Recombinant nucleoprotein-based diagnostic systems for Lassa fever: development of diagnostic assays, which do not require infectious virus for antibody and antigen detection

Short title: Recombinant NP-based diagnostics for Lassa fever

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Abstract

Diagnostic systems for Lassa fever (LF), a viral hemorrhagic fever caused by Lassa virus (LASV), such as enzyme immunoassays for the detection of LASV-antibodies and LASV-antigens, were developed using the recombinant nucleoprotein (NP) of LASV (LASV-rNP). The LASV-rNP was expressed in a recombinant baculovirus system. LASV-rNP was used as an antigen in the detection of LASV-antibodies and as an immunogen for the production of monoclonal antibodies. The LASV-rNP was also expressed in HeLa cells by transfection with the expression vector encoding cDNA of the LASV-NP gene. The immunoglobulin-G (IgG)-linked immunosorbent assay (ELISA) using LASV-rNP and an indirect immunofluorescence assay using LASV-rNP-expressing HeLa cells were confirmed to have high sensitivity and specificity in the detection of LASV-antibodies. A novel monoclonal antibody to LASV-rNP, MoAb-4A5, was established. A sandwich antigen (Ag)-capture ELISA using the monoclonal antibody and an anti-VL-rNP rabbit serum as capture and detection antibodies, respectively, was then developed. Authentic LASV nucleoprotein in serum samples collected from hamsters experimentally infected with LASV was detected by the Ag-capture ELISA. The Ag-capture ELISA specifically detected
LASV-rNP but not the rNPs of lymphocytic choriomeningitis virus or Junin virus. The sensitivity of the Ag-capture ELISA in detecting LASV antigens was comparable to that of reverse transcription-PCR in detecting LASV RNA. These rNP-based diagnostics were confirmed to be useful in the diagnosis of LF even in institutes without a high containment laboratory, as the antigens can be prepared without manipulation of the infectious viruses.
INTRODUCTION

Lassa fever (LF) is a viral hemorrhagic fever caused by Lassa virus (LASV), an Old World arenavirus. Many cases of LF occur in western Africa in countries such as Guinea, Sierra Leone and Nigeria (7, 23, 27, 29-31). It is thought that LASV infects tens of thousands of humans annually and causes hundreds to thousands of death (34). Humans become infected through contact with infected excreta, tissue or blood from the peridomestic rodent, *Mastomys natalensis*, the reservoir host of LASV (34). LASV can be transmitted to other humans via mucosal/cutaneous contact or nosocomial contamination (27). More than twenty imported cases of LF have been reported outside the endemic region in areas such as the USA, Canada, Europe and Japan (1, 2, 13, 15, 18, 24, 25). Recently the potential for the use of hemorrhagic fever viruses, including LASV, as a biological weapon has been emphasized (5, 6). Therefore, the development of diagnostic systems for LF is important even in countries free from LF outbreaks to date.

Manipulation of infectious LASV is necessary for the detection of specific antibodies. However, a high containment laboratory (Biosafety Level-4, BSL-4) is required for handling infectious LASV and therefore, the preparation of LASV antigens
cannot be implemented in institutes without a BSL-4 facility. Within this framework, it is important to develop sensitive and specific diagnostic systems for LF that eliminate the need for the manipulation of infectious LASV. In the present study, the recombinant nucleoprotein of LASV (LASV-rNP) was expressed and evaluated for its ability to detect LASV antibodies. LASV-rNP-based enzyme-linked immunosorbent and indirect immunofluorescent assays (ELISA and IFA) were developed. Furthermore, novel monoclonal antibodies to LASV-rNP were generated and used in combination with the recombinant antigen to develop a LASV-antigen (nucleoprotein, NP)-capture ELISA. This study presents an alternative strategy to develop diagnostic systems without handling infectious LASV.

