Ebola (Subtype Reston) Virus among Quarantined Nonhuman Primates Recently Imported from the Philippines to the United States


In April 1996, laboratory testing of imported nonhuman primates (as mandated by quarantine regulations) identified 2 cynomolgus macaques (Macaca fascicularis) infected with Ebola (subtype Reston) virus in a US-registered quarantine facility. The animals were part of a shipment of 100 nonhuman primates recently imported from the Philippines. Two additional infected animals, who were thought to be in the incubation phase, were identified among the remaining 48 animals in the affected quarantine room. The other 50 macaques, who had been held in a separate isolation room, remained asymptomatic, and none of these animals seroconverted during an extended quarantine period. Due to the rigorous routine safety precautions, the facility personnel had no unprotected exposures and remained asymptomatic, and no one seroconverted. The mandatory quarantine and laboratory testing requirements, put in place after the original Reston outbreak in 1989–1990, were effective for detecting and containing Ebola virus infection in newly imported nonhuman primates and minimizing potential human transmission.

Since identified, filoviruses have been responsible for both large epidemics (Marburg [MBG] virus in 1967 and Ebola [EBO] virus in 1976, 1979, 1995, and 1996) and isolated human cases with occasional secondary infections (MBG virus in 1975, 1980, and 1987 and EBO virus in 1977 and 1984) [1–4]. In most of these episodes, the source of infection for the index case has remained unknown. Despite some progress in the diagnostic evaluation, molecular virology, and epidemiology of filoviruses and in the medical management of filovirus-infected patients, the natural animal or plant reservoir of MBG and EBO viruses remains elusive.

African nonhuman primates were the source of the original MBG epidemic in 1967 [5] and of two recent EBO episodes in Côte d'Ivoire and Gabon [3, 6]. In 1989 and 1990, epizootics of EBO hemorrhagic fever in cynomolgus monkeys (Macaca fascicularis imported from the Philippines) occurred at several registered quarantine facilities in the United States and led to the description of a new member of the family Filoviridae, EBO (subtype Reston; EBO-R) virus [7, 8]. Although very pathogenic for nonhuman primates, this strain was not responsible for any disease in humans in the United States or Philippines, even though seroconversions were documented in several persons in both countries [9, 10]. In the Philippines, an epidemiologic investigation documented active transmission in the export facility that was the source of several shipments of infected primates to the United States [10]. The original source of EBO-R for the export facility remains unknown. In 1992, the same facility exported infected animals to Italy [11] despite reported depopulation measures taken in 1990.

On 30 March 1996, a cynomolgus monkey that had been imported from the Philippines and held in a licensed commercial quarantine facility in Texas died after a 3-day illness characterized by anorexia and lethargy. On 11 April, EBO virus infection was confirmed by antigen-detection testing (as required by federal regulation [12, 13]) of a liver specimen obtained from the animal, and EBO particles were seen by electron microscopy in plasma from the animal. On 8 April, a second primate housed in the same room had similar signs of illness and was euthanatized on 13 April after EBO antigen and particles were detected in the blood of the first animal. The 2 primates were part of a shipment of 100 cynomolgus monkeys from the same Philippine facility that exported the EBO-infected animals in 1989 and 1990. The 1996 shipment had been held in 2 cohorts of 50 animals each in separate isolation rooms since its arrival on 21 March 1996. At the invitation of the Texas Department of Health and the operators of the facility, a collaborative epidemiologic investigation was initiated on 15 April by the Centers for Disease Control and Prevention (CDC). The remaining 48 primates housed in the quarantine room were euthanatized on 17 April to prevent additional transmission within the room and to minimize potential exposure of employees.
Herein, we describe the laboratory investigation of the euthanatized and remaining primates, a serologic follow-up of the personnel at the quarantine facility, and the effectiveness of the quarantine regulations and requirements. During the laboratory investigation, as in 1989 and 1990, simian hemorrhagic fever virus was also isolated from some of the animals tested; the results concerning this virus will be reported elsewhere (Jahr-PB, unpublished data).

