

Ebola Virus: a Clear and Present Danger

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An epidemic of Ebola virus disease is occurring in Western Africa on a scale not seen before, particularly in the countries of Guinea, Liberia, and Sierra Leone. The continued spread is facilitated by insufficient medical facilities, poor sanitation, travel, and unsafe burial practices. Several patients diagnosed with Ebola virus disease in Africa have been evacuated to the United States for treatment, and several other patients have been diagnosed in the United States. It is important for laboratories to be aware of available tests, especially those granted emergency use authorization, as hospitals prepare protocols for the diagnosis and management of high-risk patients.

The most protracted and complicated epidemic of Ebola virus (EBOV) disease to date, and the first in western Africa, began with the report of 49 cases in Guinea on 22 March 2014 (1). Previous Ebola virus outbreaks have been confined to remote regions of Central Africa, and the largest, in Zaire (now the Democratic Republic of the Congo) in 1976, had 318 cases (2). The suspected index case in the current outbreak was believed to be a 2-year-old boy in Guinea who likely contracted the disease after exposure to an infected fruit bat and died on 6 December 2013 (1). His illness was the point source for person-to-person spread of the infection into a novel population in West Africa that was unprepared for surveillance or control. The West African countries of Guinea, Liberia, and Sierra Leone have been the most affected, and these countries continue to report increasing numbers of cases away from the epicenter, particularly in the capital cities of Monrovia, Liberia, and Freetown, Sierra Leone. Since the beginning of the outbreak, Ebola virus has infected 13,676 people (7,606 of which were laboratory confirmed) and has caused 4,910 deaths, according to a 29 October 2014 report from the World Health Organization (<http://www.cdc.gov/vhf/ebola/outbreaks/guinea/index.html>). The surge is fueled largely by insufficient medical facilities, poor sanitation, and unsafe burial practices. Traditions in parts of West Africa involve touching and washing the still-contagious bodies before burial, putting family, friends, and community members at risk.

Exact numbers of cases are difficult to determine, and the magnitude of the outbreak is thought to be significantly underestimated, with many cases and deaths not reported to current surveillance systems because of the fear of authorities and the stigma and social rejection associated with Ebola virus disease. One case in Senegal was a traveler from Guinea, and a small number of cases in Nigeria originated with a traveler from Liberia. No new cases have been reported from Senegal and Nigeria since 29 August 2014 and 5 September 2014, respectively, largely thought to be a result of rigorous control measures.

It is important to note that the incubation period, symptoms, transmissibility, duration of illness, and death rate in the current outbreak are similar to those reported for previous outbreaks. Genetic similarity across the sequenced 2014 samples suggests a single transmission from the natural reservoir, followed by human-to-human transmission during the outbreak (1, 2).

Predictive computer models to estimate the size of the West African outbreak going forward report ominous numbers on a scale never seen before with Ebola virus. A report from the Centers

for Disease Control and Prevention (CDC) estimates that if the epidemic continues without additional interventions, there will be approximately 550,000 cases by 20 January 2015 (1.4 million, when corrected for underreporting) (3).

A number of health care workers who had become infected with Ebola virus disease in West Africa have been medically evacuated for treatment in isolation wards in their countries of origin, including 4 health care workers and a freelance cameraman, who were treated in the United States in special biocontainment units at Emory University Hospital and the University of Nebraska Medical Center beginning in August 2014 and recovered. A physician who returned after working in Guinea was treated at Bellevue Hospital Center in New York, NY, and recovered. The first peripatetic case of Ebola diagnosed in the United States was a Liberian national who arrived in the United States on 20 September 2014 to visit family members in Dallas, TX, became symptomatic 4 days later, and died. Two nurses caring for this patient became infected but were treated and recovered.

The taxonomy of ebolaviruses has been modified several times since their discovery in 1976. The five known species of the genus *Ebolavirus* have nucleotide sequences that differ by 35 to 40% and are named for the region where each was originally identified (Table 1) (4). Four of the species, Bundibugyo ebolavirus (BDBV), Sudan ebolavirus (SUDV), Tai Forest ebolavirus (originally Côte d'Ivoire ebolavirus) (TAFV), and Ebola virus (originally Zaire ebolavirus) (EBOV), cause disease in humans. Reston ebolavirus (RESTV) causes disease in nonhuman primates but is not pathogenic to humans. Ebola virus is the type species of the genus. Its name was derived from the first documented outbreak in Yambuku in Zaire (now the Democratic Republic of the Congo) which lies on the Ebola River. Of the five members of the genus, Ebola virus has the highest mortality rate and has caused the greatest number of outbreaks.

