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Supporting Online Material

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Materials and Methods
References

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Discovery of Swine as a Host for the *Reston ebolavirus*

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Since the discovery of the Marburg and Ebola species of filovirus, seemingly random, sporadic fatal outbreaks of disease in humans and nonhuman primates have given impetus to identification of host tropisms and potential reservoirs. Domestic swine in the Philippines, experiencing unusually severe outbreaks of porcine reproductive and respiratory disease syndrome, have now been discovered to host *Reston ebolavirus* (REBOV). Although REBOV is the only member of *Filoviridae* that has not been associated with disease in humans, its emergence in the human food chain is of concern. REBOV isolates were found to be more divergent from each other than from the original virus isolated in 1989, indicating polyphyletic origins and that REBOV has been circulating since, and possibly before, the initial discovery of REBOV in monkeys.

Filoviruses are associated with acute fatal hemorrhagic diseases of humans and/or nonhuman primates. The family consists of two genera: *Marburgvirus*, which comprises various strains of the *Lake Victoria marburgvirus* (MARV) discovered in 1967; and the antigenically distinct genus *Ebolavirus* discovered in 1976, which comprises five species including *Sudan ebolavirus* (SEBOV), *Zaire ebolavirus* (ZEBOV), *Ivory Coast ebolavirus* [also known as Cote d'Ivoire Ebola virus (CIEBOV)], *Bundibugyo ebolavirus* (BEBOV), and *Reston ebolavirus* (REBOV) (1). REBOV is the only member of the family thus far not associated with disease in humans (2).

Since the discovery of filoviruses more than 40 years ago, ostensibly random, sporadic, and fatal outbreaks of disease in primates have evoked

interest in delineation of host tropisms, potential reservoirs for disease transmission, and persistence in nature (3). These lines of investigation have recently identified African fruit bats as potential reservoirs for ZEBOV (4, 5) and MARV (6, 7). Similar links to bats have been found for emerging infections in swine and humans involving paramyxoviruses and the severe acute respiratory syndrome (SARS) coronavirus (8, 9).

Until now, REBOV has only been associated with disease in nonhuman primates (2, 10). The virus was originally identified in 1989 in the United States from a shipment of cynomolgus monkeys (*Macaca fascicularis*) from the Philippines. Outbreaks of disease occurred in the United States in 1990 and 1996 and in Italy in 1992, which were traced back to a single facility in the Philippines (fig. S1) (11, 12). Here, we report the identification of REBOV infection in domestic swine co-infected with porcine reproductive and respiratory syndrome virus (PRRSV) that were experiencing a severe respiratory disease syndrome.

In July 2008, the Philippine Department of Agriculture requested the assistance of the U.S.

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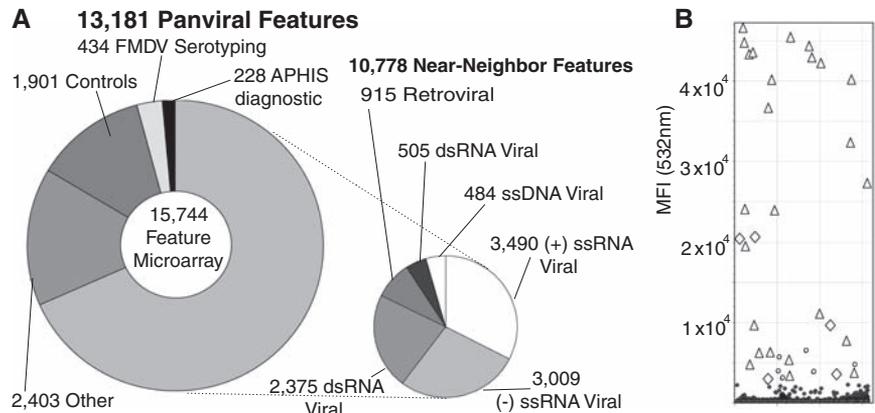


Fig. 1. Detection of REBOV in swine samples from the Philippines. **(A)** Composition of the panviral microarray used to detect REBOV. The microarray feature composition is summarized with reference to the number of unique features for identification of viral pathogens. FMDV, foot-and-mouth disease virus. **(B)** Microarray analysis of Vero cell culture of a swine lymph node from sample group A identified multiple positive features within the genus of Ebola viruses. These features corresponded primarily to sequences from REBOV with minimal reactivity toward SEBOV and ZEBOV. MFI, mean fluorescence intensity (Δ) Positive *Reston ebolavirus* spp. features; (◇) positive *Ebolavirus* genus features; (○) non-*Ebolavirus* features; and (●) negative features.