Materials and Methods

Study site. Upon arrival in the United States on 21 March 1996, 100 cynomolgus monkeys were housed 4 feet apart in parallel banks of individual stainless steel squeeze cages in two independent quarantine rooms (rooms 7 and 8; 50 animals per room). Each room had separate anteroom entrances and separate exits and independent air-handling systems with HEPA exhaust filtration systems. Standard operating procedures for newly imported animals had been maintained. Each room was considered as a single unit, and employees were required to wear separate personal protection equipment (gown, dust mist respirator, gloves, face shield) to enter each room. Separate and new needles and syringes were used for each animal and each procedure. The day after arriving, each animal was clinically examined and ear-tagged for further identification. The first of 3 tuberculin skin tests was done. A blood sample was obtained, and sera were separated and stored at −20°C.

When the animals in room 8 were euthanatized, personnel who obtained blood samples from or who did partial necropsies on the animals were built-face respirator masks (Positive Air Purifying Respirator; RACAL Health and Safety, Frederick, MD) with HEPA-filtered air input in addition to the previously described protective equipment.

During the course of the investigation, clinical and therapeutic records were analyzed, and safety and disinfection procedures were reviewed and reinforced.

Sample collection. Hereafter, the animals will be referred to by room and cage number (e.g., the animal in cage 1 in room 7 will be referred to as 7-1). Tissues (lung, heart, liver, kidney, gastrointestinal tract) and serum from the first sick animal (no. 8-2; died on 30 March) and serum from a subsequent acutely ill animal (no. 8-44) were sent, according to CDC quarantine requirements, to the Microbiological Associates, Inc. laboratory for EBO antigen–detection testing by ELISA.

On 15 April, a few animals in both rooms were anorexic; therefore, blood was obtained for testing. On 17 April, all animals in room 8 were euthanatized to avoid further transmission of the virus. Individually, the animals were anesthetized with Ketamine and samples were taken from each. Blood was obtained by intracardiac puncture. After an abdominal incision, a small piece of liver was collected, and portions were placed on dry ice and in formalin. A piece of skin was also collected from the nape of the neck and placed in formalin. Testicles were also collected from all animals and put in formalin. The animals were then humanely euthanatized by American Veterinary Medical Association–approved methods. Carcasses and potentially contaminated waste were double-bagged and disinfected with detergent solutions before being removed from the room for incineration. Specimens were then packed and sent to CDC for further processing. Packaging was done in accordance with the regulations, using infectious materials safety shippers (International Air Traffic Association and US Department of Transportation approved), and included dry ice for the sera and tissues. Formalin-fixed tissues were shipped at room temperature using the same packaging material. Animals from room 7 remained under clinical surveillance, and blood samples were obtained at multiple intervals.

Human surveillance. All personnel who were exposed to this nonhuman primate shipment, whether involved in the transportation or care and feeding, were monitored for at least 31 days after the animals in room 8 were euthanatized. Clinical symptoms, if any, and daily temperatures were recorded on a surveillance form and checked by the facility supervisor. Blood samples were obtained at the end of the surveillance period and tested by ELISA for antibody and antigen.

Serologic and virologic assays. Nonhuman primate blood samples and liver tissues were processed in a biosafety level (BSL) 4 laboratory. Aliquots of blood and 10% liver suspensions were inactivated by γ-irradiation (50,000 Gy [5 × 106 rads]) for further processing in BSL-2 facilities. A sandwich ELISA, using a mixture of mouse monoclonal antibodies for antigen capture and polyclonal hyperimmune rabbit anti-EBO virus serum for antigen detection [14], was performed on sequential dilutions of blood and liver suspensions.

IgM and IgG ELISAs. IgM antibodies were detected by capturing IgM from serum with goat anti-human μ (Tago, Burlingame, CA) adsorbed to 96-well microtiter plates and then by allowing the captured IgM to react with viral antigen (crude suspension of medium and EBO-R–infected Vero E6 cells, inactivated with 50,000 Gy, freeze-thawed twice, and sonicated), and then measuring bound antigen by the use of a hyperimmune rabbit serum and an appropriate enzyme conjugate and substrate. Each serum was tested in parallel with uninfected control antigen (uninfected cells suspension).

