference serum (kindly provided by the Onderstepoort Veterinary Institute (OVI), South Africa) were assayed in a capture ELISA as described above. After 30 min of incubation at 37°C, a non saturating dilution of hybridoma supernatants, i.e., a dilution corresponding to an OD₄₅₀ₙ₅ value ≤1.5, was added to the wells, and the plates were incubated for 1 h at 37°C. Thereafter, mAb binding was detected following a further 1 h incubation at 37°C in the presence of a 2 x 10⁻³ dilution of anti-mouse HRP-conjugated IgG (Sigma) and the assay continued as described above. Inhibition percentages were calculated according to the following equation:

\[
\frac{1 - \left( \frac{\text{mean OD}_{\text{test serum}}}{\text{mean OD}_{\text{no serum}}} \right) \times 100.}
\]

**Cloning and sequencing of nucleoprotein ORFs.** Total RNA from cells infected with the RVFV strains AR 20368, AN 1830, 252/75 and 56/74 was isolated using Tri Reagent (Sigma) and RT-PCR was performed using specific primers described previously to obtain cDNA fragments including the complete N ORF. PCR amplification products were purified and subsequently cloned into pGEM-T Easy vector system (Promega). Plasmid containing inserts were sequenced on both strands. For eukaryotic cell expression assay, the AR 20368 N sequence was subcloned in the pCMV plasmid upon digestion with Not I restriction enzyme to generated pCMV-N²⁰₃₆₈. The proper insertion of the fragment was confirmed by sequencing. pCMV-N²⁰₃₆₈ was then used to transfect BHK-21 monolayers using the protocol indicated previously.
Acknowledgements
This work was funded by the Spanish Ministry of Science-INIA grant RTA 2005-00017 and from the EU Network of Excellence EPIZONE (Contract n° CT2006-016236). R.M.’s work was funded by a fellowship from INIA. H.B. is a recipient of a Juan de la Cierva research award. We thank E. Brochi (Istituto Zooprofilattico Sperimentale Lombardia ed Emilia-Romagna) for helpful discussions and Dr. T. Gerdes (Ondersteypoort Veterinary Institute, OVI) for supplying sheep anti-RVFV sera and RVFV isolates.

Disclosure statement: mAbs D7E8 and D7D8 undergoing preliminary patent application.

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