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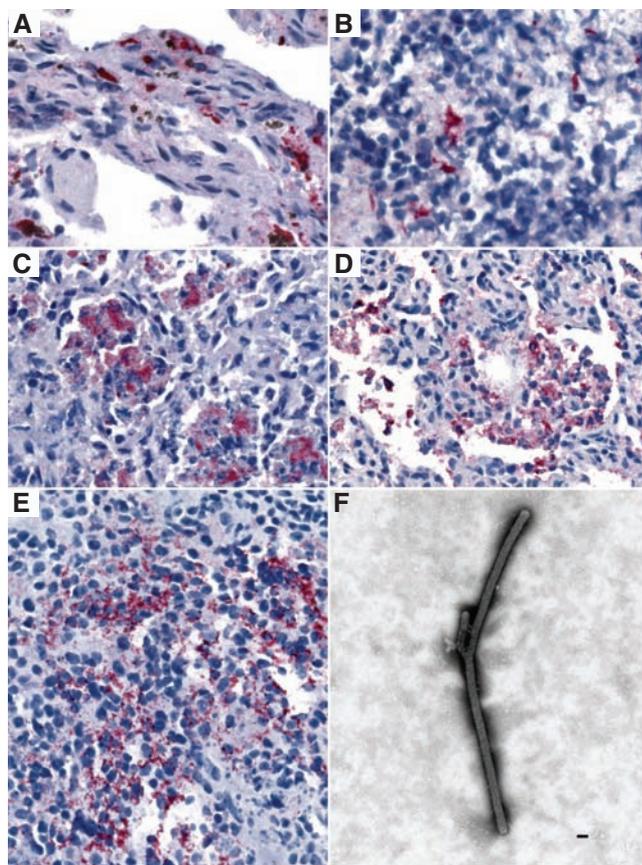
Department of Agriculture (USDA), Animal and Plant Health Inspection Service (APHIS), Foreign Animal Disease Diagnostic Laboratory (FADDL), in the diagnostic investigation of recent multiple outbreaks of a respiratory and abortion disease syndrome in swine. Clinical signs resembled a

highly pathogenic PRRSV infection, also referred to as “blue ear disease,” which has recently been spreading through Asia (13–15). Sera and tissue samples were collected from five groups of swine at two commercial premises located in Pandi, Bulacan (sample group A); Manoaag, Pangasinan

(sample groups C and E); and two inspection check points located in Sto. Nino, San Jose City, Nueva Ecija (sample group B) and Batangas (sample group D) (fig. S1). The diagnostic investigation at FADDL included diagnostics for African swine fever and classical swine fever, a directed investigation for the presence of PRRSV, and a more general search of other viral agents potentially contributing to the disease. Selected tissue samples from each group were tested and found negative for the presence of African swine fever, classical swine fever, swine vesicular disease, and foot-and-mouth disease. Consistent with a respiratory and reproductive disease syndrome, PRRSV was discovered. Sequence analysis of the *NSP2* gene revealed that it was most homologous to Chinese PRRSV isolates recently associated with blue ear disease in Asia. This determination was based on the presence of two unique deletions in the *NSP2* gene of the Philippines PRRSV isolate that are shared by recent Chinese PRRSV isolates associated with pathogenic PRRS in Asia (14, 16). Simultaneously, a lymph node from group A, cultured in Vero cells, a monkey kidney cell line nonpermissive for PRRSV, revealed cytopathic effects indicating the presence of a virus other than PRRSV.

To resolve such unexplained cases, a panviral microarray has been developed that used a near-neighbor approach for the identification of taxonomically conserved viral protein microdomains. This tool consists of tens of thousands of conserved viral genetic signature sequences microscopically arrayed on a slide and is designed to capture extracted and amplified viral nucleic acid from a query sample (Fig. 1A). It is similar in concept and design to previously published pathogen microarrays, including a panmicrobial array named the GreeneChip (17, 18) and the

Fig. 2. Immunohistopathology of EBOV and PRRSV. (A) Lymph node capsule stained for EBOV antigens. (B) Lymph node tissue stained for EBOV antigens. (C) Lung tissue stained for EBOV antigens. (D) Lung tissue stained for PRRSV antigens. (E) Lymph node germinal center stained for PRRSV antigens. (A to E) Immunoalkaline phosphatase staining, naphthol fast red substrate with light hematoxylin counterstain. (F) Filovirus particle by negative-staining electron microscopy of the E6 Vero cell culture of the lymph node. Scale bar, 100 nm.