IgG ELISAs were performed by coating the polyclonal chloride microtiter plates overnight at 4°C with a basic buffer detergent extract of uninfected and EBO-R–infected Vero E6 cells that were further inactivated by 50,000 Gy. Sera were diluted 1:100 and 4-fold through 1:6400 in 5% nonfat milk in PBS-Tween and allowed to react with the coating antigen. Bound IgG was detected with goat anti-human IgG conjugated to horseradish peroxidase. ABTS (Kirkegaard & Perry, Gaithersburg, MD) was used as substrate. For both IgG and IgM assays, optical densities at 410 nm (OD410) were recorded, and the OD410 of the negative antigen-coated well (IgG) or the negative antigen step (IgM) was subtracted from its corresponding positive antigen well to yield the adjusted OD410.

Virus isolation. Virus isolation attempts were done under BSL-4 containment in 25-cm² flasks of Vero E6 cells by absorption with 100 μL of either blood or 10% liver suspensions for 1 h at 37°C. Additional medium (Eagle MEM with Earle’s balanced salts and 5% heat-inactivated fetal bovine serum) was then added. Medium was changed after 5–7 days. Cells were observed for cytopathic effect for 2 weeks and blind passaged for another 2 weeks. All cell cultures were tested after each passage for viral antigen by indirect immunofluorescent staining with anti-EBO hyperimmune rabbit serum and fluorescein isothiocyanate–conjugated anti-rabbit made in goat.
**Polymerase chain reaction (PCR) assay.** A sensitive reverse transcriptase–PCR (RT-PCR) assay was developed to detect virus RNA in primate specimens. The assay targeted nucleoprotein gene sequences; the methods for isolating RNA and performing the RT-PCR assay are detailed elsewhere in this supplement [15]. The EBO-R primers are described in table 1 of [15].

**Immunohistochemistry.** Formalin-fixed tissues from 49 animals were routinely processed and embedded in paraffin. In all cases, routine hematoxylin-eosin–stained sections were reviewed. Immunopathologic evaluation for evidence of EBO virus infection was performed by using previously established immunohistochemical procedures [16]. Briefly, 4-μm tissue sections were predigested with proteinase-K and then incubated with a polyclonal hyperimmune rabbit anti-EBO virus serum. This step was followed by serial application of biotinylated swine anti-rabbit link antibody and an alkaline phosphatase–streptavidin conjugate (Dako, Carpenteria, CA). EBO virus antigens were then detected by using a naphthol–fast red substrate. Control tissues consisted of paraffin-embedded pellets of normal human tissues mixed with either noninfected Vero E6 or Vero E6 infected with the virus strain prototypes EBO-Z and EBO-R. Additional controls included EBO-infected and noninfected autopsy or necropsy tissues. The specificity of immunostaining was confirmed in all instances by replacing hyperimmune anti-EBO virus serum with nonimmune or different immune rabbit serum.

**Results**

**Antibody and antigen detection, virus isolation, and PCR.** Sera that were obtained from the 100 animals 1 day after their arrival (22 March) were all negative by antigen-capture, IgG, and IgM-capture ELISA (table 1). Virus isolation was attempted on the initial serum samples obtained from the animals who were subsequently found positive, but all were negative.

None of the 50 animals in room 8 (8-1 through 8-50) developed EBO-specific IgG or IgM. Animal 8-2 died on 30 March and was positive for filovirus antigen by ELISA and viral RNA by PCR in the liver and the serum; virus was isolated from the liver but not from the serum (the serum specimen had been heat-inactivated at 60°C for 1 h). Virus was isolated from the blood of animal 8-44 (euthanatized on 13 April), and virus antigen and RNA were detected. When the remainder of the animals in the room were euthanatized on 17 April, blood and liver specimens of all but 2 animals were negative by antigen detection, virus isolation, and PCR assays. The liver of animal 8-45 was positive for EBO virus by antigen detection, PCR, and virus isolation, whereas the blood was positive only by virus isolation. Animal 8-50 was found positive only by virus isolation from the blood.

