Generation and characterization of monoclonal antibodies against Rift Valley fever virus nucleoprotein

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Abstract

Due to the unpredictable and explosive nature of Rift Valley fever (RVF) outbreaks rapid and accurate diagnostic assays for low-resource settings, are urgently needed. To improve existing diagnostic assays, monoclonal antibodies (MAbs) specific for the nucleocapsid protein of RVF virus (RVFV) were produced and characterized. Four IgG2a MAbs showed specific binding to denatured nucleocapsid protein, both from a recombinant source and from inactivated RVFV, in Western blot analysis and in an enzyme–linked immunosorbent assay (ELISA). Cross-reactivity with genetically related and non-related arboviruses including Bunyamwera and Calovo viruses (*Bunyaviridae* family), West Nile and Dengue-2 viruses (*Flaviviridae* family), and Sindbis and Chikungunya viruses (*Togaviridae* family), was not detected. These monoclonal antibodies represent a useful tool for the development of rapid diagnostic assays for early recognition of RVF.

Keywords: Rift Valley fever virus; nucleocapsid protein; monoclonal antibodies, cross-reactivity.

Introduction

Rift Valley fever virus (RVFV) is an important emerging pathogen transmitted primarily by mosquitoes that affects humans and livestock in Africa and in the Arabian Peninsula (Swanepoel and Coetzer, 2004). The virus belongs to the genus *Phlebovirus*, family *Bunyaviridae* (Murphy et al., 1999) The RVFV genome consists of three single-stranded RNA segments, designated large (L), medium (M) and small (S). The L segment encodes the viral RNA-dependent RNA polymerase (L), and the M segment encodes two glycoproteins (Gn and Gc) and two non structural proteins, collectively referred to as NSm. The L and M genome segments are of negative-sense polarity, whereas the S segment is of ambisence polarity. This genome segment contains the non-structural NSs gene in the antigenomic orientation and the gene encoding the viral nucleocapsid (N) protein in the genomic orientation (Bouloy and Friedemann, 2010).

Due to the ability of RVFV to cross geographic and national boundaries and the lack of efficient prophylactic and therapeutic measures a rapid and accurate diagnosis of the disease is essential. Serological techniques can provide a very accurate way of diagnosing RVF when used in combination with clinical observations and epidemiological history and/or when seroconversion is demonstrated (Pepin et al., 2010).

Among the serological techniques for the detection of antibodies to RVFV, several ELISA formats employing whole inactivated virus as antigen source have been widely used, validated and reported as highly sensitive and specific (Paweska et al., 1995; 2003a; 2003b; 2005). However, the production of whole antigen requires containment facilities to reduce the risk of exposure of laboratory staff to infectious virus. Moreover, the antigen has shown to bind poorly to the ELISA plates. Recently, recombinant nucleocapsid protein has been used successfully as an antigen in an indirect ELISA for detection of antibodies to RVFV and has provided a very sensitive and specific method for RVF diagnosis, without risk of laboratory infection. The recombinant nucleocapsid protein was applied as antigen in indirect ELISA format that requires either a species-specific conjugate or a protein G conjugate that

recognizes immunoglobulin of various species (Fafetine et al., 2007; Jansen van Vuren et al., 2007; Paweska et al., 2007; 2008).

ELISA has also been used to capture RVFV antigens present in serum (Niklasson et al., 1983; Meegan et al., 1989) but the production of reagents was difficult and required virus growth in cell cultures. A sandwich ELISA for the detection of RVFV nucleocapsid protein based on hyperimmune serum has recently been reported as a safe and valuable tool for detection of RVFV (Jansen van Vuren and Paweska, 2009). Disadvantages of using hyperimmune serum in immunochemical assays relate to the presence of a mixed population of antibodies that can create a variety of problems (Harlow and Lane, 1988). Furthermore, polyclonal antibodies are subject to intra- and inter-laboratorial variability that results from serum batch-to-batch variation while monoclonal antibodies enable a continuous supply of large quantities of well characterized antibodies. The later can be easily standardized between different laboratories as they are usually from stable hybridoma clones (Qiu et al., 2009). Due to the high specificity for antigens, MAbs are widely used as capture antibodies for detection of different important pathogens in antigen capture ELISAs (Cai et al., 2009; Liu et al., 2010; Qiu et al., 2009; Saijo et al., 2007) and in chromatographic strip-tests (Bruning-Richardson et al., 2011; Bruning et al., 1999). Monoclonal antibodies are also used for detection of antibodies in competitive ELISAs (Martin-folgar et al., 2010) offering the advantage, over indirect ELISA, of allowing the screening of sera from different species with only one anti-mouse immunoglobulin conjugate (Saliki and Lehenbauer, 2001).

