Ebola Reston Virus Infection of Pigs: Clinical Significance and Transmission Potential

Glenn A. Marsh,[1] Jessica Haining,[1] Rachel Robinson,[1] Adam Foord,[1] Manabu Yamada,[1,a] Jennifer A. Barr,[1] Jean Payne,[1] John White,[1] Meng Yu,[1] John Bingham,[1] Pierre E. Rollin,[2] Stuart T. Nichol,[2] Lin-Fa Wang,[1] and Deborah Middleton[1]

[1]Australian Animal Health Laboratory, Livestock Industries, Commonwealth Scientific and Industrial Research Organisation (CSIRO), Geelong, Australia; and [2] Viral Special Pathogens Branch, Centers for Disease Control and Prevention, Atlanta, Georgia

In 2008, *Reston ebolavirus* (REBOV) was isolated from pigs during a disease investigation in the Philippines. Porcine reproductive and respiratory syndrome virus (PRRSV) and porcine circovirus type 2 (PCV-2) infections were also confirmed in affected herds and the contribution of REBOV to the disease outbreak remains uncertain. We have conducted experimental challenge studies in 5-week-old pigs, with exposure of animals to 10^6 $TCID_{50}$ of a 2008 swine isolate of REBOV via either the oronasal or subcutaneous route. Replication of virus in internal organs and viral shedding from the nasopharynx were documented in the absence of clinical signs of disease in infected pigs. These observations confirm not only that asymptomatic infection of pigs with REBOV occurs, but that animals so affected pose a transmission risk to farm, veterinary, and abattoir workers.

Ebola virus (EBOV) and the closely related Marburg virus (MARV) belong to the family *Filoviridae*. They are enveloped, filamentous, negative-sense RNA viruses and may be associated with severe zoonotic disease in humans. Currently, 4 species of EBOV have been reported: *Zaire ebolavirus* (ZEBOV), *Sudan ebolavirus* (SEBOV), *Ivory Coast ebolavirus*, and *Reston ebolavirus* (REBOV) [1]. A fifth species, *Bundibugyo ebolavirus*, has been proposed [2]. Four species of EBOV have caused fatal disease in humans with mortality rates of 50%–90% depending on virus strain [3, 4]. In contrast, REBOV has never been identified to cause disease in people. Several instances of humans seroconverting to REBOV have been documented; however, these were not associated with illness or death [5–8], suggesting that REBOV may be avirulent in humans.

REBOV was first identified during the investigation of fatal hemorrhagic fever involving cynomolgus macaques in a research facility in Reston, Virgina, that had been imported from the Philippines. Macaques showed sudden onset of illness with signs of anorexia, cough, nasal exudates, swollen eyelids, and enlarged spleens and kidneys [9, 10]. Macaques were determined to be coinfected with REBOV and simian hemorrhagic fever virus (SHFV; family *Arteriviridae*, genus *Arterivirus*) [9]. As SHFV is often a fatal disease in macaques, experimental infections were carried out in cynomolgus macaques and African green monkeys to determine the role of REBOV in the disease outbreak. Acute-phase viremia was observed in all challenged animals, with REBOV regularly reisolated and also detected by polymerase chain reaction (PCR) in sera and tissues for the first 15 days after challenge. REBOV was demonstrated to be less pathogenic than ZEBOV and SEBOV, with only 7 of 7 African green monkeys and 4 of 8 cynomolgus macaques surviving challenge [11]. REBOV could not be recovered from survivors 20–600 days after challenge. Following this initial outbreak in Reston, several other cases of REBOV have occurred in both the United States and Italy, all attributed to animals imported from the Philippines and all from the same monkey breeding facility [12–16].

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[a]Present affiliation/address: National Institute of Animal Health, Ibaraki, Japan.

Correspondence: Glenn Marsh, CSIRO Livestock Industries, Australian Animal Health Laboratory, PO Bag 24, Geelong, Victoria 3220, Australia (glenn.marsh@csiro.au).

