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# Mapping of conserved and species-specific antibody epitopes on the Ebola virus nucleoprotein

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ABSTRACT

Filoviruses (viruses in the genus *Ebolavirus* and *Marburgvirus* in the family *Filoviridae*) cause severe haemorrhagic fever in humans and nonhuman primates. Rapid, highly sensitive, and reliable filovirus-specific assays are required for diagnostics and outbreak control. Characterisation of antigenic sites in viral proteins can aid in the development of viral antigen detection assays such immunochromatography-based rapid diagnosis. We generated a panel of mouse monoclonal antibodies (mAbs) to the nucleoprotein (NP) of Ebola virus belonging to the species *Zaire ebolavirus*. The mAbs were divided into seven groups based on the profiles of their specificity and cross-reactivity to other species in the *Ebolavirus* genus. Using synthetic peptides corresponding to the Ebola virus NP sequence, the mAb binding sites were mapped to seven antigenic regions in the C-terminal half of the NP, including two highly conserved regions among all five *Ebolavirus* species currently known. Furthermore, we successfully produced species-specific rabbit antisera to synthetic peptides predicted to represent unique filovirus B-cell epitopes. Our data provide useful information for the development of Ebola virus antigen detection assays.



## 1. Introduction

Filoviruses are among the most lethal human pathogens recognised to date with case fatality rates up to 90%, depending on the virus species and strain (Pittalis et al., 2009; Bente et al., 2009). Filoviruses are grouped into two genera, *Ebolavirus* and *Marburgvirus*. There is one known species of *Marburgvirus*, *Marburg marburgvirus*, consisting of two viruses, Marburg virus (MARV) and Ravn virus (RAVV). In contrast, the genus *Ebolavirus* has five known species, *Zaire ebolavirus*, *Sudan ebolavirus*, *Taï Forest ebolavirus*, *Bundibugyo ebolavirus* and *Reston ebolavirus*, represented by Ebola virus (EBOV), Sudan virus (SUDV), Taï Forest virus (TAFV), Bundibugyo virus (BDBV) and Reston virus (RESTV), respectively. Furthermore, there is a newly discovered filovirus named Lloviu virus (LLOV) assigned to the proposed genus *Cuevavirus*, with one species, *Lloviu cuevavirus* (Negredo et al., 2011; Kuhn et al., 2010). The genome of filoviruses is approximately 19 kb long, and contains seven genes arranged sequentially in the order: nucleoprotein (NP), viral protein (VP) 35, VP40, glycoprotein (GP), VP30, VP24 and polymerase (L) genes (Sanchez et al., 2007).

The lack of therapeutics and vaccines for filovirus infections and the fact that other pathogens cause clinical symptoms comparable to those of Ebola and Marburg haemorrhagic fever highlights the need for rapid, sensitive, reliable and virus-specific diagnostic tests to control the spread of these viruses (Qiu et al., 2011; Sanchez et al., 2007). Rapid antigen-detection tests with filovirus-specific monoclonal antibodies (mAb) are likely one of the best ways for early diagnosis of filovirus infections in the field setting. NP may be the ideal target antigen because of its abundance in filovirus particles and its strong antigenicity (Niikura et al., 2001, 2003). The average EBOV virion, which is up to 1028 nm in length, contains about 3200 NP molecules (Bharat et al., 2012). EBOV NP consists of 739 amino acid residues, with a conserved hydrophobic N-terminus and a variable hydrophilic C-terminal part (Niikura et al., 2001;

*Abbreviations:* mAb, monoclonal antibodies; EBOV, Ebola virus; SUDV, Sudan virus; TAFV, Taï Forest virus; BDBV, Bundibugyo virus; RESTV, Reston virus; MARV, Marburg virus; RAVV, Ravn virus; NP, nucleoprotein; VP, viral protein; GP, glycoprotein; VLP, virus-like particle.

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Sanchez et al., 2007). NP plays an important role in the replication of the viral genome and is essential for formation of the nucleocapsid (Watanabe et al., 2006). The C-terminus of EBOV NP binds to VP40 while the N-terminus forms a condensed helix with the same diameter as the inner nucleocapsid helix of an EBOV particle (Bharat et al., 2012). Following expression of VP40 in cultured cells, virus-like particles (VLPs) are produced and, upon co-expression of NP, the VLP contains NP as its core (Bharat et al., 2012; Noda et al., 2007). It has been demonstrated that the C-terminal half of the filovirus NP has strong antigenicity (Saijo et al., 2001). Multiple studies have identified conformational and linear epitopes for antibodies in this NP region for several viruses within the genus *Ebolavirus* (Ikegami et al., 2003; Niikura et al., 2001, 2003).

In general, characterisation of antigenic sites in a viral protein can aid in the development of diagnostic tools, therapeutics and vaccines (Gershoni et al., 2007; Toyoda et al., 2000). Here, we identified antigenic regions within the NP molecule using mouse NP-specific mAbs and rabbit antisera to synthetic NP peptides representing viruses from all known filovirus species. Some of the identified antigenic regions are shared among multiple virus species within the *Ebolavirus* genus, whereas others are species-specific. Our data provide useful information for future development of antigen-based detection assays for the diagnosis of filovirus infections.