Since the nucleocapsid protein was shown to be the most immunodominant protein in different members of the *Bunyaviridade* family (Swanepoel et al., 1986; Vapalathi et al., 1995; Schwarz et al., 1996) and was used in ELISA as an antigen with very promising results (Fafetine et al., 2007), it represents a very good target for detection of RVFV infection. MAbs against the nucleocapsid protein may be applied for the development of different diagnostic tools for RVF, e.g. a competitive ELISA that can be validated for detection of RVFV specific antibodies in different animal species, important not only for surveillance programs but also for studying the role of different animals in the disease epidemiology. In addition, MAbs can also be used in chromatographic strip-tests for rapid detection of RVFV antigen and/or in an antigen capture ELISA format to capture RVFV nucleocapsid protein in different specimens.

Hence, the main objective of the present study was to generate and characterize monoclonal antibodies specific for the recombinant nucleocapsid protein of RVFV.

Materials and methods

Preparation of recombinant RVFV nucleocapsid protein

The nucleocapsid protein of the RVFV isolate ZIM688/78 was expressed and purified as previously described (Fafetine et al., 2007; Jansen van Vuren et al., 2007). Briefly, the recombinant nucleocapsid protein was expressed in the bacterial expression vector pET32(a)+ as an inclusion body and refolded in an appropriate buffer. The refolded protein was purified using metal affinity chromatography, and stored at -20^{o}C until use for immunization and in the different assays.

Production and purification of MAbs

MAbs were produced as previously described (Caldeira et al., 2009) by immunization of 2 female BALB/c mice, aged 4 to 6 weeks, with the recombinant RVFV nucleocapsid protein. Prior to immunization, the animals were bled for collection of pre-immune serum. The mice were then given 100µg of the recombinant RVFV nucleocapsid protein in complete Freund's adjuvant (Sigma) intraperitoneally followed by three boosts with the same amount of protein in incomplete adjuvant (Sigma) at three week intervals. Animals were bled two weeks after the third boost and the final immunization was carried out three days later with 100µg of the recombinant RVFV nucleocapsid protein. Three days after the last immunization animals were bled again. The pre-immune serum and the titer of the polyclonal antiserum after the final boost were assessed by indirect ELISA using recombinant RVFV nucleocapsid protein as antigen. The animal with the highest titer was sacrificed and its spleen cells used to fuse with Sp2/0 myeloma cells at a ratio of 1:1 in the presence of polyethylene glycol (PEG, Sigma). Hybridoma cells were selected in DMEM media (Gibco) with HAT (Sigma) and 5% (v/v) fetal calf serum (Gibco) and subsequently cloned by limiting dilution. Clones producing the highest titers of RVFV nucleocapsid protein specific antibodies, as assessed in the indirect ELISA, were selected for further use.

Indirect ELISA

The detection of secreted antibodies was done by indirect ELISA. Briefly, immunoplates (Costar 3590, Corning Incorporated, USA) were coated overnight at 4^{o}C with 0.25 µg/well of recombinant RVFV nucleocapsid protein diluted in PBS. After washing three times with TBS containing 0.05% (v/v) Tween 20 (washing buffer), plates were blocked with 100 µl washing buffer containing 5% (w/v) skim milk (Difco) then incubated for 1 h at room temperature and washed as mentioned before. Fifty microliters of hybridoma supernatant were added to each well and incubated for 1 h at 37^{o}C. The pre-immune serum and the polyclonal RVFV recombinant nucleocapsid protein specific antiserum were added as negative and positive controls, respectively. The plates were washed as described above followed by the addition of 50 µl of alkaline phosphatase conjugated anti-mouse polyvalent immunoglobulins (G, A, M) (Sigma) to each well. After incubation for 1 h at 37^{o}C, plates were washed 5 times with washing buffer and 50 µl 4-Nitrophenylphosphate disodium salt hexahydrate (AppliChem) substrate was added. Subsequently the plates were incubated at room temperature in the dark for 30 min and the optical densities (OD) determined at 415 nm in a TIM 200 Inter Med plate reader.