Due to the ease of human-to-human transmission, the severity

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TABLE 1 List of ebolaviruses

Group V negative-sense single-stranded RNA viruses
Order <i>Mononegavirales</i>
Family <i>Filoviridae</i>
Genus <i>Ebolavirus</i>
Species causing disease in humans:
Bundibugyo ebolavirus (BDBV)
Sudan ebolavirus (SUDV)
Taï Forest ebolavirus (originally Côte d'Ivoire ebolavirus) (TAFV)
Ebola virus (originally Zaire ebolavirus) (EBOV)
Species causing disease in nonhuman primates:
Reston ebolavirus (RESTV)

of the disease, the rapid onset of symptoms, and the lack of specific therapy, the ebolaviruses are classified as biosafety level 4 (BSL-4) viruses and are on the CDC's (5) category A list of potential agents of bioterrorism. Since they are enveloped RNA viruses, ebolaviruses are not stable and are relatively easy to inactivate. The Public Health Agency of Canada provides a pathogen safety data sheet with important information that indicates that ebolaviruses are susceptible to alcohol-based products and dilutions (dilutions of 1:10 to 1:100 for at least 10 min) of 5.25% household bleach (sodium hypochlorite) and calcium hypochlorite (bleach powder) as well as 3% acetic acid and 1% glutaraldehyde (6). The World Health Organization (WHO) recommends cleanup of blood or body fluid spills by flooding the spill area with a 1:10 dilution of 5.25% household bleach for 10 min and undiluted household bleach (7). For surfaces that may corrode or discolor, the area should be cleaned to remove visible stains and then exposed to a 1:100 dilution of 5.25% household bleach for more than 10 min. Ebolaviruses can also be inactivated by heating for 30 to 60 min at 60°C, boiling for 5 min, or exposure to gamma irradiation (1.2×10^6 rads to 1.27×10^6 rads) combined with 1% glutaraldehyde (6).

Laboratory studies done under environmental conditions that favor the persistence of virus found that Ebola virus titers on contaminated solid surfaces decrease rapidly at room temperature but that viability can persist for several days at 4°C (8). No virus could be recovered from metal surfaces at any time (8). Virus can remain viable in organic debris but is very sensitive to drying. The theoretical half-life (the time for 50% of the initial virus concentration to decay) for Ebola virus is 15 min, and the decay time for 99% of the initial virus concentration is 104 min (8). Ebola virus is able to survive in guinea pig sera and tissue culture media for over 46 days, with a more rapid decrease in viability when stored at room temperature than at 4°C (8). In patient care environments during an outbreak of Sudan Ebola virus in Uganda, virus was not detected by either nucleic acid amplification or culture from sites that were not visibly bloody (9). Virus was detected on a blood-stained glove and a bloody intravenous insertion site by nucleic acid amplification but not by culture. The expectation is that the risk of transmission of ebolaviruses from fomites is low when consistent daily cleaning and disinfection practices are applied (9).

There is no evidence of airborne transmission in natural settings. While genetic changes produce variants in every outbreak, Ebola virus is highly genetically stable compared to most RNA viruses (2). The observed mutation rate within the 2014 outbreak

TABLE 2 Laboratory tests for diagnosis of Ebola virus infection

Timeline of infection	Diagnostic tests ^a
Within a few days after symptoms begin	RT-PCR (including qPCR), virus isolation in cell culture, antigen detection by ELISA
Early convalescence or after recovery	IgM and IgG antibodies by ELISA
Postmortem retrospective diagnosis	RT-PCR or qPCR, virus isolation in cell culture, immunohistochemical staining

^a qPCR, real-time quantitative PCR; ELISA, enzyme-linked immunosorbent assay.

is roughly twice as high as between outbreaks, but there has been no evidence of significant functional changes (2). Although it is theoretically possible, no human virus has ever changed its mode of transmission, and it is extremely unlikely that Ebola virus would mutate to become airborne.