**MATERIALS AND METHODS**

**Cells.** A HeLa cell line was cultured in the Eagle’s minimum essential medium supplemented with 10% fetal bovine serum (FBS) and antibiotics, penicillin-G and streptomycin, (MEM-10FBS). Tn5 insect cells were used for the expression of the rNPs of arenaviruses [LASV, lymphocytic choriomeningitis virus (LCMV) and Junin virus (JUNV)] in a baculovirus system. The Tn5 insect cells were cultured as reported previously (38).
*Viruses.* LASV, strain AV, that was isolated from an imported case of LF to Germany from West Africa, was used (13). The experimental process that required manipulation of infectious LASV was carried out in the BSL-4 laboratory in the P4 laboratory, INSERM, Lyon, France. Mopeia virus (MOPV), which belongs to the Family Arenaviridae, Genus Arenavirus, was also used. Recombinant NPs of LCMV (26) and JUNV (11), designated as LCMV-rNP and JUNV-rNP, respectively, were also expressed in a baculovirus system and used in the study. A baculovirus (Ac-P), which lacks polyhedrin expression, was used as a control virus (26). The virus titer of LASV in serum samples was determined by focus forming unit assay as described previously (3).

*Sera.* Four human serum samples, 3 samples serially collected from one patient with LF and one additional sample from another patient with LF, and 96 human sera collected from Japanese subjects with no history of travel to LF-endemic areas were used as positive and negative controls, respectively. The patient with LF, from whom 3 serial serum samples were collected, was the first case of LF to be imported in Japan in 1987 (15). The other human serum sample was provided from the Special Pathogens Branch, National Center for Infectious Diseases, Centers for Disease Control and Prevention, Atlanta, GA.
Serum samples collected from 5 monkeys [*Macaca fascicularis*] subcutaneously infected with LASV strain AV at $10^3$ ffu (2 monkeys) or $10^7$ ffu (3 monkeys) and those collected from 4 monkeys with mock-infection were also used. The serum samples used in the study were collected after 4-5 weeks post challenge.

Five hamsters were subcutaneously infected with $10^3$ ffu of LASV, strain AV, and blood was drawn on days 0, 4, 11 and 16 post infection, taking the day on which the virus was inoculated as day 0. Serum fractions of the collected blood specimens were separated and tested for LASV-antigen by the Ag-capture ELISA and reverse transcription-polymerase chain reaction (RT-PCR).

Rabbit sera (polyclonal antibodies) were raised against LASV-rNP, LCMV-rNP, and JUNV-rNP by immunization of rabbits with the purified LASV-rNP, LCMV-rNP and JUNV-rNP, respectively, in the form of a mixture with the adjuvant, Inject Alum™ (Pierce). Rabbits were immunized with sufficient amount of the purified NPs of each virus three times with an interval of 2 weeks. After the confirmation of the increased titer, >10,000 times determined by indirect immunofluorescent assay, which was developed in this study, the blood was drawn from the rabbits, and serum fraction was used in the present study.

**Recombinant baculovirus.** In order to construct the transfer
vector, a cDNA clone of NP from LASV strain Josiah, was used. The cDNA was kindly provided by Dr. McCormick, former Director of the Special Pathogens Branch, National Centers for Infectious Diseases, CDC, Atlanta, GA. The complete nucleotide sequence of the NP gene is registered in GenBank under the accession number NC_004296. The DNA of the LASV-NP was amplified by PCR from the source using primers LAS-NfB (5′-GTGGATCCACACAACAATCTGG-3’; the BamHI restriction site is underlined) and LAS-NrB (5′-CCGGATCCATTACAGAACGACTC-3’). The PCR conditions were the same as previously reported (38). The 1,743-bp amplification product was digested with BamHI and subcloned into the BamHI site of pQE32 vector DNA (QIAGEN GmbH, Hilden, Germany) to construct pQE32-LASV-NP. The inserted LASV-NP DNA was sequenced using appropriate primers with an ABI PRISM™ 310 Genetic Analyzer (PE Applied Biosystems, Foster City, CA, USA), and confirmed to be in proper orientation downstream the promoter and identical to the original sequence. The DNA fragment of LASV-NP with a histidine (His)-tag was isolated from the plasmid, pQE32-LASV-NP, by digestion with EcoRI and HindIII. Then, it was blunt-repaired with Klenow enzyme and ligated into the blunt-ended BamHI site of pAcYM1 (26). The resulting recombinant transfer vector with the correct orientation with
respect to the polyhedrin promoter was constructed (pACYM1-His-LASV-NP). Tn5 insect cells were transfected with mixtures of purified *Autographa californica* nuclear polyhedrosis virus (AcMNPV) DNA and pAcYM1-His-LASV-NP following the procedures described by Kitts et al. (20), with the modification of Matsuura et al. (26). Recombinant baculovirus was then isolated. The baculovirus, which expressed His-tagged LASV-rNP (His-LASV-rNP), was designated Ac-His-LASV-NP.