Antibody purification

Supernatants of the selected clones were collected, clarified by centrifugation (20,000 x g, 10 min, 4°C), filtered through a 0.22 µm membrane filter (Millipore) and further purified on a HiTrap Protein A HP affinity column (GE HealthCare). The samples were first adjusted to the composition of the binding buffer (20 mM sodium phosphate, pH 7.0) using PD-10 columns (GE HealthCare). Before loading the sample, the column was washed with 10 volumes of binding buffer. Ten column volumes of the binding buffer were further used to remove contaminants. Bound antibodies were eluted stepwise with five column volumes of elution buffer (0.1 M citric acid, pH 3.5). Eluates were collected and pH neutralized by adding 100 µl of 1 M Tris-HCl, pH 9.0 per ml of fraction to be collected and purity was analyzed by SDS-PAGE. The antibody containing fractions were pooled and stored for further use.

Determination of the Ig class of hybridoma antibodies

Isotype identification was performed with the MonoAb ID isotyping Kit (Zymed). A 96-well microtitre plate (Costar) was coated with recombinant RVFV nucleocapsid protein overnight at 4ºC. After 3 washes with PBS containing 0.05% (v/v) Tween 20, all the wells were filled with 200 µl of washing buffer containing 5% (w/v) skim milk (Difco) and incubated for 1h at room temperature. Culture supernatants containing the selected monoclonal antibodies (100 µl) were added and further incubated for 1 h at 37°C. After incubation, the plate was washed as previously described and 100 µl of rabbit antisera specific for mouse, µ, γ_1,γ_{2a}, γ_{2b}, γ_3 heavy chains and *k* and λ light chains supplied in the kit was added to the appropriate wells. The plate was incubated for 1 h at 37°C and washed 5 times. Alkaline phosphatase conjugated goat anti-rabbit IgG was subsequently added in all wells (100 µl) and incubated at 37°C for 1 h. After washing 5 times, the substrate solution p-nitrophenyl phosphate containing 5% (w/v) skim milk (Difco), was added and the optical density read in a microplate reader as described above.

Western blot assay

A Western blot assay was used to confirm the specificity of the clones using the recombinant RVFV nucleocapsid protein as antigen on 12.5% (w/v) sodium dodecyl sulfate (SDS)-polyacrylamide gel. The recombinant RVFV nucleocapsid protein preparation (100µg/ml) was run on the gel under denaturating conditions and electrotransferred to a nitrocellulose membrane. The membranes were stained using the Ponceau staining solution, cut into strips that were placed in a multichannel reservoir and blocked at room temperature for 1 h with PBS containing 0.05% Tween 20 (v/v) and 5% (w/v) skim milk (Blocker non-fat dry milk-BioRad). Membranes were incubated with hybridoma supernatant and positive and negative controls (the same as used in ELISA) for 1.5 h at room temperature and then washed three times with 0.05% (v/v) Tween 20 in PBS. Membranes were then incubated for 1 h at room temperature with alkaline phosphatase conjugated anti-mouse polyvalent immunoglobulins (G, A, M) (Sigma). Subsequently membranes were washed as previously described and further incubated, in the dark, with AP color development buffer (BioRad).

