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A Retrospective Analysis of Ebola Virus Disease in Liberia

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A RETROSPECTIVE ANALYSIS OF EBOLA VIRUS DISEASE IN LIBERIA

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by
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APPROVAL OF DISSERTATION

We, the faculty supervising the work of Matthew Jeremiah Matson, affirm that the dissertation, *A retrospective analysis of Ebola virus disease in Liberia* meets the high academic standards for original scholarship and creative work established by the Biomedical Sciences Program and the Graduate College of Marshall University. This work also conforms to the editorial standards of our discipline and the Graduate College of Marshall University. With our signatures, we approve the manuscript for publication.

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DEDICATION

To my wife, Sarah, and my children – Arey, Ira, Elaia, and Orli – for backing me all the way, patiently enduring, and gracefully taking all things in stride. And to my parents, Mark and Rhonda, who graciously provided so many good opportunities for me.

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ABSTRACT

The outbreak of Ebola virus disease (EVD) centered in the West African countries of Guinea, Liberia, and Sierra Leone from 2013-2016 was the longest and largest such outbreak on record. At least 30,000 cases were reported, and the outbreak lasted nearly two-and-a-half years. Despite the progress made during and since that time, many questions remain unanswered. The studies here utilize a large set of nearly 750 whole blood samples from patients that were triaged and treated at the ELWA-3 Ebola treatment unit in Monrovia, Liberia in 2014 and 2015. Viral load, as approximated by qRT-PCR diagnostic assays in the field, has been shown to correlate with outcome, with higher viral loads increasing the risk of death. Here, viral load has been more accurately estimated by droplet digital PCR and shown to likely provide improved prognostic. These reanalyzed viral load data furthermore suggest that earlier initiation of supportive care, prior to peak viremia, did not improve outcomes in EVD patients at ELWA-3. Concern has also arisen regarding the possibility of bacteremia developing secondary to EVD. The samples here were analyzed using both a classic diagnostic microbiological approach and a targeting deep sequencing approach to evaluate this hypothesis, and bacteremia was found to be rare in EVD patients, with no increase in prevalence upon comparison to an EVD-negative cohort. Virological studies were also undertaken that involved isolating Ebola virus (EBOV) from the clinical samples, evaluating the isolation sensitivity of cell lines for these purposes, and analyzing the sequences obtained from EBOV isolates in comparison with sequences directly from the blood. Together, these data should further our understanding of both EVD and EBOV and possibly inform improvements in patient care.

CHAPTER 1: INTRODUCTION

In December of 2013, what would become the largest and longest outbreak of Ebola virus disease (EVD) on record most likely began with a 2-year-old boy in rural Guinea (Timothy et al., 2019). The precise circumstances surrounding the presumed zoonotic spillover of Ebola virus (EBOV) to the boy that initiated his infection remain unknown (Saez et al., 2015), as they do for all other EBOV spillover events. The young boy died of EVD, along with his pregnant mother, his sister, his grandmother, and numerous other family members and healthcare workers within a matter of weeks (Timothy et al., 2019). For nearly three months following that, spread of the virus went undetected and unabated. EVD had only historically been known 1,500 miles to the southeast in the Congo River basin and is easily confused for other severe diseases, even in places familiar with it. Thus, other infections were initially suspected. Soon cases were reported in the neighboring countries of Liberia and Sierra Leone, which would join Guinea to become the epicenter of the 2013-2016 West Africa EVD outbreak. Ultimately, the size of the outbreak was two orders of magnitude greater than any previous outbreak, with over 33,000 cases reported (Forna et al., 2020), and EVD patients were present in 15 total countries, either from spread or for treatment (Matson et al., 2020b).

From August 2014 to March 2015, more than a dozen members of the Laboratory of Virology at the NIH/NIAID Rocky Mountain Laboratories (RML) volunteered to staff the on-site diagnostic laboratory at the Eternal Love Winning Africa 3 (ELWA-3) Ebola treatment unit (ETU) in Monrovia, Liberia together with other volunteers from the CDC. This remains the largest ETU ever built to date, and provided care to over 1,800 EVD patients – a staggering figure considering that from the discovery of EBOV in 1976 (WHO, 1978) until the West Africa outbreak, a cumulative total of 1,395 EVD patients had been documented (Matson et al., 2020b).

Samples collected for diagnostic purposes at ELWA-3 were stored on-site until they could be transported to secure biosafety level 4 (BSL-4) facilities for long-term storage and possibly further study. A subset of these samples was transported to RML and stored at -80 °C and accompanied by a deidentified patient database containing demographic, geographic, diagnostic, and outcome data. The samples remained untouched prior to the studies undertaken and reported here.

Ebola virus

EBOV, member of species *Zaire ebolavirus*, is in the family *Filoviridae* (collectively referred to as 'filoviruses'), named for the unique filamentous shape of the viral particles. Other clinically relevant members of the family are Sudan virus (SUDV), Bundibugyo virus (BDBV), and Taï Forest virus (TAFV) – each member viruses of different species within the genus *Ebolavirus*, which together comprise the ebolaviruses. Marburg virus (MARV) and Ravn virus (RAVV), both members of species *Marburg marburgvirus*, constitute the remainder of the currently known clinically relevant viruses in the family *Filoviridae* (Figure 1.1). All of these viruses are enveloped and possess a single-stranded, non-segmented, negative-sense RNA genome. The clinical disease caused by these viruses is similar, but case fatality rates differ, with EBOV thought to be the deadliest.

The EBOV genome is approximately 19,000 nucleotides in length and is comprised of 7 genes: nucleoprotein (NP), viral protein 35 (VP35), viral protein 40 (VP40), glycoprotein (GP, which primarily produces the transcript for secreted GP [sGP], but also undergoes transcriptional

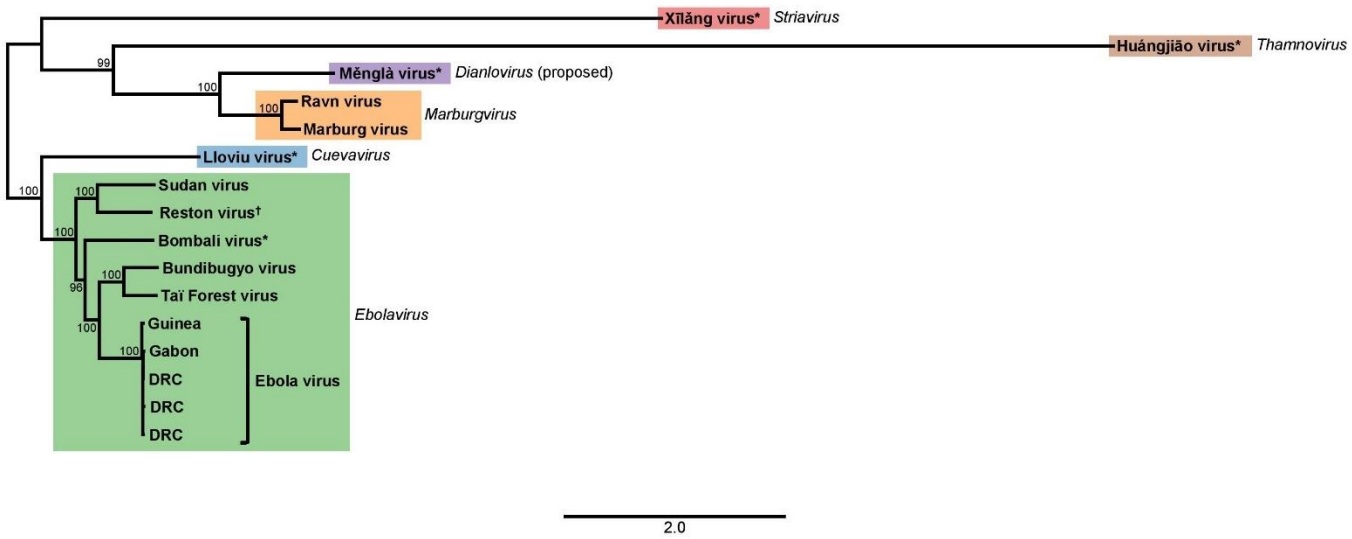

Figure 1.1. Phylogenetic tree of viruses in the *Filoviridae* family.