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From September 2007 to May 2008, disease outbreaks were reported on several pig farms in the Philippines. Pigs of all ages were affected, with a wide variety of clinical signs of disease including fever, coughing, and skin lesions. Samples from 3 different farms in which disease was observed were sent to the Foreign Animal Disease Diagnostic Laboratory, United States Department of Agriculture in Plum Island, New York, for isolation and identification of the causative agent for the pigs' disease. Initial analysis of the samples resulted in the isolation of porcine reproductive and respiratory syndrome virus (PRRSV; family *Arteriviridae*, genus *Arterivirus*) and porcine circovirus type 2 (PCV-2; family *Circoviridae*) [17]; these pathogens are regularly associated with disease clinically similar to that reported from the Philippines. Sequence characterization of the NSP2 gene of the PRRSV isolate showed it was most closely related to Chinese PRRSV isolates recently associated with blue ear disease in Asia. In addition, REBOV was isolated from lung and lymph nodes of pigs from each of the 3 farms. Full genome sequences were obtained for each of the REBOV isolates, with distinct sequences from each location showing approximately 4% nucleotide difference in sequence identity between isolates and no discernable grouping with the REBOV isolates obtained from macaques [17]. The differences in swine isolates suggest distinct spillover events from an as yet unidentified host or that pigs act as asymptomatic hosts with virus circulating in them for many years. Bats have been implicated as reservoirs for filoviruses in Africa, including ZEBOV and MARV [18–22], and further investigations are necessary to determine whether REBOV is present in bats in the Phillipines. In addition to evidence of infection in swine, 141 people were also tested for the presence of antibodies to REBOV. Six individuals tested positive for immunoglobulin G (IgG), all of whom worked on pig farms or with swine products, suggesting the potential transmission from pigs to humans [17]. Further serological testing of individual with and without exposure to pigs is necessary for further investigation of the spillover of REBOV from pigs to humans.

The isolation of REBOV from swine may indicate emergence of a filovirus in a new mammalian host. REBOV infection of swine raises important biosecurity concerns about the potential for disease emergence in humans and other livestock animals. In previous outbreaks of REBOV in monkey facilities, there have been no incidences of disease in humans despite several people seroconverting to the virus. Whether the signs of disease seen in the pigs from the original outbreak are a consequence of the REBOV infection or the result of coinfection with PRRSV and PCV-2 was not determined.

The natural transmission of REBOV to pigs was a novel finding and has raised questions regarding the role that this virus played in the disease observed. To address these questions, we have commenced a series of experimental infections of pigs with a Philippines swine isolate of REBOV under Biosafety Level 4 (BSL4) conditions at the Australian Animal Health Laboratory, Geelong, Australia (AAHL). The objectives of the studies reported here are to document (1) the nature of clinical disease in REBOV-infected pigs, (2) the site(s) of virus replication in the pig, and (3) the routes of virus shedding, and thus possible routes of transmission. A greater understanding of REBOV infection in pigs will permit more informed assessment of the risks to swine and human health posed by this viral infection.

METHODS

Animals

Clinically healthy (male and female) non-specific, pathogen-free pigs, 5 weeks of age, were purchased from a commercial breeder and housed in a single room at BSL4. Room temperature was maintained at 22°C with 15 air changes per hour; humidity varied between 40% and 60%. For each study, 8 pigs were kept in 2 groups of 4, in 2 pens with one side modified for movement along a track to provide a ''squeeze cage'' for administration of chemical restraint. There was no direct contact between the 2 pens of pigs. Before inoculation or collection of specimens, animals were immobilized with a mixture of ketamine hydrochloride (20 mg/kg) (Ketamil; Ilium) and medetomidine (60 μg/kg for pigs) (Domitor; Novartis) by intramuscular injection. For reversal, atimepazole (Antisedan; Novartis) was given intramuscularly at 50% of the doses used for medetomidine. Animal studies were conducted with approval from the Commonwealth Scientific and Industrial Research Organisation (CSIRO) Australian Animal Health Laboratory Animal Ethics Committee. Staff wore fully encapsulating suits with breathing apparatus while in the animal room.

Virus Preparation and Challenge

A Philippines 2008 porcine isolate of REBOV was transferred from the Centers for Disease Control and Prevention (CDC) to AAHL. Virus was then propagated in a single passage and titrated by median tissue culture infective dose ($TCID_{50}$) in Vero cells. In each study, inoculum (10^6 $TCID_{50}$ of REBOV) was administered to 4 pigs by drops into the nostrils and throat and to the remaining 4 by single subcutaneous injection over the lateral thorax.

Housing, Monitoring, and Sample Collection

After challenge, pigs were inspected daily for signs of disease.