All animals in room 7 (7-1 through 7-50) remained alive during the extended quarantine period. All animals were tested for IgG, IgM, and antigen, and were found negative on 23 April (32 days after their arrival) and 8 May (47 days after their arrival). During this period, a few animals were tested again when they appeared ill (7-5, 7-21, 7-29 to 7-31, and 7-45 to 7-48); all were negative (ELISA antigen, IgG and IgM, virus isolation).

**Human surveillance.** Ten persons handled the animals, the shipping crates, or the potentially contaminated material from this shipment. In-place disease control protocols and personal protective equipment were used in every case so that no persons had any unprotected direct contact. No one developed suspicious clinical symptoms or fever. No antibody or antigen was detected in the blood samples collected at the end of the surveillance period.

**RT-PCR identification of the EBO virus strain.** In the liver specimen from 8-2 and the blood from 8-44, a 337-bp sequence was amplified, detected, and sequenced. The virus was considered to be closely related or identical to the EBO-R virus. Furthermore, the complete glycoprotein gene sequence of this 1996 EBO virus has 98.9% nucleotide identity with the original 1989 EBO-R virus (Sanchez et al., this issue [15]).

**Immunohistochemistry.** Lung, kidney, heart, and gastrointestinal tract specimens from the first EBO virus–positive animal (8-2) were available for immunohistochemistry and were found to be positive for EBO. Extensive amounts of viral antigens were seen in these tissues, localized primarily within mononuclear phagocytic and endothelial cells (figure 1A, B).

All but 2 liver specimens from the 48 remaining animals in the affected room were negative for EBO by immunohistochemistry. Animals 8-45 and 8-50 both showed focal immunostaining of Kupffer’s cells (figure 1C). Skin specimens were examined to evaluate the skin-biopsy technique in primates, following the recent information on the presence of EBO antigen in skin tissues of a fatal human case [16], but they were negative for all 48 animals, including those found positive by other laboratory tests. Testicular tissues from all 48 animals were also negative for EBO.

**Discussion**

As shown during the 1989–1990 Reston outbreak and more recently during the 1995 human EBO epidemic in the Democratic Republic of the Congo, the only accurate diagnostic tests for acute EBO disease are the detection of antigen, virus, or part of the viral genome in the blood or tissues of dead or dying subjects. All three methods have positive aspects and should be used in combination. The ELISA is a quick, easy, and very robust diagnostic test and is particularly adapted for the processing of a large number of specimens. The isolation of virus requires a minimum of 7–14 days and a BSL-4 laboratory, but it offers an opportunity for further study of the isolated virus. The amplification of EBO RNA directly from the tissues or blood can be done in a few hours by a well-equipped and specialized laboratory and provides genetic information useful for epidemiologic and phylogenetic analyses. In this case, the sequence of the glycoprotein gene was shown to be nearly identical to the original 1989 EBO-R virus. Specimens for
Table 1. Results of Ebola laboratory testing of samples from a shipment of 100 imported nonhuman primates housed in a US-registered quarantine facility, April 1996.

