from cynomolgus macaques in the RESTV epizootic showed higher IL-1ra responses in the convalescent phase than in the non-convalescent phase (Figure 1). There were no significant differences in the concentration of IL-10 between the two groups (data not shown). This suggests that, unlike other ebolaviruses infections, RESTV does not induce MARS, which is characterized by an elevated induction of IL1ra in the acute phase.

In conclusion, we have shown that the anti-$GP_{1,2}$ responses, rather than the anti-NP responses, in cynomolgus macaques naturally infected with RESTV were specifically detected in the convalescent stage of RESTV infection. In addition, a high concentration of proinflammatory cytokines/chemokines was observed in the convalescent phase. Therefore, the anti-$GP_{1,2}$ response and the upregulation of the specific proinflammatory response might be useful indicators of convalescence from RESTV infection in cynomolgus macaques.

Conclusions

In this study, we analyzed the humoral responses in cynomolgus macaque serum samples collected during the 1996 Reston outbreak in the Philippines and demonstrated that the anti-RESTV $GP_{1,2}$ response and the proinflammatory innate response play significant roles in the convalescence from RESTV infection in cynomolgus macaques.

Methods

Sera

Twenty-seven cynomolgus macaque serum samples were obtained from the cynomolgus macaque facility in the Philippines where the 1996 RESTV epizootic occurred [27]. The serum specimens in the affected facility were collected under quarantine of the Philippines. Nineteen of the 27 samples were previously subjected to an antigen capture ELISA. Nine of the samples were found to be RESTV antigen-positive, and the remaining 10 were considered to be antigen-negative [15]. The serum specimens were treated at 56°C for 30 minutes and virus in the cynomolgus macaque serum samples were inactivated. As negative controls for the IgG-ELISA and IFA, we used serum samples from 102 cynomolgus macaques collected at the Tsukuba Primate Research Center (TPRC) in Japan. As positive controls for the IgG-ELISA and IFA, two rabbits were immunized four times with the histidine-tagged ectodomain of the RESTV glycoprotein ($GP_{1,2}$) (RESTV $GP_{1,2}\Delta TM$). The histidine-tagged RESTV $GP_{1,2}\Delta TM$ of a 1996 RESTV [28] was prepared and purified as described below. The sera were collected from the rabbits, inactivated, and stored at 4°C until use. The experiments with animals were performed in accordance with the Animal Experimentation Guidelines of the National Institute of Infectious Diseases. The protocol was approved by the Institutional Animal Care and Use Committee of the institute (Permit number: 990163 and 109075).

Expression and purification of the recombinant RESTV $GP_{1,2}$ ectodomain

A recombinant baculovirus that expresses RESTV $GP_{1,2}\Delta TM$ was used to prepare recombinant RESTV $GP_{1,2}$ for the IgG-ELISA [16]. Briefly, a recombinant baculovirus carrying the ectodomain of RESTV $GP_{1,2}$ (DDBJ accession no. AB050936) with histidine-tag sequences at its 3'-terminus was infected into Tn5 cells at a multiplicity of infection (moi) of 1. The cells were collected, washed with PBS, and then lysed in PBS containing 1% Nonidet P40 (NP-40) on ice for 15 min. After being centrifuged, the recombinant RESTV $GP_{1,2}\Delta TM$ was purified with Ni^{2+}-agarose beads (QIAGEN, Hilden, Germany) and His Bind Kits (Novagen, Darmstadt, Germany). The purified recombinant RESTV $GP_{1,2}\Delta TM$ was used for the IgG-ELISA specific for RESTV $GP_{1,2}$. Lysates of Tn5 cells infected with baculovirus with a deleted polyhedrin gene, Ac-ΔP, were similarly processed and then used as negative control antigen in the IgG-ELISA described below.

RESTV $GP_{1,2}$-specific IgG-ELISA

Ninety-six well plates were coated with the RESTV $GP_{1,2}\Delta TM$ or with negative control antigen in 100 μl of PBS and incubated overnight at 4°C. The plates were washed three times with PBS containing 0.05% Tween 20 (PBS-T), and then 200 μl of PBS-T containing 5% skim milk (SKIM-PBS-T) was added to each well and incubated for 2 hr at 37°C. The cynomolgus macaque sera were diluted at 1:100, 1:400, 1:1,600, and 1:6,400 in SKIM-PBS-T, and the hyperimmune rabbit sera were four-fold serially diluted from 1:1,000 to 1:64,000 in SKIM-PBS-T. One hundred microliters of each serum dilution was added to the antigen-coated wells and incubated for 1 hr at 37°C. After they were washed three times with PBS-T, the wells were further reacted with either HRP-conjugated goat anti-human IgG (H + L) (Lot:60504974, ZyMED) or HRP-conjugated goat anti-rabbit IgG (H + L) (Lot:398581A, ZyMED) at a dilution of 1:1,000 in SKIM-PBS-T. After being washed three times again with PBS-T, the ABTS substrate (Roche Diagnostics) was added to the wells. Then, the plates were incubated for 30 minutes at 37°C, and the OD values of the wells at 405 nm were measured. Adjusted OD values were calculated by subtracting the OD value of the wells coated with the negative control antigen from that of the wells coated with RESTV $GP_{1,2}\Delta TM$.

RESTV NP-specific IgG-ELISA

The NP-specific IgG-ELISA, which is similar to the $GP_{1,2}$-specific ELISA except for the purified recombinant

RESTV NP with a histidine tag at the C-terminus, has been previously reported [6,16].

ELISA index and determination of the cut-off value for the IgG-ELISA

The sum of the OD values of serum dilutions at 1:100, 1:400, 1:1,600, and 1:6,400 for each specimen was calculated and designated as an "ELISA index" in the IgG-ELISA. The mean plus three standard deviations (SD) of the ELISA indices for the IgG-ELISAs was calculated using serum samples from uninfected TPRC cynomolgus macaques and was used as the cut-off value for the IgG-ELISAs.

Indirect immunofluorescent antibody assay (IFA) specific for RESTV NP and $GP_{1,2}$

The IFA specific for RESTV NP was reported previously [8]. In the present study, a RESTV $GP_{1,2}$-specific IFA was established using stably RESTV $GP_{1,2}$-expressing HeLa cells. HeLa cell line was purchased from the American Type Culture Collection and used. The RESTV $GP_{1,2}$ cDNA of a 1996 RESTV was subcloned into a mammalian expression plasmid, pKS336, to generate pKS336-RESTV-$GP_{1,2}$. The HeLa cells expressing RESTV $GP_{1,2}$ were selected in a medium containing 2 μg/ml of blasticidin-S-hydrochloride (Sigma, St. Lois, MO) after transfection with pKS336-RESTV-$GP_{1,2}$ using the FuGENE HD Transfection Reagent (Roche Diagnostics, Germany). The cells were trypsinized, washed with PBS, and mixed with normal HeLa cells, and were then spotted on 14 well Teflon-coated glass slides, air dried, and fixed with acetone at room temperature for 5 min. The slides were stored at -80°C until use.

The slides were thawed and dried just before use. The serum specimens were 2-fold serially diluted in PBS, and a 20 μl aliquot of each dilution was applied to the wells of the antigen slides and incubated at 37°C for 1 hr in a humidified chamber. Then the antigen slides were washed with PBS and reacted with 20 μl per well of FITC-conjugated goat anti-human IgG (H + L) (ZyMax lot: 415460A, Invitrogen, CA, U.S.A.) for cynomolgus macaque sera and FITC-conjugated goat anti-rabbit IgG (H + L) (ZyMax lot: 402686A, Invitrogen, CA, U.S.A.) for rabbit hyperimmune sera at a dilution of 1:100. After incubation at 37°C for 1 hr, the slides were washed with PBS and covered with micro cover glasses. The slides were examined for the staining pattern under a fluorescent microscope. The antibody titer in the IFA was determined as the reciprocal of the highest dilution showing positive staining.

RESTV neutralization (NT) assay using VSV-RESTV-$GP_{1,2}$/GFP

The VSV pseudotype bearing RESTV $GP_{1,2}$, VSV-RESTV-$GP_{1,2}$/GFP was generated essentially according to the method described for the VSV pseudotype bearing SARS-CoV S protein [29], except that pKS336-RESTV-$GP_{1,2}$ was used in the present study [17]. Briefly, 293 T cells were prepared in 24 well plates at 20-30% confluency. The cells were transfected with pKS336-RESTV-$GP_{1,2}$ using FuGENE HD. The cells were then cultured for 24 hr and inoculated with VSV ΔG*/GFP pseudotyped with the VSV-G protein at a moi of 5, adsorbed for 1 hr at 37°C, and then washed with DMEM-5% FCS and cultured for 24 hr. The culture supernatants were collected and centrifuged at 1,000 rpm to remove cell debris. Thereafter, the supernatants were stored at -80°C as VSV-RESTV-$GP_{1,2}$/GFP. The infectivity titer of VSV-RESTV-$GP_{1,2}$/GFP, harboring the VSV ΔG*/GFP genome, was determined by counting the number of GFP-positive cells under a fluorescent microscope upon infection into Vero E6 cells, as described previously. Briefly, VSV-RESTV-$GP_{1,2}$/GFP was 3.2 (0.5 $\log_{10}$)-fold serially diluted with DMEM-5% FCS and then inoculated to Vero E6 cells seeded in 96 well culture plates. The cells were incubated at 37°C in a CO_2 incubator for 24 hr. Then, GFP-positive cells were detected and counted under a fluorescent microscope (BZ-9000; KEYENCE, Osaka, Japan), and the infectious units (IU) of the pseudotyped VSV were calculated.

The serum samples were serially diluted in DMEM-5% FCS, and a 50 μl aliquot of each dilution was mixed with the same volume of DMEM-5% FCS containing 1,000 IU of VSV-RESTV-$GP_{1,2}$/GFP and incubated for 1 hr at 37°C. The mixture was inoculated into Vero E6 cells and incubated for 24 hr. The number of GFP-positive infected cells was counted, and serum dilutions with 50% neutralization (NT_{50}) were identified.

Multiplex assay for cytokines and chemokines in the cynomolgus macaque sera

Eleven RESTV-infected cynomolgus macaque serum samples were inactivated at 56°C for 30 min, diluted 1:10 in the assay diluent supplied with the Human Cytokine 25-Plex antibody bead kit (Invitrogen, CA), and were subjected to a multiplex cytokine analysis using a Luminex 100 instrument (Luminex Co., Austin, TX) according to the manufacturer's instructions. This Human Cytokine 25-Plex antibody bead kit was previously used to cynomolgus macaque sera and the cross-reactivity was confirmed [30]. As negative controls, we used sera from 13 cynomolgus macaques bred at the TPRC and investigated the cytokine concentrations of these serum samples.

Abbreviations

Ag: Antigen; BDBV: Bundibugyo ebolavirus; EBOV: Ebola virus; ELISA: Enzyme-linked immunosorbent assay; $GP_{1,2}$: Glycoprotein; $GP_{1,2}$ΔTM: Ectodomain of the RESTV glycoprotein; VHF: Viral hemorrhagic fever;
IFA: Immunofluorescent antibody assay; MARS: Mixed anti-inflammatory response syndrome; NP: Nucleoprotein; NP-40: Nonidet P40;
NT: Neutralization; PBS-T: PBS containing 0.05% Tween 20; RESTV: Reston

virus; SD: Standard deviation; SHFV: Simian hemorrhagic fever virus; SKIM-PBS-T: PBS-T containing 5% skim milk; TPRC: Tsukuba Primate Research Center; VSV: Vesicular stomatitis Indiana virus; VSV-RESTV-$GP_{1,2}$/GFP: VSV pseudotype bearing RESTV $GP_{1,2}$.