The baculovirus, Ac-LCMV-NP, which expressed LCMV-rNP, was used in the study (26).

The recombinant baculovirus that expressed JUNV-rNP, Ac-JUNV-NP, was generated as follows. The gene encoding the NP of JUNV (strain MC2) was reconstructed from cloned cDNA. The nucleotide sequence of the interest gene was deposited in GenBank under accession number D10072 (12). A complete NP gene with the initiation and stop codons amplified by PCR using appropriate primers, which possessed BamHI restriction sites. The entire DNA product of JUNV-NP was digested with BamHI and ligated into the transfer vectors, pAcUW2B (28). Clones containing the insert in the correct orientation were selected and the plasmid DNA was used for co-transfection in Sf21 cells.
with a polyhedrin positive AcMNPV DNA, and the supernatant culture was screened for a polyhedrin negative phenotype by plaque assay (19). Finally, recombinant baculovirus clones over-expressing JUNV-rNP were obtained after three successive plaque purifications. One of them, designated AcMNPV-Jun-N122, was used in the present study and is referred to in the rest of the text as Ac-JUNV-NP.

**Expression and purification of His-LASV-rNP, LCMV-rNP and JUNV-rNP.** Tn5 cells infected with Ac-His-LASV-NP were incubated at 26 °C for 72 h. The cells were then washed twice with cold phosphate-buffered saline (PBS) solution. A preliminary study demonstrated that most of the Tn5 cellular proteins were solubilized in PBS containing 2M urea (PBS-2M urea), but that the His-LASV-rNP was insoluble and that the LASV-rNP could be solubilized in PBS containing 8M urea (PBS-8M urea). Therefore, the Tn5 cells infected with Ac-His-LASV-NP were first suspended in PBS-2M urea. After the centrifugation of the cell suspensions at 15,000 g for 10 min, the pellet fractions were collected and then were solubilized in PBS-8M urea. After the centrifugation of the samples, the supernatant fractions were subjected as the purified antigens. LCMV-rNP and JUNV-rNP showed similar dissolution characteristics in urea to that of His-LASV-rNP;
therefore, LCMV-rNP and JUNV-rNP were also fractioned in the same way as the His-LASV-rNP. The control antigen was produced from Tn5 cells infected with Ac-•P in the same manner as that for the positive antigens. The His-LASV-rNP was also purified using the Ni²⁺-column purification method as reported previously (38). The source for His-LASV-rNP-purification was the supernatant fraction of the PBS-8M urea-treated Tn5 cells infected with Ac-His-LASV-NP after sufficient dilution with PBS in order to reduce the urea concentration.

**SDS-PAGE.** Expression and purification efficiency of His-LASV-rNP, LCMV-rNP and JUNV-rNP were analyzed on sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) gels (12 % polyacrylamide) after staining with Coomassie blue.

**Establishment of monoclonal antibodies.** Monoclonal antibodies (MoAbs) were generated as previously described (32, 41). BALB/c mice were immunized with purified His-LASV-rNP in the present study. Isotypes of the MoAbs were determined by using a mouse MoAb isotyping kit (Life Technologies).

**Expression of truncated NPs of LASV.** In order to determine the epitope of the MoAbs to the His-LASV-rNP, truncated LASV-rNPs were expressed as a form of fusion protein with glutathione S-transferase (GST) as shown in Figs. 1. The DNA
corresponding to each of the truncated NP fragments was amplified with the designed primers. The amplified DNA was subcloned into the BamHI and EcoRI cloning sites of plasmid pGEX-2T (Amersham Pharmacia Biotech, Buckinghamshire, England). Each insert was sequenced and confirmed to be in the correct frame and identical to the original sequence. The GST-tagged NP fragments were expressed in an E. coli (BL21) system.