<table>
<thead>
<tr>
<th>Room no.</th>
<th>Month/day</th>
<th>Animal no.</th>
<th>Sample</th>
<th>Ag</th>
<th>IgM</th>
<th>IgG</th>
<th>Isolate</th>
<th>PCR</th>
<th>IHC</th>
</tr>
</thead>
<tbody>
<tr>
<td>7</td>
<td>3/22</td>
<td>7-1 to 7-50</td>
<td>Serum</td>
<td>–</td>
<td>–</td>
<td>–</td>
<td>ND</td>
<td>ND</td>
<td>NS</td>
</tr>
<tr>
<td>4/15</td>
<td>7-45 to 7-48</td>
<td>Serum</td>
<td>–</td>
<td>–</td>
<td>–</td>
<td>–</td>
<td>ND</td>
<td>ND</td>
<td>NS</td>
</tr>
<tr>
<td>4/17</td>
<td>7-5, 7-21, 7-29 to 7-31, 7-44 to 7-48</td>
<td>Serum</td>
<td>–</td>
<td>–</td>
<td>–</td>
<td>–</td>
<td>ND</td>
<td>ND</td>
<td>NS</td>
</tr>
<tr>
<td>4/23</td>
<td>7-1 to 7-50</td>
<td>Serum</td>
<td>–</td>
<td>–</td>
<td>–</td>
<td>–</td>
<td>ND</td>
<td>ND</td>
<td>NS</td>
</tr>
<tr>
<td>5/8</td>
<td>7-1 to 7-50</td>
<td>Serum</td>
<td>–</td>
<td>–</td>
<td>–</td>
<td>–</td>
<td>ND</td>
<td>ND</td>
<td>NS</td>
</tr>
<tr>
<td>8</td>
<td>3/22</td>
<td>8-1 to 8-50</td>
<td>Serum</td>
<td>–</td>
<td>–</td>
<td>–</td>
<td>ND</td>
<td>ND</td>
<td>NS</td>
</tr>
<tr>
<td>3/30</td>
<td>8-2</td>
<td>Serum</td>
<td>+</td>
<td>NS</td>
<td>NS</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>NS</td>
</tr>
<tr>
<td>4/11</td>
<td>8-44</td>
<td>Serum</td>
<td>+</td>
<td>–</td>
<td>–</td>
<td>+</td>
<td>–</td>
<td>–</td>
<td>NS</td>
</tr>
<tr>
<td>4/15</td>
<td>8-17 to 8-19, 8-49, 8-50</td>
<td>Serum</td>
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<td>–</td>
<td>–</td>
<td>–</td>
<td>–</td>
<td>–</td>
<td>NS</td>
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<td>–</td>
<td>–</td>
<td>–</td>
<td>–</td>
<td>–</td>
<td>–</td>
<td>NS</td>
</tr>
<tr>
<td>5/8</td>
<td>8-50</td>
<td>Serum</td>
<td>–</td>
<td>–</td>
<td>–</td>
<td>–</td>
<td>–</td>
<td>–</td>
<td>NS</td>
</tr>
<tr>
<td>8-1, 8-3 to 8-43, 8-46 to 8-49</td>
<td>Serum</td>
<td>–</td>
<td>–</td>
<td>–</td>
<td>–</td>
<td>–</td>
<td>–</td>
<td>NS</td>
<td></td>
</tr>
</tbody>
</table>

NOTE. Ag = ELISA antigen detection, IgM = IgM capture ELISA, IgG = IgG ELISA, Isolate = isolation on Vero cells, PCR = genome detection by polymerase chain reaction, IHC = Ebola virus–specific immunohistochemistry test; ND = not done, NS = test not suitable on tissue or serum.

* IHC was also positive on heart, kidney, lung, and gastrointestinal tract of this animal.

Serum was heat inactivated.

immunohistochemistry are easy to collect, formalin fixation renders them safe for transport, and the results are important for understanding the pathogenesis of the disease. The immunohistochemistry data obtained in this study showed that the skin-biopsy test cannot be used to assess animals early in the incubation phase and should be reserved for use on dead or dying animals.

The adequacy of the current quarantine procedures are well illustrated by this outbreak. The fact that none of the sera collected on arrival were positive shows that testing blood samples of animals before exportation or immediately on arrival in the United States is unlikely to detect asymptomatic EBO-infected animals. The nonspecific signs (e.g., fever, anorexia, lethargy) observed in the first 2 EBO virus–infected animals of this outbreak were identical to the signs observed during the 1989–1990 outbreak [7, 10, 17, 18]. This strongly supports both the need to monitor imported nonhuman primates for illness during the mandatory 31-day quarantine and the need to test animals that die or become ill with such signs during quarantine.