Competing interests
The authors declare that they have no competing interests.

Authors' contributions
ST, TI, SF, and SM designed the experiments and analyzed the experimental data. ST and SF prepared the manuscript. SM supervised the experiments and helped draft the manuscript. YS, SW, and II helped to perform the experiments. NN performed the multiplex assay. YY and MM prepared the serum samples. TI, TM, YI, MS, HA and SK supervised the experiments. All authors have read and approved the final manuscript.

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RESEARCH ARTICLE Open Access

A seroepidemiologic study of Reston ebolavirus in swine in the Philippines

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Abstract

Background: Ebola viruses cause viral hemorrhagic fever in humans and non-human primates and are endemic in Africa. Reston ebolavirus (REBOV) has caused several epizootics in cynomolgus monkeys (*Macaca fascicularis*) but is not associated with any human disease. In late 2008, REBOV infections were identified in swine for the first time in the Philippines.

Methods: A total of 215 swine sera collected at two REBOV-affected farms in 2008, in Pangasinan and Bulacan, were tested for the presence of REBOV-specific antibodies using multiple serodiagnosis systems. A total of 98 swine sera collected in a non-epizootic region, Tarlac, were also tested to clarify the prevalence of REBOV infection in the general swine population in the Philippines.

Results: Some 70 % of swine sera at the affected farms were positive for REBOV antibodies in the multiple serodiagnosis systems. On the other hand, none of the swine sera collected in Tarlac showed positive reactions in any of the diagnosis systems.

Conclusions: The high prevalence of REBOV infection in swine in the affected farms in 2008 suggests that swine is susceptible for REBOV infection. The multiple serological assays used in the study are thought to be useful for future surveillance of REOBV infection in swine in the Philippines.

Keyword: Reston ebolavirus, Antibody, Swine, Philippines

Background

The Ebola virus (EBOV) is an enveloped, negative-strand RNA virus belonging to the family *filoviridae* in the order of *mononegavirale* [1]. Four of the five ebolavirus species, Zaire (ZEBOV), Sudan, Tai Forest, and the recently discovered Bundibugyo ebolavirus, are endemic in continental Africa and cause a severe form of viral hemorrhagic fever with high mortality in humans and non-human primates [2-6]. Reston ebolavirus (REBOV) is sporadic in the Philippines and has caused several epizootics in cynomolgus macaques [7]. REBOV was first isolated in 1989 from cynomolgus macaques imported from the Philippines for medical research in the United States [7-10]. About 1,000 monkeys died or were euthanized in a quarantine facility in Reston, Virginia. Subsequently, 21 animal handlers at the Philippine exporter and four employees of the quarantine facility were found to have antibodies to the virus, indicating that they had been infected [11,12]. Epizootics in monkeys in the Philippines were then reported in 1992 and 1996, and all the epizootics have been traced back to a single monkey facility, in Calamba, Laguna in the Philippines [11,13-16]. Since the closure of the facility in 1997, no REBOV epizootics in cynomolgus monkeys have been reported.

In October 2008, REBOV infection was confirmed for the first time in swine associated with multiple epizootics of respiratory and abortion-related diseases in the Philippines [17]. In several pools of swine samples collected from geographically distant swine farms, co-

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infection with REBOV and porcine reproductive and respiratory syndrome virus (PRRSV) was confirmed [17]. Serological studies of limited scale on 13 swine sera in the affected farms failed to detect REBOV antibodies in ELISA, although PRRSV antibodies were detected [17]. It is still unclear how REBOV was spread among swine during the epizootic. Moreover, it is not clear if REBOV infection in the swine population is either sporadic and incidental or common in the Philippines. To try to answer these questions, we prepared multiple serodiagnosis systems for detecting REBOV infection in swine and analyzed swine sera obtained from the affected farms and from farms not associated with any epizootics in the Philippines. The results showed a high prevalence of REBOV infection in swine in the affected farms at the epizootics in 2008; however, REBOV antibodies were not detected in the swine population not associated with the epizootics, indicating that REBOV infection in swine in the Philippines is not common, at least in some parts of Tarlac.

Results

Detection of REBOV-NP and -GP antibodies in swine using IFA

Swine sera were analyzed for the presence of REBOV-NP and -GP antibodies in IFA specific to REBOV-NP and -GP, respectively. In the IFA, none of the 49 swine sera collected in Japan showed a positive reaction (data not shown), and so they were considered to be REBOV-NP and -GP antibody negative. In the IFA specific to REBOV-NP, antibody positive swine sera showed characteristic granular staining patterns in the cytoplasm (Figure 1A), which were indistinguishable from those of REBOV-infected cynomolgus monkey sera [18] and REBOV-NP immunized rabbit sera (data not shown). Antibody-negative swine sera showed no reaction (Figure 1B). In the IFA specific to REBOV-GP, antibody positive swine sera showed characteristic cellular surface staining patterns (Figure 1C), which were indistinguishable from those of REBOV-infected cynomolgus monkey sera and REBOV-GP immunized rabbit sera (data not shown). Antibody-negative swine sera showed no reaction (Figure 1D). In Bulacan, 104 (71.2%) and 115 (78.8%) of the 146 swine sera showed positive reactions in the NP- and GP-specific IFA, respectively. In Pangasinan, each 54 (78.3%) of the 69 swine sera showed positive reactions in the NP- and GP-specific IFA. In total, 158 (73.5%) and 169 (78.6%) of the 215 swine sera collected at the affected farms were REBOV-NP and -GP antibody positive in the IFA, respectively (Table 1). On the other hand, none of the 98 swine sera collected in Tarlac in the Philippines showed any positive reaction (Table 1).

Detection of REBOV neutralizing antibodies in swine

The VSV-pseudotype bearing REBOV-GP efficiently infected Vero E6 cells which are known to be susceptible to REBOV infection. The infectious titer of the VSV-pseudotype reached 3.6 x 10^6 IU/mL when measured on Vero E6 cells. Infection with the VSV-pseudotype was neutralized with rabbit serum to REBOV-GP (data not shown). Swine sera were analyzed in the NT using the VSV-pseudotype. In the NT, none of the 49 swine sera collected in Japan neutralized the infection with the VSV-pseudotype on Vero E6 cells at serum dilutions of 1 in 100. These were therefore considered to be REBOV NT antibody negative. In the affected farms, 108 (74.0%) of the 146 swine sera in Bulacan and 46 (66.7%) of the 69 swine sera in Pangasinan showed positive reactions in the NT, respectively at a serum dilution of 1 in 100. In total, 154 (71.6%) of the 215 swine sera in the affected farms showed NT antibody positive (Table 1). NT titers of the sera were then obtained for the 34 NT antibody positive sera in the affected farms. NT titers ranged between 100 and 12,800 with average of 790 and median of 400 (data not shown). In Tarlac, none of the 98 swine sera showed any positive reaction (Table 1).

Detection of REBOV-NP and -GP specific antibodies in swine using IgG-ELISA

IgG antibodies to the REBOV-NP and -GP were detected by recombinant REBOV protein-based IgG-ELISA. The OD values to the NP and GP of each serum sample at a dilution of 1 in 100 were plotted (Figure 2A and B), and ROC and TG-ROC curves were also drawn (Figure 2C to F). Since the OD values of negative samples were very low, the cut off values defined as the values at intersection points of the sensitivity curve and specificity curve were 0.077 and 0.104 for REBOV-NP and -GP, respectively. At the cut off values, sensitivity and specificity of the assay are over 95% in both IgG-ELISAs (Figure 2E and F). In Bulacan, 115 (78.8%) and 119 (81.5%) of the 146 swine sera showed positive reactions in the NP- and GP-specific IgG-ELISA, respectively. In Pangasinan, 62 (89.9%) and 46 (66.7%) of the 69 swine sera showed positive reactions in the NP- and GP-specific IgG-ELISA, respectively. In total, 177 (82.3%) and 165 (76.7%) of the 215 swine sera collected at the affected farms were REBOV-NP and -GP antibody positive in the IgG-ELISA, respectively (Table 1). Conversely, 1 of the 98 swine sera collected in Tarlac in the Philippines showed a positive reaction in the GP specific IgG-ELISA and 1 of the 49 swine sera collected in Japan showed a positive reaction in the NP- and GP-specific IgG-ELISA (Table 1). However, these results are considered to be false positives because the OD values were close to the cut off level defined by the assay, additionally all the

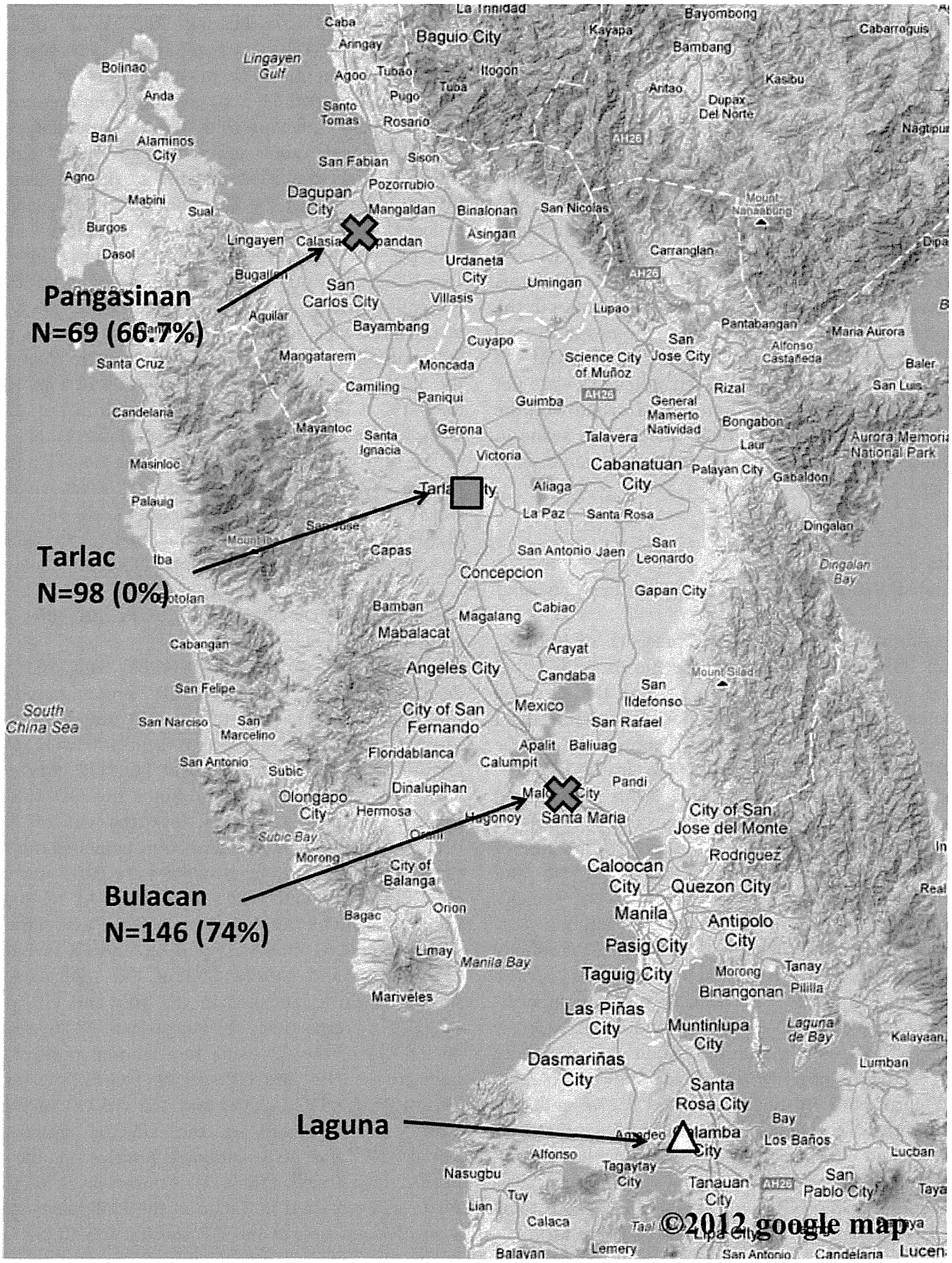

Figure 1 Detection of REBOV antibodies in IFA. Immunofluorescence staining pattern of HeLa cells expressing REBOV-NP (**A**) and REBOV-GP (**C**) with a REBOV antibody positive swine serum at a dilution of 1 in 160. Negative staining pattern of HeLa cells expressing REBOV-NP (**B**) and REBOV-GP (**D**) with a REBOV antibody negative swine serum (B and D).