**Western blotting.** The MoAbs were tested for reactivity to His-LASV-rNP and its fragments by Western blotting as reported previously (17, 32, 41).

**Pepscan analyses.** ELISA was performed as reported previously with the purified rNP or partial NP peptides as the antigen (33). The peptides were shifted by 1 amino acid (aa), with a consecutive overlap of 9 aa to cover the entire LASV-NP1 (aa residues from 1 to 100) and LASV-NP5 (aa residues from 361 to 460) fragments. Linear epitopes on the NP were determined by using Pepscan (Chiron Technologies, Clayton, Australia), according to the manufacturer’s instructions. Ninety-six peptides were prepared as 14-aa biotinylated peptides, including a 4-aa spacer sequence (SGSG) at the amino-terminal end, according to each of the amino acid sequences of the
LASV-rNP1 and LASV-rNP5 of the LASV Josiah strain. The methods were previously described in detail (33).

**IgG-ELISA.** IgG-ELISA was performed as previously described except for the antigen preparation (38, 39). Briefly, ELISA plates (96-well type plate, PRO-BIND™, Falcon, Becton Dickinson Labware, Franklin Lakes, NJ) were coated with the predetermined optimal quantity of purified His-LASV-rNP, LCMV-rNP, or JUNV-rNP (approximately 100 ng/well) at 4 °C overnight. Then, each well of the plates was inoculated with 200 µl of PBS containing 5% skim milk and 0.05% Tween-20 (M-T-PBS) and incubated for 1 h for blocking. The plates were washed 3 times with T-PBS and then inoculated with the test samples (100 µl/well), which were diluted 4-fold from 1:100 to 1:6400 with M-TPBS. After a 1 h incubation period, the plates were washed 3 times with T-PBS, and then the plates were inoculated with goat anti-human IgG antibody labeled with HRPO (1:1000-dilution; ZYMED LABORATORY). After a further 1 h incubation period, the plates were washed and 100 µl of ABTS solution (Roche Diagnostics, Mannheim, Germany) was added to each well. The plates were incubated for 30 min at room temperature and optical density (OD) was measured at 405 nm against a reference of 490 nm. The adjusted OD was calculated
by subtracting the OD of the negative antigen-coated wells from that of the corresponding wells. The mean and standard deviation (SD) were calculated from those of the 96 control sera. The cutoff value for the assay was defined as the mean plus 3 SD.

**Immunofluorescence.** The pQE32-LASV-NP was digested with BamHI and the insert was subcloned into the BamHI site of the pKS336 vector (40). The LASV-NP gene that was inserted into the pKS336 vector, pKS336-LASV-NP, was confirmed to be in the correct orientation to the promoter, tested for nucleotide sequencing as described above, and the nucleotide sequence of the gene was confirmed to be identical to the original sequence. HeLa cells were then transfected with pKS336-LASV-NP using FuGENE™6 transfection reagent (Roche Diagnostics) according to the manufacturer’s instructions. The cells transfected with the plasmid were selected with 3 µg/ml of blasticidin S-hydrochloride in MEM-10FBS. The HeLa cell clones were analyzed for the expression of LASV-rNP by indirect immunofluorescence assay (IIFA) using the rabbit serum raised against His-LASV-rNP. The cells expressing LASV-rNP were subcloned and used as IIFA antigens.

**Antigen-capture ELISA.** Ag-capture ELISA was performed as previously described (32, 41). The purified monoclonal antibody
to His-LASV-rNP, MoAb-4A5, produced in this study was diluted in PBS solution and 100 µl adsorbed overnight at 4°C onto the immunoplates (96-well type plate, PRO-BIND™, Falcon, Becton Dickinson Labware). Purified MoAb-4A5 was coated on the immunoplates at a concentration of approximately 100 ng/well in 100 µl of PBS. The difference in the procedures between the Ag-capture ELISA in this study and those in the previous studies (32, 37, 41) is that the MoAb, MoAb-4A5, and rabbit serum raised to His-LASV-rNP were used as capture and detector antibodies, respectively. The procedure for the Ag-capture ELISA is as follows: the ELISA plate was coated with capture monoclonal antibody, followed by blocking of the plate with M-T-PBS, addition of the samples to the ELISA plate, detection of the captured LASV-NP with rabbit serum raised to His-LASV-rNP, detection of rabbit IgG antibody that reacted with the captured antigen with goat anti-rabbit IgG antibodies conjugated with HRPO (Zymed Laboratories), and substrate reaction. In each run of the Ag-capture ELISA, the negative control antigen (M-T-PBS) was also tested. Serially diluted samples were added to the monoclonal antibody-coated wells. The OD\textsubscript{405} values of each well were adjusted by subtracting the OD\textsubscript{405} value of the negative control antigen from the corresponding well. The adjusted OD\textsubscript{405}
was taken as a measure of the amount of antigen specifically bound. All samples were treated with 1% Nonidet-P40 (NP-40) in PBS to destroy the LASV virion and expose the NP in the LASV virion.