Sequences were aligned using Clustal Omega and a maximum-likelihood phylogenetic tree was generated with PhyML using a generalized time-reversible substitution model and 1,000 bootstrap replicates. Multiple variants of Ebola virus (i.e. isolates obtained from spillover events in Guinea, Gabon, and DRC) are shown for context.

editing to produce transcripts for transmembrane GP and small secreted GP [ssGP] – the functions of which are thought to include immunomodulation) (Mehedi et al., 2011a; Sanchez et al., 1996), viral protein 30 (VP30), viral protein 24 (VP24), and polymerase (L) (Figure 1.2). The only surface protein present on the EBOV virion is GP, which is trimer of heterodimers that facilitates viral entry as part of a series of incompletely understood mechanisms. This is thought to take place primarily by macropinocytosis via interactions of C-type lectins with glycosylated regions of GP in conjunction with TIM and TAM receptor binding of phosphatidylserine present in the EBOV viral envelope (Feldmann et al., 2001; Kuroda et al., 2015). Following internalization of the EBOV virion and pH-dependent proteolytic processing of GP, GP binds with the intracellular endosomal receptor, Niemann-Pick C1 (NPC1), and facilitates fusion of the virion with the endosomal membrane and release of the viral nucleocapsid into the cytoplasm of the host cell (Hoenen et al., 2019; Moller-Tank & Maury, 2015). Transcription of the viral genome is then initiated by EBOV's RNA-dependent RNA polymerase, together with viral co-

factors including VP35, VP30, and NP. Seven monocistronic EBOV mRNA transcripts are produced in a decreasing gradient from 3' to 5', as the polymerase only associates with the genomic RNA template at a single binding site at the 3' terminus and often dissociates from the template upon reaching stop codon during transcription (Muhlberger, 2007). Replication of the viral genome begins with production of a positive-sense reverse complement (complementary RNA or cRNA) of the genomic RNA that serves as a template for producing negative-sense genomic RNA that will be packaged into virions with viral proteins for release from the host cell (Muhlberger, 2007).

Ebola virus disease

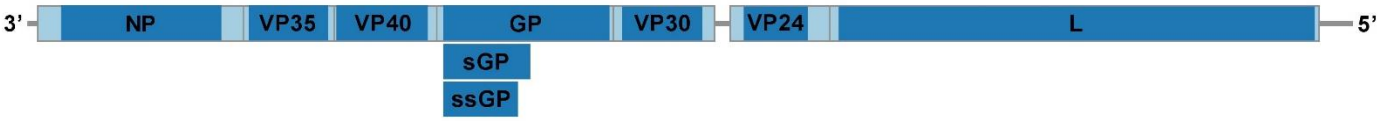

Figure 1.2. Ebola virus genome.

Dark blue regions indicate approximate CDS; light blue regions indicate untranslated regions. In addition to the large un-transcribed intergenic region (143 nts) between VP30 and VP24 that is visible in the diagram, very short un-transcribed intergenic regions that are not visible in the diagram are also present between NP and VP35 (5 nts), VP40 and GP (5 nts), and VP24 and L (4 nts).

EVD Prior to the 2013-2016 West Africa outbreak, EVD was referred to as 'Ebola hemorrhagic fever' (EHF) (Chertow et al., 2014). While hemorrhagic features (e.g. petechiae, epistaxis, bleeding from gums, bloody diarrhea, oozing from venipuncture sites, etc.) are sometimes present in EVD, they are relatively rare overall. EVD is characterized by non-specific constitutional signs and symptoms initially, followed by voluminous diarrhea, vomiting, vascular leakage, coagulopathy, systemic inflammation, multiorgan failure, and shock (Chertow et al., 2014). Case fatality rates average 60-70% (Matson et al., 2020b). The incubation period is typically 3-13 days, and up to 21 days (Kortepeter et al., 2011).

EVD has no pathognomonic features and cannot be reliably diagnosed on clinical grounds alone. During the first EVD outbreak in 1976 in DRC (Zaire at the time), EBOV was identified as the etiological agent and as a previously unknown virus using electron microscopy and immunofluorescence antibody testing (Breman et al., 2016; "Ebola haemorrhagic fever in zaire, 1976," 1978). Since the mid-1990's, PCR-based approaches have been the gold-standard for EVD diagnostics. Initially, conventional reverse-transcription PCR (RT-PCR) was employed until the development of real-time reverse-transcription PCR (qRT-PCR) in the early 2000's. ELISA-based approaches, designed to detect IgM, IgG, and/or viral antigen have also been historically used for diagnostic purposes (Broadhurst et al., 2016). More recently, antigen-based rapid diagnostic tests (RDTs) have been developed and offer faster results and increased portability compared to qRT-PCR (Couturier et al., 2020), although RDT results are currently considered presumptive until confirmed by qRT-PCR. Importantly, only cell-culture based diagnostic approaches, which are virtually never used given the necessity of BSL-4 containment, can assess for the presence of viable, infectious virus.

Human-to-human transmission of EBOV is thought to primarily occur via direct contact with the bodily fluids (primarily blood, vomit, and feces) of an infected individual, with transmission from respiratory droplets and fomites also possibly playing a role (Bausch et al., 2007; Judson et al., 2015). Epidemiological evidence does not support the transmission of EBOV via an aerosol route in a typical community-based setting. During the 2013-2016 West Africa outbreak, strong evidence implicated sexual transmission via the semen of persistently infected, convalescent males to females. In one instance in Liberia, a woman likely became infected with EBOV following a sexual encounter with a male that had recovered from EVD nearly 6 months earlier (Mate et al., 2015); in another case in Guinea, a woman is thought to have been infected

from a sexual encounter with a male that had recovered from EVD well over a year prior (Diallo et al., 2016). Viable, infectious EBOV has also been recovered from other bodily fluids following convalescence, including cerebrospinal fluid (Jacobs et al., 2016), aqueous humor (Varkey et al., 2015), and urine (Kreuels et al., 2014), though no transmission is thought to have occurred in these instances.

Treatment for EVD has historically been limited to basic supportive care. During the West Africa outbreak, more advanced levels of supportive care were standardized and included both oral and intravenous fluid resuscitation, antiemetics and antidiarrheals, vasopressors, electrolyte and glucose monitoring and management, and empiric treatment of potential malarial and bacterial co-infections. During the West Africa outbreak, experimental drugs, including both small-molecule antivirals and monoclonal antibodies, were administered under compassionate use protocols (Uyeki et al., 2016). More recently, during the EVD outbreak in eastern DRC from 2018-2020, many of these drugs were assessed using randomized controlled trials, with the monoclonal antibodies Mab114 and REGN-EB3 showing most promise (Mulangu et al., 2019).