## 2. Materials and methods

### 2.1. Plasmid construction

Plasmids expressing GP, VP40 and NP were constructed as described previously (Nakayama et al., 2010; Nidom et al., 2012). Briefly, viral RNAs were extracted from the supernatant of Vero E6 cells infected with EBOV (Mayinga), SUDV (Boniface), TAFV (Côte d'Ivoire), BDBV (Bundibugyo), RESTV (Pennsylvania) or MARV (Angola). Full length NP, VP40 and GP cDNA were amplified by RT-PCR using KOD-plus-Neo polymerase (Toyobo) and cloned into TOPO® vector using the Zero Blunt® TOPO® PCR Cloning Kit (Invitrogen). After sequence confirmation, the cloned genes were inserted into the mammalian expression vector pCAGGS.

### 2.2. Preparation of purified VLPs and NP

Human epithelial kidney 293T cells were grown in Dulbeco's modified Eagle's medium (DMEM), supplemented with 10% FCS, penicillin (100 unit/ml) and streptomycin (100 μg/ml). VLPs were produced by transfection of 293T cells with plasmids expressing NP and VP40 together with or without the plasmid expressing GP as described previously (Licata et al., 2004; Urata et al., 2007). Forty-eight hours after transfection, VLPs in the supernatant were purified by centrifugation through a 25% sucrose cushion at 28,000 × *g* and 4 °C for 1.5 h. The pelleted VLPs were resuspended in PBS and stored at −80 °C. For the preparation of purified NP, 293 T cells transfected with the plasmid encoding EBOV NP were lysed, and the NP fraction was collected by discontinuous CsCl gradient centrifugation of the lysates as described previously (Bharat et al., 2012; Noda et al., 2010).

### 2.3. Mouse mAb production

On day 0, six-week-old female Balb/c mice were immunised intramuscularly with 100 μg of EBOV VLPs consisting of NP and VP40 with complete Freund's adjuvant (Difco). The animals were boosted intramuscularly on day 14 with 100 μg of the same EBOV VLPs and incomplete Freund's adjuvant. After a final intravenous boost with 100 μg of the same EBOV VLPs without adjuvant on day 39, spleen cells were harvested on day 42 and fused to P3-U1 myeloma cells according to standard procedures (Shahhosseini et al., 2007). Hybridomas were maintained in Roswell Park Memorial Institute medium 1640 containing 20% FCS, penicillin (100 unit/ml), streptomycin (100 μg/ml), L-glutamine (4 mM) and 2-mercaptoethanol (55 μM). Hybridoma supernatants were screened by an enzyme linked immunosorbent assay (ELISA) for secretion of NP-specific antibodies using purified EBOV NP and VLP as antigens. Specificity and cross-reactivity of mAbs were also confirmed by Western blotting. Selected hybridoma cells were then cloned twice performing limiting dilution.

### 2.4. Production of rabbit antisera

Genetyx ver6.0 for Windows (GENETYX CORPORATION) was used to predict B-cell epitopes in the NPs of EBOV, SUDV, TAFV, BDBV, RESTV and MARV, and the amino acid (aa) positions around 630–650 were selected. Synthetic peptides corresponding to this aa region in NP were produced (Sigma). Rabbits were then immunised with keyhole limpet haemocyanin-conjugated synthetic peptides by the standard procedure, and antisera were obtained on day 49.

### 2.5. ELISA

Ninety six-well ELISA plates (Nunc®, Maxisorp) were coated with 50 μl PBS containing purified EBOV NP (2 μg/ml), VLPs (2–5 μg/ml) or synthetic peptides (100 μg/ml) per well overnight at 4 °C. ELISA was carried out as described previously (Nakayama et al., 2011), using mouse antisera, hybridoma supernatants, purified mAbs or rabbit antisera as primary antibodies and goat anti-mouse IgG (H + L) or donkey anti-rabbit IgG (H + L) conjugated with peroxidase (Jackson ImmunoResearch) as secondary antibodies.

### 2.6. Western blotting

Vero E6 cells cultured in DMEM supplemented with 10% FBS, penicillin (100 unit/ml), streptomycin (100 μg/ml) and L-glutamine (4 mM) were infected with EBOV (Mayinga), SUDV (Boniface), TAFV (Cote d'Ivoire), BDBV (Bundibugyo), RESTV (Pennsylvania), MARV (Angola, Musoke, Ozolin and Ci67) or RAVV (Ravn) at a multiplicity of infection of 1 and maintained for 72 h. Cell culture supernatants were subjected to SDS-PAGE. For the screening of hybridoma supernatants (see above), VLPs were used instead of authentic virus lysates. After electrophoresis, separated proteins were blotted on a polyvinylidene difluoride membrane (Millipore) or Immobilon-P transfer membrane (Millipore). Mouse mAbs and rabbit antisera were used as primary antibodies. The bound antibodies were detected with peroxidase-conjugated goat anti-mouse IgG (H + L) or donkey anti-rabbit IgG (H + L) (Jackson ImmunoResearch), followed by visualisation with Immobilon Western (Millipore).

### 2.7. Ethics and biocontainment statements

Animal studies were carried out in strict accordance with the Guidelines for Proper Conduct of Animal Experiments of the Science Council of Japan. The protocol was approved by the Hokkaido University Animal Care and Use Committee. All efforts were made to minimise the suffering of animals. All infectious work with filoviruses was performed under high containment complying with standard operating procedures approved by the Institutional Biosafety Committee in the BSL4 Laboratories of the Integrated Research Facility at the Rocky Mountain Laboratories, Division of Intramural Research, National Institute of Allergy and Infectious Diseases, National Institutes of Health, Hamilton, Montana, USA.

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