A very low infectious dose of 1 to 10 virions is sufficient to cause infection in nonhuman primates after aerosol exposure (10). The incubation period ranges from 2 to 21 days, with an average of 8 to 10 days. The incubation period may be related to the route of infection and has been reported at 6 days in cases that resulted from a needle stick injury (5). During the incubation period, the virus actively replicates, and by the time symptoms develop, the individual is considered contagious. After gaining access to the body, Ebola virus and other filoviruses can infect and replicate in a wide range of cell types. Initially infected cells include cells of the mononuclear phagocytic system, with dendritic cells being more affected than monocytes or macrophages. From there, fibroblastic reticular cells of regional lymph nodes become infected, and virions can readily enter the lymphatic system or bloodstream, with subsequent infection of fixed and mobile macrophages in the liver, spleen, and other organs and tissues. Virions released from these cells infect neighboring cell types, with a predilection for endothelial cells, hepatocytes, and mononuclear phagocytes. Viral replication is associated with disruption of the immune and vascular systems (11). Extensive focal necrosis that is most severe in the liver, spleen, lymph nodes, kidney, lung, and gonads, fluid shifts, microvascular coagulation, and interstitial hemorrhage all contribute to the development of shock and death (11). The inability of the disabled host immune system to clear the infection is a major factor responsible for the dramatic course of the disease (11).

Symptoms tend to appear suddenly and are similar to those of other infectious diseases that occur more frequently, such as malaria, typhoid fever, dysentery, or influenza. The most common early symptoms include fever, general malaise, loss of appetite, vomiting, diarrhea, headache, and abdominal pain. Specific hemorrhagic symptoms are rarely reported (in <1% to 5.7% of patients). Unexplained bleeding, however, has been reported in 18.0% of cases after the third day of illness. Seroepidemiological investigations suggest that mild or asymptomatic infections can occur (7, 12).

The laboratory tests that are useful in making a diagnosis of Ebola virus depend largely on availability and the stage at which the patient presents during the course of infection (Table 2). In fatal infections, patients usually die before developing a measurable antibody response. IgM antibody is generally detectable at about 10 to 18 days after presumed exposure, and IgG is measurable at about 1 week after the appearance of IgM (13). These find-

ings suggest that serologic tests may be useful for the diagnosis of infection in patients who survive long enough to mount an immune response or recover, but not for those who present early in the course of infection. IgM, as detected by capture immunoassay using whole-cell sonicates, disappears by 60 to 80 days after the onset of infection (14). Survivors of Ebola virus infection have persistent detectable IgG up to at least 12 years after infection (14, 15). Survivors are presumed to be immune to the virus strain with which they were infected, although the duration of immunity is not known. Serum-neutralizing activity and the ability to produce high levels of cytokines in response to stimulation with viral protein GP1-649 were also detectable after 12 years (15).

High viral titers are present in acute illness, and diagnostic tests for patients who present within a few days of development of symptoms include virus isolation in cell culture, PCR, and antigen capture enzyme-linked immunosorbent assay (ELISA) (16).

To establish a diagnosis after death, autopsy tissues can be tested for the presence of Ebola virus antigen using immunohistochemical staining or *in situ* hybridization techniques. Virus culture or PCR-based methods can be used if fresh tissue is available. Electron microscopy using conventional negative-staining procedures has also been used to identify filoviruses in tissue but is not readily available.

Isolation of Ebola virus and other hemorrhagic fever viruses in culture is a high-risk procedure and should be performed only in BSL-4 facilities, such as are available at the CDC. Ebola virus has been cultured from specimens such as serum or postmortem tissues. Monolayers of several cell types have successfully been used. Cultures using Vero or Vero E6 cells are typically held for 14 days, with cytopathic effect generally visible at about 7 days after inoculation. Vero cells of low passage number show a cytopathic effect as soon as 3 days, and culture medium without serum may generate a more complete cytopathic effect (17). Virus growth can be identified by electron microscopy, fluorescein-labeled polyclonal Ebola virus-specific antibodies prepared from the sera of mice or rabbits challenged with Ebola virus, or PCR of culture supernatant. Antigens may be detectable by these techniques prior to the development of cytopathic effects and have been found as early as 3 days after inoculation (18). Similarly, human dendritic cells prepared from peripheral blood monocytes and CV-1 African green monkey kidney cells support the growth of Ebola virus, with virus particles detectable by electron microscopy at 3 days postinoculation before the appearance of a cytopathic effect (18, 19). Ebola virus replication has been found to be particularly efficient in non-human primate alveolar macrophage cell cultures, with high virus titers and cytolysis evident within 24 h of inoculation (20).