EVD vaccines, which had previously only been in preclinical development, were also assessed in clinical trials for the first time in an outbreak setting at the very end of the West Africa outbreak. One of the vaccines used, a recombinant vesicular stomatitis vector expressing EBOV Kikwit glycoprotein (GP) (rVSVΔG-ZEBOV-GP) manufactured by Merck that can be delivered as a single dose or as a homologous prime-boost regimen, was widely utilized during the 2018-2020 eastern DRC outbreak, and has since received FDA and European Medicines Agency (EMA) approval (Callaway, 2019; FDA, 2019b). Additionally, Johnson and Johnson's human adenovirus serotype 26 vector expressing EBOV Mayinga GP that is followed by a multivalent modified vaccinia virus Ankara vector expressing EBOV Mayinga GP, SUDV Gulu

GP, MARV virus Musoke GP, and TAFV nucleoprotein as a heterologous prime-boost was also used in the 2018-2020 DRC outbreak, and received EMA approval in July, 2020 (Ilunga Kalenga et al., 2019; MRC/UVRI, 2019).

Ebola virus: a uniquely challenging hemorrhagic fever virus

EBOV is considered to be the prototypical hemorrhagic fever virus (Feldmann & Geisbert, 2011). As discussed above, the term 'hemorrhagic fever' is somewhat of a misnomer, however, as overt hemorrhage is rarely present in the disease manifestations of these viruses. Nevertheless, the nomenclature has remained as a useful way to categorize viruses that can elicit a similar, severe disease manifestation. The hemorrhagic fever viruses are found on every continent except Antarctica (Figure 1.3) and include a diverse array of more than 30 different viruses from six different viral families (Figure 1.4). They range from extremely rare viruses that have only caused a handful of known infections (e.g. SBAV, LUJV) to the perennial scourges of YFV and DENV. All are single-stranded RNA viruses, though both positive-sense and negative-sense species are present. Many are arboviruses, transmitted to humans by the bites of mosquitoes or ticks, while others require close contact with an infected reservoir or intermediate host species. All are maintained or presumed to be maintained in wildlife or domesticated animals. Some of the viruses that are technically considered hemorrhagic fever viruses only very rarely cause a severe disease in patients, instead tending to elicit milder signs and symptoms with CFRs < 1%; EBOV, on the other hand, often causes very severe disease with case fatality rates of > 50% (Figure 1.4) (Chertow et al., 2014; "Ebola haemorrhagic fever in zaire, 1976," 1978; Feldmann & Geisbert, 2011; Ilunga Kalenga et al., 2019; Kratz et al., 2015; Maganga et al., 2014; Muyembe-Tamfum et al., 1999; Nsio et al., 2020; WHO, 1978).

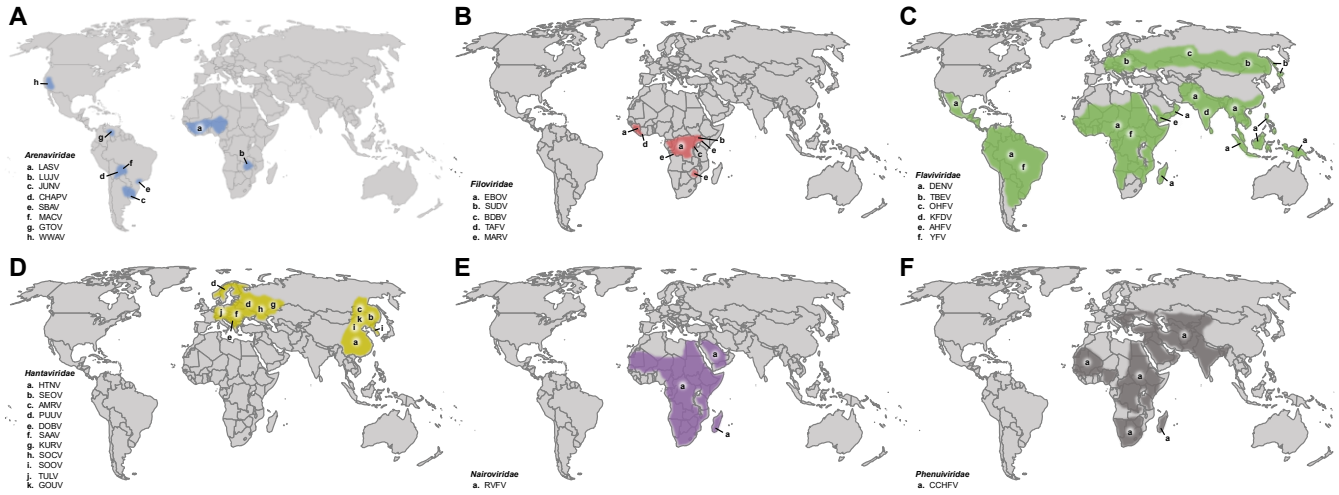

Figure 1.3. Approximate geographic range of the hemorrhagic fever viruses by family.

Some of the ranges overlap for the specific viruses, indicated by the lowercase letters, particularly amongst the flaviviruses (C) and the hantaviruses (D). A, *Arenaviridae*; B, *Filoviridae*; C, *Flaviviridae*; D, *Hantaviridae*; E, *Nairoviridae*; F, *Phenuiviridae*. For full information regarding virus abbreviations, see Appendix 2.

Based on the locations of where spillovers to humans have occurred, EBOV and the other filoviruses have a relatively narrow geographic range compared to the other hemorrhagic fever viruses (Figure 1.3). However, the biodiversity within this range is extraordinarily high, and the ecology of EBOV is particularly enigmatic. EBOV is presumably maintained in a zoonotic reservoir, but after more than 40 years since the first known outbreak in 1976, it is yet to be isolated from any animal, and not for lack of effort. Many resources have been devoted to sampling wildlife in the search for the EBOV reservoir(s), with tens of thousands of samples collected and analyzed from vertebrates and arthropods over the decades since its discovery (Breman et al., 1999; De Nys et al., 2018; Groseth et al., 2007; Leirs et al., 1999; Leroy et al., 2005; Reiter et al., 1999). Various bat species are suspected EBOV reservoirs, as at least nine different species, both insectivorous and frugivorous, have been shown to carry anti-EBOV