Table 1 Detection of REBOV-antibodies in swine

	NP		GP		NT
	IgG-ELISA	IFA	IgG-ELISA	IFA	
Bulacan	115/146 (78.8%)	104/146 (71.2%)	119/146 (81.5%)	115/146 (78.8%)	108/146 (74.0%)
Pangasinan	62/69 (89.9%)	54/69 (78.3%)	46/69 (66.7%)	54/69 (78.3%)	46/69 (66.7%)
Tarlac	0/98 (0%)	0/98 (0%)	1/98 (1.0%)	0/98 (0%)	0/98 (0%)
Japan	1/49 (2.0%)	0/49 (0%)	1/49 (2.0%)	0/49 (0%)	0/49 (0%)

samples collected in Tarlac and Japan tested using the alternative serological assays were negative.

Agreement between the serological assays detecting REBOV antibodies

We next examined the agreement between the serological assays used in the present study. The data obtained in each assay were binarized to either positive or negative result. These binarized data were then statistically analyzed by pair-wise comparisons in nonparametric one-way ANOVA. This analysis showed that there were no significant differences between the serological assays ($p > 0.05$, data not shown). The result supports the contention that all of the five serological assays used in the present study posses similar discriminatory capacity. Thus we are confident that many REBOV antibody positive samples showed a positive reaction in all of the five assays.

Discussion

ZEBOV and Marburg virus were identified in African fruit bat species [19,20]. More recently, Marburg virus was successfully isolated from a fruit bat, *Rousettus aegyptiacus* [21], suggesting that fruit bats are reservoir animals of filoviruses. In the Philippines, we have recently demonstrated that an Asian fruit bat, *Rousettus amplexicaudatus,* has antibodies to REBOV [22]. Since the REBOV genome has not yet been detected in the bat, conclusive evidence that the bat species is a reservoir, or one of the reservoir animals, of REBOV is not available. Nevertheless, it is possible that REBOV was transmitted to swine from these bats since these bats inhabit many areas of the country, including the regions around the affected facilities both in Pangasinan and Bulacan.

In this study, we have aimed to clarify how REBOV infection was spread among swine during the REBOV

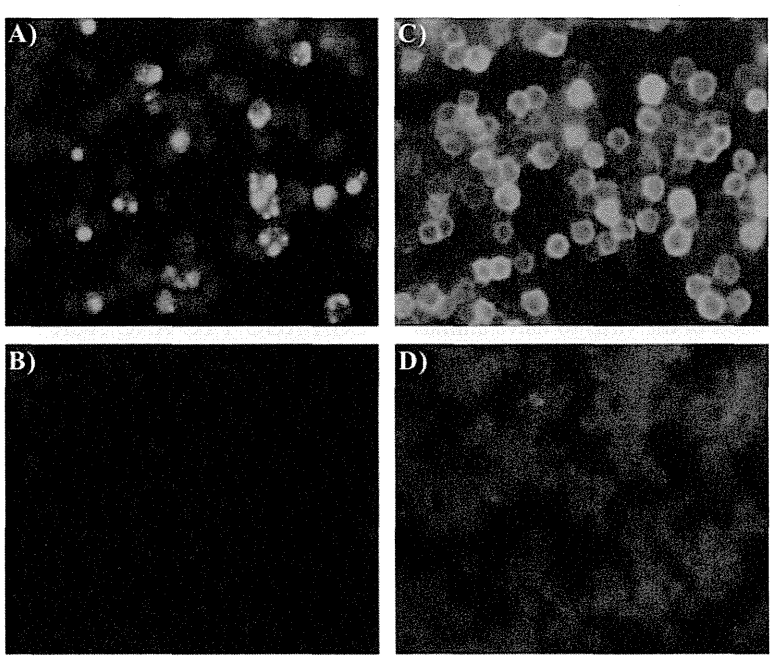

Figure 2 Distribution of OD values and ROC and TG-ROC analyses of the IgG-ELISA specific for REBOV-NP and -GP. OD values to the NP (**A**) and GP (**B**) of each serum sample at a dilution of 1 in 100 were plotted for swine group showing either IF and NT antibody positive or negative. ROC and two graph-ROC (TG-ROC) curves were analyzed and drawn using Stat Flex software.

epizootics. We have tested 215 swine sera collected from the REBOV affected farms using IFA and IgG-ELISA specific for REBOV-NP and -GP, and NT. NT is the gold standard of serological assay in many virus infections. However, since REBOV needs to be cultured in high-containment laboratories, we performed an alternative NT using the VSV-pseudotype bearing REBOV-GP. This avoided the use of infectious REBOV, enabling the work to be carried out at low containment. Previously it has been shown that VSV-pseudotype bearing ebolavirus GP mimicks ebolavirus infection [23], Approxymately 70% of the swine sera from REBOV affected farms were REBOV antibody positive. This indicated that swine are susceptible to REBOV infection. Unfortunately, we could not analyze the IgM antibody responses in swine, since after the sera were heat inactivated the gamma globulin fractions were precipitated with ammonium sulfate, and reconstituted in PBS prior to be testing. An indication of the IgM responses to REBOV would have provided evidence of a recent infection. Thus, it is still unclear if REBOV infection was spread during epizootics or whether a population of the animals in the farms was infected with REBOV prior to the epizootics. The swine not associated with the epizootics, in Tarlac, are considered to be free from REBOV infection. These samples were collected in 2010, over 2 years after the epizootic, and from animals born after the epizootic. Moreover, we could not analyze the swine specimens near the affected farms in 2008. Thus, in this study, it is not clear if REBOV infection in 2008 was limited in the affected farms. Further study is necessary to conclude if the swine population in the Philippines is generally free from REBOV infection.

Recently, it has been shown that the experimental infection of swine with REBOV alone resulted in subclinical infection with rapid clearance of the virus [24]. Alternatively, ZEBOV has been shown to replicate to high titers in experimentally infected swine and to cause severe lung pathology resulting in transmission of the virus to naïve animals [25]. Thus, swine has been shown experimentally to be highly susceptible to ZEBOV infection. Furthermore, some amino acid mutations in NP and/or VP24 in ZEBOV resulted in adaptation of the virus to guinea pigs and mice [26,27]. Thus, we cannot rule out the possibility that mutations introduced in the REBOV genome during serial transmission in swine will result in adaptation of the virus to swine in future. In this regard, a regular serological survey of REBOV infection in swine in the Philippines is desirable. The serodiagnosis systems presented in this study might be useful for such a survey.

Conclusions

The high prevalence of REBOV infection in swine at the affected farms in 2008 suggests that swine are susceptible for REBOV infection. The multiple serological assays used in the study are thought to be useful for future surveillance of REOBV infection in swine in the Philippines.

Methods

Swine serum specimens

A total of 215 swine sera were collected from two REBOV affected pig farms, located in Pangasinan and Bulacan in 2008 (Figure 3). Of these, 146 sera were collected from swine at the farm in Bulacan, and 69 were collected from those in Pangasinan. Swine samples in the affected farms were collected under quarantine of the Philippines. The sera were kept frozen at the Research Institute for Tropical Medicine (RITM) in the Philippines until use. Ninety-eight swine sera were collected from July to September 2010 from swine aged between 2 and 20 months (median of 4.5 months) in Tarlac in the Philippines, where no swine epizootic has been documented. The swine specimens at Tarlac were collected and used under approval of IRB (No. 2009-018) of RITM, and informed consent was obtained from the farm owners. Forty-nine swine sera collected at slaughterhouse in 2006-7 in Japan, kindly supplied by Dr. T-C Li at the National Institute of Infectious Diseases, were used as REBOV antibody negative control sera. Swine sera were inactivated at 56°C for 30 minutes, and then the gamma globulin fractions were precipitated with ammonium sulfate and reconstituted in phosphate buffered saline (PBS).

Immunofluorescence assay (IFA) specific to REBOV-NP and -GP using HeLa cells expressing the recombinant protein

The entire cDNA of REBOV-GP ORF was amplified from the swine lymph node specimen used for amplification of the cDNA of NP by RT-PCR using the primers REBOV-GP/F (5'-CGA AGC TTC GAA CAT GGG GTC AGG ATA TCA ACT-3') and REBOV-GP/R (5'-CGA AGC TTC AAC ACA AAA TCT TAC ATA TAC AAA G-3') (the *Hind* III site is underlined). The complete nucleotide sequence of the amplicon was determined to be identical to that of Reston 08A (GenBank accession number FJ621583), indicating that the specimen was collected from the same farm as that of sample group A from which Reston 08A was isolated [17]. The *Hind* III fragment of the amplicon was subsequently cloned into pKS336 [28], and the pKS336 plasmid with REBOV-GP, pKS336-pREBOV-GP, was used for transfection. HeLa cells were transfected with pKS336-pREBOV-GP using a FuGENE HD (Roche Diagnostics, Mannheim, Germany) according to the manufacturer's instructions. The cells transfected with the plasmids were selected with 3 μg/mL of blasticidin S-hydrochloride (Invitrogen, CA, USA) in Dulbecco's