**RT-PCR.** RT-PCR was performed as previously described (10). The primers used in the RT-PCR were 36E2 (5′-acccgggatcctaggcatt-3′) and 80E2 (5′-atataatgatgactgttgttctttgtgca-3′). The RT-PCR reaction was carried out with a Ready-to-Go RT-PCR tube (Pharmacia). The amplified PCR products were visualized with ethidium bromide in 2% agarose gel after electrophoresis.

**RESULTS**

**Expression of His-LASV-rNP.** Tn5 cells infected with each of the recombinant baculoviruses, Ac-His-LASV-NP, Ac-LCMV-rNP, and Ac-JUNV-rNP were suspended in PBS-2M urea. Most of the cell proteins were solubilized by this treatment, while the rNPs of these viruses remained insoluble. After centrifugation at 15,000 g for 10 min, pellet fractions were collected. The rNPs, which were still present in the pellet fractions, were completely solubilized in PBS-8M urea. The samples were then centrifuged at 15,000 g for 10 min, and the supernatant
fractions of the PBS-8M urea were confirmed to contain highly purified recombinant rNPs of arenaviruses (Fig. 2).

**Development of indirect immunofluorescence.** The LASV-rNP was expressed in HeLa cells by transfection with the expression vector, pKS336-LASV-NP. The transfected cells were stained by anti-His-LASV-rNP rabbit serum and human serum samples from LF-patients (Fig. 3). All 4 serum samples collected from 2 LF-patients showed a positive staining, but 96 control serum samples did not. The LASV-rNP-based IIFA was also evaluated using serum samples collected from monkeys experimentally infected with LASV. All the sera collected from 5 LASV-infected monkeys showed a positive staining, but those from 4 mock-infected monkeys did not.

**Development of His-LASV-rNP-based IgG-ELISA.** Four serum samples collected from LF-patients were determined to be positive by His-LASV-rNP-based IgG-ELISA, whereas 94 of the 96 control serum samples were determined to be negative. Thus, the sensitivity and specificity of the ELISA were 100% and 96%, respectively. All serum samples collected from 5 LASV-infected monkeys were determined to be positive, whereas those from 4 mock-infected monkeys were negative.

In order to examine cross-reactivity among arenaviruses in
the LASV-rNP-based IgG-ELISA, antisera against LASV-rNP, LCMV-rNP, or JUNV-rNP were examined (Fig. 4). The anti-LASV-rNP serum showed a strongly positive reaction, and anti-LCMV rNP and anti-JUNV-rNP sera showed strongly positive reactions in the IgG ELISA using the respective antigens (Fig. 4A, B and C). Anti-LCMV-rNP and anti-JUNV-rNP sera showed a less strongly positive reaction in the His-LASV-rNP-based IgG-ELISA than anti-LASV-rNP serum (Fig. 4A). Anti-LASV-rNP and anti-JUNV-rNP also showed a less strongly positive reaction in the His-LCMV-rNP-based IgG-ELISA than anti-LCMV-rNP serum (Fig. 4B). However, anti-LASV-rNP and anti-LCMV-rNP sera showed a negative reaction in the JUNV-rNP-based IgG-ELISA (Fig. 4C). Human sera from LF-patients showed a highly positive reaction in the LASV-rNP-based IgG-ELISA, but sera from AHF-patients did not (Fig. 4D). Serum from an AHF-patient showed a highly positive reaction in the JUNV-rNP-based IgG-ELISA (Fig. 4E). These results suggest that cross-reactive antibody among arenaviruses may be detected by the newly developed LASV-rNP-based IgG-ELISA.