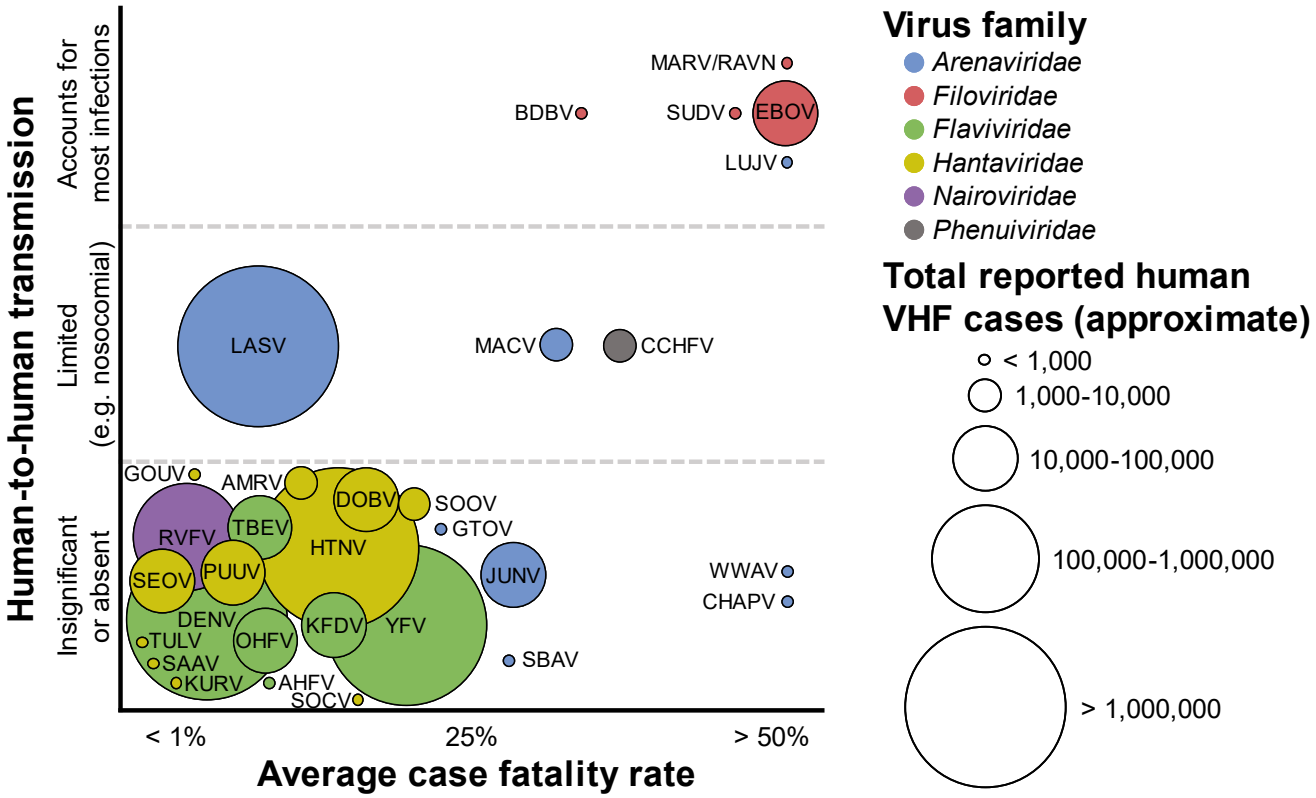

Figure 1.4. Level of human-to-human transmission and approximate case fatality rates and overall cases for the hemorrhagic fever viruses.

Some of the hantaviruses are extremely similar with many only recently classified as distinct members of separate species, thus have likely historically been mistaken for one another. Case counts should therefore be interpreted cautiously. Positioning along the x-axis is continuous – the relative position of the viruses indicates their approximate case fatality rates of anywhere from < 1% to greater than 50%. Positioning along the y-axis is categorical – the height of a virus within one of the three given categories is irrelevant. Case numbers are extremely small (< 50) for some viruses (e.g. WWAV, LUJV, etc.), so their case fatality rates and human-to-human transmissibility are general estimates only.

antibodies and three of these have also tested positive for EBOV RNA (Olival & Hayman, 2014). EBOV is suspected to naturally infect other mammalian species as well, particularly non-human primates, and has been implicated in massive die-offs of some of these animals (Bermejo et al., 2006). These are thought to be dead-end hosts for EBOV, however, and conclusive evidence elucidating the true reservoir(s) remains elusive. In contrast, natural reservoirs have been identified for virtually all of the other hemorrhagic fever viruses, and in many cases their discovery has facilitated efforts to mitigate spread to humans. Given that EBOV only

sporadically spills over into humans, it is unclear whether definitively identifying the natural reservoir(s) will offer significant public health benefit, as preventing the already rare event of EBOV spillover may prove untenable. Thus, EVD outbreaks are likely to pose a continued threat, and indeed have been occurring with increasing frequency over the past two decades.

The West Africa EVD outbreak

The West Africa outbreak raised countless questions, many of which remain unanswered. Perhaps the most pervasive and contentious questions center on the role of supportive care in the management of EVD. Historically, the modus operandi of EVD outbreak response was containment, not treatment. However, during the West Africa epidemic, increasingly sophisticated and standardized levels of supportive care were introduced to ETUs, with the goal of significantly improving patient outcomes while also containing the spread of the virus. This supportive care generally consisted of fluid resuscitation (oral and/or intravenous), anti-diarrheal and anti-emetic medication, vasopressors, glucose and electrolyte monitoring and management, and empiric treatment with antibiotics and antimalarials (WHO, 2019d), although the specific protocols at each ETU varied. The hope was that early initiation of these interventions would give patients the edge needed to fight the infection and recover. This new standard of care was generally hailed as a success, with many speculating it played a large role in reducing the naïve CFR reported to be well below historical norms at approximately 40% – a figure that is still widely reported today (Fischer et al., 2019; Furuse et al., 2017; Lamontagne et al., 2018; Langer et al., 2018; Schieffelin et al., 2014; Uyeki et al., 2016; WHO, 2019d; Zhang et al., 2015).

However, recent analyses of the data have called this CFR figure into question and have convincingly shown that it was likely well within the historical norms of 60-70%, and possibly even higher. This, if true, undermines the narrative that supportive care, at the level broadly

available in West Africa, significantly reduced EVD mortality. Nevertheless, the most advanced supportive care available (including mechanical ventilation, dialysis, extracorporeal membrane oxygenation, etc.) – which was not available in West Africa – likely does offer a survival benefit for EVD, as the CFR for the 27 EVD patients treated in the US or Europe was 18.5% (Uyeki et al., 2016). However, the benefit even here is somewhat uncertain, as 23 of these 27 patients received one or more investigational therapies (e.g. antivirals and antibody-based drugs) together with advanced supportive care.

The questions addressed

We decided to investigate two primary questions related to these topics utilizing the sample set from ELWA-3. First, as the viral load in EVD has been shown to correlate with outcome, but has only been inferred from calculations using C_t values from a large array of different assays, we decided to more accurately and consistently estimate the viral load in as many of the EVD patient samples as possible using a novel droplet digital PCR (ddPCR) assay. The findings of this would then be correlated with outcome and various other epidemiological parameters to further explore the role of viral load on the course of disease, particularly in relation to the time at which supportive care was initiated relative to viral load.

Secondly, as part of the supportive care regimen for EVD includes the empiric administration of broad-spectrum antibiotics out of a concern that bacterial sepsis may routinely complicate EVD, we sought to evaluate the sample set for the presence or absence of pathogenic bacteria. The role of sepsis in severe EVD is yet to be systematically studied, although the hypothesis that bacterial may translocate from the gut through compromised mucosa has widely discussed in the literature (Carroll et al., 2017; Kreuels et al., 2014; Lamb et al., 2015; Lamontagne et al., 2018; Matson et al., 2020a; Reisler et al., 2018; Wolf et al., 2015). Similar

phenomena have been reported with infections of *P. falciparum* parasites, including an increased occurrence of gram-negative sepsis in patients with severe malaria (Decuypere et al., 2016). For EVD, profound inflammation capable of disrupting the intestinal architecture would offer a plausible explanation for bacterial translocation. However, this is typically not what is observed in EVD; the voluminous watery diarrhea is more consistent with a secretory process, and minimal inflammation is present in the lamina propria of the small intestine upon autopsy (Martines et al., 2015). Nevertheless, clinically significant bleeding from the gastrointestinal tract is observed in a small subset of patients (Chertow et al., 2014), and could provide evidence for a combined secretory and inflammatory process, which would seemingly be more consistent with the development of sepsis. To look for evidence of bacterial translocation in the blood samples, both classical microbiology and targeted deep sequencing would be employed.