Because of the risks associated with virus isolation in culture, other diagnostic tests that can be performed in laboratories where high-containment systems are not available have been used. The Ebola virus genome encodes seven proteins, including a nucleoprotein, four virion structural proteins (VP35, VP40, VP30, VP24), glycoprotein (GP), and RNA-dependent RNA polymerase (L protein). Antigen capture tests have been reported in the literature using monoclonal antibodies against several Ebola virus targets, including the envelope glycoprotein, nucleoprotein, and VP40. Sensitivities of these assays are in the range of 10^3 to 10^4 PFU/ml serum (21).

PCR-based tests have emerged as the preferred methods and have been used in both conventional and real-time formats, with successful use of different gene targets. In an outbreak of Sudan

ebolavirus in Uganda, reverse transcription-PCR (RT-PCR) of specimens collected early in the course of infection was able to detect ebolavirus 24 to 48 h prior to detection by an antigen capture assay (22). Similarly, in an outbreak of Ebola virus in Gabon, only 83% of cases identified by RT-PCR were identified by the antigen capture assay (23). In addition, RT-PCR was able to detect Ebola virus in peripheral blood mononuclear cells during the 1- to 3-week convalescent period after the disappearance of symptoms when antigen was undetectable (12).

Laboratory-developed RT-PCR assays have shown high clinical sensitivity for the detection of virus from early acute disease throughout early recovery (24, 25). Analytical sensitivities have been reported at about 478 to 2,647 genome eq/ml of plasma (24, 25). Primers are most successfully used when revised in light of published sequence information of currently circulating strains (24, 25).

A study in Uganda showed that high viral RNA concentrations of 10^8 copies or higher per ml of serum by the second day of illness were predictive of poor outcome in a missionary hospital isolation ward setting (22). RNA copy levels were 2 logs lower in patients who survived.

There are no nucleic acid amplification tests to detect Ebola virus that are approved by the U.S. Food and Drug Administration (FDA). Several tests, however, have received emergency use authorization (EUA) to accommodate testing during the current outbreak. On 5 August 2014, and with the request amended on 10 October 2014 to include more specimen types, EUA was granted for the EZ1 real-time TaqMan RT-PCR assay developed by the Naval Medical Research Center for the Department of Defense (<https://federalregister.gov/a/2014-22086>). The EZ1 test is intended to detect Ebola virus in patients suspected of being infected based on clinical and epidemiological screening criteria. The test uses two primer/probe sets, one that detects the Ebola virus target specifically as well as the EZ1 synthetic positive template control and another that detects human RNase P and is used as an extraction control. Positive and negative controls validate the test run (<http://www.fda.gov/downloads/MedicalDevices/Safety/EmergencySituations/UCM408334.pdf>). The test is approved for blood or plasma specimens. Nucleic acid extraction is done using the Qiagen QIAamp viral RNA minikit, followed by real-time RT-PCR using the Applied Biosystems 7500 Fast Dx system (Thermo Fisher Scientific, Waltham, MA), Roche LightCycler (Roche Diagnostics, Indianapolis, IN), or Biofire Defense Joint Biological Agent Identification and Diagnostic System (JBAIDS) instrument (BioFire Defense, Salt Lake City, UT). The limit of detection (LOD) using inactivated virus is 5,000 PFU/ml on the 7500 Fast Dx system and LightCycler and 7,500 PFU/ml on the JBAIDS instrument. The LOD using live virus under BSL-4 containment conditions was verified at 1,000 PFU/ml on all instruments. A positive test result indicates that the patient is presumptively infected with the Ebola virus. Use of the EZ1 assay is limited to laboratories designated by the Department of Defense.

On 10 October 2014, two tests developed by the Centers for Disease Control and Prevention (5), the CDC Ebola virus NP real-time RT-PCR assay and the CDC Ebola virus VP40 real-time RT-PCR assay, were also granted EUA status (<http://www.fda.gov/downloads/MedicalDevices/Safety/EmergencySituations/UCM418807.pdf> and <http://www.fda.gov/downloads/MedicalDevices/Safety/EmergencySituations/UCM418812.pdf>). The NP test targets the nucleoprotein gene, and each test is internally controlled