**Development of LASV Ag-capture ELISA.** Three clones of a hybridoma that excreted a monoclonal antibody to His-LASV-rNP were established. The isotype of the 3 MoAbs were identified
as IgG. These MoAbs were designated MoAb-4A5, MoAb-6C11 and MoAb-2-11. Of these MoAbs, MoAb-4A5 was most efficient in capturing His-LASV-rNP in the Ag-capture ELISA format. The Ag-capture ELISA using MoAb-4A5 detected His-LASV-rNP concentrations as low as 800 pg/ml (Data not shown). Furthermore, the Ag-capture ELISA detected the MOPV-NP but not the rNPs of LCMV and JUNV (Data not shown).

All the sera collected from 5 LASV-infected hamsters on days 11 and 16 post infection were Ag-positive in the Ag-capture ELISA using MoAb-4A5 as a capture antibody, whereas the sera collected on days 0 and 4 were negative. The OD₄₅₀ values in the ELISA were the highest on day 11. The reactivity patterns in each hamster in the ELISA were similar to the viremia levels (Fig. 5). The sera collected on days 11 and 16 were found to be LASV genome-positive by RT-PCR (10). Thus, the sensitivity of the Ag-capture ELISA was similar to that of RT-PCR.

**Determination of the epitope recognized by the monoclonal antibodies.** The epitope recognized by MoAbs was determined. The MoAb-4A5 reacted in Western blotting with GST-LASV-rNP1-6 (full length LASV-rNP), GST-LASV-rNP1-5 and GST-LASV-rNP1-4, but not with the other truncated LASV-rNPs shown in Table 1, suggesting that MoAb-4A5 reacted with a conformational epitope located on
the amino-terminal portion of LASV-rNP. The epitope was maintained when the extreme amino-terminal portion, LASV-rNP1, was present, but was lost when LASV-rNP1 was removed. These results suggest that the extreme amino-terminal portion, LASV-rNP1, is essential for the maintenance of the conformational epitope. The MoAb-4A5 and MoAb-2-11 reacted in Western blotting with GST-LASV-rNP1 and GST-LASV-rNP5, respectively (Table 1).

The pepscan analyses indicated that MoAb-6C11 and MoAb-2-11 recognized linear epitopes. 6C-11 and 2-11 MoAbs recognized GLDFSEV (aa position 41-47) within LASV-rNP1 and FATQP (amino acid position 439-443) within LASV-rNP5, respectively (Fig.6).

**DISCUSSION**

The present paper reports the development of diagnostic systems (antibody and antigen detection systems) for LF using LASV-rNP. The LASV-rNP-based IgG-ELISA was sensitive and specific in detecting anti-LASV-IgG. Although the data were not shown, IgM-capture ELISA using purified LASV-rNP as an antigen was developed in the same way as that shown in previous reports and detected LASV-IgM antibody (42, 43). All sera collected from LF-patients and monkeys infected with LASV showed positive
reactions in the LASV-rNP-based IIFA. The staining patterns of
the rNP with these sera were granular in the IIFA (Fig. 3), making
it easy to distinguish positives from negatives. IIFA using
LASV-rNP-expressing HeLa cells was also highly sensitive and
specific in detecting LASV-IgG. In the preliminary study,
approximately 15% of the sera collected from 334 Ghanaians and
only less than 1% of 280 Zambians showed positive reactions in
the LASV-rNP-based IgG ELISA (our data). The results are
considered to be compatible with the fact that LF is endemic
to the western African region including Ghana but not to the
eastern African region. The LASV-rNP-based antibody detection
systems such as ELISA and IIFA were suggested to be useful not
only in the diagnosis of but also in the seroepidemiological
study on LF.

The LASV-rNPs were expressed by a transformation system
in Escherichia coli or by recombinant baculovirus systems and
have already been applied as antigens in ELISA, Western blotting,
and IIFA for detection of antibodies to LASV (4, 14, 16, 22,
23, 44). In the present study, Ag-capture ELISA using MoAbs to
LASV-rNP was also developed. Furthermore, detection of the
cross-reactive antibody by LASV-rNP-based IgG-ELISA was
examined. The results for cross-reactivity indicate that the
LASV-rNP-based IgG-ELISA detects not only the antibodies to LASV but also those to LCMV.