A third question which was also approached utilizing the ELWA-3 samples sought to address more virological questions. Every EBOV isolate that is used for animal modeling and in vitro studies has come from human clinical samples, where an aliquot of the sample (usually blood) is placed on suitable cells (usually Vero E6 cells, derived from African green monkey kidneys) and virus is harvested after replicating to high titers in the cell culture. Well-document adaptations can occur in EBOV following serial passage on cell lines (including Vero E6 cells and others), but whether adaptations are evident immediately upon isolation from clinical specimens had not been previously addressed. These adaptations can occur both at a consensus genetic level and at the level of single nucleotide variants (SNVs), which reflect the so-called ‘quasispecies’ diversity that is present. Adaptations at both of these levels can have important implications for the phenotypic characteristics of the virus, both in vivo and in vitro. We were ideally situated to attempt to address some of these questions with the large set of EVD patients

in storage. Furthermore, these findings would inform the suitability of using sequences from isolated viruses for phylodynamic analysis. This had always been the approach prior to West Africa – EBOV was first isolated from samples to create a pure, high-titer sample that could easily be sequenced and then used for phylogenetic analysis and other genetic studies. However, during the West Africa outbreak, the vast majority of sequences were produced directly from patient samples thanks to advances in deep sequencing technology. It was thus unknown if virus isolation would immediately induce genetic changes in the virus that would render the sequences unsuitable for comparisons within the larger framework of West Africa EBOV phylogeny. Sequencing directly from the stored ELWA-3 samples was found not to be an ideal approach given the degradation that had occurred in the samples.

Specific aims

The aims of this dissertation were as follows:

1. To quantify the EBOV genome copy number in the ELWA-3 sample set with a droplet digital PCR assay, and correlate the findings with outcome and other epidemiological parameters
2. To determine the prevalence of bacteremia in the ELWA-3 sample set, with EVD-negative patients as a control population, using both a classical microbiological approach and targeted deep sequencing
3. To isolate and sequence EBOV from as many of the clinical samples as possible and utilize the sequence data to investigate adaptations, or a lack thereof, upon isolation and perform a phylodynamic analysis of the outbreak in Monrovia and the surrounding area

Chapter outline

Chapters 2, 3, and 4 are comprised of the bulk of the experimental data obtained from working with the stored ELWA-3 patient samples and constitute the core of this dissertation. Chapters 2 and 3 are each divided into two sections. The first sections describe the development and optimization of the protocols that were utilized for the given chapter, as these chapters each required a moderate amount of preliminary work prior to initialization of the primary studies. The second sections consist of the focal questions being addressed and the study or studies undertaken with the EVD patient samples. In Chapter 2, aim 1 is addressed and the viral loads of EVD patients are explored in detail, utilizing a droplet digital PCR platform with a novel assay developed for this study designed to more accurately quantify viral load by excluding viral mRNA. The viral load data is then utilized to explore a number of epidemiological questions.

Chapter 3 addresses aim 2. A retrospective cross-sectional approach is employed, with the prevalence of bacteremia in EVD-positive patients compared with that in EVD-negative patients. Only triage (first-draw) samples were utilized in order to avoid the confounding factor of antibiotics, which were administered empirically to all EVD-positive patients following admission. We analyzed the entire sample set using a classic microbiological approach, and analyzed a set of 90 age, sex, and time-from-symptom-onset matched samples (45 EVD-positive and 45 EVD-negative) using a state-of-the-art targeted deep sequencing approach. While our 'control' population (the EVD-negative group) was not comprised of healthy controls (to have a sample taken at an ETU, certain signs and symptoms consistent with EVD had to be present), it had the advantage of being matched geographically and demographically with the EVD patients.

Chapter 4 addresses aim 3, although focuses almost exclusively on the first half of the aim (isolation and sequencing, and investigation of adaptations). The coronavirus disease 2019

(COVID-19) pandemic shifted our research priorities, and we had to delay the sequencing of approximately 140 remaining EBOV isolates on which the phylodynamic analysis (the second half of the aim) depended. That study will hopefully resume in the near future and will undoubtedly provide useful and interesting data.

In Chapter 5, the West Africa EVD epidemic is put in context with all the other EVD epidemics since 1976. In it, we make the very basic, yet novel, observation that the initial period in which EVD outbreaks go undetected has a profound impact on how the outbreak is likely to progress in terms of size and duration. Moreover, we also find that very little, if any, progress has been made in detecting EVD outbreaks more quickly, despite the diagnostic revolution offered by qRT-PCR over two decades ago. Indeed, many of the longest delays in recognizing EVD have come in the past decade, including the West Africa epidemic. This is a fundamental public health shortcoming, and addressing it offers benefits that likely far outweigh whatever improvements we can make in the treatment of patients once infected, whether with supportive care or antibiotics or new drugs.

Finally, chapter 6 provides a brief summarizing discussion of the most salient points of the previous chapters and suggests ways that the questions being addressed here may continue to be probed with future studies.

CHAPTER 2: ELWA-3 EBOLA VIRUS DISEASE VIRAL LOAD AND EPIDEMIOLOGY

§ 1 – Development and Optimization of Protocols

Aliquots from the whole blood human EVD samples in long-term storage in the BSL-4 facilities at RML were to be first inactivated according to standard operating procedures and then removed from BSL-4 for extraction and processing in BSL-2. A limited number of reagents are currently approved for the inactivation of blood samples with the downstream intent of nucleic acid extraction and include AVL/ethanol (as part of the Qiagen Viral RNA Mini Kit) and TRIzol reagent. Typically, AVL/ethanol is preferred for its simplicity and less hazardous nature. However, we had two priorities when optimizing the protocols for this study: (1) maximize yield of viral RNA extracted, and (2) simultaneously extract DNA to use for possible future studies (e.g. host polymorphisms). Maximizing the yield of the viral RNA that was extracted was deemed much more of a priority, so a series of mock extractions was performed to test the AVL/ethanol protocol and the TRIzol protocol side-by-side. A 10-fold serial dilution of stock EBOV-Makona C07 was made in plain DMEM, from undiluted through to a 1:1,000,000 dilution. 100 µL of each dilution was then added to 900 µL of whole non-human primate blood in EDTA. Inactivation and extraction were performed in triplicate for both AVL/ethanol and TRIzol on each of the 7 virus-spiked blood samples. For AVL/ethanol, the manufacturer's instructions (Qiagen Viral RNA Mini Kit) were followed, which included using 140 µL of sample and eluting in 60 µL of the elution buffer provided (Buffer AVE). For the TRIzol samples, the 140 µL of sample was also extracted using 1.26 mL TRIzol reagent, 252 µL chloroform for phase separation, Phasemaker gel separator tubes, and Thermo Fisher PureLink extraction columns – rather than the traditional precipitation-based approach for nucleic acid

extraction from TRIzol, which is less reliable for very low concentrations of nucleic acids. Elution for the TRIzol samples was performed with three sequential washes of 35 µL of 10 mM Tris pH 8.0, for a total eluent volume of 105 µL.

The amount of EBOV RNA in the extracts was then assessed by the laboratory's standard one-step qRT-PCR targeting a region in the L (polymerase) gene. Total RNA was not assessed (e.g. by NanoDrop, Qubit, etc.), as host RNA was not of interest. C_t values were consistently lower from the TRIzol extracted samples throughout the range of dilutions, particularly at the higher dilutions. At the maximum dilution (1:1,000,000), the EBOV RNA was still detected in all three replicates of the TRIzol-extracted samples but was only detected in one of the AVL/ethanol extracted samples (Figure 2.1). Thus, the TRIzol-based inactivation and extraction protocol was selected as the preferred method.