Assessment of monoclonal antibodies cross-reactivity

The Western blot assay described above was also used, to assess whether the monoclonal antibodies recognized antigens from other arboviruses of the families *Flaviviridae* (West Nile Virus and Dengue virus II) and *Togaviridae* (Sindbis virus and Chikungunya virus) and other members of the *Bunyaviridae* family, namely from the genus *Orthobunyavirus* (Bunyamwera virus and Calovo virus). Briefly, 10^7 sub-confluent Vero cells were infected at a m.o.i. 0.1-1 PFU/cell of these viruses and collected when a generalized cytophatic effect became evident (24-48 hr p.i.). Infected cells were lysed in 20 mM Tris-HCl, pH 7.0, 150 mM NaCl, 1 mM EDTA and 1% (v/v) Triton X-100. Extracts from cells infected with different viruses were tested by Immunoblot as described above using the inactivated whole RVFV as a positive control (SPU 00201) and non-infected cell extract as a negative control.

Results

Twenty three hybridoma cell lines producing recombinant RVFV nucleocapsid proteins specific antibodies were selected based on indirect ELISA data using the recombinant RVFV nucleocapsid protein as antigen. Hybridomas were cloned by limiting dilution and those clones showing the highest optical densities when analyzed by ELISA were selected for further assays. The specificity of the monoclonal antibodies as confirmed by Western blot analysis has shown that ten out of the twenty three MAbs bound to the antigen, four of which (Fig. 1: lanes 3, 6, 7 and 9) showed a strong reaction with the recombinant RVFV nucleocapsid protein. The antiserum against the recombinant RVFV nucleocapsid protein used as positive control also revealed a strong single band of approximately 62 kDa (Fig. 1, lane 1). Some of the MAbs that demonstrating positive results in the indirect ELISA did not recognize the nucleocapsid protein in the Western blot assay as can be observed in figure 1. The monoclonal antibody isotype determination, showed that the clones named (A12G, 4D3, B3H and D3G) all belong to the class IgG, subclass IgG2a. These results were further used for definition of the purification strategy; a protein A affinity column allowed the purification of the selected clones without major contaminations from other immunoglobulins that might be present, namely IgM, IgA and IgE. The monoclonal antibodies were further tested for recognition of RVF inactivated virus using Western blotting, as referred before. The same

assay was used to assess the cross-reactivity of the generated monoclonal antibodies with extracts from cells infected with West Nile virus, Dengue virus II, Sindbis virus, Chikungunya virus, Bunyamwera virus and Calovo virus. As can be seen in figure 2, the monoclonal antibody 4D3 specifically recognized the RVF inactivated virus producing an expected band of approximately 62 kDa. However, no reaction was observed neither on other viral extracts nor on the negative control extract. Similar binding patterns were obtained for the other MAbs, hence all four monoclonal antibodies proved specific for RVFV nucleocapsid protein.