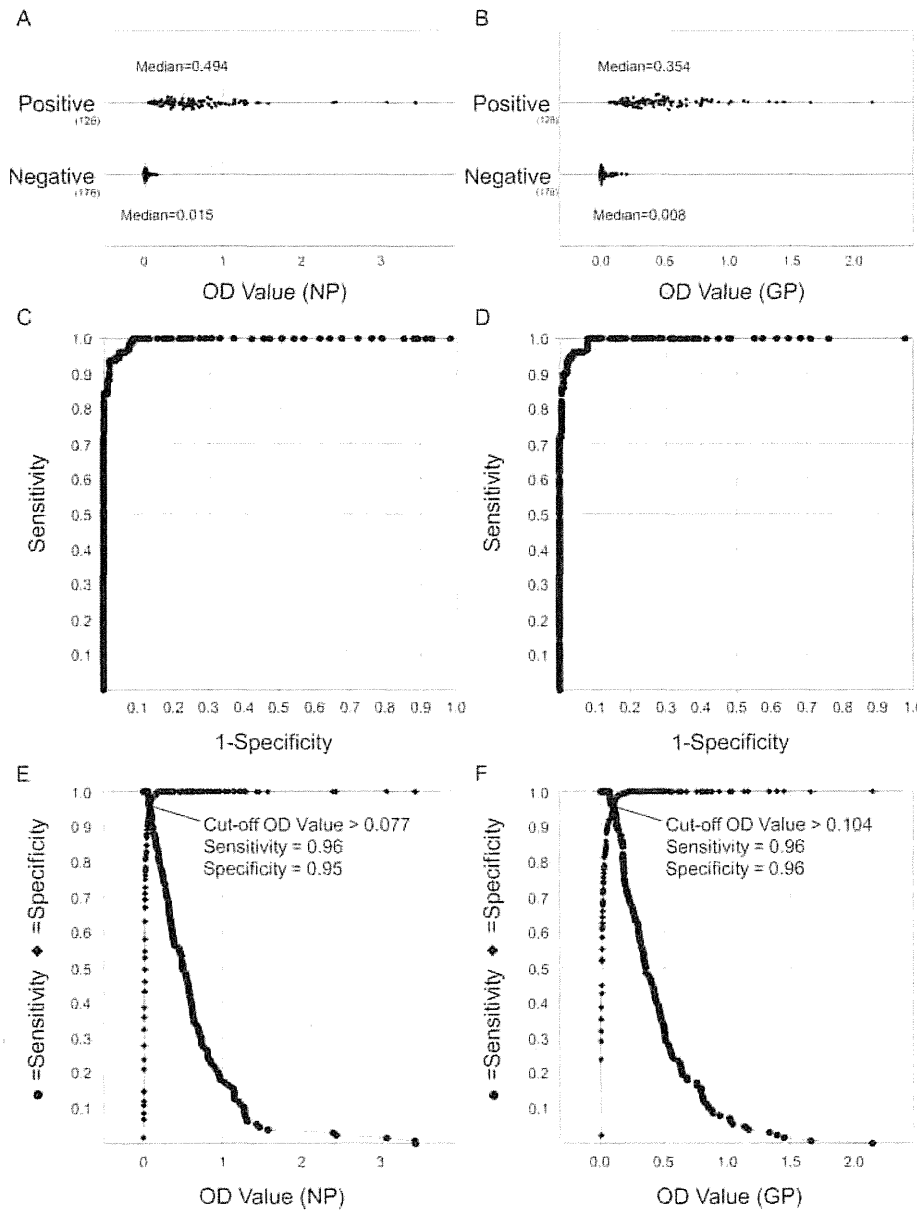

Figure 3 A topographical map showing locations in the Philippines where swine serum samples were obtained. Swine serum samples were collected at farms in three regions in the Philippines, Bulacan, Pangasinan and Tarlac. These regions were showed the numbers of specimens and percentage of positivity using NT. The three locations and Laguna, where the Ferlite monkey facility used to be located, are shown on a Google map (©2011 Google Map data ©2011 AND Europa Technologies).

modified Eagle medium (DMEM) supplemented with 5% fetal calf serum (FCS). The selected cells were analyzed for the expression of REBOV-GP in IFA using anti-REBOV-GP rabbit serum. The cells expressing REBOV-GP were spotted on multiwell slide glasses, fixed in acetone, and used as antigens for IFA specific to REBOV-GP. The IFA specific to REBOV-NP was performed as described previously [18], with slight modifications. Mock-transfected HeLa cells were used as negative control antigens in the IFA. The antigen slides were incubated with serially diluted swine sera under humidified conditions at 37°C for 1 hour. The antigen slides were washed in PBS, then reacted with rabbit anti-pig IgG conjugated with fluorescein isothiocianate (FITC) (Bethyl, TX, USA) at a dilution of 1 in 100 at 37° C for 1 hour. The slides were washed in PBS, covered with cover glasses, and examined for staining patterns under a fluorescence microscope fitted with appropriate

barrier and excitation filters for FITC visualization. The antibody titer in the IFA was determined as the reciprocal of the highest dilution showing positive staining.

Generation of vesicular stomatitis virus (VSV)-pseudotype bearing REBOV-GP

Generation of VSV-pseudotype bearing REBOV-GP was performed as described previously [29-31]. Briefly, 293 T cells were transfected with pKS336-pREBOV-GP and cultured for 24 hours, and then the cells were infected with VSVΔG* (kindly supplied by Prof. M.A. Whitt, University of Tennessee) [30,31] and cultured for 24 hours. The culture supernatants were then collected, filtered through a 0.22 μm-pore-size filter, and stored at -80°C until use. The infectious titer (infectious unit, IU) of VSV-pseudotype on Vero E6 cells was determined by counting the number of Green Fluorescent Protein (GFP)-expressing cells.

Neutralization test (NT)

The sera were diluted twofold from 1 in 100 with DMEM containing 5% FCS and 1,000 IU of VSV-pseudotype bearing REBOV-GP. The mixture was incubated for 1 hour at 37°C, then inoculated onto Vero E6 cells seeded on 96-well plates. The numbers of VSV-pseudotype infected cells were determined by counting of the number of GFP-positive cells according to the methods described previously [23,29,30]. NT titers of the tested sera were defined as the reciprocals of the highest dilutions at which more than 50% inhibition of infectivity was observed. Serum samples were considered to be NT antibody negative when less than 50% inhibition of infectivity was observed at a dilution of 1 in 100.

Recombinant baculoviruses expressing recombinant REBOV proteins

cDNA of REBOV nucleoprotein (NP) open reading frame (ORF) from a swine lymph node specimen collected at the farm in Bulacan was amplified by reverse transcription polymerase chain reaction (RT-PCR), and the nucleotide sequence of the amplicon was determined to be identical to that of Reston 08A. The cDNA was subcloned into pGEM-Teasy (Promega, WI, USA) and used for the following PCR template. PCR was performed using the plasmid clone to add *Bam*HI linker with primers REBOV-NP/F (5'-GGG CTA GCG GAT CCA AGT CGA TAT GGA TCG TGG GAC C-3') and REBOV-NP/R (5'-TTG CGG CCG CGG ATC CCT GAT GGT GCT GCA AGA TTG-3') (the restriction site is underlined). A *Bam*HI-digested fragment of the plasmid was then subcloned into pAcYM1-C-His plasmid, a derivative of pAcYM1 plasmid [32] carrying eight histidine coding sequences just downstream of the *Bam*HI site, to construct pAcYM1-His-pREBOV-NP. A recombinant baculovirus, Ac-His-pREBOV-NP, was generated using a previously described method [33]. The recombinant baculovirus, which expresses the ectodomain of REBOV-glycoprotein (GP) with a histidine-tag at its carboxyl terminus [22], was used to prepare recombinant REBOV-GP for IgG-ELISA. A baculovirus (Ac-ΔP) that lacks the polyhedorin gene was used to prepare a negative-control antigen in insect cells.

Expression and purification of recombinant NP and GP of REBOV in baculovirus

Tn5 insect cells were infected with Ac-His-pREBOV-NP and Ac-His-REBOV-GP and then incubated at 26°C for 72 hours and 48 hours, respectively. The cells were washed in PBS, lyzed in PBS containing 1% NP40 and 8 M urea, and then clarified by centrifugation at 8,000 rpm for 10 min. The supernatant fraction was collected, and recombinant REBOV-NP and REBOV-GP were purified using a Ni2 + -resin purification system (QIAGEN, Düsseldorf, Germany) according to the manufacturer's instructions. The expression and purification of REBOV-NP and REBOV-GP were confirmed in 10% sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) and Coomassie Brilliant Blue R-250 staining Tn5 cells infected with Ac-ΔP were processed similarly and used as negative control antigen in the ELISAs. The purified antigens and negative control antigen were kept at -80°C until use.

IgG-ELISA

Swine sera were analyzed for the presence of antibodies to REBOV-NP and REBOV-GP in IgG-ELISA, essentially as described previously [22,34,35]. Briefly, half of the wells of the ELISA plates (Falcon, NJ, USA) were coated with a predetermined optimal quantity (approximately 100 ng/well) of each purified recombinant protein, and the rest of the wells were coated with the negative control antigens. After washing the plates three times in PBS containing 0.05% Tween-20 (T-PBS; SIGMA-ALDRICH, MO, USA), each well was incubated with 200 μL of T-PBS containing 5% skim milk (MT-PBS; Yukijirushi, Hokkaido, Japan) for 1 hour at 37°C. After washing the plates three times in T-PBS, the antigen-coated and negative-control-antigen–coated wells were inoculated with the test samples (100 μL/well) in MT-PBS at a dilution of 1 in 100, incubated for 1 hour at 37°C, and washed in T-PBS. Then, each well was incubated with a Protein A/G conjugated with horseradish peroxidase at a dilution of 1 in 1,500 (PIERCE, IL, USA) in MT-PBS for 1 hour at 37°C. The plates were washed three times, and 100 μL of ABTS solution (Roche Diagnostics, Mannheim, Germany) was added to each well. The plates were incubated for 30 min, and the optical density (OD) was measured at 405 nm with a reference at 490 nm. The adjusted ODs for each tested sample

were calculated by subtracting the ODs of the negative-control-antigen–coated wells from those of the corresponding REBOV-antigen–coated wells.

Statistical methods

Sensitivity, specificity and predictive values for positive and negative tests were calculated by standard methods. Sensitivity and specificity were defined as the probability that the target assay result was positive when the IFAs specific to REBOV-NP and -GP and NT showed positive and the probability that the target assay result was negative when the IFAs and NT showed negative, respectively. Receiver operating characteristics (ROC) and two graph-ROC (TG-ROC) curves were analyzed using Stat Flex software (Artech Co. Ltd., Osaka, Japan) [36,37]. To examine the agreement between the serological assays used in the present study, we employed Friedman test with Dunn's post-hoc test [38,39] using GraphPad Prism using pair-wise comparisons in nonparametric one-way ANOVA.

Competing interests

The authors declare that they have no competing interests.

Authors' contributions

YS participated in the study design, the experimental work, the analysis interpretation of the data and drafted the manuscript. CD, MS (Tohoku University), RA, ST, SF, II, TM, IK, FFMJ, SL, DPC, SBA, RGM, ELL, KRCD, MSC, RO, MS and HO participated in the study design, the experimental work, and helped draft the manuscript. TY participated in the statistical analysis of the data. SM conceived and designed the study and participated in the analysis and interpretation of the data and writing of the manuscript. All authors read and approved the final manuscript.

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The Dominant-Negative Inhibition of Double-Stranded RNA-Dependent Protein Kinase PKR Increases the Efficacy of Rift Valley Fever Virus MP-12 Vaccine

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Rift Valley fever virus (RVFV), belonging to the genus *Phlebovirus*, family *Bunyaviridae*, is endemic to sub-Saharan Africa and causes a high rate of abortion in ruminants and hemorrhagic fever, encephalitis, or blindness in humans. MP-12 is the only RVFV strain excluded from the select-agent rule and handled at a biosafety level 2 (BSL2) laboratory. MP-12 encodes a functional major virulence factor, the NSs protein, which contributes to its residual virulence in pregnant ewes. We found that 100% of mice subcutaneously vaccinated with recombinant MP-12 (rMP12)-murine PKRN167 (mPKRN167), which encodes a dominant-negative form of mouse double-stranded RNA (dsRNA)-dependent protein kinase (PKR) in place of NSs, were protected from wild-type (wt) RVFV challenge, while 72% of mice vaccinated with MP-12 were protected after challenge. rMP12-mPKRN167 induced alpha interferon (IFN-α) in sera, accumulated RVFV antigens in dendritic cells at the local draining lymph nodes, and developed high levels of neutralizing antibodies, while parental MP-12 induced neither IFN-α nor viral-antigen accumulation at the draining lymph node yet induced a high level of neutralizing antibodies. The present study suggests that the expression of a dominant-negative PKR increases the immunogenicity and efficacy of live-attenuated RVFV vaccine, which will lead to rational design of safe and highly immunogenic RVFV vaccines for livestock and humans.