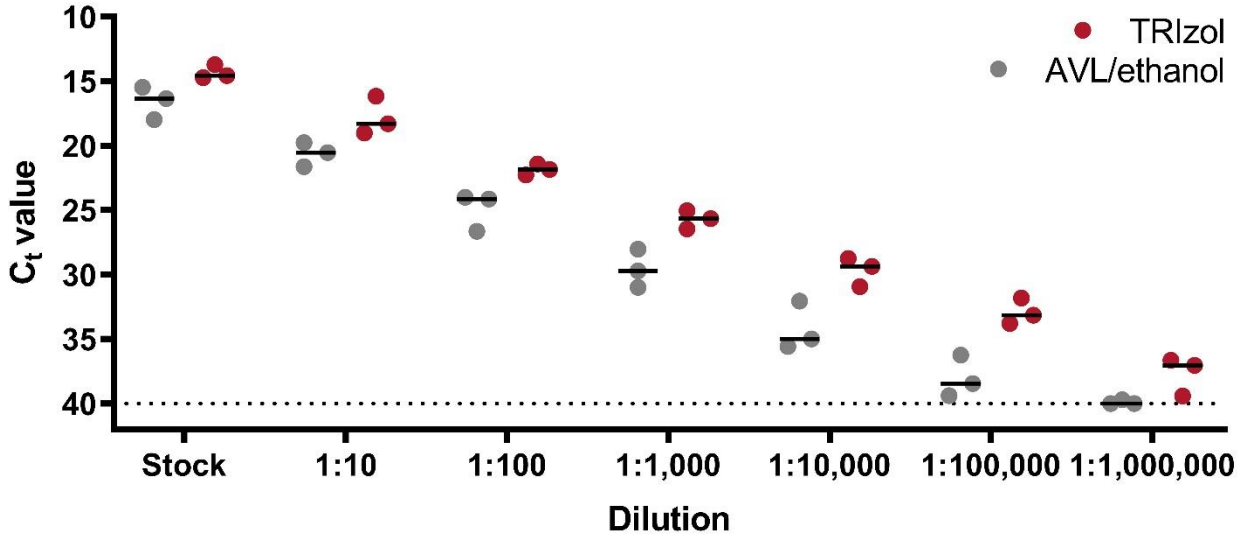

Figure 2.1. Comparison of AVL/ethanol with TRIzol extraction.

Serial 10-fold dilutions of stock EBOV-Makona C07 were made and added to whole non-human primate blood (100 µL virus dilution to 900 µL blood). Extractions for each protocol were performed in triplicate and extracts were analyzed by a one-step qRT-PCR assay targeting a region of the L gene.

Next, a one-step vs. two-step protocol was assessed on the ddPCR instrument. A one-step protocol is advantageous in that it is quicker, but a two-step assay allows for optimization of the cDNA generation step. Extracted EBOV viral RNA from a Makona C07 stock was first diluted in a 10-fold series. For the one-step assay, the Bio Rad One-Step RT ddPCR Advanced Kit for Probes was used and is the only kit available that is recommended for use by the manufacturer of the ddPCR instrument. For the two-step assay, cDNA was first generated from the viral RNA extract using the Invitrogen SuperScript IV kit with random hexamers for priming, followed by an RNase H step after reverse transcription to remove the RNA from the RNA/DNA hybrid molecules. The cDNA was then analyzed by ddPCR using the Bio Rad ddPCR Supermix for

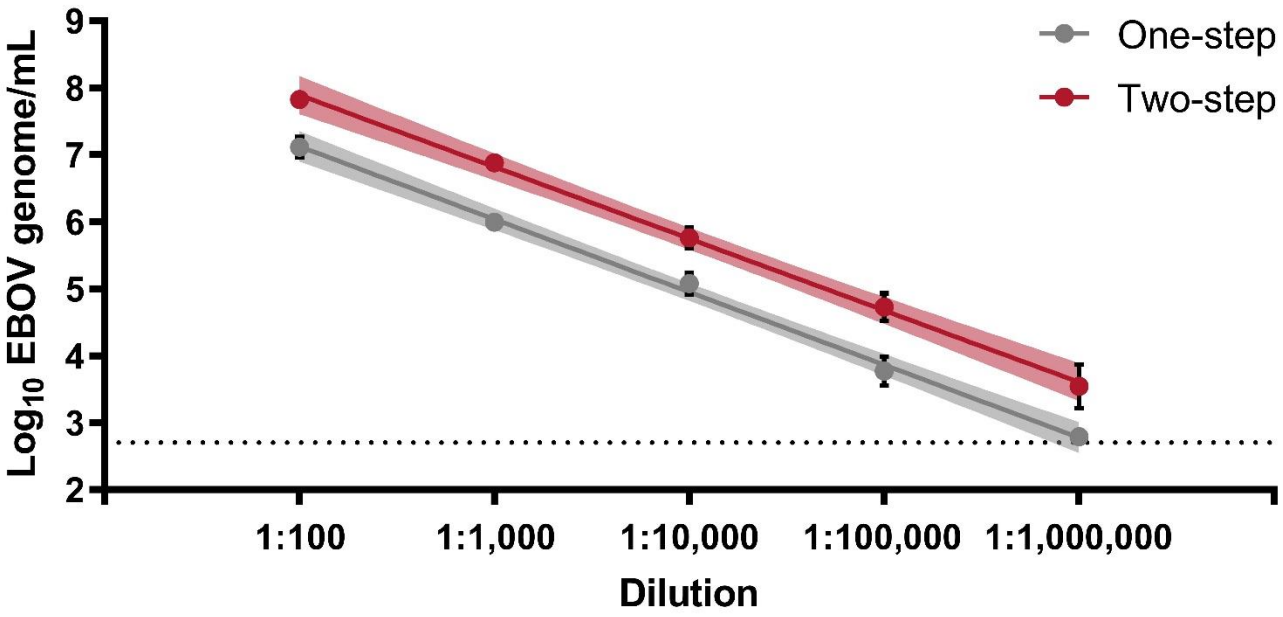

Figure 2.2. Comparison of a one-step and a two-step ddPCR assay for the quantification of EBOV RNA.

Dilutions prior to 1:100 are not shown, as they were outside of the dynamic range of the ddPCR instrument. Each point represents the mean of three independent replicates, and error bars show standard error of the mean.

Probes with no dUTP. Both the one-step and two step assays targeted the same region of the L gene as the qRT-PCR assay and each dilution was tested in triplicate. The two-step ddPCR assay

provided higher quantification across all dilutions tested and was selected to be used for the study to maximize quantification efficiency (Figure 2.2). This is likely due to an increased efficiency of the SuperScript IV reverse transcriptase.

Finally, development of a semi-strand specific assay was undertaken to more accurately approximate EBOV viral genome copy number. A typical qRT-PCR (or ddPCR) assay that targets genomic regions of EBOV is unable to discriminate between the negative sense viral (genomic) RNA, the positive sense viral copy RNA (which is used as a template for produce negative sense genomic RNA molecules that will be packaged into virions), and positive sense viral mRNA (which is translated by ribosomes into viral proteins). Thus, quantification of all three viral RNA species simultaneously is subject to factors such as the viral replication cycle. Furthermore, EBOV produces mRNA transcripts of its 7 genes unevenly, with decreasing transcription frequency from 3' to 5'. The consequence of this is that quantification assays that target different regions of the genome (e.g. NP, VP40, L – all of which were used during the West Africa outbreak) will yield differing results simply based on the transcription frequency of the genes they target.