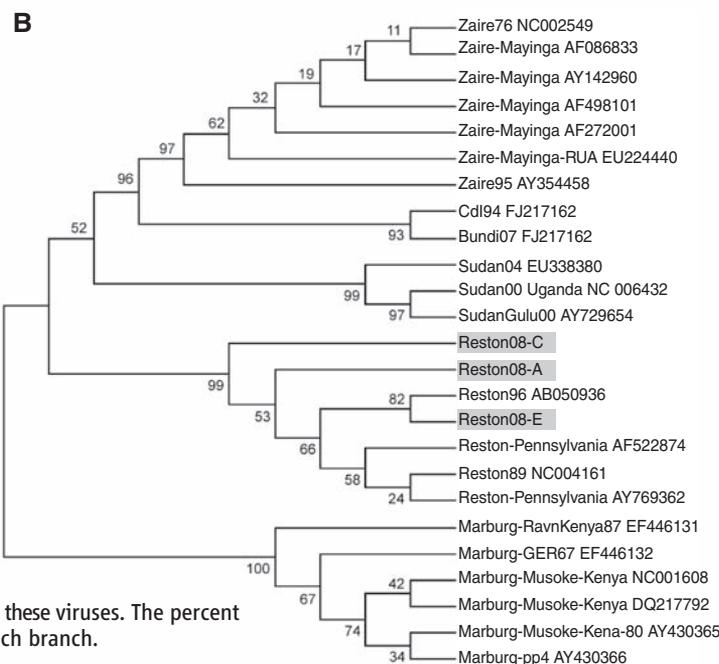


A

Nucleotide Similarity	Reston08-A	Reston08-C	Reston08-E	Reston96
Reston08-A				
Reston08-C	95.5%			
Reston08-E	96.9%	95.8%		
Reston96	97.7%	96.5%	98.70%	
Reston89	97.7%	96.9%	97.90%	98.80%

Amino acid changes	Reston08-A	Reston08-C	Reston08-E	Reston96
Reston08-A 5209 aa				
Reston08-C 5201 aa	193			
Reston08-E 5201 aa	122	193		
Reston96 5201 aa	98	171	64	
Reston89 5201 aa	105	148	92	61

Fig. 3. Phylogeny of REBOV. (A) Full-length genomic sequences for Reston08-A, Reston08-C, and Reston08-E were experimentally determined, with the exception of the defined 5' and 3' termini, and aligned. Nucleotide similarity scores and the number of predicted amino acid changes between swine and monkey REBOV genomes are shown. (B) A consensus neighbor-joining tree drawn without distance topology illustrates the independent branching of the three 2008 Philippine swine viruses within the REBOV clade, demonstrating the divergence between each of these viruses. The percent branching out of 1000 random bootstrap iterations is indicated above each branch.



ViroChip used to characterize the SARS coronavirus (19).

To identify the unknown virus, the Vero cell culture was subjected to microarray analysis. Results revealed positive signals for 28 out of 28 distinct array features present in a 3.7-kb span of the REBOV *L* gene (Fig. 1B). By contrast, only 3 out of 30 and 2 out of 30 features were positive for the ZEBOV and SEBOV species, respectively. No other notable signals other than controls were positive (Fig. 1B). Because microarray results are sequence dependent, polymerase chain reaction (PCR) primers designed from the features themselves were used to PCR amplify and sequence the viral cDNA captured by the microarray slide. This sequence analysis confirmed that the captured viral sequences were more than 95% identical to the *L* gene of all previously sequenced REBOV isolates. Because REBOV is classified as a biological safety level 4 select agent, samples were transferred to the Special Pathogens Branch at the Centers for Disease Control and Prevention (CDC) (Atlanta, Georgia), and identification of REBOV was confirmed by Ebola-specific real-time reverse transcription (RT)-PCR analysis (table S1), antigen enzyme-linked immunosorbent assay, immunohistochemistry, and virus isolation in E6 Vero cell culture.

REBOV was only found in sample groups that also tested positive for PRRSV (table S1). Histopathological and immunohistochemical examination of lymph nodes from animals infected with REBOV and PRRSV from groups A and C showed different patterns of antigen localization and pathology (Fig. 2, A, B, and E). REBOV antigens were seen focally in lymphoid and lymph node capsule tissues with minimal necrosis (Fig. 2, A and B), whereas PRRSV antigens were seen in the germinal centers of lymphoid follicles displaying germinal cell hyperplasia and focal necrosis (Fig. 2E). Immunostaining of lung tissues for REBOV and PRRSV revealed localization of both viral antigens in areas displaying mixed inflammatory cells and sloughed necrotic debris in alveolar spaces consistent with interstitial pneumonia (Fig. 2, C and D). Negative-staining electron microscopy of the E6 Vero cell culture of the lymph node from the Bulacan site (group A) revealed filamentous virus particles and partially assembled intermediate particles characteristic of filoviruses (Fig. 2F). Serological studies on 13 swine sera from groups A, B, and D for the detection of antibodies to REBOV were negative. In contrast, antibodies to PRRSV were detected in swine sera from each of the tested sample groups A, B, and D.

