Development and validation of serological assays for viral hemorrhagic fevers and determination of the prevalence of Rift Valley fever in Borno State, Nigeria

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Background: Rift Valley fever (RVF) is endemic to the tropical regions of eastern and southern Africa. The seroprevalence of RVF was investigated among the human population in Borno State, Nigeria to determine the occurrence of the disease in the study area in comparison with that of Lassa fever and Crimean-Congo Hemorrhagic fever.

Methods: Recombinant nucleoprotein (rNP)-based IgG-ELISAs for the detection of serum antibodies against RVF virus (RVFV), Lassa fever virus (LASV), and Crimean-Congo hemorrhagic fever virus (CCHFV) were used to test human sera in Borno State, Nigeria. The presence of neutralizing antibody against the RVFV-glycoprotein-bearing vesicular stomatitis virus pseudotype (RVFVpv) was also determined in the human sera.

Results: Of the 297 serum samples tested, 42 (14.1%) were positive for the presence of RVFV-IgG and 22 (7.4%) and 7 (2.4%) of the serum samples were positive for antibodies against LASV and CCHFV, respectively. There was a positive correlation between the titers of neutralizing antibodies obtained using RVFVpv and those obtained using the conventional neutralization assay with the attenuated RVFV-MP12 strain.

Conclusions: The seroprevalence of RVF was significantly higher than that of LASV and CCHF in Borno State, Nigeria. The RVFVpv-based neutralization assay developed in this study has the potential to replace the traditional assays based on live viruses for the diagnosis and seroepidemiological studies of RVF.

Keywords: Nigeria, Rift Valley fever, Seroprevalence

Introduction

Rift Valley fever virus (RVFV) is a zoonotic mosquito-borne virus belonging to the genus Phlebovirus in the Family Bunyaviridae. It causes severe diseases in humans and livestock throughout Africa1 and the Arabian Peninsula2. RVFV is also considered to be a potential bioterrorism agent. In the last few decades, Rift Valley fever (RVF) outbreaks have been reported in eastern and southern Africa (e.g., Kenya, Somalia, United Republic of Tanzania, Madagascar and South Africa).3,4 In contrast, there have been very few reports on the recent occurrence of RVF in western and central Africa. Significant high- and low-prevalence clusters of RVF in sub-national areas on the African continent have been reported.5 Since the spread of RVFV largely depends on the mosquito vectors and the translocation of animal hosts, an endemic situation usually occurs in the restricted geographical areas inhabited by their hosts and vectors. In Nigeria, RVFV antibodies have been found in sheep, goats, cattle, horses and camels in the northern states of Kaduna and Sokoto7 and in the plateau area8 suggesting that the virus may be enzootic in Nigeria. In addition, serological studies conducted on human sera have confirmed the existence of the disease in Nigeria.9 The specific geographical location of Borno State in northeastern Nigeria, which shares international borders with three other African countries (Cameroon, Chad and Niger), makes it vulnerable to the trans-boundary spread of various diseases, including viral hemorrhagic fevers (VHFs). In addition, Borno State has been reported as the niche for Lassa fever virus (LASV) and possibly other VHFs. However, the epidemiology of RVF and other VHFs has not been extensively investigated in Borno State. A detailed and accurate investigation of the seroprevalence is necessary to ascertain the occurrence and spread of RVF in this area.
RVFV possesses a single-stranded tripartite RNA genome composed of three segments: S, M and L. The S segment encodes the nucleocapsid protein (NP) and non-structural (NS) protein, using an ambisense strategy. The M segment encodes the precursor for the glycoproteins Gn and Gc and two non-structural proteins of 78 kDa and 14 kDa. The L segment encodes the L protein. The nucleotide sequence of the NP gene is highly conserved among various RVFV strains. Serum antibodies against NP are readily detected early after infection and in convalescent individuals, providing a basis for the diagnosis of RVF.

The traditional diagnostic assays for VHF are based on immunoassays that use live viruses as the source of capture antigens. The use of highly attenuated RVFV (RVFV-MP12) does not require stringent biosafety measures and could readily be adopted in laboratories in developing countries where infrastructures for biosafety level 3 or 4 containments are lacking. The usefulness of recombinant viral nucleoprotein (rNP)-based serological assays, such as IgG-ELISAs and immunofluorescence assays (IFAs) for the detection of antibodies against VHSFs such as Crimean-Congo hemorrhagic fever virus (CCHFV) and LASV have been reported. Recombinant protein technology does not require high containment biosafety facilities and could readily meet the demand for a simple and reliable system not only for diagnosis of VHFV but also for comparative seroepidemiology of various VHSFs in a cohort study.