To address these concerns, an assay was designed to target the intergenic (IG) region between VP30 and VP24 (see below, §2 of this chapter). This allows for mRNA to be excluded from quantification and produces a closer approximation to the true viral genome copy number. However, positive-sense viral copy RNA is not excluded, so the IG assay will still overestimate the viral copy number by some amount.

To validate the IG assay, Vero E6 cells were inoculated with EBOV at an moi of 0.1. At 24-, 48-, and 72-hours post-inoculation, both culture supernatant and cells were collected in triplicate for analysis by both the IG assay and the L assay. The culture supernatant should be primarily composed of virions, with a relatively low abundance of replicative intermediates (viral cRNA and viral mRNA, which would only be present in the supernatant from lysed cells). The cellular fraction, on the other hand, contains all viral RNA species (vRNA, cRNA, and mRNA). Thus, comparing the quantification results of the IG assay and the L assay between the two sample types should reveal both the IG assay's ability to compare favorably with the L assay while effectively excluding viral mRNA (Figure 2.3).

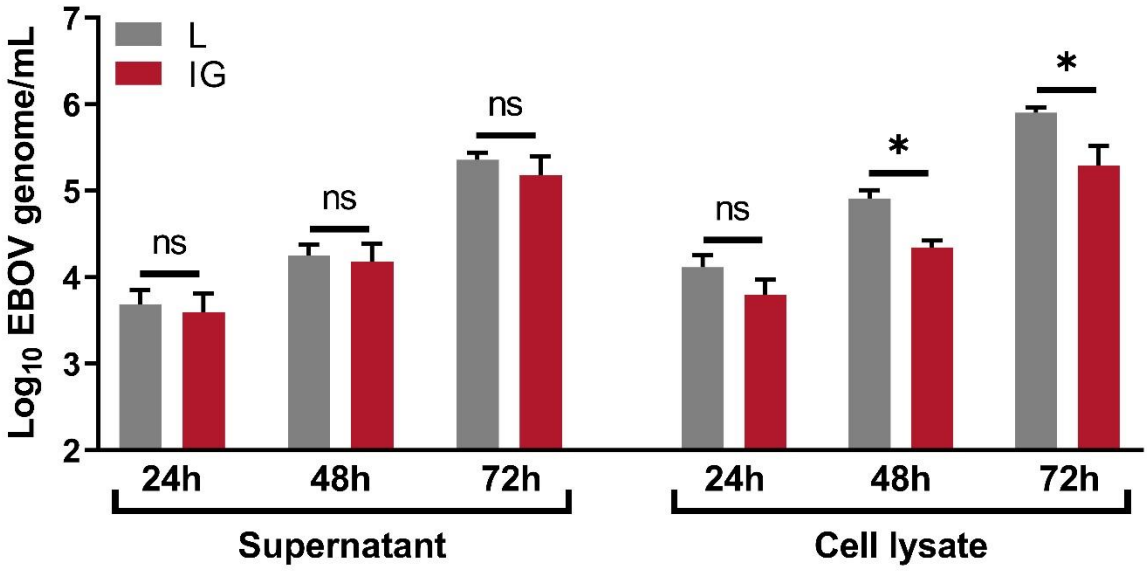

Figure 2.3. Comparison of a ddPCR assay targeting an intergenic (IG) region of the genome with an assay targeting a region of the polymerase (L) gene.

Each time point was analyzed in triplicate by both the IG and L assays. The error bars represent the standard deviation. The single asterisks (*) represent a *p*-value of $0.01 > x < 0.05$ when comparing the means by a *t*-test.

This was found to be the case following the analyses, with only slight differences present between IG and L in the quantification of EBOV RNA in supernatant, but significant differences present at two of the three timepoints when quantifying EBOV RNA from the cell lysate (Figure

2.3). A protocol was then established to back-calculate the copy numbers obtained from the ddPCR instrument to the original patient blood sample (Figure 2.4).

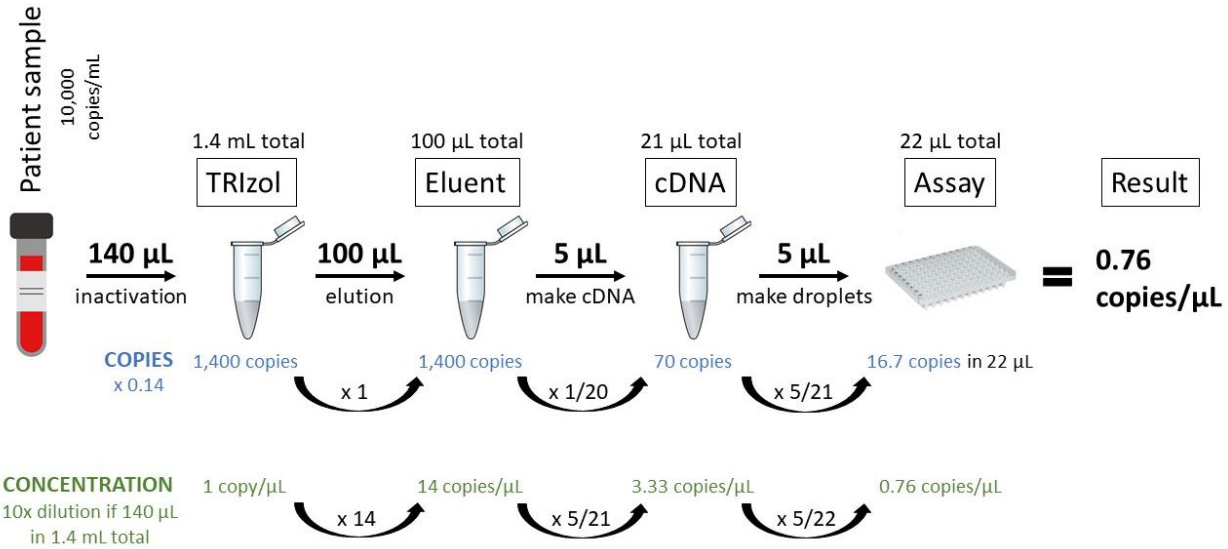

Figure 2.4. Method for quantification of viral load using ddPCR and back-calculating to the original blood sample.

The values shown are strictly for illustrative purposes. In some initial testing for optimization and development of the assay, different volumes of RNA were used to generate cDNA, and different volumes of cDNA were analyzed by ddPCR. Calculations were adjusted as necessary in those circumstances.

§ 2 – The Study

Reevaluation of triage Ebola virus load in Liberia by droplet digital PCR: epidemiological findings and methodological advantages

A manuscript in preparation

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INTRODUCTION

Ebola virus disease (EVD) is a viral hemorrhagic fever caused by Ebola virus (EBOV), a filovirus that is enzootic in central and West Africa (Feldmann & Geisbert, 2011; Munster et al., 2018). EVD is characterized initially by constitutional signs and symptoms, followed by weakness, severe diarrhea and vomiting, coagulopathy with or without overt hemorrhage, multi-organ failure, and sometimes neurological involvement. Case fatality rates during outbreaks are typically 60-70%. Most EVD outbreaks have been relatively small (< 100 cases) and short (< 4 months) (Matson et al., 2020b), but from December 2013 until June 2016, the West African countries of Guinea, Liberia, and Sierra Leone suffered an EVD outbreak which is the largest and longest on record (Team et al., 2016). Monrovia, the capitol of Liberia, was an epicenter of the outbreak, and the Eternal Love Winning Africa 3 (ELWA-3) Ebola treatment unit (ETU) located on the outskirts of the city remains the largest facility of its kind ever built (Abramowitz et al., 2015; de Wit et al., 2016b; Nyenswah et al., 2016), where more than 1,800 EVD patients were ultimately diagnosed and received care (Chertow et al., 2014; de Wit et al., 2016b; Rosenke et al., 2016). In the last six years following the beginning of the West Africa outbreak, five additional EVD outbreaks have occurred, each in the Democratic Republic of the Congo (DRC) (Ilunga Kalenga et al., 2019; Maganga et al., 2014; Matson et al., 2020b; Nsio et al., 2020; WHO, 2018d, 2020). Amongst these was the second longest and deadliest EVD outbreak on record in North Kivu province and the surrounding area. Thus, EVD continues to be a perennial threat to the people and the fragile public health infrastructure in developing regions of central and West Africa and knowledge gaps must continue to be filled to improve patient care.

