BSL-4: Authorized Personnel Only

by

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Part I – Background*

No Good Deed Goes Unpunished

Liberia, West Africa, 27 July 2014

"I don't want to die here," Claire whispered to the nurse as she gazed at the rows of beds where dying patients lay around her. The nurse's eyes drifted to the floor as she tried to think of something positive to say.

Just three days ago Claire was working with the decontamination team at the clinic. One by one, she carefully sterilized the personal protective equipment worn by individuals coming and going from the quarantine tents; doctors, nurses, family members of visiting patients. There were so many. All it took was a single unnoticed error; a microscopic puncture in her hazmat suit, or maybe she incorrectly removed her gloves or facemask? Now she was a patient at the clinic.

The nurse reached out and laid a gloved hand on Claire's feverish, rash-covered arm, being careful not to disturb the IV. "Are you feeling up to having a bit of your favourite Liberian potato soup?" she said as cheerfully as she could muster. Claire nodded no through a glassy stare.

"Claire, we all have hope that this experimental serum will work. Your condition is grave but stable at present, and we've just received confirmation from the CDC that you are getting a first-class ticket home to continue your treatment. You must stay strong now, you hear me?" the nurse insisted.

Claire slowly turned her ghost-like gaze back to the nurse, willing a slight smile as she recognized the all too familiar training in bedside manner. *Am I really going to make it home? How did this happen? I was so careful during the decontaminations.* Claire thought of the hundreds of sick patients that she had seen admitted to clinic in the past weeks before she herself became ill. She squinted her blood shot eyes as she grimaced in pain. Her body ached and was burning up. Her head pounded and she barely had enough strength to speak. *This is just the beginning,* she thought.

She tried not to think of the telltale symptoms of deterioration that could rapidly develop and that so many others had painfully suffered in their final days; nausea, vomiting, diarrhea, and eventually, internal bleeding. Claire's thoughts turned to Kurt Preston. *I can't believe Dr. Preston insisted that I get the only dose of experimental serum available when he himself is at risk of the same fate. He is truly a hero. I pray that the passive immunity treatment he has received from the convalescent boy's blood is effective.*

"When do I leave?" Claire asked.

The nurse took a deep breath. "Dr. Preston's flight will return to the U.S. on Saturday. Your flight will hopefully be very soon after that. The medical evacuation of infected U.S. aid workers is top priority now." Claire nodded.

Just then, the nurse's attention was drawn to a group of workers entering the room dressed head-to-toe in the same white rubber protective gear. They were accompanying five new patients into the ward, with their family members suited in hazmat gear, weeping in tow. "You should go tend to the others." The nurse nodded and turned to leave.

I feel so grateful, yet so guilty that my ability to go home brings some hope to my condition. These people are home and there is little hope. They are living in a hellish nightmare. The number of cases just keeps increasing. This outbreak is out of control. We need more help...

Claire closed her eyes and tried to rest.

This case is based on true events. In July of 2014, two U.S. volunteer aid workers became violently ill when they contracted the disease that they had set out earlier in the previous month to help fight in the West African country of Liberia. As of the twenty-seventh of that month, the fate of the lives of those two individuals was still very uncertain. At this point in the story, these two heroic people were among hundreds of others who were fighting for their lives. So many more had lost their fight, including Patrick Sawyer, the first American victim of the outbreak (1) and Dr. Skeik Umar Khan, a top virologist in one of Liberia's neighboring countries, Sierra Leone, where the outbreak had spread from its onset in Guinea just weeks into the New Year (2).

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* The scenario presented in this case study is based on factual information gathered from various news sources as the outbreak was unfolding. However, "Claire" and the "nurse" character, as well as the feelings and sentiments of these characters, are fictitious.

Learning Goals and Your Role

Are you wondering what became of the infected U.S. humanitarian workers? What disease they caught? Why and how the disease outbreak happened? If there was a cure? Before you find out, this case will take you on a journey through this nightmarish outbreak to gain a better understanding of the complex and arduous challenges that public health officials face in characterizing, containing, and combating deadly infectious disease epidemics.

In this case study, you will play the role of an Infectious Disease Specialist in training at the Center for Disease Control and Prevention (CDC) headquarters in Atlanta, Georgia. You will be helping in the response efforts to the infectious disease outbreak happening in Africa. Along the way, you will learn about selected molecular biology approaches used in the detection and diagnosis of disease epidemics and apply this knowledge using molecular biology software, such as *Case It*, a free molecular biology laboratory experiment simulation, and online bioinformatics tools. (Bioinformatics is the science of collecting and analyzing complex biological data, such as DNA and protein sequences.) Additionally, throughout the case study you will analyze and evaluate social and cultural aspects of infectious disease control.

A Long-Standing Battle of Microscopic Proportions

> *[In 1974], my professor of social medicine grabbed my shoulder firmly, to make sure I was paying attention. "There's no future in infectious diseases," he stated flatly, in a tone that bore no argument. "They've all been solved." But I wanted to go to Africa. I wanted to save lives. And it seemed to me that infectious disease might be just the ticket and full of unresolved scientific questions. So I ignored him.*
> —Peter Piot, *No Time to Lose: A Life in the Pursuit of Deadly Viruses*

Humans have battled with infectious diseases since antiquity. From the plagues that swept across villages of ancient times, to the global human immunodeficiency virus (HIV/AIDS) pandemic of the twenty-first century, infectious diseases have had an irrefutably enormous impact on human history (3).

Interestingly, the primary cause of most infectious disease outbreaks in the human population is due to the impact that humans have had on the environment, in particular, through ecological encroachment. Throughout history, human population dynamics (growth, expansion, urbanization) have disquieted the "slumber" of zoonotic pathogens, facilitating zoonoses when humans cross the animal-human ecosystem interface. This has been true for over half of the nearly 1500 agents known to cause infectious disease in humans (4).

Of these pathogens, "viruses [have] exact[ed] an enormous toll on the human population and are the single most important cause of infectious disease morbidity and mortality worldwide" (5). However, with improvements in nutrition, sanitation, and healthcare, particularly with the advent of vaccines, the incidence of outbreaks and the mortality rates due to viral agents in developed countries has been significantly reduced (3). A problem "solved" even? Perhaps from a westerner's perspective. However, despite widespread advances in medical science, viruses, among other infectious agents, continue to have devastating effects for human populations in many developing countries (3).

Of the challenges to medical science, monitoring and responding to the emergence (and re-emergence) and spread of deadly tropical febrile (fever-causing) infectious diseases markedly illuminates the devastation that can occur (4). In countries and regions characterized by limited resources and impoverished conditions, and where many illnesses share the same symptoms in the early stages, it is extremely difficult to differentiate between diseases based solely on clinical observations, let alone treat and control viral disease outbreaks. Consequently, scientific progress in our understanding of viral infections is paramount in the detection, management, and eradication of disease outbreaks caused by viruses in developing countries.