The Ag-capture ELISA using MoAb-4A5 was confirmed to be useful in the detection of authentic LASV antigen in sera serially collected from hamsters infected with LASV. The sensitivity of the MoAb-4A5-based Ag-ELISA was similar to that of conventional RT-PCR, the efficiency of which in the diagnosis of LF was previously reported (10). Therefore, the MoAb-4A5-based Ag-capture ELISA is regarded as useful in the diagnosis of LF. Unfortunately, the efficacy of the MoAb-4A5-based Ag-capture ELISA in the diagnosis of LF was not evaluated using serum samples from patients. Thus, further study is still required. The 3 MoAbs including MoAb-4A5 were characterized and the corresponding amino acid residues within the NPs of LASV, MOPV, LCMV and JUNV to the epitope of MoAb-6C11 and MoAb-2-11 are summarized in Panel C of Fig. 6. It was of interest that LASV, MOPV, LCMV and JUNV might be identified by analyses of the reactivity patterns of MoAb-4A5, MoAb-6C11 and MoAb-2-11 to the NPs of each virus. The NP of all LASV strains circulating in the western and central parts of Africa would be detected by the MoAb-4A5-based Ag-capture ELISA, as this ELISA was able to detect MOPV-NP that was different from LASV.
in terms of genetic and evolutional characteristics.

We have so far reported the development of antibody- and Ag-detection systems using the recombinant nucleoprotein of the viruses for EHF, MHF and CCHF (32, 33, 36-42). Recently, a number of highly pathogenic emerging virus infections in humans appeared, such as Nipah virus encephalitis (8), SARS-coronavirus infections (21, 35), and highly pathogenic avian influenza virus infections (9, 45, 46). The strategy shown herein might be applicable to the development of diagnostic systems for severe viral infections whose etiologic agents are highly pathogenic to humans, as an alternative method to those using infectious viruses.

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Table 1. Reactivity of the MoAb developed in the present study with the GST-tagged truncated LASV-rNP in western blotting.

<table>
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<tr>
<th>Truncated LASV-rNP</th>
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<td>LASV-rNP1</td>
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<td>N.D.</td>
<td>-</td>
<td>N.D.</td>
</tr>
<tr>
<td>LASV-rNP5-6</td>
<td>N.D.</td>
<td>-</td>
<td>N.D.</td>
</tr>
</tbody>
</table>

*1 and *2: "+" and "-" indicate positive and negative reactions, respectively.

35
*3: "N.D." indicates "not determined".
*4: LASV-rNP1-6 indicates LASV-rNP.

Table 2. Reactivity of the MoAbs developed in the present study with the NPs of LASV, MOPV, LCMV and JUNV in Western blotting.

<table>
<thead>
<tr>
<th>MoAb</th>
<th>NP</th>
<th>LASV</th>
<th>MOPV</th>
<th>LCMV</th>
<th>JUNV</th>
</tr>
</thead>
<tbody>
<tr>
<td>4A5</td>
<td></td>
<td>+</td>
<td>+</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>6C11</td>
<td></td>
<td>N.D.</td>
<td>-</td>
<td>+</td>
<td>-</td>
</tr>
<tr>
<td>2-11</td>
<td></td>
<td>+</td>
<td>N.D.</td>
<td>-</td>
<td>-</td>
</tr>
</tbody>
</table>

*1 and *2: "+" and "-" indicate positive and negative reactions, respectively.
*3: "N.D." indicates "not determined". The reactivity of the MoAb-6C11 and MoAb-2-11 was not evaluated with MOPV-NP. However, theoretically, MoAb-6C11 should be reactive with MOPV-NP, due to the presence of the amino acid residues that can react with MoAb-6C11, but MoAb-2-11 should be non-reactive with MOPV-NP, due to the absence of the amino acid residues that can react with MoAb-2-11.
Figure legends

Fig. 1. Schematic representation of truncated LASV-rNP expressed as a form of GST-fusion protein in *E. coli* transformed with the corresponding expression vector. The description “LASV-NP1-6” in panel A indicates full-length LASV-rNP.