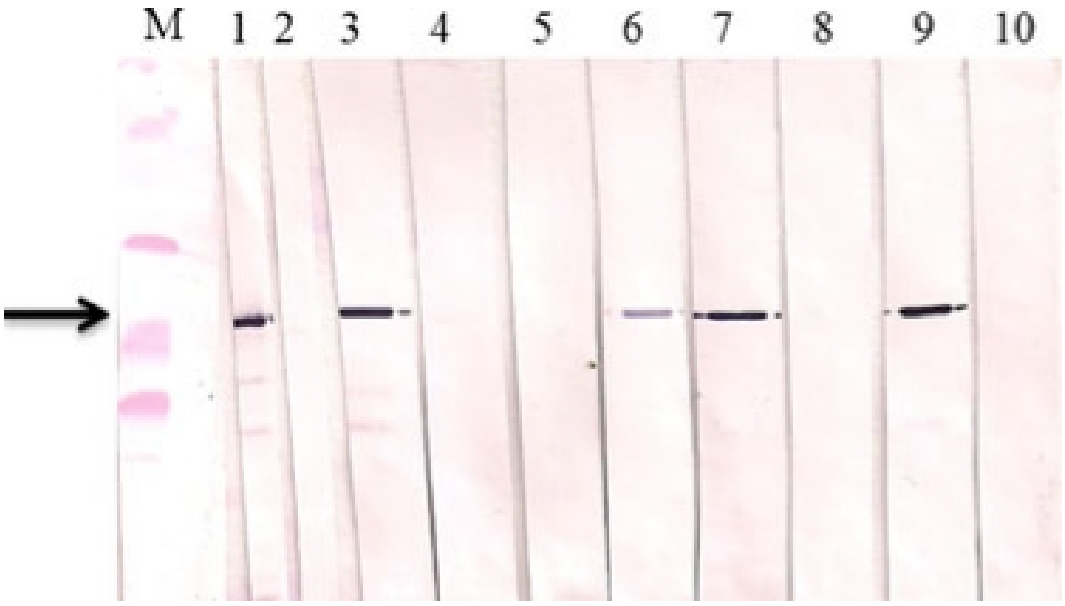

Fig. 1. Western blotting screening of different hybridoma supernatants with recombinant 12 RVFV nucleocapsid protein. Lane M: Low Molecular Weight calibration kit for SDS electrophoresis (GE Healthcare); lane 1: anti-recombinant RVFV nucleocapsid protein serum 1:1000; lane 2: serum from PBS immunized mouse 1:1000; lanes 3–10: different hybridoma supernatants. The arrow indicates positive reaction (62 kDa recombinant RVFV nucleoprotein band).

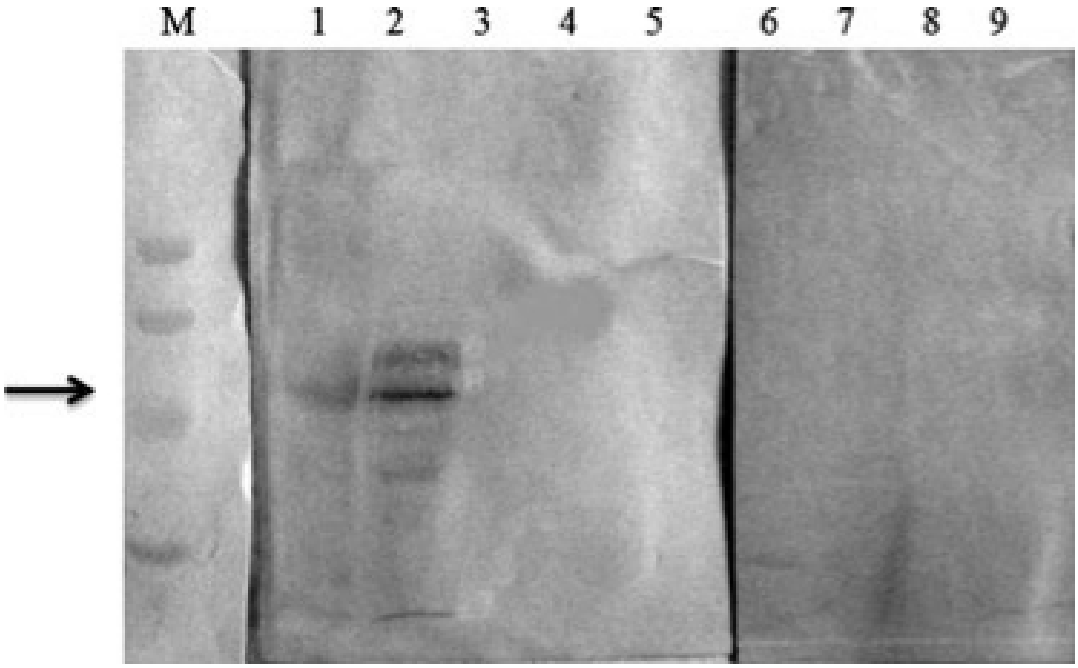

Fig. 2. Western blot analysis of MAb 4D3 specificity. Inactivated RVFV and lysates of Vero cells infected with several arboviruses were probed with MAb 4D3. Lane M: Low Molecular Weight calibration kit for SDS electrophoresis (GE Healthcare); lane 1: positive control; lane 2: RVF inactivated virus; lane 3: control extract; lanes 4–9 different viral extracts: West Nile, Dengue virus II, Sindbis, Chikungunya virus, Bunyamwera virus and Calovo. The arrow indicates positive reaction (62 kDa band).