There is much research that still needs to be done in areas ranging from the molecular and cell-to-cell interaction level, to the level of tissues and systems, and extending to the intersections of epidemiological and ecological interfaces (3). Indeed, there is no time to lose in this battle.

Part II – Disease Detective

As previously mentioned, many tropical diseases in their early stages present with a set of similar symptoms, making diagnoses, treatment, and containment a challenge for medical health officials. One way to narrow down the list of potential pathogens is to classify pathogenic agents by various criteria. Infectious diseases can be classified in many ways, such as treatment, transmission, and preventative measures.

Analysis Activity A

The focus of your first training task is disease detection at the macro-level. Begin by analyzing and comparing the symptomatic and situational characteristics of the disease outbreak presented in this case to the known characteristics of pathogens on record at the CDC. Using the list of diseases below, carefully select the *top three* pathogens that you suspect could be responsible for the disease outbreak in West Africa.

To conduct this analysis, review the details presented in the story so far and conduct research on the diseases in the following list using this CDC website link: http://www.cdc.gov/DiseasesConditions/.

Febrile tropical diseases that present with similar symptoms:

- Alkurma hemorrhagic fever (AHF)
- Crimean-Congo hemorrhagic fever (CCHF)
- Dengue hemorrhagic fever (DHF)
- Ebola hemorrhagic fever (EHF)
- Influenza (H1N1)
- Lassa fever (LF)
- Legionnaire's disease (LD)
- Lujo hemorrhagic fever (LHF)
- Malaria
- Marburg hemorrhagic fever (MHF)
- Plague
- Typhoid fever (TF)
- Yellow fever (YF)

Questions

1. Which of the classification criteria are especially helpful in narrowing down your list? Justify your answer.

2. Prepare your list of the top three suspected pathogens. Share your list with a partner or another group and discuss how they compare. Be prepared to justify your selections.

Sound the Alarm! We've Got a "Hot Zone"

When local health officials recognize a group of disease cases presenting with similar characteristics, they notify national and international public health organizations. At the onset of the disease outbreak in March, the World Health Organization's (WHO) Regional Office for Africa released the following situation report. Situation reports are official publications that convey the details and updates related to a particular event, such as a natural disaster or disease outbreak.

> *World Health Organization—Situation Report Guinea, West Africa, 28 March 2014 (adapted excerpt from (6))*
>
> Cases of an unknown disease presenting with an acute febrile illness characterised by diarrhoea, vomiting, and bleeding in some individuals, were first reported from Guéckédou district, Guinea, West Africa, in February 2014, presumed to be a viral haemorrhagic fever or other severe gastrointestinal disease. The outbreak investigation subsequently retrospectively identified earlier suspected cases that fell ill and died in December 2013 (including "patient zero," the first reported case (7)).

Now Entering BSL-4 Microbiological Laboratory—Authorized Personnel Only

Your next training task will take place in Unit #70 of the Viral Special Pathogens Branch (VSPB) at the CDC. The disease responsible for this outbreak appears to be highly virulent, and in order to confirm the identification of this pathogen you must conduct micro-level analyses in the Biosafety Level 4 (BSL-4) laboratory. The blood serum samples from "patient zero," Claire, Dr. Preston, and the five new patients admitted to the isolation ward of the treatment center in Monrovia, Liberia, have been transported to the CDC under strict BSL-4 containment protocols. Before you enter the lab, you must successfully complete the training on the four levels of biological safety and review the principles and procedures for the laboratory disease detection assays.

BSL-4 MICROBIOLOGICAL LABORATORY

Go to: **http://www.cdc.gov/training/quicklearns/biosafetyce/biosafetyCE.html**

Overview of the ELISA

The enzyme-linked immunosorbent assays (ELISA, pronounced eh-LY-suh) allows for the detection and quantification of analytes such as antibodies, antigens, peptides, proteins, and hormones in serological samples. ELISAs are usually performed using a multi-well micro-plate. There are four typical ELISA formats (direct, indirect, sandwich, and competition/inhibition); with the end result for all types showing wells with color intensities varying in proportion to the amount of analyte in the original sample (8). The general steps for the indirect ELISA method are explained below and illustrated in Figure 1 on the next page.

Step 1: In an indirect ELISA, an antigen (a substance that causes the immune system to create antibodies) of interest is coated to the wells of the plate.

Step 2: Samples of the patient's serum are then added to the wells. If specific antibodies against the antigen are present, they will bind to the antigen fixed to the bottom of the wells. The excess unbound antibodies are washed away (not shown).

Step 3: A solution of animal antibodies against human antibodies that is conjugated (linked) to an enzyme is added. These antibodies will bind to the human antibodies that are bound to the antigen. The wells are washed again.

Step 4: Finally, a colourogenic (colour-producing) solution containing a substrate that will react with the enzyme is added. The interaction of the substrate with the enzyme on the second antibody generates a visible colour that can be quantified with an electronic plate-reader and is expressed as optical density (OD). The darker the colour, the higher the level of antibody present. A test is considered positive if the sample's OD is twice that of the negative control.

Figure 1: Indirect ELISA method.
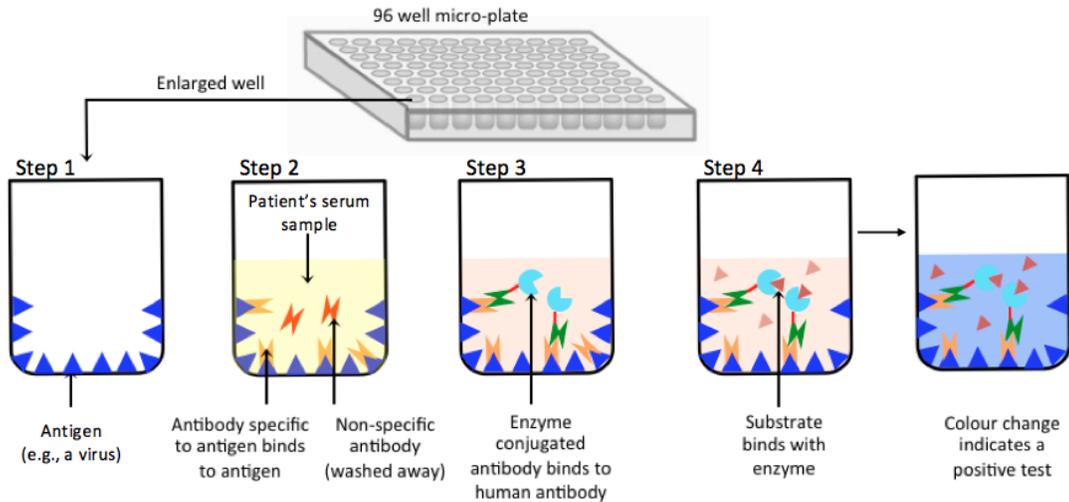
Overview of PCR

Polymerase chain reaction (PCR) tests are used to detect the presence of nucleic acid in blood or tissue samples. The test essentially takes a segment of genetic information (i.e., a gene of interest) and makes billions of copies of that segment. Reverse-transcription PCR (RT-PCR) is one variant of the PCR method used to detect ribonucleic acid (RNA) in samples. Since many virus genomes consist of a single strand of RNA, the enzyme, reverse transcriptase, may be used to create a strand of complementary DNA (cDNA) prior to conducting the PCR test. Using a PCR method performed on a 96-well plate, the amount of virus in the sample can then be quantified and expressed as viral load (VL). The VL value corresponds to the number of copies of virus RNA or DNA in the sample. Temporal VL measurements can be used to monitor the progress of the disease and to determine the effectiveness of medical interventions (e.g., drug or other treatments).