Study 1: Pigs were anesthetized on days 2, 4, 6, 8, 10, and 13 after exposure to REBOV and sampled for shed virus by deep nasal, throat, and rectal swabs, and blood was taken to monitor for viremia and antibody production. Urine was also collected from the floor of the pens to monitor for the presence of shed virus. Duplicate swabs were collected, with one placed in MagMax extraction buffer (Ambion) for RNA extraction and the other in phosphate-buffered saline containing 1% bovine serum albumin, 100 U penicillin G, and 100 μg streptomycin sulfate.

This sample was stored at −80°C until processing for virus isolation. Blood samples were also collected on days 15, 17, 20, and day 28 when pigs were euthanized and tissues collected for real-time PCR analysis and virus isolation. At sampling times pigs were weighed and rectal temperatures recorded.

Study 2: Based on timelines determined from the outcome of study 1, pigs were similarly exposed to 10^6 $TCID_{50}$ of REBOV and 2 animals from each group were euthanized on days 6 and 8 following either oronasal or subcutaneous challenge. At necropsy, an extensive range of tissues, swabs, and blood were collected for real-time PCR and virus isolation (including 10 sites in the lung reflecting proximal and distal areas of each lobe) and histology.

Real-time PCR and Virus Isolation

To detect the presence of virus in samples, collected RNA was extracted using the MagMAX-96 viral RNA isolation kit (Ambion). TaqMan real-time reverse-transcription polymerase chain reaction (RT-PCR) was carried out using the AgPath-ID 1-step RT-PCR kit (Applied Biosystems), targeting the nucleoprotein (NP) gene of REBOV according to a method supplied by the CDC. Positive results were defined by a cycle threshold value of ≤37. Virus isolation from a selection of specimens positive in PCR was attempted by inoculation of Vero cells with homogenized tissues. Isolated virus was detected by staining cell cultures with rabbit polyclonal sera generated against the REBOV NP protein at 7 days after infection. All samples were blind-passaged once to detect low levels of isolated virus.

Serology

Serology for detection of antibodies to REBOV was conducted using an in-house-developed indirect enzyme-linked immunosorbent assay (ELISA) based on a recombinant *Escherichia coli*–expressed NP protein of REBOV, with antibody binding on serial dilutions of sera detected using an antiporcine IgG-horseradish peroxidase conjugate (USBiologicals). ELISAs were developed using 3,3',5,5'- tetramethylbenzidine and read at a wavelength of 450 nm. Cutoff values for positive samples were set at mean +3 standard deviations of the prebleed sera.

Pathology and Immunohistology

Specimens fixed in 10% neutral buffered formalin were routinely processed, cut, and stained with hematoxylin and eosin. Immunohistochemistry was performed using a high-titer rabbit polyclonal anti-REBOV nucleoprotein antiserum and the Dako EnVision FLEX detection system.

RESULTS

Animals

All animals in each study remained clinically well following exposure to REBOV. Specifically, fever, signs of respiratory disease, and skin lesions were not identified.

Real-time PCR and Virus Isolation

In study 1, REBOV genome was detected in nasopharyngeal secretions of pigs from 2 to 8 days following exposure by the oronasal route (Table 1), with virus reisolated from days 4 to 8 after infection. Genome or virus was not found in rectal swabs, urine, or blood of these animals at any time. Tissue samples collected at euthanasia on day 28 after infection were negative by real-time PCR for REBOV.

In Study 2, REBOV genome was found in nasopharyngeal secretions of 7 of 8 pigs on days 6 and 8, as well as rectal swabs (5 of 8 pigs), blood (7 of 8 pigs), and most other organ and tissue samples including skeletal muscle (Figure 1). In lung, the highest levels of genome were generally associated with regions of pneumonia. Virus was regularly reisolated from superficial (submandibular, axillary, inguinal) and internal (bronchial, mesenteric) lymph nodes, nasal turbinates, muscle, and lung of pigs challenged both by subcutaneous and oronasal routes.

Serology

In study 1, all animals challenged by either oronasal or subcutaneous routes showed seroconversion to REBOV by day 10 after infection, with generally higher titers in animals exposed by the subcutaneous route (Table 2). In study 2, antibody was detected in all pigs, with an increase in antibody at day 8 compared with day 6.