In this study, the seroprevalence of RVFV infection in humans in Borno State, Nigeria, was determined using rNP-based IgG ELISAs, and the prevalence of RVFV antibody was compared with those of other hemorrhagic fever virus infections including LASV and CCHFV. In addition, we developed virus neutralization assays using vesicular stomatitis virus (VSV) pseudotype virus-bearing glycoproteins of RVFV, and the usefulness of the VSV pseudotype system was determined for a high throughput screening of neutralizing antibodies against RVFV.

Materials and methods

Serum samples

Two hundred and ninety-seven serum samples were collected between September 2011 and February 2012 from patients attending health facilities (government hospitals, private hospitals or clinics) in 10 out of the 27 local government areas (LGAs) in Borno State in northern Nigeria. A simple random sampling technique was used to obtain human sera from the selected LGAs, which consisted of at least three LGAs from each of the three senatorial districts (North, Central and South), and also from the town of Lassa.

Expression and purification of rNPs

Insect Tn5 cells were infected with recombinant baculoviruses expressing rNPs of RVFV, LASV or CCHFV to produce recombinant His-tagged RVFV-rNP, LASV-rNP or CCHFV-rNP, respectively. The rNPs were purified by Ni\textsuperscript{2+} column chromatography (QIAGEN GmbH, Hilden, Germany), as described previously. The negative control antigen (ΔP) was prepared from a baculovirus (Ac-ΔP) that lacks polyhedrin expression using the same protocols as for the rNPs. Expression of the His-tagged rNPs and ΔP was analyzed by SDS-PAGE gels (12% polyacrylamide) stained with Coomassie blue (Bio-Rad Laboratories, Hercules, CA, USA) (Supplementary Figure 1).

IgG-ELISA

IgG-ELISA was performed as described previously. Briefly, 96-well ELISA plates were coated with the predetermined optimal quantity of purified RVFV-rNP, LASV-rNP or CCHFV-rNP (approximately 100 ng/well) at 4°C overnight. Each well of the plates was then covered with 200 μl of PBS containing 5% skim milk and 0.05% Tween 20 (Sigma, St. Louis, MO, USA) (PBST-M), followed by incubation for 1 h at 37°C for blocking. The plates were washed three times with PBS containing 0.05% Tween 20 (PBST) and then inoculated with test serum (100 μl/well), which was diluted 1:400 and 1:1600 with PBST-M. After a 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 the optical density at 405 nm (OD\textsubscript{405}) was measured against a reference of 490 nm. The adjusted OD\textsubscript{405} value was calculated by subtracting the OD\textsubscript{405} value of the negative Ag-coated wells from that of the corresponding wells. The mean plus three standard deviations (mean+3SD) of the ELISA indices for the IgG-ELISAs were calculated using human serum samples that were confirmed to be negative for infection with the respective viruses by IFA (data not shown) and was used as the cut-off value for the IgG-ELISAs. In order to minimize false-positive results that could occur with single serum dilution, IgG response was considered to be positive if the sample showed adjusted OD\textsubscript{405} values above the cut-off at both 1:400 and 1:1600 dilutions.

Conventional neutralization assay

The conventional neutralization assay using infectious RVFV (RVFV-MP12 strain) was performed as described previously. Briefly, heat-inactivated serum samples were diluted three-fold (from 1:40 to 1:1080) with Eagle’s minimum essential medium (MEM, Sigma, St. Louis, MO, USA) containing 2% FBS (Invitrogen, Carlsbad, CA, USA). Each test sample (50 μl) was then mixed with the same volume of RVFV-MP12 at an infectious dose of 100 plaque forming units. The mixture was then incubated for 1 h at 37°C for neutralization. After incubation, the mixtures were tested for neutralization by the cytopathic effect inhibition assay using Vero E6 cells. The neutralization antibody (NAb) titer was defined as the reciprocal of the highest dilution at which no cytopathic effect was observed.

Generation of VSV pseudotyped with RVFV-glycoprotein

The glycoprotein (GP) cDNA of RVFV-MP12 was cloned into the pKS336 vector to construct an RVFV-GP expression plasmid, designated as pKS336-RVFV-GP. To generate the RVFV-GP-bearing VSV pseudotype (RVFVpV), a *G-VSVΔG/luc encoding luciferase gene (kindly provided by Dr. M. A. Whitt), instead of the VSVG gene, was inoculated into human kidney 293 T cells transfected
with pKS336-RVFV-GP. After 24 h the culture supernatants were collected and used as a working seed for the RVFVpv.

**Neutralization test using RVFVpv**

The dilution of RVFVpv used was calculated to produce approximatley $10^5$ relative light units in control wells. Serum samples were mixed with RVFVpv at a dilution of 1:50 in MEM (Sigma) supplemented with 2% FBS (Invitrogen). Then, the mixture was incubated at 37°C for 1 h for neutralization. The serum-RVFVpv mixture was transferred to 96-well plates containing monolayers of Vero E6 cells. After 24 h the cells were lysed, and the luciferase activities were measured using the Bright-Glo Luciferase Assay System (Promega, Madison, WI, USA) according to the protocol recommended by the manufacturer.