Figure 2: PCR procedure by which virus RNA can be amplified to allow for identification. (Adapted from: Smith, T. 2011. *Ebola and Marburg Viruses*, 2nd ed. Infobase Publishers, New York, NY.)
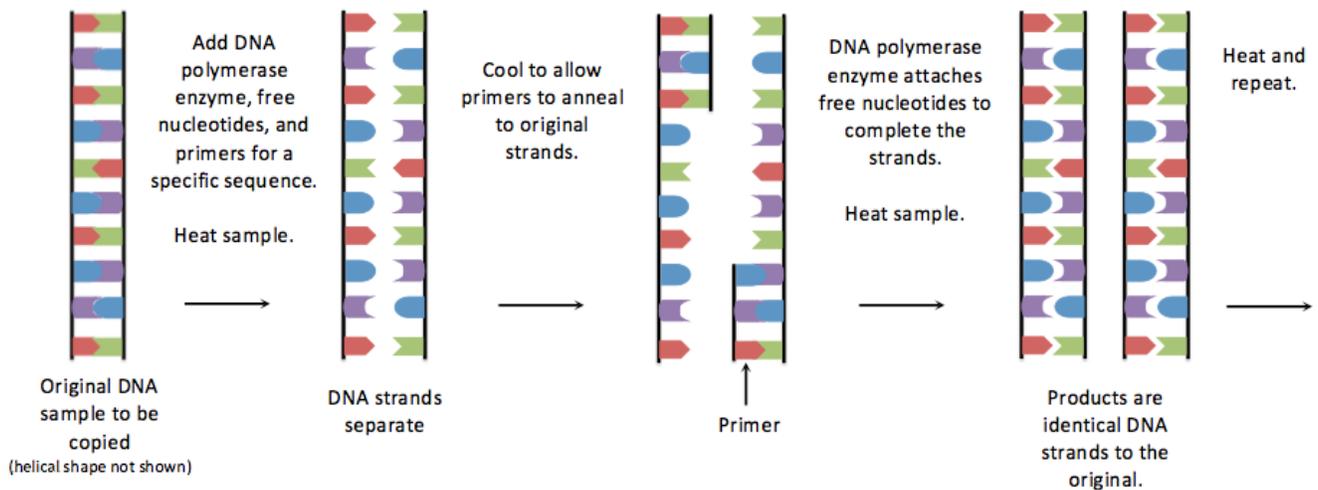
Lab Activity A

To conduct this analysis:

1. First, determine the identification of the causative agent from the top three suspected pathogens (MHF, EHF, CCHF). Using the *Case It* software, run an ELISA on the proteins in the blood sample from "patient zero."

2. Then, run a 96-well plate PCR test on the cDNA isolated from the sample. Create a data table to record your results.

3. Next, determine the infection status of the rest of the patients. Run an ELISA on the proteins in the blood samples collected from these patients, followed by a 96-well plate PCR test. Create a data table to record your results.

Protein and DNA samples:

|			
Patient zero	Toimu	Sarifina	Positive control for EFH
Claire	Konah	Bindu	Positive control for MHF
Dr. Preston	Finda	Negative control	Positive control for CCHF

Prior to conducting the ELISA test, you will need to determine which ELISA format is the most suitable for this case. To do this, read about the four types of ELISA formats (see, for example, https://www.abdserotec.com/elisa-types-direct-indirect-sandwich-competition-elisa-formats.html). Then, compare this information to the data on serological markers for the top three suspected pathogens (MHF, EHF, CCHF) presented in Figure 3 (next page). You will need to incorporate what you have learned in order to correctly execute the ELISA using the *Case It* software.

Questions

1. Which ELISA format is most suitable for this case? Explain.

2. Based on the test results from patient zero's sample, which virus caused the outbreak? Explain.

3. Based on the test results from all of the patient samples, what do you conclude? How would you counsel these patients?

4. Compare the results of the ELISA test to the results of the PCR test for each patient. What issues are raised?

5. Is there anything unusual or concerning about any of the patients' results? Explain. If so, how would you counsel these patients? What further recommendations would you make and why?

6. Would you recommend re-testing any of the samples using a measurement of viral load over time? Explain. If you answered yes, conduct the test and report your conclusion and recommendation.

7. Why do you suppose that conducting both an ELISA and a PCR test is necessary? Why not just run one or the other? Make reference to the ELISA and PCR data that you collected from all of the patients in your answer.

8. On February 20, 2015, the WHO announced the approval of the use of a 15-minute test for Ebola virus infection called the ReEBOV Antigen Rapid Test Kit. The test is similar to a blood glucose strip test, except instead of the need for a device that reads the results digitally, the user waits for a reaction to occur in a test tube. Read the media release article on the new test published by *CBC News* (http://www.cbc.ca/news/health/ebola-outbreak-rapid-test-approved-1.2965177) and compare and contrast the new test to the traditional methods (i.e., ELISA/PCR). Should the new method replace the old methods in the detection of the virus? Why or why not?