Rift Valley fever (RVF) is a mosquito-borne zoonotic disease caused by Rift Valley fever virus (RVFV) that belongs to the genus *Phlebovirus*, family *Bunyaviridae* (68). RVF has been endemic in sub-Saharan African countries for more than 80 years and has spread to Egypt, Madagascar, Saudi Arabia, and Yemen (6, 7, 12, 68, 71). RVFV causes a high rate of abortion and acute lethal infection in newborn ruminants, such as sheep, goats, and cattle, and febrile illness in humans (13, 68, 71). Although most human patients recover from the disease without any complications, some develop lethal hemorrhagic fever or encephalitis, whereas approximately 1 to 10% of patients experience blindness for undefined periods (25, 61). RVFV is transmitted by mosquitoes; floodwater *Aedes* species maintain RVFV in areas of endemicity through transovarial transmission, whereas other mosquito species belonging to the genus *Culex* or *Anopheles* that bite both ruminants and humans can also transmit RVFV during outbreaks and work as amplification vectors (41, 59). The spread of RVFV into countries where it is not endemic may occur through the spread of RVFV-infected mosquitoes, movement of animals, or travel of humans infected with RVFV or intentional attacks with biological agents (9, 72, 73). RVFV is a risk group 3 pathogen and an overlap select agent of the Department of Health and Human Services (HHS) and the U.S. Department of Agriculture (USDA) and a category A high-priority pathogen of the National Institute for Allergy and Infectious Diseases (NIAID) in the United States (44, 45).

The genome of RVFV is comprised of a tripartite negative-strand RNA genome with S, M, and L segments (68). The S segment encodes the nucleocapsid (N) protein and nonstructural NSs protein in an ambisense manner. The M segment encodes a single M mRNA, and the precursor protein can be cotranslationally cleaved into the 78-kDa protein, the nonstructural protein NSm, and viral envelope proteins Gn and Gc. The L segment encodes the RNA-dependent RNA polymerase. Neither NSs nor NSm is essential for viral replication, and recombinant RVFV lacking both NSs and NSm is still viable (4). The lack of NSm does not affect viral replication in type I interferon (IFN)-competent cells, and the virus still retains its virulence in the rat model (5). On the other hand, lack of NSs abrogates RVFV competency to replicate in type I IFN-competent cells (29, 56), which results in the attenuation of RVFV in animals (10, 14, 74), suggesting that NSs is a major virulence factor of RVFV.

Vaccination of susceptible ruminants and humans is the only effective way to prevent the spread of RVFV during an outbreak (26). Currently, there are no licensed vaccines or therapeutics available outside countries where the virus is endemic. Randall et al. developed a formalin-inactivated vaccine for Rift Valley fever (64). The original inactivated candidate vaccine has been improved in terms of safety by using FRhL-2 cells instead of primary rhesus or African green monkey kidney cells. The improved vaccine, TSI-GSD-200, was produced with the virulent Entebbe strain, and the manufacturing capability at a high-containment

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facility is very limited. Pittman et al. demonstrated that vaccination with TSI-GSD 200 on days 0, 7, and 28 (subcutaneously [s.c.]) elicits a geometric neutralizing antibody titer of 1:237, while the half-life of the neutralizing antibody is 287 days and the titer decreased below 1:40 (62). Because of the requirement for repeated immunization to gain sufficient neutralizing antibody titer and the short half-life of the resulting neutralizing antibodies, it would be ideal to prepare a vaccine candidate that will induce rapid and long-term protective immunity in both humans and ruminants with a single administration, i.e., a live-attenuated vaccine. However, there is concern that live-attenuated vaccine strains may revert to virulence and cause unexpected diseases among vaccinees. Candidate live-attenuated vaccines, the MP-12 strain (11) and the clone 13 strain (C13) (56), have been shown to be immunogenic in ruminants and sufficiently safe for veterinary use (14, 48, 50–55), while the safety evaluations of these vaccines in humans has not been completed. At present, MP-12 is the only RVFV strain that is a risk factor 2 pathogen and that is excluded from the select-agent rule. The MP-12 strain carries attenuated M and L segments, while the S segment encodes a virulent phenotype due to the functional NSs gene (2, 67, 75). The C13 strain carries wild-type RVFV M and L segments, while the S segment encodes NSs with a 69% truncation, which abolishes all functions of NSs (3, 21, 37, 38, 56). Using a reverse genetics system for the MP-12 strain, a recombinant MP-12 (rMP12) with a 69% truncation of the NSs gene that is identical to that of strain C13 NSs was generated and designated rMP12-C13type (29). rMP12-C13type carries attenuated M and L segments of MP-12, while the immunogenicity and efficacy of rMP12-C13type in animals and humans have not been characterized.

RVFV inhibits host general transcription, including beta interferon (IFN-β) mRNA synthesis (3, 37, 38). Transcription factor IIH (TFIIH) is an essential transcription factor for host RNA polymerases I and II (24, 43) and is composed of 10 subunit proteins: XPD (gene defective in xeroderma pigmentosum patient complementation group D), p8, p34, p44, p52, p62, XPB, ménage-à-trois 1 (MAT1), cyclin H, and cdk7 (18, 69). NSs suppresses the general host transcription by sequestering TFIIH p44 subunit proteins (37) and by promoting the degradation of TFIIH p62 subunit proteins (32). In addition to suppressing broad-host-range transcription through interference with TFIIH function, NSs can also bind to Sin3A-associated protein 30 (SAP30) on the IFN-β promoter, which may repress the activation of the promoter (38).

In addition to general transcription suppression, NSs also promotes the degradation of double-stranded RNA (dsRNA)-dependent protein kinase (PKR) (21, 27). During the replication of RNA viruses, PKR binds to dsRNA or 5′-triphosphated single-stranded RNA (ssRNA) at its dsRNA-binding domain, located at the N terminus, which causes a conformational change exposing the kinase domain at the C terminus (17). Active PKR is a dimer and phosphorylates the eIF2α subunit protein (17). eIF2-GTP transports methionine-tRNA to the 43S preinitiation complex to initiate translation, while eIF2B is responsible for converting eIF2-GDP into eIF2-GTP (16). The phosphorylated eIF2α binds to eIF2B at high affinity and sequesters the limited eIF2B molecule (16). Cells with phosphorylated eIF2α cannot initiate translation; however, RVFV NSs degrades PKR and prevents the phosphorylation of eIF2α during viral replication (27). Since suppression of PKR leads to the active synthesis of viral proteins during infection, we hypothesized that MP-12, encoding a dominant-negative form of PKR, would inhibit PKR-mediated eIF2α phosphorylation without suppressing host innate immune responses and effectively induce protective immunity against RVFV. We generated rMP12-murine PKRN167 (mPKRN167), which encodes a dominant-negative form of mouse PKR in place of NSs, and tested its efficacy and immunogenicity in mice.

MATERIALS AND METHODS

Media, cells, and viruses. BHK/T7-9 cells that express T7 RNA polymerase (30) were grown in minimal essential medium alpha (MEM-alpha) containing 10% fetal calf serum (FCS). Penicillin (100 U/ml) and streptomycin (100 μg/ml) were added to the medium. BHK/T7-9 cells were selected in medium containing 600 μg/ml hygromycin. rMP12-C13type, which carries the NSs gene with a 69% in-frame truncation (29), was described previously. rMP12-mPKRN167 encodes the N terminus of mouse PKR from amino acids (aa) 1 to 167 with an N-terminal Flag tag in place of MP-12 NSs. These recombinant MP-12 viruses were recovered from BHK/T7-9 cells as described previously (29) and passaged once in Vero E6 cells. Viral titers were determined by plaque assay in Vero E6 cells. Lyophilized MP-12 vaccine was reconstituted and passaged once either in MRC-5 cells or in Vero E6 cells. RVFV strain ZH501 stock was generated after one Vero E6 cell passage of an original ZH501 reference collection vial (serial number JM1137) at the University of Texas Medical Branch (UTMB) (49).

Plasmids. The N terminus of mouse PKR (aa 1 to 167) with a Flag tag was amplified by PCR from first-strand cDNA, derived from total RNA of wild-type (wt) mouse embryonic fibroblasts (MEFs) that were infected with rMP12-C13type (7 h postinfection [p.i.]), with HpaFlagmPKR-F (5′-TGT CGT TAA CAT GGA TTA CAA GGA TGA CGA CGA TAA GAT GGC CAG TGA TAC CCC AGG T-3′) and Spe-m167-PKR-R (5′-AGG AAC TAG TTC AAG TTT TCG GCG GGC TCT TTA ACA-3′) (restriction enzyme sites are underlined). Then, the PCR fragments that were cut with HpaI and SpeI were cloned into the NSs open reading frame (ORF) of pProT7-vS(+) that was cut with HpaI and SpeI as described previously (29).

Recovery of recombinant MP-12. The rMP12-mPKRN167 virus was recovered in BHK/T7-9 cells by using a plasmid combination of pProT7-S(+)-mPKRN167, pProT7-M(+), pProT7-L(+), pT7-IRES-vN, and pT7-IRES-vL, as described previously (29).

Western blotting. MEFs were mock infected or infected with MP-12, rMP12-C13type, or rMP12-mPKRN167 at a multiplicity of infection (MOI) of 3, and cells were collected into 2× sample buffer at 12 or 16 h p.i. Western blot analysis was performed as described previously (27). The membranes were incubated with anti-eIF2-alpha antibody (Cell Signaling Technology), anti-phospho-eIF2-alpha (ser51) antibody (Cell Signaling Technology), anti-RVFV mouse polyclonal antibody (a kind gift from R. B. Tesh, UTMB), anti-FLAG M2 monoclonal antibody (Sigma), and anti-actin polyclonal antibody (I-19; Santa Cruz Biotechnology).

Immunization and virus challenge. For testing humoral immune responses, 5-week-old female CD1 outbred mice were inoculated subcutaneously with phosphate-buffered saline (PBS) (mock) ($n = 5$) or 1×10^5 PFU of MP-12 ($n = 10$), rMP12-C13type ($n = 10$), or rMP12-mPKRN167 ($n = 10$). At 1, 2, 3, 30, 90, and 180 days p.i., less than 100 μl of blood was collected from the retro-orbital vein, and serum samples were obtained for IFN-α enzyme-linked immunosorbent assay (ELISA), virus plaque assay, or a plaque reduction-neutralization test ($PRNT_{80}$). For testing the efficacy of MP-12 NSs mutants, 5-week-old female CD1 outbred mice were inoculated subcutaneously with PBS (mock) ($n = 10$) or 1×10^5 PFU of MP-12 ($n = 11$), rMP12-C13type ($n = 10$), or rMP12-mPKRN167 ($n = 9$). Sera were collected at 42 days p.i., and the mice were challenged with 1×10^3 PFU of wt RVFV strain ZH501 (intraperitoneally [i.p.]) at 44 days p.i. The challenge experiment was performed at an animal biosafety level 4 (BSL4) facility at the UTMB Shope laboratory. The mice were observed for 21 days, and body weight was monitored daily. Survival curves of mice (Kaplan-Meyer method) were analyzed with the

Graphpad Prism 5.03 program (Graphpad Software Inc.). For testing viral antigen accumulation at draining lymph nodes, 5-week-old female CD1 outbred mice were inoculated in the footpad (30 μl) with PBS (mock) ($n = 3$) or 1×10^5 PFU of MP-12 ($n = 4$), rMP12-C13type ($n = 4$), rMP12-mPKRN167 ($n = 4$), or heat-inactivated MP-12 ($n = 3$). The draining lymph nodes (popliteal and inguinal) were collected at 1 day p.i. and used for antigen capture ELISA. Mice that were vaccinated with MP-12 ($n = 3$) were also tested at 2 and 3 days p.i. For testing cytokines and chemokines in mouse sera, 5-week-old female CD1 outbred mice were inoculated in the footpad (30 μl) with PBS (mock) ($n = 3$) or 1×10^5 PFU of MP-12 ($n = 4$), rMP12-C13type ($n = 4$), or rMP12-mPKRN167 ($n = 4$). Sera were collected at 2 days p.i. and used for Bio-Plex analysis.