The apparent transmission of EBO virus between the animals within the room (figure 2) raises several questions. The first animal (8-2), which was probably in the incubation phase during the trip and the initial days of the quarantine, became symptomatic on 27 March 1996 (6 days after arriving) and died on 30 March. The second animal (8-44) became symptomatic on 8 April, 19 days after arriving and 9 days after the death of the first animal. These 2 animals were not housed close to each other (figure 2), and no instruments were shared between them, thus eliminating an obvious possible mode of transmission. If infection occurred prior to arrival, the 19-day incubation period for the second animal is certainly close to the limit, but there are few experimental data on incubation times following a low-dose inoculation with EBO-R.

These animals also differed in the duration of disease; the first one died after a 3-day illness, while the second was euthanatized after a 5-day illness (on confirmation of EBO virus disease in the first animal). Animal 8-44 was euthanatized on 13 April, and an asymptomatic adjacent animal (8-45) was determined to be positive on necropsy on 17 April. Direct contact between both animals or between infected-animal excreta and the naive animal was possible and could explain this transmission. Fifteen days separated the death of the first animal and illness in the last EBO-positive animal found at necropsy (8-50). These 2 animals were housed opposite each other and had no direct contact that might have led to disease transmission. Other possible modes of transmission between noncontiguous cages are possible: projection of excreta at the bottom of the cages; carry-over of infectious secretions on animal caretakers’ gloves that were not changed between procedures, such as tube feedings; and remaining infectious secretions on the nasogastric tubes despite disinfection.

Due to the successive exportations of EBO virus–infected animals into the United States and Italy and the concerns
Figure 1. Immunostaining of Ebola viral antigens in various organs of cynomolgus monkeys as determined by immunohistochemistry. A, Lung of monkey 8-2. Viral antigens are seen predominately within mononuclear phagocytic cells and pulmonary microvasculature. Original magnification, ×158. B, Intestine of monkey 8-2. Note extensive necrosis and abundant extracellular viral antigens in lamina propria. Original magnification, ×100. C, Liver of monkey 8-45. Viral antigens are seen in cytoplasm of several Kupffer’s cells. Naphthol–fast red substrate with light hematoxylin counterstain. Original magnification, ×158.

Figure 2. Animal distribution in quarantine room (room 8) with Ebola-infected animal and time line of infection. No. indicated on some cages corresponds to cage and animal nos. in room. D = death, E = euthanatized, and shaded areas = clinical phase of the disease.
of the research community, animal rights groups, and the regulatory agencies, the regulations related to the exportation of nonhuman primates from the Philippines were changed. For several years, exporters in most countries have been allowed to sell only purpose-bred animals, not wild-caught animals. However, wild-caught animals continued to be used as breeding stock. The same Philippine exporter was the supplier of the primates involved in the 1989–1990 Reston outbreak and the Italian EBO episode in 1992 [1, 11]. In May 1996, an epidemiologic investigation at the export facility in the Philippines revealed ongoing transmission among the animals inside the facility (Miranda et al., this issue [19]). In response to the US epizootics in 1989 and 1990, additional disease control measures were required by CDC for importation of nonhuman primates [13], and modifications have been made as recently as 25 March 1996 (table 2). The number of registered importers has been reduced, and a special import permit is required for rhesus, cynomolgus, and African green monkey shipments.

The 1996 Texas episode demonstrated that the current requirements were appropriately designed and effective: (1) Isolation and appropriate quarantine of recently imported animals prevented further dissemination of disease, (2) use of personal protective equipment by animal handlers prevented unprotected contact, (3) diagnostic testing of ill animals coupled with an effective laboratory testing program provided an early warning of EBO virus disease in the shipment, and (4) close coordination occurred among the importer, the diagnostic laboratory, the Texas Department of Health, and CDC. Following the euthanasia of all animals in room 8, 2 additional EBO-positive animals were found among the still asymptomatic animals, providing evidence that after EBO is introduced into an animal room, spread of virus to other animals is likely unavoidable; thus, strict depopulation measures are justified. In addition, the strict application of facility and personal isolation procedures between quarantine rooms stopped the spread of the virus and spared other animals in the facility, including the second cohort of 50 animals from the same shipment. Inclusion of other infectious agents, such as MBG virus, in the diagnostic testing requirements and application of similar requirements for the importation of other nonhuman primate species are under consideration.

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References