Figure 3: Serological Markers of Acute Viral Hemorrhagic Fever Infections in Cases of MHF, EHF, and CCHF
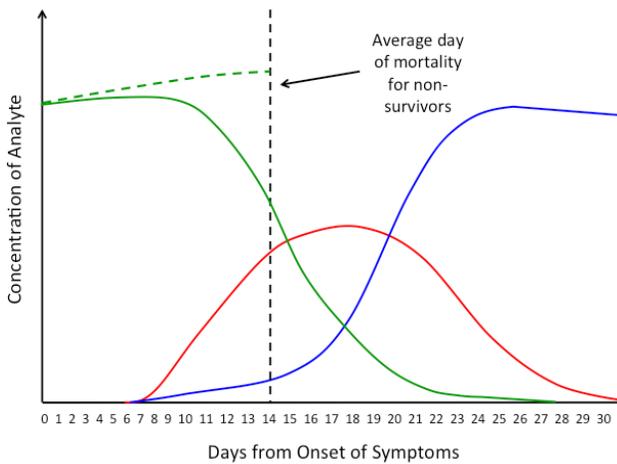
A. Marburg Hemorrhagic Fever Virus
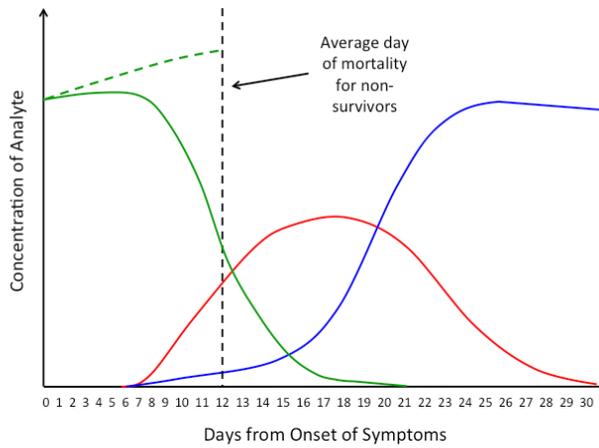
B. Ebola Hemorrhagic Fever Virus
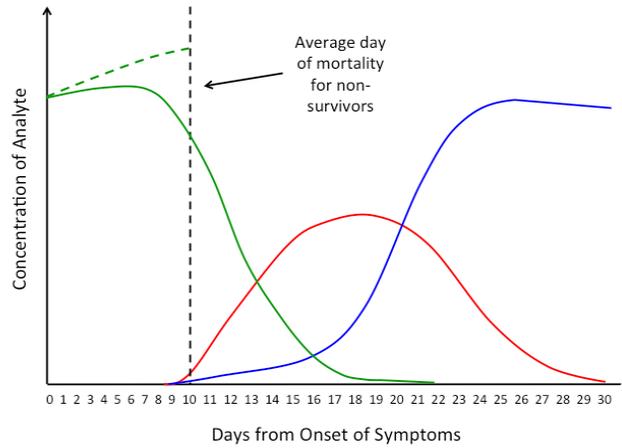
C. Crimean-Congo Hemorrhagic Fever Virus

Legend

— Viral load (survivors)
--- Viral load (non-survivors)
— IgM*
— IgG*

*survivors only shown

Source: The graphs in Figure 3 were created using information gathered from (9–12). Relative concentrations may not be exactly accurate. In some cases of non-survivors, IgM and IgM may not be detected prior death due to the immunosuppressive effects of the virus.

CDC Headquarters, VSPB, Atlanta, Georgia, 1 August 2014

Just as you finish checking over your final report summary on the patient samples, your internship supervisor enters the lab. "So, have you correctly identified the pathogen?"

You glance at your paper a final time, take a deep breath, and hand it over. She peers down at it, adjusting her glasses to read your report. As she silently reviews it, you nervously inspect her facial expression for motions that hint to your success or failure. When she finally looks up from the report, you smile awkwardly. The few eyebrow raises, eye squints, and subtle mouth gestures have given you little indication of your performance.

"Excellent work, intern!" she exclaims. "You have correctly identified the pathogen as the Ebola virus."

You are shocked and delighted all at once. Just as you are about to go for the high five, your supervisor quirks her head to the side and says, "Not so fast there, grasshopper. We still need to know which type of Ebola virus we are dealing with. The patients' prognoses depend on this information."

You stand there looking confused. "Oh? There's more than one type? Why does that matter?"

A Brief History of an Old Disease

Most literature reviews detailing the horrific aftermath of the emergence of the Ebola virus into the human world reference the grave period in the fall of 1976 that began when the index case of the outbreak, a 44-year-old school instructor named Mabalo, presented with febrile symptoms thought to be malaria (13). Just nine years prior, the first outbreak of another previously uncharacterized virus of (the later classified) *Filoviridae* family to which Ebola belongs, the Marburg virus (MARV), was reported. Together these two African-cradled hemorrhagic fever-causing RNA viruses have made their way into history books as two of the deadliest human pathogens on Earth (14).

In contrast to other historically devastating diseases like smallpox and the bubonic plague, the emergence of this filovirus about 40 years ago makes Ebola a *new* disease, right? Interestingly, scientists have detected the mark of filovirus infections as integrated genetic elements in the genomes of small mammals (15). Evidence from phylogenetic and sequencing analyses suggest "that the association of mammals with filoviruses is likely tens of millions of years older (15) than previously thought (16)." Thus, the zoonotic transfer of filoviruses in the second half of the twentieth century appears to have made a very, very long awaited debut into a human host. This raises the question of how did a family of pathogens that have evolved such virulent properties remain hidden from human knowledge for so long? To better understand this question, it is important to examine the ecological characteristics of these pathogens' existence from an evolutionary perspective,* in addition to their microscopic or molecular properties. Despite the tremendous effort of expert and dedicated researchers, Ebola's natural reservoir has not yet been definitively identified. However, recent evidence from field studies reveals that three species of fruit bat carry the disease asymptomatically, implicating them as likely candidates in the route of primary transmission from nature to humans (17–19).

Despite the enigmatic nature of the Ebola virus zoonoses, there is a growing body of evidence surrounding the five characterized species, ranging from geographic distribution and virulence patterns in human and animal populations, to structural and genetic diversity. As mentioned, Ebola first manifested itself in the human population in 1976 when it first emerged almost simultaneously in the African countries of Sudan and the Democratic Republic of Congo (DRC, formerly Zaire) (20). The two outbreaks were later identified as two distinct species of Ebola virus named Sudan Ebola virus (SEBOV) and Zaire Ebola virus (ZEBOV), named after the Ebola River in Zaire (20). In 1994, a new species was discovered in the Ivory Coast, namely Côte d'Ivoire Ebola virus (CIEBOV) (20,21), and another, Bundibugyo Ebola virus (BEBOV) (20,22), appeared in 2007 in Uganda. A fifth strain, Reston Ebola virus (REBOV), was identified in 1989, when infected laboratory monkeys were imported from the Philippines to a quarantine facility in Reston, Virginia.

All Ebola virus species are disease-causing agents in humans (20), with the exception of the REBOV strain, whose pathogenic effects appear to be limited to certain animal populations (23, 24). Not all Ebola virus strains are created equal, and they exhibit distinctive phenotypes with respect to virulence due to variations at the genotype level. Elucidating the underlying molecular mechanisms for the differences in virulence observed between the five Ebola strains in humans (with the REBOV strain being non-pathogenic in humans) has proven particularly difficult in the animal models currently established, and results demonstrated *in vitro* have not been reproducible in *in vivo* models of disease pathogenesis (25). Nevertheless, data collected outlining the chronology of Ebola virus disease outbreaks at the macro-level can reveal insight into the genetic differences of this disease under current investigation at the micro-level (26, 27).

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* Another case study entitled "Hunting the Ebola Reservoir Host" by Allison Black and Annie Prud'homme-Généreux, and the review article "The Ecology of the Ebola Virus" by Allison Groseth et al., are two excellent resources to explore for further investigation on this topic.