Pathology and Immunohistology

No gross or histologic abnormalities were detected in animals from study 1 that were euthanized 28 days after infection.

In study 2, gross abnormalities were confined to lymphoid and respiratory systems (Table 3). In 7 pigs there was lymphadenomegaly affecting submandibular, retropharyngeal, and bronchial lymph nodes. Consolidation of lung lobes was observed in 4 animals including 3 pigs exposed to REBOV by a parenteral route and 1 that had received nonparenteral exposure. Areas of grossly affected lung included the hilus (1 pig), left apical and left cardiac

Table 1. Detection of Shed REBOV in Nasal and Tonsil Swabs Following Experimental Challenge of Pigs

		Days after challenge						
Challenge route	Pig	0	2	4	6	8	10	13
Oronasal	1	…	N T	N^+	N^+ T^+	N^+	…	…
	2	…	…	N T	N^{++} T^{++}	N^+	…	…
	3	…	…	N^+	N^{+++} T^+	…	…	…
	4	…	N^+	N^{++}	N^{+++}	…	…	…
Subcutaneous	5	…	…	…	…	…	…	…
	6	…	…	…	…	…	…	…
	7	…	…	…	…	…	…	…
	8	…	…	…	…	…	…	…

NOTE. Source of swab material is nasal (N) or tonsil (T); polymerase chain reaction positives are indicated by the inclusion of N or T. Isolation of virus is indicated by "+" with increasing numbers of + indicating a higher virus load. REBOV, *Reston ebolavirus*.

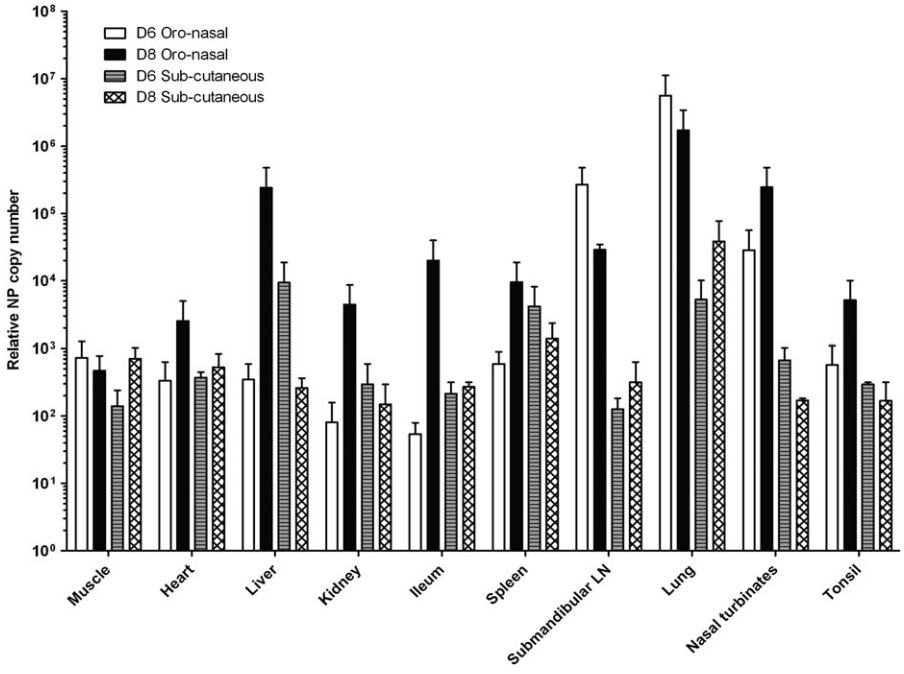

Figure 1. Real-time reverse-transcription polymerase chain reaction testing of pig tissues for the presence of REBOV nucleoprotein (NP) sequence. Two pigs at each time point were euthanized and tissues collected. Results are relative NP copy numbers with error bars indicating standard error. LN, lymph node.