**Statistical methods**

The sensitivity, specificity and predictive values for positive and negative tests were calculated by standard methods. Spearman’s rank correlation coefficient test, ROC curves and two-graph-ROC (TG-ROC) curves were analyzed using the Stat Flex ver. 5 software program (Artech Co. Ltd., Osaka, Japan).

**Results**

**rNP-based IgG-ELISA**

In order to determine the seroprevalence of RVFV in humans in Borno State, Nigeria, sera were first subjected to an His-RVFV-rNP-based IgG-ELISA. An IgG response was considered to be positive if the sample had adjusted OD_{405} values higher than cut-off values at both 1:400 and 1:1600 dilutions. Of the 297 serum samples analyzed, 42 (14.1%) were positive for RVFV IgG (Table 1). Simultaneously, the serum samples were also tested for the presence of antibodies against LASV and CCHFV using the rNP-based IgG ELISAs, and a total of 22 (7.4%) and 7 (2.4%) of the samples were positive for antibodies against LASV and CCHFV, respectively (Table 1). The result indicated a high prevalence rate of RVF in the study area.

|               | IgG-ELISAa | LASV-rNP ELISA | CCHFV-rNP ELISA | NAb assay   |
|---------------|------------|----------------|-----------------|-------------|
|               | RVFV-rNP ELISA |               |                 | RVFVpv NAbb | RVFV-MP12 NAb |
| No. positive (%) | 42 (14.1)    | 22 (7.4)       | 7 (2.4)         | 43 (15.5)   | 34 (12.6)      |
| No. negative (%) | 255 (85.9)   | 275 (92.6)     | 290 (97.6)      | 235 (84.5)  | 237 (87.4)     |
| Total          | 297 (100)    | 297 (100)      | 297 (100)       | 278 (100)   | 271 (100)      |

CCHFV-rNP: Crimean-Congo hemorrhagic fever virus recombinant nucleoprotein; LASV-rNP: Lassa fever virus recombinant nucleoprotein; NAb: serum neutralization antibody; RVFV: Rift Valley fever virus; RVFV-MP12: highly attenuated MP12 strain of RVFV; RVFV-rNP: RVFV recombinant nucleoprotein; RVFVpv: RVFV-glycoprotein-bearing vesicular stomatitis virus pseudotype.

a IgG response considered positive if the sample had a positive titer at both 1:400 and 1:1600 dilutions.
b >75% inhibition considered positive.
the sensitivity and specificity curves, the cut-off value was 75% neutralization, and the sensitivity and specificity were 94% and 95%, respectively. If the cut-off was defined as 50% neutralization, the sensitivity would increase to 100%, but specificity would decrease to 89% (Figure 1). There was a positive correlation between the titers of neutralizing antibodies obtained using RVFVpv and those obtained using the conventional neutralization assay with RVFV-MP12. Spearman’s rank correlation coefficient ($r_s$) was calculated to be 0.77 (Figure 2). Thus, NAb’s against RVFV can be screened by the RVFVpv-based neutralization assay using the single serum dilution format.

### Discussion

In this study, the seroprevalence of RVF was investigated among human population in Borno State, Nigeria to determine the occurrence and spread of the disease in comparison with those of Lassa fever and CCHF. Since recombinant protein-based immunoassays with high sensitivity and specificity have been demonstrated to be useful for the diagnosis of VHF’s in humans,15–17,25,26 we have used rNP-based IgG-ELISAs for the detection of serum antibodies against RVFV, LASV, and CCHFV. Of the 297 serum samples tested for RVFV-IgG, a total of 42 (14.1%) showed positive results (Table 1). The antibody prevalence observed in this study is in agreement with the results of a surveillance study carried out in the 1980s, when 14.8% of the sera collected in more than 30 locations throughout Nigeria were found to be positive for a hemagglutination-inhibiting antibody against RVFV.11 In this study a significant difference in the prevalence of antibodies was observed (RVFV rNP-ELISA [14.1%], LASV rNP-ELISA [7.4%] and CCHFV rNP-ELISA [2.4%]), and the highest prevalence was noted for RVFV rNP-ELISA antibody. In addition, more than 12% of the samples tested had NAb activity against RVFV. These results indicate that RVFV is more actively circulating in the study area compared with LASV and CCHFV. It is therefore important to undertake a risk assessment of RVFV infection in humans in Nigeria.