Analysis Activity B

Questions

1. Analyze Table 1, which contains information on Ebola virus disease outbreak chronology. Which Ebola virus strain is considered to be (by case fatalities):
   a. the least virulent? Explain.
   b. the most virulent? Explain.

Table 1: Chronology of previous Ebola virus disease outbreaks. (*Source:* Adapted from WHO, http://www.who. int/mediacentre/factsheets/fs103/en/; 2014 data has been omitted.)

Year	Country	Ebola virus species	Cases	Deaths	Case fatality
2012	Democratic Republic of Congo	Bundibugyo	57	29	51%
2012	Uganda	Sudan	7	4	57%
2012	Uganda	Sudan	24	17	71%
2011	Uganda	Sudan	1	1	100%
2008	Democratic Republic of Congo	Zaire	32	14	44%
2007	Uganda	Bundibugyo	149	37	25%
2007	Democratic Republic of Congo	Zaire	264	187	71%
2005	Congo	Zaire	12	10	83%
2004	Sudan	Sudan	17	7	41%
2003 (Nov–Dec)	Congo	Zaire	35	29	83%
2003 (Jan–Apr)	Congo	Zaire	143	128	90%
2001–2002	Congo	Zaire	59	44	75%
2001–2002	Gabon	Zaire	65	53	82%
2000	Uganda	Sudan	425	224	53%
1996	South Africa (ex-Gabon)	Zaire	1	1	100%
1996 (Jul–Dec)	Gabon	Zaire	60	45	75%
1996 (Jan–Apr)	Gabon	Zaire	31	21	68%
1995	Democratic Republic of Congo	Zaire	315	254	81%
1994	Cote d'Ivoire	Taï Forest	1	0	0%
1994	Gabon	Zaire	52	31	60%
1979	Sudan	Sudan	34	22	65%
1977	Democratic Republic of Congo	Zaire	1	1	100%
1976	Sudan	Sudan	284	151	53%
1976	Democratic Republic of Congo	Zaire	318	280	88%

2. Figure 4 (next page) depicts Ebola virus outbreaks by species and affected population size between the time of the emergence of the disease in 1976 and the last outbreak in 2012. An "X" has been added to mark the affected regions (i.e., Guinea, Sierra Leone, Liberia, Nigeria) in the 2014 outbreak (as of August 2014). Does the map offer any insight into which strain(s) caused the 2014 outbreak? Why or why not?

Figure 4: Ebola Hemorrhagic Fever Distribution Map.
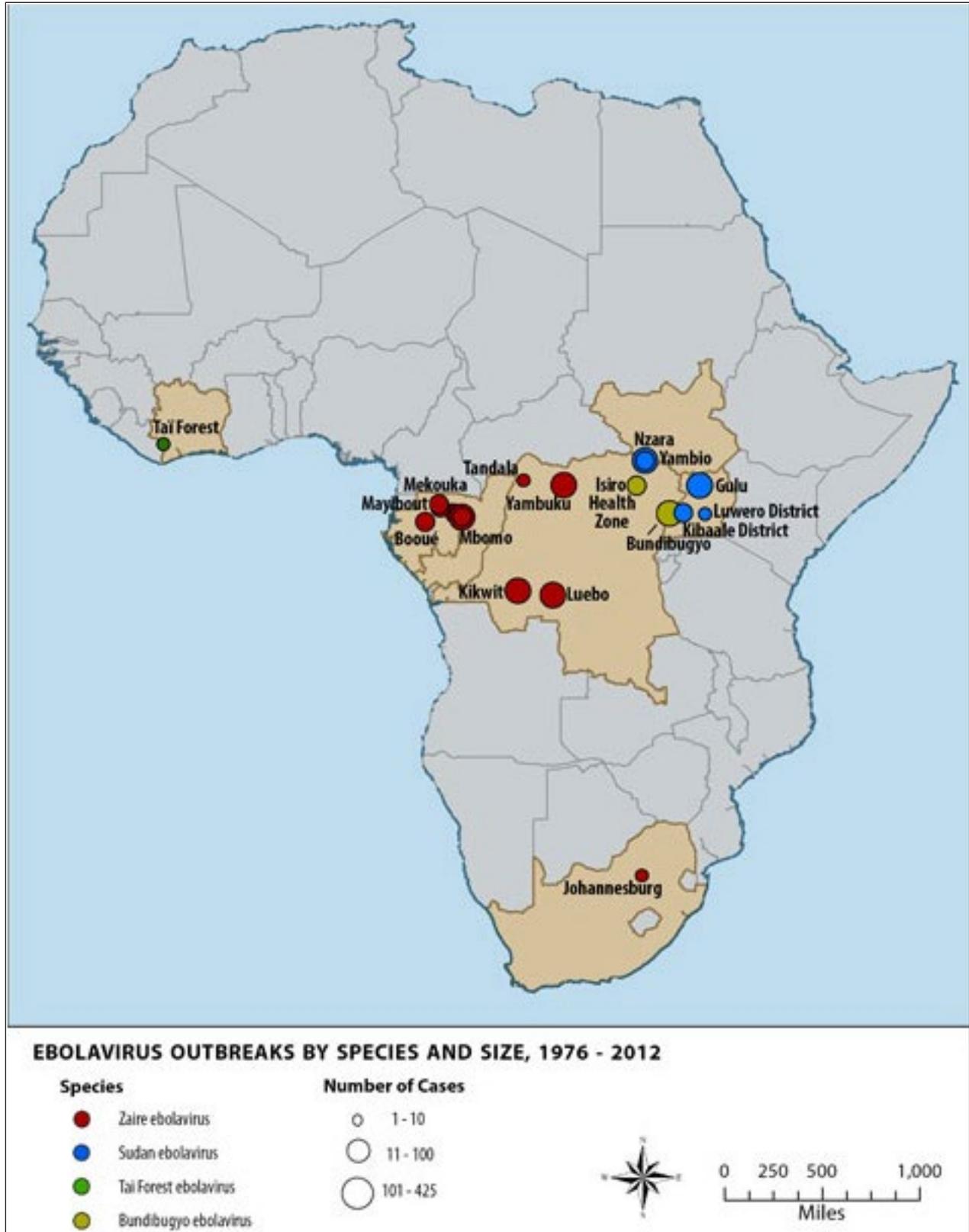
Lab Activity B

For your final laboratory task, your internship supervisor at the VSPB has requested that you use bioinformatics to elucidate the strain of Ebola responsible for the outbreak in West Africa. To do this, you will first generate a *multiple sequence alignment* to compare the DNA sequence of the unknown 2014 Ebola virus strain to the known sequences of Ebola virus strains isolated from previous outbreaks. Then, you will create a *phylogenetic tree* from your sequence alignment to determine the evolutionary relatedness of the strains.†

Accession numbers for the complete genomes of previously sequenced Ebola virus strains are provided in Table 2. An accession number is a unique identifier given to a DNA sequence, which can be entered into the National Centre for Biotechnology Information's (NCBI) genetic sequence database, Genbank (http://www.ncbi.nlm.nih.gov/genbank/). Once the DNA sequences are obtained from Genbank, you can use a bioinformatics tool like BLAST or Clustal Omega to run the multiple sequence alignment and build the phylogenetic tree. In this activity, you will be using Clustal Omega.