Detection of cytokines and chemokines in mouse sera. Serum IFN-α was measured by using a VeriKine Mouse Interferon Alpha ELISA Kit (catalog number 42120; PBL International) according to the manufacturer's instructions. A standard curve of mouse IFN-α control (12.5 to 400 pg/ml) was plotted, and the absorbance (optical density [OD]) at 450 nm of serum samples (1:10) was fit onto the standard curve using Graphpad Prism software.

Detection of cytokines and chemokines in mouse sera. Cytokines and chemokines in mouse sera that were collected at 2 days p.i. were measured by Bio-Plex Pro Mouse Cytokine 23-Plex Assay (Bio-Rad) according to the manufacturer's instructions. Cytokine reference standards (2 to 32,000 pg/ml) that were provided by the manufacturer were included in the assay. Mouse sera (15 μl) and universal sample diluents in the kit (45 μl) were mixed and loaded onto a 96-well plate containing beads (each 50 μl). The raw data were first statistically compared by two-way analysis of variance (ANOVA), and the fold difference from mock-infected samples was analyzed subsequently.

Construction of recombinant baculovirus carrying the RVFV N protein gene. A PCR fragment carrying the MP-12 N ORF was cloned into the baculovirus transfer vector pBACgus-1 (Novagen) under the control of the very late strong polyhedrin promoter. This vector carries the GUS reporter gene, which allows selection of recombinant viruses. The RVFV N gene was cloned in frame with a C-terminal His tag fusion sequence presented in the transfer vector. In order to increase the affinity of the recombinant protein for Ni-nitrilotriacetic acid (NTA) resin during the purification process, an additional 6-His sequence was added, separating the vector-derived His tag with a 15-amino-acid linker (34). The constructed transfer vector was designated pBACgus-RVN. Sf9 cells were cotransfected with BacVector-3000 Triple Cut Virus DNA (Novagen) and the transfer vector pBACgus-RVN by using ExGene 500 transfection reagent as described previously (40, 57). Direct plaque purification was used to select recombinant baculovirus clones. X-GlcA substrate was added to the agarose overlay, and blue plaques were selected. The expression of the RVFV N protein was confirmed by indirect immunofluorescence assay (IFA) with anti-RVFV antibody, and the resulting recombinant baculovirus was designated rAcMNPV-RVN.

Expression and purification of RVFV N protein. HighFive cells were infected at an MOI of 5 and collected at different time points, starting at 3 days p.i. The maximum RVFV N protein expression was determined at 6 days p.i. Fifty milliliters of HighFive cell culture was infected at an MOI of 5, and cells were harvested at 6 days p.i. and lysed on ice for 30 min in lysis buffer (8 M urea, 0.1 M NaH_2PO_4, 0.01 M Tris-HCl, pH 8.0) at a 1:5 ratio of cells to buffer. The lysate was clarified by centrifugation, and Ni-NTA HisBind Superflow Resin (EMD4BioSciences) was added to the supernatant. After overnight incubation, the resin was washed with washing buffer 1 (8 M urea, 0.1 M NaH_2PO_4, 0.01 M Tris-Cl, pH 6.3) twice. Nonspecifically bound protein was further removed from the resin by washing with washing buffer 2 (8 M urea, 0.1 M NaH_2PO_4, 0.01 Tris-Cl, pH 5.9), and RVFV N protein was eluted with elution buffer (8 M urea, 0.1 M NaH_2PO_4, 0.01 M Tris-Cl, pH 4.5). The purified N was diluted with PBS for IgG ELISA.

Expression and purification of RVFV NSs protein. The PCR fragment encoding the partial NSs at the C terminus (aa 219 to 265) was amplified with the primer set GST-NSdel3F (5′-CTC AGG ATC CCC ATG GAG GAG AGC CTG ATG CTG CGC TC-3′) (the BamHI site is underlined) and GST-NSdel3R (5′-GCC TGA ATT CCT AAT CAA CCT CAA CAA ATC CAT CAT CAT C-3′) (the EcoRI site is underlined) and cloned downstream of GST between BamHI and EcoRI in the pGEX-3X plasmid (GE Healthcare). Purification of the partial NSs GST fusion protein was performed as described previously (28).

IgG ELISA. RVFV N or NSs protein was expressed and purified as described above, and 96-well ELISA plates were coated overnight at 4°C at a concentration of 100 ng/well. After washing 3 times with PBS containing 0.1% Tween 20 (PBS-T), the wells were blocked with PBS-T containing 5% skim milk at 37°C for 2 h. Then, the wells were incubated with serum samples at 37°C for 1 h. The wells were washed 3 times with PBS-T and reacted with horseradish peroxidase (HRP)-conjugated anti-mouse IgG (Santa Cruz Technologies) at 37°C for 1 h. After washing with PBS-T 3 times, 2,2′-azinobis[3-ethylbenziazoline-6-sulfonic acid] (ABTS) was added to the wells. The plate was incubated at room temperature for 30 min, and the optical density at 415 nm was recorded. The cutoff value, 0.36, was defined as the mean plus 2 times the standard deviation of 36 normal mouse serum samples (1:400) for anti-N IgG, while the cutoff value of 0.144 was defined as the mean plus 3 times the standard deviation of 24 normal mouse serum samples (1:100) for anti-NSs IgG. The highest dilution of sera that gave an OD value larger than the cutoff was designated the anti-N antibody titer. Because the anti-NSs antibody level was low, an OD value of 1:100 dilution was used for demonstrating the presence of anti-NSs antibody.

Antigen-capture ELISA. Mock-infected or RVFV-infected MEFs (2.5×10^5 cells) were incubated with PBS containing 1% Triton X-100 and 0.05% Tween 20 for 10 min at room temperature and centrifuged at 15,000 × *g* at 4°C for 5 min. The supernatants were mixed 1:1 with PBS containing 0.05% Tween 20 and 1% skim milk, and 4-fold serial dilutions were performed. Mouse popliteal and inguinal lymph nodes were collected separately into tubes containing 120 μl of PBS with 1% Triton X-100, 0.05% Tween 20, and 1% skim milk on ice. After homogenization, samples were centrifuged at 15,000 × *g* at 4°C for 5 min. Then, 100 μl of lysate sample was used for ELISA. Antigen capture ELISA was performed as described previously (15), and a 96-well flat-bottom plate was coated with 125 ng of monoclonal antibody (D5-59) against RVFV N in PBS overnight at 4°C. After washing 3 times with PBS-T, the wells were blocked with PBS-T containing 5% skim milk for 2 h at room temperature. Subsequently, lymph node lysates were added to the well and incubated at 37°C for 1 h. The plate was washed with PBS-T 3 times, and rabbit anti-RVFV serum (1:1,000) was added as a detection antibody and incubated at 37°C for 1 h. After washing with PBS-T 3 times, the wells were reacted with HRP-labeled anti-rabbit IgG at 37°C for 1 h. After washing with PBS-T 3 times, ABTS substrate (ABTS tablet and buffer; Roche Diagnostics) was added to the wells. The plate was incubated at room temperature for 30 min, and the optical density at 415 nm was recorded. The cutoff value was determined as the mean plus 3× the standard deviation of 24 wells (MEF cell samples) or 40 wells (lymph node samples) per plate that reacted to PBS instead of the samples.

Plaque reduction neutralization test. Mouse sera (6 μl) and Dulbecco's modified Eagle's medium (DMEM) containing 10% fetal bovine serum (FBS) (42 μl) were mixed and subsequently serially diluted 4-fold. Each 20-μl aliquot of diluent was transferred into flat-bottom 96-well plates containing 5 μl of MP-12 virus (50 PFU/well; final dilutions of sera, 1:10, 1:40, and 1:160). The plate was incubated at 37°C for 1 h, and 150 μl of DMEM with 10% FBS was added to the well. Then, 150 μl of the mixture was transferred into a 24-well plate with confluent Vero E6 cells, and the plate was incubated at 37°C for 1 h. After removal of the inocula, 0.5 ml of a 1:1 mixture of 1.2% Noble agar in water and 2× MEM (Gibco) was overlaid in each well. At 2 days p.i., 0.5 ml of the second overlay, containing neutral red, was added to each well and incubated overnight. The average of the plaque numbers in 6 different wells with added mock-vaccinated mouse sera was used as the cutoff number (typically 8 to 9).

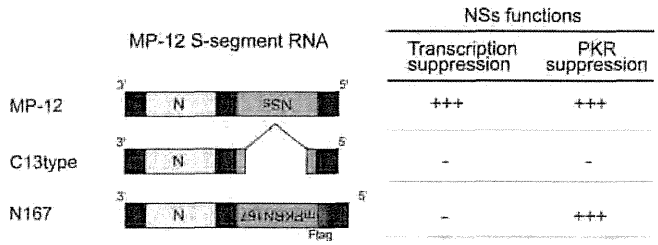

FIG 1 Schematics of S segments of MP-12, rMP12-C13type, and rMP12-mPKRN167. rMP12-C13type (C13type) lacks 69% of the NSs ORF, as described previously (56). rMP12-mPKRN167 (N167) encodes a dominant-negative form of mouse PKR in place of NSs. The expected phenotype corresponding to each S segment is also presented. +++ and − represent the presence and absence of suppression, respectively.

The highest dilution of sera that produced a number of plaques below the cutoff number was designated the $PRNT_{80}$ neutralizing antibody titer.

Detection of RVFV N antigens at popliteal lymph nodes by IFA. Popliteal lymph nodes were collected from mice that were mock vaccinated with PBS or subcutaneously vaccinated with 1×10^5 PFU of MP-12, rMP12-C13type, rMP12-mPKRN167, or heat-inactivated MP-12 (56°C for 30 min) at 1 day p.i. These lymph nodes were fixed overnight with 4% paraformaldehyde, and the fixative was replaced with PBS. The PBS was gradually replaced with 30% sucrose at 4°C and embedded into OCT compound (Sakura Finetek). Cryosectioning was performed at 5-μm thickness, and the sections were fixed with cold acetone-methanol (1:1) for 1 min. Antigens were retrieved with proteinase K (ready to use; Dako catalog number S3020) for 5 min and stained with anti-RVFV N rabbit polyclonal antibody (1:500) (49) or biotin-conjugated anti-CD45R/B220 rat IgG2a antibody (1:500) (clone RA3-6B2; BioLegend) for 2 h at room temperature. Signals were detected by fluorescein isothiocyanate (FITC)-conjugated donkey anti-rabbit IgG (1:1,000; Poly4064; BioLegend) or streptavidin-DyLight 594 (1:1,000; catalog number 405222; BioLegend) by incubating for 30 min at room temperature. Normal rabbit serum (catalog number R9133; Sigma) or biotin rat IgG2a(κ) isotype control (clone RTK2758; BioLegend) was used as the control stain. Specific signals were detected under an Olympus IX71 microscope with a DP71 camera, and the image was detected in DP Manager software.