Discussion

RVF is a disease of both medical and veterinary interest due to its impact on public health and economy in the regions where it occurs. Moreover it is an emerging disease that recently spread beyond regions of its historical occurrence and therefore needs to be carefully monitored both in endemic and RVF-free regions. For this reason the development of new rapid and accurate diagnostic tools is a priority.

The nucleocapsid protein is the most immunodominant protein in different members of the *Bunyaviridade* family (Swanepoel et al., 1986; Vapalathi et al., 1995; Schwarz et al., 1996) and has been applied successfully in indirect ELISA formats for the detection of RVF specific antibodies (Fafetine et al., 2007; Jansen van Vuren et al., 2007; Paweska et al., 2007; 2008). Production of specific monoclonal antibodies against this structural protein may further contribute to the development of improved assays for detection of the RVFV infection.

The MAbs selected in the present study revealed a specific ability to recognize the recombinant nucleocapsid protein both based on an indirect ELISA and recognition of antigens in a Western blot assay. However, only four out of ten chosen monoclonal antibodies

based on ELISA results gave a strong reaction in Western blot. This could be explained by the different reactivity against hidden epitopes, weak affinity of the monoclonal antibodies (Daginakatte et al., 1999) or different conformation adopted by the recombinant nucleocapsid protein in the solid phase (Al-Yousif et al., 2000). Serological techniques applied for the detection of antibodies to RVFV have been reported to be cross-reactive with other viruses (Scott et al., 1986; Swanepoel et al., 1986). To assess whether the produced monoclonal antibodies were specific for RVFV only, they were tested against extracts of cell infected with other members of the *Bunyaviridae* family (Bunyamwera virus and Calovo virus from the genus *Orthobunyavirus*) and other RNA viruses from the families *Flaviviridae* (West Nile Virus and Dengue virus II) and *Togaviridae* (Sindbis virus and Chikungunya virus). No cross-reactivity was found using Western blot for analyzing the four selected monoclonal antibodies. Recently, Raymond et al. (2010) reported that the phlebovirus nucleocapsid protein has a novel protein fold that differs substantially from the nucleocapsid of other negative-sense RNA viruses, a fact that can explain the absence of cross-reaction with the Bunyamwera and Calovo viruses.

Monoclonal antibodies against RVFV were previously described by Zaki et al. (2006) and by Martin-Folgar et al. (2010). Zaki et al. (2006) produced monoclonal antibodies after immunization of BALB/c mice with the whole inactivated RVFV and tested them against two RVFV strains (KEN97 and ZH548) representative of two different phylogenetic lineages. The monoclonal antibodies could successfully detect RVFV from cell culture. Martin-Folgar et al. (2010) developed monoclonal antibodies against RVFV nucleocapsid protein, evaluated them against different strains of RVFV and applied them effectively in ELISA for detection of antibodies and RVFV antigen. In addition to what was done in these two studies, the monoclonal antibodies described in our study were also tested against other viruses of the *Bunyaviridae* family and arboviruses from two other families adding value to its assessment.

Based on the results described previously, the monoclonal antibodies generated and characterized in this study could be used in a competitive ELISA to detect specific RVFV antibodies in the sera from different animal species with the use of one anti-mouse immunoglobulin conjugate. This contrasts the currently available indirect ELISA that requires species-specific immunoglobulin conjugates. The assay would be particularly important in studying the role of different species in the epidemiology of the disease since RVFV is known

to affect different animal species including wildlife (Anderson and Rowe, 1998; Evans et al., 2008; Swanepoel and Coetzer, 2004) and buffaloes have been shown to play a role in the inter-epidemic maintenance of the virus (LaBeaud et al., 2011). The high specificity of the monoclonal antibodies for antigens could also be exploited to capture RVFV nucleocapsid protein in an antigen capture ELISA and/or chromatographic strip-test. This might provide rapid diagnostic tools for detection of RVFV needed to mitigate the impact of RVF and to reduce its transmission between animals and humans, especially in those countries where the use of molecular based techniques like PCR or virus culture is problematic.

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