PCR products of the complete genome for the unknown 2014 Ebola virus strain have been isolated from a sample from patient zero. Obtain the sequence from your laboratory supervisor.

Lineage information about each Ebola virus strain can be viewed by entering the Taxonomy ID numbers found in Table 2 into the NCBI's Taxonomy Browser (http://www.ncbi.nlm.nih.gov/Taxonomy/Browser/wwwtax.cgi).

To conduct this analysis:

1. To obtain the DNA sequences for the known Ebola virus strains isolated from previous outbreaks: enter the first accession number into the *Nucleotide* search bar and click *Search* (Figure 5).
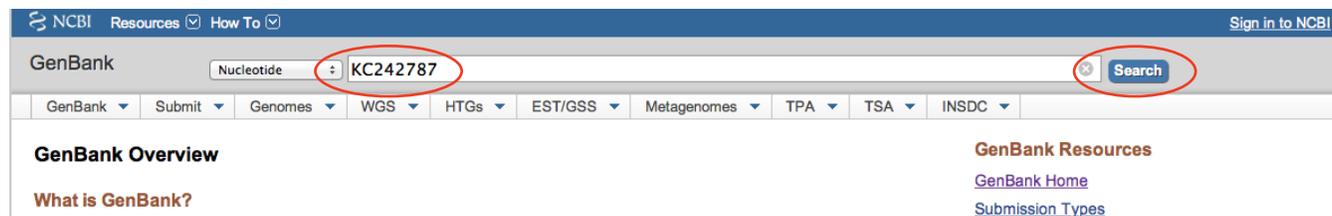
Definitions

Multiple sequence alignment: The process of comparing two or more DNA or protein sequences to one another by aligning the sequences and looking for similarities and differences.

Phylogenetic tree: A branching diagram or "tree" showing the evolutionary relationships among various species, based on similarities and differences in their physical and/or genetic characteristics. The lengths of the branches on the tree are proportional to the amount of time since the two organisms (or sequences) diverged from one another.

BLAST (Basic Local Alignment Search Tool) and *Clustal Omega:* Online bioinformatics tools used to compare DNA or protein sequences to one or more other sequences, or to compare DNA or protein sequences to a collection of sequences found in databases.

Table 2: NCBI Ebola Virus Genome and Taxonomy Identifiers

Ebola Virus Strain	Accession Number	Taxonomy ID
EBOV Luebo_DRC, 2007	KC242787	186538
EBOV Kikwit_DRC, 1995	AY354458	186538
EBOV Mayinga Yambuku_DRC, 1976	AF272001	186538
BEBOV Ebobund-112 Isiro_DRC, 2012	KC545393	565995
BEBOV Bundibugyo_Uganda, 2007	FJ217161	565995
REBOV Pennsylvania_Philippines, 1989	AF522874	386032
REBOV Reston08-A_Philippines, 2007	FJ621583	129003
SEBOV Sudan_Maleo, 1979	KC242783	186540
SEBOV Sudan_Kibaale_Uganda, 2012	KC545389	186540
CIEBOV Tai Forest 1994	FJ217162	186541
Marburgvirus 1980 (outgroup)	NC_001608	11269

Figure 5.

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† Definitions and procedure adapted from: https://www.nwabr.org/sites/default/files/Genetic_Research_Lesson3_NWABR.pdf

2. Scroll down the page to explore the information available about the virus strain. For example, the accession number KC242787 corresponds to a Zaire strain of Ebola isolated from an outbreak that occurred in Leubo, DRC in 2007. The genome of this virus consists of 18,958 base pairs (bp).

3. After you are done exploring the information, click on the *FASTA* link in the upper left hand corner (Figure 6). The FASTA link contains the nucleotide sequence format used for conducting multiple sequence alignments in BLAST or Clustal Omega.

Figure 6.

4. Open the file provided by your lab supervisor that contains the DNA sequence for the unknown 2014 Ebola virus strain. From the FASTA link you previously opened, copy the entire DNA sequence for the first accession number, including the title (*>gi|436409299|gb|KC242787.1| Zaire ebolavirus isolate EBOV/H.sapiens-tc/COD/2007/23 Luebo,* complete genome). Paste it into the file after the DNA sequence of the unknown 2014 Ebola virus strain. Leave one space between the end of the sequence and the title of the next sequence.

5. Repeat this process until you have copied and pasted all of the DNA sequences for the Ebola virus strains contained in Table 2. You should have 12 sequences in total. *Note:* The Marburg virus sequence is included as the *outgroup*. An outgroup is a sequence included in phylogenetic trees from an older or more distantly related species, and is used as a point of reference and to mark the evolutionary time. Inclusion of an outgroup is said to *root* the tree.

6. Open the Clustal Omega website (http://www.ebi.ac.uk/Tools/msa/clustalo/) in a new window in your browser. Go back to your file containing the 12 DNA sequences and copy all of the sequences (including their titles). Paste the sequences into the box labeled *STEP 1—Enter your input sequences* (Figure 7) on the Clustal Omega *Input form* page.

Figure 7.

7. Change the drop down menu to display *DNA* instead of *PROTEIN* and click *Submit* (Figure 7). *The sequence alignment may take several minutes to complete. Please be patient.*

8. Once the results are complete, click on the tab that says *Phylogenetic Tree* (Figure 8). Scroll down to the *Phylogram* image. This will reveal the evolutionary relationships of all of the viruses used in this query. If you wish to save the tree, take a screen shot and paste it into a document or draw it in your notebook. It may be helpful to write in the names of the virus strains next to each respective identifier to make your analysis easier.

Figure 8.
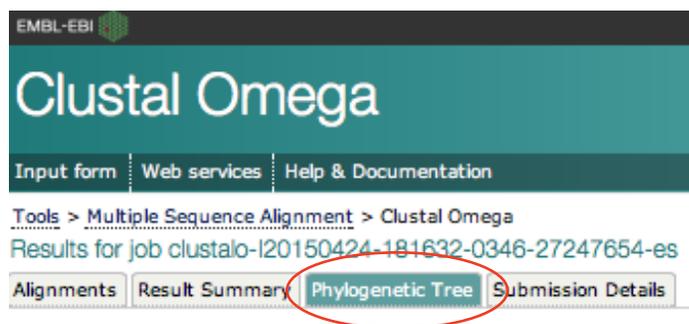
Questions

1. Based on the phylogenetic tree, which strain is the virus isolated from patient zero's sample most closely and least closely related to? Explain.

2. Do the tree results support the initial conclusions about the strain that you made in Question 2 of Analysis Activity B? Explain.