Statistical analysis. The statistical analyses were performed using the Graphpad Prism 5.03 program (Graphpad Software Inc.). The Mann-Whitney U test and unpaired Student's *t* test were used for the comparison of two groups, while a Kruskal-Wallis test following Dunn's posttest was performed to compare more than three groups. Survival curves of mice were analyzed by a log rank (Mantel-Cox) test.

Ethics statement. Mouse studies were performed in facilities accredited by the Association for Assessment and Accreditation of Laboratory Animal Care (AAALAC) in accordance with the Animal Welfare Act, NIH guidelines, and U.S. federal law. The animal protocol was approved by the UTMB Institutional Animal Care and Use Committee (IACUC) (protocol numbers 0904027 and 1007038). All the recombinant DNA and RVFV were created upon approval of the Notification of Use by the Institutional Biosafety Committee at UTMB. The wt RVFV ZH501 strain was used at the Robert E. Shope BSL4 laboratory at the UTMB in accordance with NIH guidelines and U.S. federal law.

RESULTS

Generation of recombinant MP-12 encoding dominant-negative mouse PKR. Recombinant RVFV strain MP-12 was generated by a reverse genetics system (29). The schematics of MP-12 S segments are shown in Fig. 1. rMP12-C13type carries a 69% in-frame truncation of the NSs gene in the MP-12 backbone (29). rMP12-mPKRN167 encodes the N-terminal 167 amino acids of mouse PKR in place of NSs, which is similar to a dominant-negative form of human PKR, PKRΔE7 (27, 39). To confirm the dominant-negative effect of the mouse PKR N terminus, MEFs were mock infected or infected with MP-12, rMP12-C13type, or rMP12-mPKRN167 at an MOI of 3. Cells were collected at 12 h p.i. or 16 h p.i., and the increase of eIF2α phosphorylation was analyzed. At 12 h p.i., Flag-tagged mouse PKR N terminus was expressed, and the phosphorylation level of eIF2α was similar to that of mock- or MP-12-infected cells, while eIF2α was significantly phosphorylated in cells infected with rMP12-C13type, which lacks a functional NSs gene (Fig. 2A). At 16 h p.i., no increase of eIF2α phosphorylation was observed in cells infected with rMP12-mPKRN167 (Fig. 2B). The results suggest that rMP12-mPKRN167 expresses a dominant-negative form of mouse PKR and inhibits PKR-mediated eIF2α phosphorylation, which is induced in MP-12-infected cells in the absence of NSs.

We noted an apparent increase in N abundance in cells infected with rMP12-mPKRN167 (Fig. 2A). To confirm this, we performed antigen capture ELISA to detect N proteins (15). MEFs were mock infected or infected with MP-12, rMP12-C13type, or rMP12-mPKRN167 at an MOI of 3. Cell lysates were collected at 1 h p.i. and 16 h p.i., and the N abundances were tested by ELISA. At 1 h p.i., similar amounts of N protein were observed among cells infected with MP-12, rMP12-C13type, or rMP12-mPKRN167 (Fig. 2C). At 16 h p.i., more N proteins were accumulated in cells infected with rMP12-mPKRN167 than in those infected with MP-12 or rMP12-C13type (Fig. 2D). The N proteins were not increased in the absence of viral replication (Fig. 2E). These results suggest that the expression of a dominant-negative form of mouse PKR increases the accumulation of N proteins in infected cells. Next, we compared the immunogenicity and efficacy of rMP12-mPKRN167 with those of parental MP-12 or rMP12-C13type.

Efficacy and immunogenicity of rMP12-mPKRN167. To know whether the expression of dominant-negative PKR supports the immunogenicity of MP-12 vaccine, we first analyzed the levels of IFN-α, viremia, and neutralizing antibodies in vaccinated mice. Mice were subcutaneously vaccinated with PBS or 1×10^5 PFU of MP-12, rMP12-C13type, or rMP12-mPKRN167. Viruses were inoculated in the common dorsal subcutaneous area so that the efficacy data would be comparable to those of other vaccine candidates using subcutaneous inoculation. All the mice vaccinated with rMP12-mPKRN167 induced detectable IFN-α in the sera at 1 day p.i., whereas 0 or 55% of mice vaccinated with MP-12 or rMP12-C13type, respectively, induced IFN-α (Fig. 3A). Viremia levels in sera at 1, 2, and 3 days p.i. were tested by plaque assay. Three of 10 mice that were vaccinated with MP-12 developed viremia of 100 to 400 PFU/ml at 3 days p.i., while no mice that were vaccinated with rMP12-C13type or rMP12-mPKRN167 virus showed detectable viremia of 100 PFU/ml or more (Fig. 3B). These results suggest that a lack of NSs in MP-12 resulted in the induction of type-I IFN in mice and prevented viremia.

We tested the development of neutralizing antibody in vaccinated mice at day 30, day 90, and day 180 to determine the effect of dominant-negative PKR expression on long-term immunity (Fig. 3C). The mean neutralizing antibody titers of mice that were vaccinated with MP-12, rMP12-C13type, or rMP12-mPKRN167 were highest at 90 days p.i. and were 1:2,988, 1:1,056, or 1:2,497, respectively, while the mean neutralizing antibody titers were decreased to 1:1,068, 1:216, or 1:961, respectively, at 180 days p.i. All mice vaccinated with rMP12-mPKRN167 induced neutralizing antibody at 90 days p.i., while 3 or 1 mouse vaccinated with MP-12

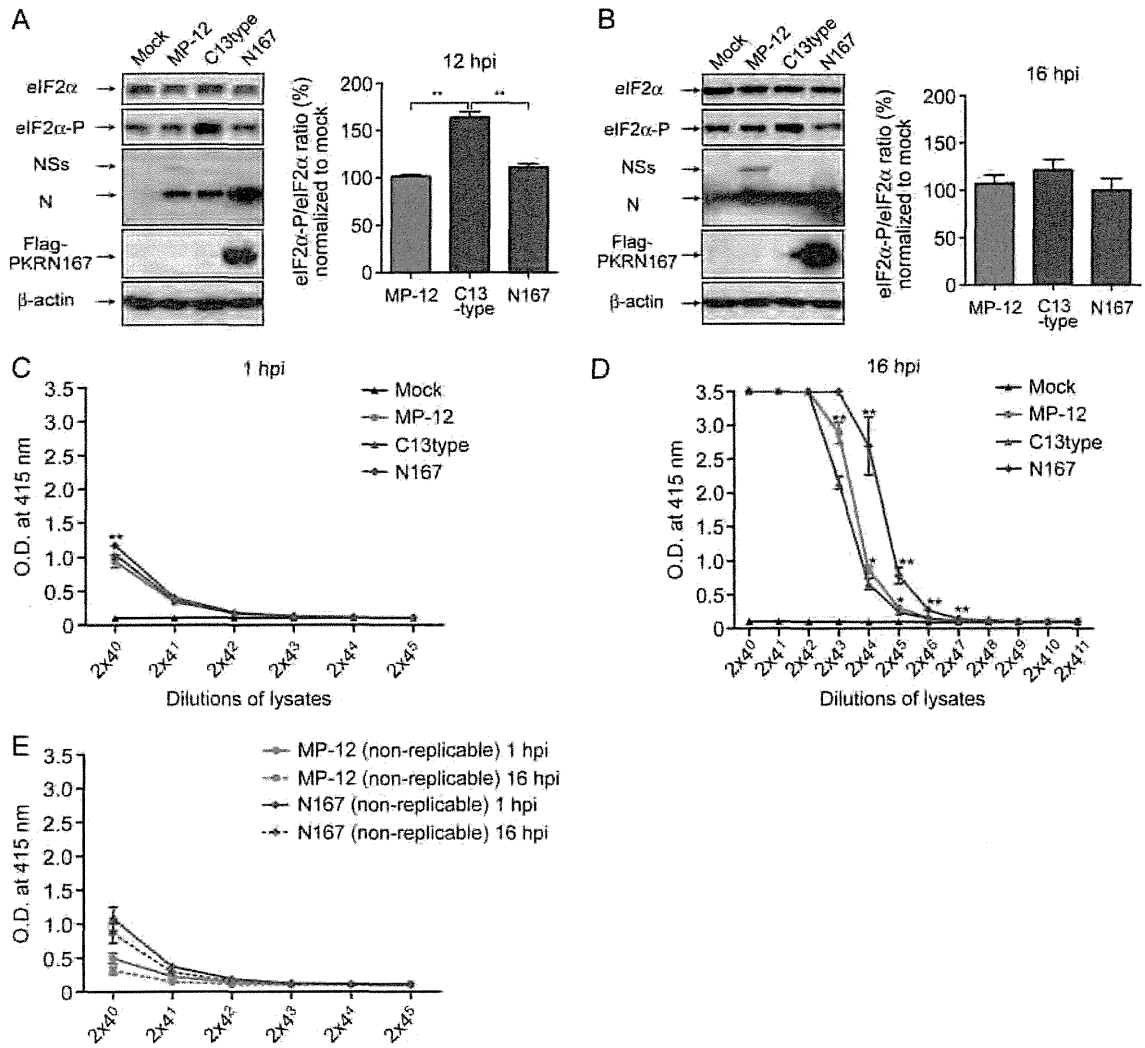

FIG 2 Dominant-negative effect of rMP12-mPKRN167. Wild-type MEFs were mock infected or infected with MP-12, rMP12-C13type, or rMP12-mPKRN167 at an MOI of 3. Cells were collected at 12 h p.i. (A) or 16 h p.i. (B), and the abundance of eIF2α, phosphorylated eIF2α (eIF2α-P) (Ser 51), NSs, N, Flag-PKR N167, and β-actin were analyzed by Western blotting. The mean ratios and standard deviations of the density of phosphorylated eIF2α and eIF2α were determined from 3 independent experiments. The asterisks represent statistical significance (Student's unpaired *t* test; $P < 0.01$). (C and D) MEFs were mock infected or infected with MP-12, rMP12-C13type, or rMP12-mPKRN167 at an MOI of 3. Cell lysates were collected at 1 h p.i. (C) and 16 h p.i. (D), and the abundance of N proteins was measured by antigen capture ELISA for RVFV N proteins (15). The asterisks represent statistical significance (Mann-Whitney U test; *, $P < 0.05$; **, $P < 0.01$ versus rMP12-C13type at each dilution). (E) As a control to monitor initial N protein derived from inocula, heat-inactivated MP-12 or rMP12-mPKRN167 was also tested.

or rMP12-C13type, respectively, did not induce neutralizing antibodies. These results suggest that the expression of dominant-negative PKR is an effective way to successfully induce long-term neutralizing antibodies by using MP-12 vaccines lacking NSs.