Fig. 2. SDS-PAGE analyses of the purification of His-LASV-rNP using the Ni²⁺-column purification method (A) and of the semi-purification strategy based on the hydrophobic property of arenavirus NPs (B). The supernatant fractions of the Ac-•P-, Ac-His-LASV-NP, Ac-LCMV-NP, or Ac-JUNV-NP-infected Tn5 cells treated with PBS-2M urea (B, left part). The pellet fractions of these cells treated with PBS-2M urea were further solubilized with PBS-8M urea (B, right part).

Fig. 3. Staining patterns of LASV-rNP-expressing HeLa cells by a LF-patient’s (A) and a non-LF-patient’s (B) sera in IIFA.

Fig. 4. Reactivity of antibodies to arenaviruses (LASV, LCMV and JUNV) to the rNPs of these viruses. Reactivity of rabbit sera raised to LASV-rNP (●), LCMV-rNP (■), or JUNV-rNP (▲) with the antigens, His-LASV-rNP (A), LCMV-rNP (B) and JUNV-rNP.
(C) in IgG-ELISA is shown. Reactivity of the sera collected from patients with LF (D) and AHF (E) with the antigens, LASV-rNP (●), LCMV-rNP (■), JUNV-rNP (▲), and negative control antigen (◆) in IgG-ELISA is also shown.

Fig. 5. Detection of the LASV genome by the RT-PCR (A), LASV-NP by the LASV-NP-Ag-capture ELISA (B), and the infectious dose of LASV (C) in serially collected sera of hamsters experimentally infected with LASV. The OD$_{405}$ values in panel B are those obtained at a dilution level of 1:40.

Fig. 6. Pepscan analyses to determine the epitopes of MoAb-6C11 (A) and MoAb-2-11 (B). The vertical bar indicates the amino acid residues with amino acid position within the LASV-NP. MoAb-6C11 was confirmed to react with 7-amino acid residues positioned from 42nd to 48th, GLDFSEV, within LASV-NP1 (A). MoAb-2-11 was confirmed to react with 5-amino acid residues positioned from 439th to 443rd, FATQP, within LASV-NP5 (B). The corresponding amino acid residues to the epitope of the MoAb-6C11 and MoAb-2-11 among MOPV, LCMV and JUNV are shown (C). The GenBank accession numbers for the S-genes of LASV, MOPV, LCMV and JUNV are NC_004296, AY772170, AY847350 and DQ272266, respectively.
The epitope of the MoAb-6C11 is present not only in the NP of LASV, but also in those of MOPV and LCMV, but not in that of JUNV.
(A) Purified LASV-rNP

(B) 2M urea

2M urea

8M urea

ΔP
JUNV-NP
LASV-NP
LCMV-NP
ΔP
JUNV-NP
LASV-NP
LCMV-NP
OD_{405} measured in the Ag-capture ELISA

Viremia level (ffu/ml)
(C) Amino acid sequence of the corresponding region

<table>
<thead>
<tr>
<th>Arenavirus-NP</th>
<th>MoAb-6C11</th>
<th>MoAb-2-11</th>
</tr>
</thead>
<tbody>
<tr>
<td>LASV-NP</td>
<td>31-VVKDAQALLHGLDFSEVNVQRLMRKERRD</td>
<td>431-HGIDVTDLFATQPGGLTSAVI</td>
</tr>
<tr>
<td>MOPV-NP</td>
<td>31-VIKDAQALLHGLDFSEVANVQRLMRKEKRD</td>
<td>432-HGIDIQDLFSVQPGGLTSAVI</td>
</tr>
<tr>
<td>LCMV-NP</td>
<td>31-VIKDATNLLNLGLDFSEVSNVQRMKEKRD</td>
<td>424-HGMDLADELNAQPGLTSSVI</td>
</tr>
<tr>
<td>JUNV-NP</td>
<td>31-VLKDAKLADSIDFNQVAQVQRALRKTKRG</td>
<td>422-HGILMKDIEDAMPGVLSYVI</td>
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</tbody>
</table>