3. Based on the tree results, how would you counsel the infected patients?

4. Patient zero in the 2014 outbreak was traced back to a 2 year-old child living in Meliandou Village, Guéckédou (a prefecture of Guinea) in December of 2013. From there, further outbreaks appeared in neighboring districts, and subsequently, in the neighbouring countries of Liberia, Sierra Leone, and Nigeria. Additionally, in late August of 2014, the DRC declared an Ebola outbreak, becoming the fifth African country that year with confirmed cases. If you wanted to perform a genetic analysis of the Ebola virus circulating within the human population to determine if the outbreaks in West Africa and the DRC were caused by multiple introductions of the virus from an unknown natural reservoir, how would you test this?

5. Using the information you have learned about past Ebola outbreaks and the results from the lab tests you have conducted, make predictions about the following characteristics of this outbreak:
   a. the potential Case Fatality Rate (CFR)—a proportion of deaths within a population of cases (expressed as a percentage).
   b. the potential duration of the outbreak.

Part III – Hide and Seek, Game No. 15

A quick survey of the chronology of Ebola virus outbreaks (Table 1) revels an interesting feature; Ebola plays a game of hide and seek. After the virus initially surfaced in 1976, it appeared a few more times up until 1979, after which, it seemingly disappeared for the next 15 years. However, since the virus resurfaced in 1994 and has subsequently appeared in approximately regular one-to-two year intervals, we know that it must have a "hiding place" somewhere out there. Although the exact reservoir for the virus in nature has not been conclusively identified, several studies that have tested animals in Africa for Ebola virus infections are suggestive of a bat-to-human transmission of the virus (17–19, 28). Another important phenomenon in this game of hide and seek, is that the severity of the impact on the human population from the most recent outbreak was incomparable relative to past outbreaks. What started off as a relatively contained epidemic, comprised of just over 100 cases in late March, had spiraled out of control into what the WHO declared to be "an international public health emergency" by early August. As of the fifth of November 2014, the fifteenth outbreak of Ebola had reached a frightening magnitude; 13,042 cases and 4,818 deaths (29) and the end of the outbreak seemed nowhere in sight.

A visual representation published by *The New York Times*, in which the 2014 outbreak is compared to other large outbreaks in the past, clearly depicts the horrifying reality of the epidemic. View the figure at: http://www.nytimes.com/interactive/2014/07/31/world/africa/ebola-virus-outbreak-qa.html?_r=0

Analysis Activity C

Questions

1. How do the predictions you made in Question 5 of Lab Activity B compare to:
    a. the outbreak data as of November 5, 2014?
    b. the current (most recent) outbreak data? (Internet research is required.)

2. What important characteristic(s) would allow for bats to be a likely reservoir for the Ebola virus?

3. Why do you think this outbreak is the worst ever on record? In other words, what is different about this outbreak compared to past outbreaks? In groups of six, gather research that reflects the following factors: (i) physiological/biological, (ii) environmental/ecological, (iii) demographic/geographical, (iv) socioeconomic, (v) cultural, and (vi) scientific/technological.

Part IV – Hot Air

West Africa, 2 August 2014

Dr. Preston's evacuation team gathered at dawn to review the protocol one last time. They could afford nothing less then absolute precision.

The lead medical official lowered the phone and covered the receiver with his hand. "I have the Director of the CDC on the sat phone. He is going to address the team before departure," he said, putting the phone on speaker.

The director's voice filled the small meeting tent. "Good morning everyone," she said. "As you are all well aware, this evacuation operation will be very difficult logistically in terms of containment and care. The safety of all personnel is of the utmost priority, from the time of departure to the moment that you release the patient into the care of the medical team at Emory University Hospital in Atlanta. The evacuation jet is outfitted with multiple layers of protection to contain the virus. The patient's gurney will be placed within a secured plastic tent and the patient will be enclosed in another layer of plastic. All personnel are to be suited in full hazmat gear at all times, without exception. Of course, you know this, you've all done the drills."

Everyone looked around at each other. Nodding heads signaled to the lead medical officer that they were ready to deploy. The lead gave the confirmation.

"Oh, and one more thing," the director added. "Immediately upon landing, you have a debrief meeting after the decontamination protocol. The subject of the meeting is to discuss today's evacuation and make any necessary adjustments for Claire's evacuation next Tuesday. Any questions?"

A junior evacuation specialist nervously raised her hand and spoke up. "This is the first time that an infected individual with Ebola will be stepping on U.S. soil and the social media has exploded into a frenzy of fear and conspiracy theories about this undertaking." Her voice shuddered with unease. "How can we be sure that we are not risking more lives and a potential cross-continental outbreak, just to save these two people? Is this the right decision?" All eyes turned toward her as she awaited a response.

Analysis Activity D

In addition to the two U.S. aid workers, many other Ebola patients have been transported out of Africa to their home countries for treatment. As of January 26, 2015, at least 24 cases had been treated in Europe and the United States with a recovery rate of 79 percent (5/24 patients died). For a map and case list of patients treated outside of Africa, go to http://www.nytimes.com/interactive/2014/07/31/world/africa/ebola-virus-outbreak-qa.html?_r=0.

Questions

1. "We are aiding and abetting Ebola through good intentions." What do you think this statement means? Do you agree or disagree with this statement? Explain.

2. Do you think that trying to save the life of these 24 individuals justifies the potential risk to an unknown number of other lives? Explain.

3. Nancy Writebol of Charlotte, North Carolina, and Dr. Kent Brantly of Morristown, Texas, were the actual, real-life volunteer aid workers who became the first Ebola-infected patients to set foot on U.S. soil in August of 2014. Conduct research to determine the outcome of their cases. What factors do you think contributed to the outcome of their cases?

4. Drawing on comparisons to Africa, what factors prevented the U.S. from becoming the next Hot Zone?

5. What do you think is the likelihood of the Ebola virus becoming established in a reservoir in North America? Explain.

6. Propose reasons to explain why Africa is the "cradle" to so many viruses.

Part V – Life After Ebola

When you hear the word *cases* in the various forms of media that report on infectious disease outbreaks, it must not be forgotten that each case represents a life. The following is a true story.

It started on the March 14th when Mohamed's older brother came from his village 450 km away to seek medical care in Conakry. Naturally, he stayed with his brother and family. Zena also was there and took care of her cousin. The sick man was diagnosed with malaria and typhoid fever in his village, and suffered vomiting, fever, respiratory difficulties, and fatigue. A physician, also a member of the family, arrived to provide first aid in the home. The brother's condition worsened and he was evacuated to the hospital, where he passed away on the 18th of March. The family took the body to the village. People who took care of him, helping in the funeral rituals by washing the body, and people who treated him, all became ill. The sick included Zena on her return to Conakry, Mohamed in the village, the cousin's physician who took care of the patient at the beginning and three uncles who washed the body.