We next tested the efficacy of rMP12-mPKRN167 in mice. Outbred CD1 mice were mock vaccinated with PBS or vaccinated with 1×10^5 PFU of MP-12, rMP12-C13type, or rMP12-mPKRN167 via the subcutaneous route. At 44 days p.i., mice were challenged with 1×10^3 PFU of wt RVFV ZH501 (i.p.), and survival was monitored for 21 days. Mock-vaccinated mice succumbed to RVFV-induced disease within 10 days p.i. (mean survival time, 8 days), while all mice vaccinated with rMP12-mPKRN167 were protected from wt RVFV challenge (Fig. 4A). On the other hand, 72% or 80% of mice vaccinated with MP-12 or rMP12-C13type, respectively, were protected from challenge (Fig. 4A). We did not find statistical significance by the log rank test in the survival curve among the vaccinated mice. The daily weight change showed a continuous decrease in body weight in mock-vaccinated mice, while surviving mice that were vaccinated with MP-12, rMP12-C13type, or rMP12-mPKRN167 did not show a significant decrease in body weight after wt RVFV challenge (Fig. 4B). Further, we determined neutralizing antibody titers prior to challenge with wt RVFV at 42 days postimmunization. Importantly, all of the vaccinated mice that succumbed to disease lacked detectable neutralizing antibodies at 42 days p.i. (Table 1). These results suggest that neutralizing antibody plays an important role in protecting vaccinated animals from wt RVFV challenge. We also noticed that one mouse that was vaccinated with rMP12-mPKRN167 (number 10-1) survived without detectable neutralizing antibodies at 42 days p.i. (Table 1). The sera were

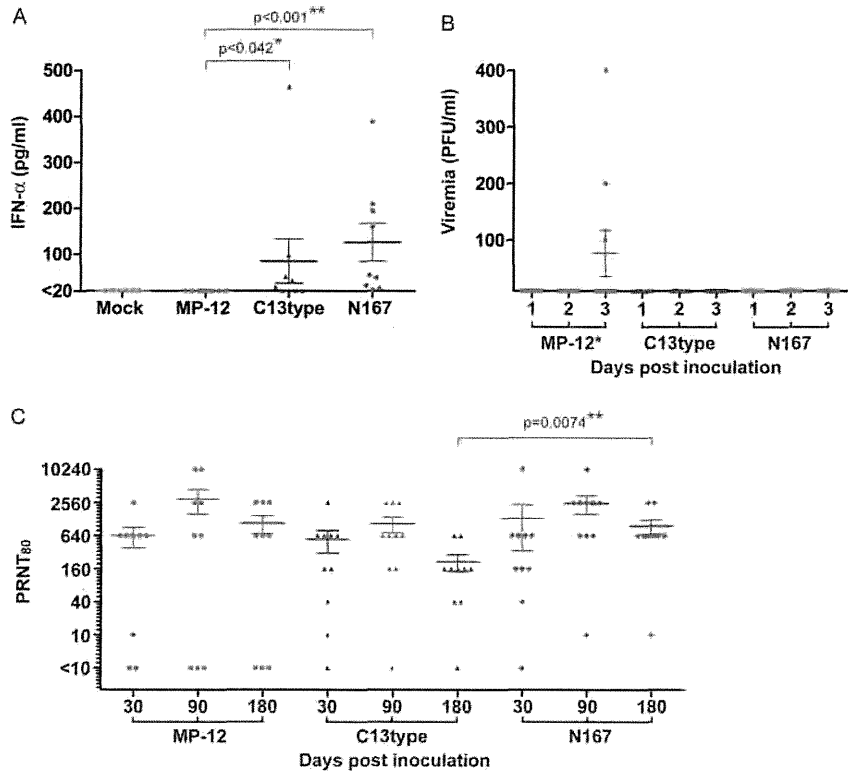

FIG 3 Immunogenicity of rMP12-mPKRN167. Five-week-old CD1 mice were mock vaccinated with PBS ($n = 6$) or vaccinated subcutaneously with 1×10^5 PFU of MP-12, rMP12-C13type (C13type), or rMP12-mPKRN167 (N167) ($n = 9$). Sera were collected at 1, 2, 3, 30, 90, and 180 days p.i. (A) Abundance of IFN-α in mouse serum samples (mock, $n = 6$; other groups, $n = 9$) at 1 day p.i. Serum IFN-α was measured with the VeriKine Mouse Interferon Alpha ELISA Kit (PBL International). A Mann-Whitney U test was performed for statistical analyses (*, $P < 0.05$; **, $P < 0.01$). (B) Viral titers of mouse serum samples ($n = 10$ per group). The viral titers in serum samples at days 1, 2, and 3 were determined by plaque assay in Vero E6 cells. *, $P < 0.05$ by the Kruskal-Wallis test. (C) Neutralizing antibody titers of serum samples: MP-12 ($n = 9$), C13type ($n = 10$), or N167 ($n = 10$). A $PRNT_{80}$ was performed to determine the neutralizing antibody titer. A Mann-Whitney U test was performed for statistical analyses between $PRNT_{80}$ titers at 180 days p.i. (*, $P < 0.05$; **, $P < 0.01$). The error bars indicate standard deviations.

further analyzed with an IgG ELISA system to detect anti-N-specific antibodies. We found that the survivor (number 10-1) lacking neutralizing antibody had a 1:1,600 titer of anti-N antibodies (Table 1). It was also found that three dead mice that were vaccinated with MP-12 (number 3-1, 3-4, and 4-4) lacked both detectable neutralizing antibodies and anti-N IgG (Table 1), indicating that MP-12 failed to stimulate the host immune system in those mice.

In addition to immunogenicity, the ability to differentiate infected from vaccinated animals (DIVA) is important for vaccine development. MP-12 lacks a marker for DIVA, and antibody responses cannot be discriminated from natural infection. rMP12-

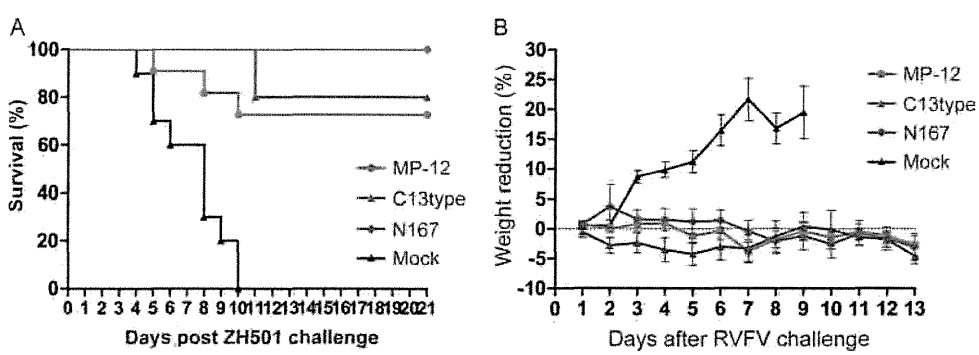

FIG 4 Protection efficacy of rMP12-mPKRN167 in mice. Five-week-old CD1 mice were mock vaccinated ($n = 10$) or vaccinated subcutaneously with 1×10^5 PFU of MP-12 ($n = 10$), rMP12-C13type (C13type) ($n = 10$), or rMP12-mPKRN167 (N167) ($n = 9$). Sera were collected at 42 days p.i., and mice were challenged with 1×10^3 PFU of wt RVFV strain ZH501 (i.p.) at 44 days p.i. The mice were observed for 21 days postchallenge. (A) Kaplan-Meier survival curves of vaccinated mice after wt RVFV challenge. (B) Daily weight changes of mock-vaccinated mice or surviving vaccinated mice after challenge. The error bars indicate standard deviations.

TABLE 1 Antibody titers pre- and post-wt RVFV challenge

Virus	Mouse no.	Pre-challenge[a]			Death day	Postchallenge[b] anti-NSs OD value[c]
		$PRNT_{80}$ titer	Anti-N titer	Anti-NSs OD value[c]		
MP-12	3-1	<10	<1:100	0.123	8	
	3-2	10,240	1:102,400	0.238		0.190
	3-3	640	1:102,400	0.175		0.207
	3-4	<10	<1:100	0.098	10	
	3-5	2,560	1:51,200	0.105		0.134
	4-1	2,560	1:102,400	0.436		0.353
	4-2	10,240	1:25,600	0.105		0.155
	4-3	2,560	1:51,200	0.098		0.143
	4-4	<10	<1:100	0.106	5	
	4-5	2,560	>1:204,800	0.111		0.173
	11-1	10,240	1:3,200	0.106		0.178
C13type	5-1	2,560	1:102,400	0.105		0.297
	5-2	<10	1:3,200	0.127	10	
	5-3	160	1:12,800	0.103		0.159
	5-4	40	1:12,800	0.104		0.247
	5-5	<10	1:12,800	0.110	10	
	6-1	640	1:12,800	0.120		0.356
	6-2	40	1:12,800	0.108		0.217
	6-3	40	1:400	0.120		0.118
	6-4	640	1:12,800	0.106		0.187
	6-5	10	1:3,200	0.114		0.134
N167	9-1	2,560	>1:204,800	0.104		0.228
	9-2	40	1:6,400	0.105		0.179
	9-3	40	1:3,200	0.106		0.188
	9-4	160	1:3,200	0.098		0.158
	9-5	10,240	1:102,400	0.108		0.179
	10-1	<10	1:1,600	0.104		0.344
	10-2	40	1:6,400	0.102		0.125
	10-3	160	1:3,200	0.098		0.117
	10-4	2,560	1:6400	0.101		0.204

[a] Prechallenge, 42 days postimmunization.
[b] Postchallenge, 21 days postchallenge.
[c] OD value of sera diluted at 1:100. The cutoff value was determined to be 0.144 in this experiment.

C13type and rMP12-mPKRN167 encode a negative DIVA marker (lack of the NSs gene). We tested whether vaccinated mice induce anti-NSs antibody. For this purpose, we developed an IgG ELISA by using truncated NSs proteins that were tagged with GST at the N terminus to detect IgG that was specific to NSs. NSs is a nonstructural protein and is expressed only when virus replicates in cells. Although MP-12 encodes NSs, only 3 out of 11 mice vaccinated with MP-12 raised anti-NSs IgG (Table 1), while none of the mice vaccinated with rMP12-C13type or rMP12-mPKRN167 developed detectable anti-NSs antibodies before challenge. These results suggest that rMP12-C13type and rMP12-mPKRN167 are superior to MP-12 in DIVA. After wt RVFV challenge, 6 out of 8 (75%) or 7 out of 9 (77.7%) mice vaccinated with rMP12-C13type or rMP12-mPKRN167 raised detectable anti-NSs antibodies (Table 1). The OD value of anti-NSs antibodies in pre- and postchallenge sera were statistically significant (Mann-Whitney U test; $P < 0.01$) in mice vaccinated with rMP12-C13type and rMP12-mPKRN167, but not in those vaccinated with MP-12.

Impact of dominant-negative PKR on viral-protein accumulation. PKR suppression promotes viral-protein accumulation and replication of RVFV in cell culture (27). To understand why rMP12-mPKRN167 has high efficacy in mice, we tested the accumulation of viral proteins at the local draining lymph nodes. We chose to observe popliteal and inguinal lymph nodes after vaccination in the footpad, because the antigen accumulation at those draining lymph nodes is well characterized in mice (22). Outbred CD1 mice were mock vaccinated or vaccinated with 1×10^5 PFU of MP-12, rMP12-C13type, or rMP12-mPKRN167 subcutaneously in the footpads. Then, popliteal and inguinal lymph nodes were separately collected to measure the abundance of RVFV N antigens. Before the assay, we analyzed the amounts of RVFV N antigens in virus inocula by using antigen capture ELISA, which can specifically detect RVFV N (15). Figure 5A shows the OD values of virus inocula containing MP-12, rMP12-C13type, rMP12-mPKRN167, or heat-inactivated MP-12 (a control virus that cannot replicate). We confirmed that N abundances were similar among the inocula. The cutoff value of the ELISA was determined for each plate as the mean plus 3 times the standard deviation of 40 wells that reacted to PBS instead of samples. At 1 day p.i., the popliteal lymph nodes of mice vaccinated with C13type (4 out of 4; 100%) or N167 (4 out of 4; 100%) showed significant N protein accumulation (Fig. 5B), whereas the N proteins were only marginally increased in popliteal lymph nodes of mice that were vaccinated with MP-12 (3 out of 4; 75%) (Fig. 5B).