RVFV is transmitted through contact with body fluids from infected humans and animals or by mosquito bites and/or aerosols. The particular location of Borno State, with its shared geographical borders with three other African countries, indicates that a regional epidemiological study should be conducted not only in the LGAs in Nigeria, but also in the neighboring countries, to identify the possible risk factors for transboundary RVFV infection. The borders are porous and unrestricted human and animal

### Table 2. The relationship between the results of the authentic virus (RVFV-MP12 strain)-based and RVFVpv-based neutralization assays (n=270)

| NAb for RVFV-MP12 | % Neutralization |
|-------------------|-----------------|
|                   | >75  | 50–75 | <50  |
| Positive          | 32 (11.9%) | 2 (0.7%) | 0 (0%) |
| Negative          | 11 (4.1%) | 14 (5.2%) | 211 (78.1%) |
| Total             | 43 (15.9%) | 16 (5.9%) | 211 (78.1%) |

NAb: serum neutralization antibody; RVFV-MP12: highly attenuated MP12 strain of Rift Valley fever virus; RVFVpv: RVFV-glycoprotein-bearing vesicular stomatitis virus pseudotype.

### Figure 1. The results of the ROC and two-graph (TG)-ROC analyses of the vesicular stomatitis virus pseudotype virus-bearing glycoproteins of Rift Valley fever virus, (RVFVpv)-based neutralization assay. In (A) the ROC analysis graph, the specificity values are deducted from 1.0 in the x-axis, and the sensitivity vs 1-specificity data are plotted as dots. In (B) the TG-ROC analysis graph, sensitivity data are plotted as dots and specificity data are plotted as diamonds.
We also developed a novel serum neutralization test using RVFVpv to detect serum antibody against RVFV. The reliability and usefulness of the assay were evaluated by comparing the results of the assay with those obtained from the widely used ‘gold-standard’ neutralization assay using infectious RVFV. Of the 43 serum samples that showed more than 75% neutralization by the RVFVpv-based assay, 11 were negative by the conventional RVFV-MP12 neutralization assay (Table 2). It is possible that the results of the neutralization assay using RVFVpv are more sensitive than the standard neutralization assay using RVFV-MP12. This assumption is supported by the observation that the NAB titers measured using pseudotyped VSV bearing the GP of Nipah virus or SARS-coronavirus are higher than those obtained by using infectious viruses.\textsuperscript{21,29,30}

The ROC and TG-ROC analyses indicated an appropriate cut-off value for the percent neutralization (75%) to demonstrate that the NABs in the sera had high sensitivity and specificity. The conventional assay for measuring NABs requires serial-dilutions of the test serum and takes several days for the virus to replicate to a level which results in plaque-forming or cytopathic effects in infected cells. However, the new assay based on RVFVpv employs a single serum dilution (1:50) and has a quantitative nature, where the luciferase activity can be determined one day after inoculation onto cells. Finally, pseudotyped VSVs do not produce infectious progeny viruses, thereby ensuring their safe use as diagnostic tools. Taken together, our results indicate that the RVFVpv-based assay for measuring NAB has safe, rapid and high-throughput diagnostic capabilities.

Our study has limitations, one of which is the relatively small sample size compared to the previous study, carried out in the 1980s, employing more than 3000 human sera from the different ecological zones in Nigeria.\textsuperscript{11} In addition, we could not access detailed information on the subjects (age, profession, history of illness etc.). Although our data were obtained using the most recent serological procedures, the lack of demographic information on the study subjects makes it difficult to provide an advanced epidemiological understanding of VHF s in Nigeria. However, this study has important strengths: it provides information on RVFV infection with a high prevalence in human population in Borno State and demonstrates the usefulness of the VSV-based neutralization assay for the epidemiological investigations.

Conclusions

The results of rNP-based ELISA have shown that approximately 14% of the study population in Borno State, Nigeria, have a history of RVFV infection, and the seroprevalence of RVF was higher than those of other viral hemorrhagic fevers such as Lassa fever and CCHF. In addition, the RVFVpv-based NAB assay developed in this study has the potential to replace traditional assays based on live viruses for the diagnosis and seroepidemiological analysis of RVF in endemic and non-endemic countries.

Supplementary data

Supplementary data are available at Transactions Online (http://trstmh.oxfordjournals.org/).

Authors’ contributions: DNB, FK and SSB conceived the study; SF and MS designed the study protocol; DNB, HT, TY, ST, KI and AF carried out the serological assays, and analysis and interpretation of the data. DNB and SF drafted the manuscript; MS, SM, MS and SSB critically revised the manuscript for intellectual content. All authors read and approved the final manuscript. SF and MS are guarantors of the paper.

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Competing interests: None declared.

Ethical approval: This study protocol was approved by the Borno State Ministry of Health, Nigeria and the Ethics Committee of the National Institute of Infectious Diseases, Tokyo, Japan [No. 371].

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