RT-PCR revealed REBOV nucleic acid in animals from groups C and E, at the Pangasinan site, and from group A at the Bulacan site (fig. S1 and table S1). Samples from groups B and D did not test positive for REBOV or PRRSV; however, PCR revealed porcine circovirus type 2 (PCV-2) among samples from groups A, B, and D (table S1), and microarray analysis further revealed *Porcine teschovirus 1* from the SK6 porcine kidney cell culture of a tonsil from group D.

Viral genomes for REBOV identified from three samples—designated Reston08-A, Reston08-C, and Reston08-E—at two geographically distinct locations were ~18.9 kb in length and confirmed that the viruses were REBOV species (Fig. 3). The Reston08 viruses were significantly more divergent from each other (3.93% mean difference in nucleotide identity) than from the prototypical reference isolate from 1989 (2.5% mean difference in nucleotide identity), indicating polyphyletic origins of the REBOV infections in swine at both locations (Fig. 3A).

The lack of a phylogenetic clade, distinct from viruses in macaques, for the recent REBOV infections in swine (Fig. 3B) suggests that REBOV has been circulating since, and possibly before, the initial discovery of REBOV in monkeys exported from the Philippines in 1989. The isolation of REBOV from swine represents an extension in the known host tropism. The interisolate divergence of the three recent swine isolates is greater than that observed among the monkey isolates obtained from the single implicated primate export facility (Fig. 3A). Given the broader genetic diversity and geographic distribution of REBOV in swine, it is possible that REBOV spilled over to monkeys and swine from an as yet unidentified host. Bats have been implicated as reservoirs for other filoviruses, including ZEBOV and MARV, and may also represent a candidate reservoir for REBOV.

Of 141 tested individuals, we identified 6 individuals who worked on pig farms or with swine products that had positive serum immunoglobulin G (IgG) titers to REBOV, confirming the potential transmission from pigs to humans. The remaining 135 individuals tested negative for IgG titers to REBOV. Given the observed sequence divergence between the Reston08 viruses, a broader surveillance program is being planned. REBOV infection in domestic swine raises concern about the potential for emerging disease in humans and a wider range of livestock. However, as in previous REBOV incidents, there is no evidence of disease in humans despite the apparent occurrence of human infections evidenced by seropositive titers of REBOV-specific antibody.

The role of swine as either an incidental host or an integral part of the virus's transmission cycle has yet to be determined. Because evidence of coinfection with PRRSV, an arterivirus, was found with REBOV, we can speculate about a link between coinfection and disease in swine. This possibility is of interest in light of the atypical, highly pathogenic infections in swine by PRRSV that are currently spreading through Asia (14–16). Simian hemorrhagic fever virus, a well-known pathogen of captive primates and also an arterivirus of previously characterized pathogenicity, was identified in a coinfection of monkeys during the first detected outbreak of REBOV (20), although later studies clearly demonstrated the pathogenicity of REBOV as a single agent in experimentally infected monkeys (21).

There is concern that its passage through swine may allow REBOV to diverge and shift its potential for pathogenicity. Moreover, REBOV infections in swine highlight the need for investigations into the pathogenesis of REBOV in coinfections or in immunocompromised hosts. Through domestic and international interdisciplinary cooperation and collaboration, it is expected that future epidemiology and pathogenesis studies will shed light on the potential reservoirs, mode(s) of transmission, mechanisms of pathogenesis, prevalence of REBOV in nature, and its consequences for agricultural industries and trade.

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Materials and Methods

Fig. S1
Table S1
References

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Discovery of Swine as a Host for the *Reston ebolavirus*

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Not Reston at All

Reston ebolavirus is named, mistakenly perhaps, for Reston, Virginia, where it was discovered in the 1970s in imported macaques. After some alarm it was found not to be virulent in humans, uniquely among the ebola viruses, which are characteristically fatal causing a horrific spectrum of symptoms. Using a panviral detection assay, *Reston ebolavirus* has been rediscovered by **Barrette et al.** (p. 204) in domesticated pigs in the Philippines in association with other viruses that cause respiratory illness. The strains involved are closely related to the original macaque strain and, given how little variance there is among the viruses, it appears that it is freely circulating between these species possibly, like several other zoonotic viruses, having a reservoir in bats. Serological assays indicated that farm workers have become infected, although no obvious symptoms of human disease have been reported.

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