lobes (1 pig), hilus and left apical lung lobe (1 pig), and left apical lobe (1 pig). Histopathology confirmed reactive hyperplasis of enlarged lymph nodes and acute bronchopneumonia in grossly abnormal lung. REBOV NP antigen was identified in lymphoid tissues (Figure 2) of all 4 pigs receiving REBOV by subcutaneous injection as well as the pig exposed by the oronasal route that had bronchopneumonia. REBOV antigen was detected in the lung of 3 of the animals with pulmonary consolidation (Figure 2), where it was associated primarily with alveolar macrophages and also bronchoalveolar epithelial cells. Although REBOV antigen was detected throughout the lung tissue of these animals including areas distant from pneumonic lesions, the most striking immunopositivity colocalized with the numerous alveolar macrophages in areas of bronchopneumonia. Mild acute rhinitis was identified in all pigs, but REBOV antigen was only detected in submucosal lymphoid tissue—not the respiratory epithelium—of the 3 pigs with parenteral exposure to REBOV that also had pulmonary consolidation. Focal necrosis of tonsillar epithelium associated with neutrophil infiltrates was seen in all pigs; this was not associated with REBOV antigen. No histological abnormalities were detected in the spleen, liver, kidney, heart, intestine, or brain of any pig although REBOV antigen was detected in the spleen of a pig exposed by the subcutaneous route.

DISCUSSION

The experimental infection with REBOV of domestic pigs described in this paper demonstrates the potential of REBOV to replicate subclinically in pigs, with infection established by both parenteral and nonparenteral routes. This subclinical infection

Table 2. ELISA Immunoglobulin G Titers Against Recombinant REBOV Nucleoprotein Antigen

		Days after challenge										
Challenge route	Pig	0	2	4	6	8	10	12	15	17	20	28
Oronasal	1	<100	<100	<100	<100	400	800	800	800	3200	1600	3200
	2	<100	<100	<100	<100	<100	200	800	800	400	100	100
	3	<100	<100	<100	<100	200	100	100	800	400	100	400
	4	<100	<100	<100	<100	<100	200	200	200	400	<100	100
Subcutaneous	5	<100	<100	<100	<100	200	3200	3200	3200	3200	3200	NT
	6	<100	<100	<100	<100	800	12 800	12 800	12 800	12 800	6400	NT
	7	<100	<100	<100	<100	800	3200	3200	3200	1600	1600	NT
	8	<100	<100	<100	100	800	800	1600	800	1600	1600	NT

NOTE. ELISA, enzyme-linked immunosorbent assay; NT, not tested; REBOV, *Reston ebolavirus*.

Table 3. Study 2—Summary of Gross and Histopathological Findings in Pigs Exposed to REBOV

		Lesions/antigen									
Days after exposure	Challenge route	Lymph node	Lung	Nasal turbinates	Spleen	Liver	Kidney	Heart	Tonsil	Intestine	Brain
6	Subcutaneous	+/+	+/+	+/+[a]	−/−	−/−	−/−	−/−	−/−	−/−	−/−
6	Subcutaneous	+/+	+/−	+/+[a]	−/−	−/−	−/−	−/−	−/−	−/−	−/−
8	Subcutaneous	+/+	−/−	+/−	−/−	−/−	−/−	−/−	−/−	−/−	−/−
8	Subcutaneous	+/+	+/+	+/+[a]	−/+	−/−	−/−	−/−	−/−	−/−	−/−
6	Oronasal	+/+	−/−	+/−	−/−	−/−	−/−	−/−	−/−	−/−	−/−
6	Oronasal	+/−	−/−	+/−	−/−	−/−	−/−	−/−	−/−	−/−	−/−
8	Oronasal	+/−	−/−	+/−	−/−	−/−	−/−	ND	−/−	−/−	−/−
8	Oronasal	+/−	+/+	+/−	−/−	−/−	−/−	−/−	−/−	−/−	−/−

NOTE. ND, Not done; REBOV, *Reston ebolavirus*.

[a] Antigen detected in submucosal lymphoid tissue.

in pigs highlights the ongoing need to understand the infection process to better assess the risks posed to humans.

Following exposure of 5-week-old pigs, REBOV replication was confirmed in many tissues. The highest levels of virus replication were observed in lung and lymphoid tissue, the tissues from which virus was isolated in the original disease investigation [17]. Virus isolation was also performed on muscle tissue with infectious virus being isolated from 6 of the 8 pigs in the second study. The infection profiles were consistent with an acute infection, with virus clearance associated with seroconversion occurring within 10–12 days after challenge. No evidence was found indicating virus persistence in any tissue, as demonstrated by negative real-time PCR tests on tissues collected at necropsy on day 28. Shedding of virus was identified most consistently via the nasopharynx, suggesting that this might provide a route of transmission by contact (aerosol or droplet) from pig to pig, which may be facilitated if concurrent respiratory disease is present. Shedding via the fecal route was observed in some circumstances and this may provide additional sources of contamination or transmission. As virus was also observed in blood from 1 study, there is also the potential for spread of infection by contaminated vaccine needles, blood collection apparatus, or insects. Risks to handlers and abattoir workers by droplet/aerosol exposure and penetrating lesions of the skin are also present and are a potential source of introduction of virus to the human population.