by detection of the human RNase P gene to indicate adequate nucleic acid extraction. Nucleic acid extraction is done using the MagMAX pathogen RNA/DNA kit and Dynal BeadRetriever (Life Technologies, Thermo Fisher Scientific, Waltham, MA). Individual reactions for positive, negative, and extraction controls are included in each run. Whole blood collected with EDTA as the anticoagulant, plasma, and serum are acceptable specimen types. Urine can also be tested but only in conjunction with a corresponding whole-blood, plasma, or serum specimen. The test is run on the ABI 7500 Fast Dx system with positive, negative, and extraction controls. The limit of detection was not affected by TRIzol and was determined to be 3 to 30 50% tissue culture infectious doses (TCID₅₀) per reaction mixture using inactivated virus and 10- to 100-fold lower using live virus (<http://www.fda.gov/downloads/MedicalDevices/Safety/EmergencySituations/UCM418810.pdf>). The second EUA CDC test targets the viral matrix protein VP40 as well as RNase P. The test is authorized for the same specimen types and for the same extraction and amplification methods as the NP test and includes positive, negative, and extraction controls. The limit of detection using live virus was determined to be 1 to 3 TCID₅₀/reaction mixture, with a 10- to 100-fold-increased LOD using inactivated virus (<http://www.fda.gov/downloads/MedicalDevices/Safety/EmergencySituations/UCM418815.pdf>). Both tests are intended for the presumptive identification of Ebola virus in patients with signs and symptoms of Ebola virus infection and/or appropriate epidemiological risk factors. These tests may be used only by qualified laboratories that are designated by the CDC. Positive results of tests performed in laboratories outside the CDC must be confirmed at the CDC.

On 25 October 2014, EUA was granted for the BioFire Defense FilmArray BioThreat-E test and the FilmArray NGDS BT-E assay (<http://www.fda.gov/downloads/EmergencyPreparedness/Counterterrorism/MedicalCountermeasures/MCMLegalRegulatoryandPolicyFramework/UCM420416.pdf> and <http://www.fda.gov/downloads/EmergencyPreparedness/Counterterrorism/MedicalCountermeasures/MCMLegalRegulatoryandPolicyFramework/UCM420421.pdf>). The FilmArray instrument is a fully automated, enclosed platform that performs real-time PCR, with all reactions performed in a bubble pouch that is sealed after addition of the specimen. One sample is tested at a time, with results returned in about an hour. The procedure consists of nucleic acid purification followed by reverse transcription, two-stage nested PCR in triplicate, and high-resolution melt curve analysis to detect the presence of Ebola virus. An internal RNA process control ensures successful performance of all processes. Reference material will be available from the manufacturer in the near future. The RNA of Zaire ebolavirus, variant Mayinga, can also be purchased from B.E.I. Resources, Manassas, VA (catalog no. NR-31806). The standard specimen type is whole blood. Urine can also be tested in combination with a matched whole-blood specimen from the same patient. The EUA version of the test has been optimized by adding primer sequence degeneracy to improve detection of the strain currently in circulation. Studies of the earlier versions of the test using inactivated older Ebola virus strains in whole blood showed the LOD to be 6×10^5 PFU/ml (<http://www.fda.gov/downloads/EmergencyPreparedness/Counterterrorism/MedicalCountermeasures/MCMLegalRegulatoryandPolicyFramework/UCM420424.pdf>). Loss of multiple logs of sensitivity is expected due to damage from the inactivation process, and synthetic template

studies as well as our experience with real-world specimens (to be reported in a separate publication) show significantly improved sensitivity comparable to those of other assays. The NGDS BT-E version of the assay is designated for use at sites specified by the Department of Defense that currently perform testing with the FilmArray system. LOD studies show 1×10^4 PFU/ml with inactivated virus and at least 1×10^3 PFU/ml with live virus (<http://www.fda.gov/downloads/EmergencyPreparedness/Counterterrorism/MedicalCountermeasures/MCMLegalRegulatoryandPolicyFramework/UCM420419.pdf>).

Recommendations from the CDC indicate that patients who present less than 72 h after developing symptoms and have a negative RT-PCR test on a blood specimen should be cleared based on clinical and laboratory criteria and on the facility's ability to monitor the patient after discharge (<http://www.cdc.gov/vhf/ebola/hcp/considerations-discharging-pui.html>). More information will be available as a result of ongoing epidemiological and genomic surveillance to monitor viral mutations. These studies will help to ensure continued accurate diagnosis and will guide research on the development of antiviral agents. The international response has allowed for intensified public health strategies, with the expectation of controlling and ending the ongoing outbreak of Ebola virus disease in West Africa.

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