Ebola hit Mohamed and Zena's family hard. By the time it was over, nine members of one family were infected and six had died. The older brother of Mohamed died, followed by his wife and a 10-year-old son. Mohamed was admitted to a hospital in Conakry on the 21st of March along with his wife. There he found Zena and other members of the family. All together they were admitted to the same isolation ward, in difficult conditions. Zena said that they vomited so much that they thought they would never survive. Mohamed said he would never forget the people who took care of them.

One of their doctors back in March was Dr. Rob Fowler, a physician from Canada who was seconded to the WHO and has been treating patients in the field. After Dr. Fowler reunited with Zena and Mohamed, it was very emotional. They huddled closely and recalled their moments together in the hospital. They talked about those who lived and those who died. Zena, a 24-year-old woman who was a school teacher, and Mohamed, a 34-year-old civil servant, both lost their jobs. Zena said she received a phone call from the school telling her not to come back because parents and children were afraid of Ebola contamination. It made her sad because she loved her work and the students. Mohammed said people told him they think that being an Ebola patient means that you are dead, you will never recover, that it will kill you, no matter what you do. And for a time, Zena and Mohamed felt this was true. But through their journey to recovery, they have found new purpose and a way to help others.

Zena and Mohamed now work in their communities, going from place to place to talk to people, families, relatives of the ill, and others about Ebola. They work with Medecins Sans Frontieres (Doctors Without Borders) and others to give talks about what Ebola is, how to prevent infections, and emphasize that people who go to health centres can survive. They are living examples. Stigma and fear still exist but they are proud to give back some of their time and experience to communities. They contribute their personal stories and experience to save lives and help control the outbreak.

Source: Adapted from WHO article at http://www.who.int/features/2014/life-after-ebola/en/

Although each Ebola outbreak has claimed many more lives then there have been survivors, people do survive. With no known cure, treatment largely relies on supportive care, the quality of which can vary greatly among treatments centres depending on the availability of supplies and medical staff, which in turn can greatly influence disease progression. Indeed, the issue of standard of care was a major factor that influenced the decisions to evacuate non-African aid workers. In the cases of Nancy Writebol and Dr. Brantly, both patients were fortunate enough to be provided with the experimental drug, ZMapp, in addition to receiving a high quality of care. However, it is almost impossible to determine the individual impact this treatment made on their recovery.

While the WHO made the decision in August to sanction the use of an experimental drug in light of the severity of the outbreak, only a limited number of doses had ever been produced at that time. This shortage would prove much to the detriment of Africans who were unquestionably affected the most by the outbreak. Africa was left with the extremely difficult choice of deciding which Ebola patients would receive the handful of doses of ZMapp left in the world. In fact, it was not until mid-February of 2015 that the U.S. government was able to begin shipping small batches of ZMapp for testing in West African patients (30, 37).

While waiting for the months it would take to manufacture even a small stock, could there have been another way to treat Ebola patients? In Part I of this case study, you learned that Dr. Brantly also received a blood transfusion as part of his treatment. The blood was donated from a 14-year-old male Ebola patient who had been under his care, and survived. The principle behind the treatment is that the blood plasma of a convalescent (survivor), concentrated with antibodies from an immune response proven in surmounting the infection, may "passively" confer the same immunity when transfused into the blood stream of another infected patient.

Interestingly, in the last phase of the 1995 Ebola epidemic in Kikwit, DRC, the treatment of a small cohort (n=8) of humans with blood transfusions donated from convalescent patients demonstrated a survival rate of almost 90 percent, where transfusions were performed an average of 10 days (range 4–15 days) after the onset of symptoms (31). In the case of ZMapp, the efficacy of the drug has only been tested in non-human primates, and achieving success that exceeds the average survival rate of infected patients depends largely on the drug being administered within one hour post-infection (32). Similar to the (currently limited) use of ZMapp in humans, there is virtually no way to prove a causative relationship between survival rate and treatment in the Kikwit case.

Notwithstanding this unanswered question of efficacy, there is another issue to ponder. Considering the prospect of the Kikwit results and the potential to achieve them within a much larger window of opportunity for treatment administration compared to the ZMapp trials, and taking into account the considerable delay in the future production and distribution of ZMapp and the extremely virulent nature of the virus, why haven't the WHO and the CDC considered convalescent blood transfusions to be a viable treatment option worth employing against Ebola? Interestingly, passive immunotherapy is the standard of care for other infectious diseases such as influenza (33, 34), and blood transfusions are simple and common procedures. Could Ebola survivors be the solution hiding in plain sight?

Analysis Activity E
Questions

1. Suppose that medical officials at the WHO did not sanction the use of an experimental intervention in the Ebola outbreak. Do you think that infected Ebola patients should have the right to receive an experimental drug or treatment if they give their consent?

2. ZMapp was the first experimental treatment to be administered to humans infected with the Ebola virus.
   a. Although the WHO authorized the use of the drug, what ethical issues does administering an experimental treatment to humans raise?
   b. Do the potential benefits outweigh the possible risks? Explain.

3. Passive immunity is the transfer of active antibodies against a disease from one person to another. One recommendation made from the 1995 Kikwit case study was for researchers "to prepare for a more thorough evaluation of passive immunity therapy during new Ebola virus outbreaks." However, before treatment interventions can be approved for use in humans, they must pass rigourous testing standards in animal experimental models.
   a. Design an experiment to determine the effects of passive immunotherapy in animals infected with Ebola. The experiment should yield results that can be used to justify the use of this therapy in humans. Consider the following in your experimental design:
      • Which animal model would be the best to use and why?
      • What observations and measurements should be taken?

- What are the controls in the experiment?
- What confounding factors could affect the results and how can they be addressed in the experimental design?
- What criteria would signify that the experiment was successful?
- What safety precautions are necessary to conduct such an experiment?

b. Experimentally testing the merits of passive immunotherapy in humans may be virtually impossible, considering that, historically, Ebola outbreaks have occurred sporadically, with few infections (relative to other infectious diseases), and have killed the majority of those infected. Thus, obtaining enough plasma from convalescent patients during an outbreak to treat others involved in that same outbreak is highly unlikely (35). Propose a possible solution to overcome this.

4. Currently, there are no approved and effective drug treatments or vaccines against the Ebola virus.
    a. Propose reasons to explain this, despite the virulent nature of the Ebola virus and the fact that there have been repeated outbreaks over the past forty years.
    b. What recommendations would you make for future research efforts in the fight against the Ebola virus in Africa? Consider the following aspects: surveillance, detection, diagnosis, containment, and treatment.

5. Peter Piot was one of the co-discoverers of the Ebola virus back in 1976 when the first outbreak surfaced in Africa. Throughout his career in various global health initiatives aimed at fighting infectious diseases, he has always maintained the philosophy that it is beneficial to protect those who do not have the resources to protect themselves. By protecting them, we (those who have resources) would be protecting ourselves.
    a. Do you agree or disagree with this philosophy? Explain.
    b. To what extent does Canada's (or your country's) involvement in international infectious disease initiatives reflect Peter Piot's philosophy?

☙

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