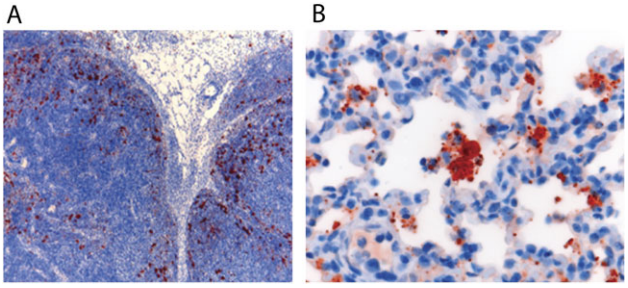

Figure 2. Immunohistochemical staining of REBOV NP antigen in tissues from experimentally infected pigs. (*A*) Lymph node of pig following oronasal challenge. (*B*) Lung of pig with dense viral antigen staining (red) of alveolar macrophages.

Differences with respect to virus shedding and blood-borne virus were observed in the 2 studies. Shedding was not documented following parenteral exposure in study 1, but occurred in study 2. The reasons for this observation are as yet unknown; however, it is of interest that the first experimental infection was undertaken in the spring and study 2 was carried out in the winter. It is possible, although clearly unproven, that a higher prevalence of subclinical respiratory infections in the winter may have predisposed this cohort to a more florid infection with REBOV. Although the Australian pig herd is free of PRRS, and pigs used in these experiments were negative for PCV-2, other respiratory pathogens are present in commercial herds. In study 2, grossly pneumonic lesions were observed in lungs of some pigs. Virus was reisolated on occasion from all areas of lung that were sampled including grossly normal tissue although antigen was only detected in the lung of pigs with pneumonia; at present the cause and/or effect relationship between pneumonia and REBOV is unclear. One possibility for future consideration is that preexisting subclinical respiratory infections caused by organisms such as *Mycoplasma* may predispose animals to enhanced REBOV replication as increased populations of alveolar macrophages may support higher levels of virus replication, Unfortunately, lack of capability for identification of nonviral pathogens at BSL4 currently precludes detailed examination of such interactions. In any event, it is recommended that in future studies aimed at establishing coinfection models—such as PRRS combined with REBOV—careful attention is paid to cohort selection with respect to time in the study design. Those pigs with preexisting subclinical respiratory infections may represent an increased transmission risk to other susceptible pigs, other animals, and people. The legacies of pneumonia, REBOV antigen, or genome, were not observed in lung tissue from any pig from study 1, suggesting that any virus-induced pulmonary disease occurring in these animals during the acute phase of

infection had been mild, and this is consistent with the generally less florid infection seen in this group. Had pigs in study 1 developed pulmonary pathology similar to that in pigs from study 2, residual pathology would have been expected to be identifiable when they underwent postmortem examination 28 days after challenge.

Interestingly, in the original disease outbreak investigation, REBOV was only observed in pigs coinfected with PRRSV [17]. This supports the theory that preexisting respiratory disease allows for an increase in virus replication in lung tissue in infected pigs. Further experiments are planned to examine the relationship of REBOV, PCV2, and PRRSV in terms of increase in systemic spread in REBOV and increase in shedding. It is possible that this relationship may be very complex and the mechanism for increased viral loads may not be able to be elucidated with ease in the pig infection model.

The evidence of virus shedding and replication of virus in internal organs in the absence of clinical disease represents a potential source of infection to farm, veterinary, and abattoir workers. This appears to be an unprecedented emergence of filovirus infection in a new host that may have important biosecurity implications for both livestock health and emergence in the human food chain. Although REBOV has not been seen to result in any human disease, the basis for this observed attenuation remains unknown. The consequence of REBOV becoming pathogenic in humans is serious, and ongoing undetected infections and replication in pigs and other animals with REBOV may result in the emergence of viruses that are more pathogenic in humans and/or livestock.

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