Higher viral load, as estimated by cycle threshold (C_t) values from quantitative reverse transcription polymerase chain reaction (qRT-PCR) analysis, has been shown to correlate with

increased mortality in EVD and is an important prognostic indicator (Crowe et al., 2016; de La Vega et al., 2015; Faye et al., 2015; Furuse et al., 2017; Lanini et al., 2015; Li et al., 2016; Vernet et al., 2017; Zhang et al., 2015). However, different assays, platforms, and protocols were used at ETUs during the West Africa outbreak, and the viral load estimations that are sometimes provided from standard curves report a range of units that are not comparable (e.g. 'arbitrary units' or 'genome equivalents'). Furthermore, given the inherent limitations and variability of qRT-PCR for viral load estimation (Hayden et al., 2017; Hayden et al., 2012), considerable heterogeneity may be present in the estimations both between and within ETUs (Hayden et al., 2008).

Here our aim was to improve our understanding of the relationship between absolute viral load and patient outcome by quantifying the viral loads in a large set of Liberian EVD patient samples that were collected at the ELWA-3 ETU in Monrovia in 2014 using improved diagnostic tools and methods. This is the first such rigorous viral load assessment in a Liberian cohort (de La Vega et al., 2015; Lanini et al., 2015; Li et al., 2016; Vernet et al., 2017) and allows for a number of important epidemiological, diagnostic, and virological observations to be made.

MATERIALS AND METHODS

Sample acquisition and processing

Whole blood samples were collected from patients at the ELWA-3 ETU in Monrovia, Liberia in 2014 for diagnostic purposes and provided with a unique sample identification number. Multiple samples from a patient were linked using a unique ministry of health identifier (MOH-ID). At the ETU, viral RNA was extracted from aliquots of the samples using the Qiagen Viral RNA Mini Kit, and a region of the polymerase (L) gene was amplified by qRT-PCR.(de Wit et al., 2016b) A subset of these whole blood samples was transported to the NIH/NIAID

Rocky Mountain Laboratories further analyses in the biosafety level 4 (BSL-4) facilities. Samples were selected using two criteria: the presence of sufficient volume and origination from an ELWA-3 patient, as the laboratory supporting ELWA-3 also served as a regional diagnostic center and processed samples from numerous other ETUs.

Total RNA was extracted from whole blood samples in EDTA using TRIzol reagent (10 parts TRIzol to 1-part blood), Phasemaker tubes with chloroform phase separation, and PureLink RNA columns (Invitrogen, Thermo Fisher Scientific, Waltham MA USA) according to manufactorer's instructions. Elution was performed with 3 x 35 µL (105 µL total) 10 mM Tris-HCl pH 7.5. To assess the quality of the samples and their suitability for copy number analysis following storage, and to facilitate further comparisons of the assays (L vs IG) and platforms (qRT-PCR and ddPCR) used, 75 of the TRIzol extracts were randomly selected and analyzed with the same assay (one-step qRT-PCR targeting a portion of the L gene) and instruments used in the field (initially a Cepheid SmartCycler, which was used for the majority of the samples in the field and which 47 of the samples were reevaluated; later a Roche LightCycler, for which 28 of the samples were reevaluated) (Figure 2A-D) (de Wit et al., 2016b).

Absolute viral genome copy number was measured by a two-step droplet digital PCR (ddPCR) assay using a QX200 Droplet Digital PCR System with automated droplet generation by an AutoDG instrument (Bio Rad, Hercules, CA USA). Viral cDNA was generated with SuperScript IV (Thermo Fisher, Waltham, MA USA) using random hexamers according to the manufacturer's protocol. To exclude viral mRNA and more accurately estimate the viral genome copy number, primers and probes were designed to target the intergenic region between VP30 and VP24, in a region where mRNA is never transcribed, as follows: forward, 5'-TGACGGAACATAAATTCTTTCTGC-3'; reverse, 5'-CGGTCACAATATACCTCCTGAAA-

3'; probe, 5'-FAM-TGTGGAGGAGGTCTATGGTATTCGCT-3'. Undiluted and 1:100 diluted aliquots of cDNA from each sample were analyzed by ddPCR to ensure an accurate reading within the dynamic range of the instrument, and values obtained were back calculated to reflect the viral genome copy numbers in the original blood samples. Values are shown with 95% Poisson confidence intervals that are back calculated from data generated by the ddPCR software.

Database cleaning

The diagnostic C_t value from each patient sample was recorded in a field notebook along with patient demographic information, as available. This included sex, age at onset, patient recalled symptom onset date, date of ETU admission, and date of sample draw. Data was entered into an Excel spreadsheet, and the MOH-ID was used to link data on patient outcomes collected by Médecins Sans Frontières (MSF). The data was reviewed for missing and conflicting information (i.e. patient age or sex, symptom onset dates, outcome, etc.) and where necessary, compared to the original field notebooks for verification. Where resolution was not possible, these variables were considered missing for that patient. After cleaning, any remaining patient identifiers were removed from the final analyzed database. Data cleaning was conducted using R version 3.6.x.

Statistical methods

Correlation between variables was assessed using Spearman's ρ and general trends amongst the variables are depicted with regression models that include 95% confidence intervals. Other statistical methods were used as appropriate. Differences in the mean ddPCR $\log_{10}$ EBOV genome copies/mL of admission samples between survivors and non-survivors was compared using multiple regression, controlling for age, sex, and days from onset to admission. Changes in

the ddPCR $\log_{10}$ EBOV genome copies/mL at presentation and time from symptom onset to admission were also analyzed by comparing the corresponding mean values of each from the first half of the sampling period (< 59 days after August 22, 2014) with the second half of the sampling period (≥ 59 days after August 22, 2014) using Student's t-test with Welch's correction. Statistical significance was assessed at $p \leq 0.05$. Slopes of lines of best fit were compared with ANCOVA. The optimal cutoff value for the prediction of patient outcome using either the ddPCR $\log_{10}$ EBOV genome copies/ml or the qRT-PCR diagnostic C_t values was determined using receiver operating characteristic (ROC) curve analysis and minimizing the absolute value of the difference between sensitivity and specificity using the OptimalCutpoints package. The change in case fatality rate over time was assessed by computing the average number of deaths per three-week period from August 28, 2014 to December 11, 2014 according to date of ETU admission. Multivariate linear regression was used to assess changes in log-transformed viral load, time from symptom onset to ETU presentation, and time from ETU admission to death or discharge, controlling for patient age, sex, and days from symptom onset to admission, and viral load (as appropriate) over the course of the outbreak. Statistical analyses were performed with R version 3.6.x and GraphPad Prism 8.

Ethical review

This study made use of de-identified patient specimens and matched clinical data received a determination of 'not human subjects research' by the NIH Office of Human Subjects Research Protection (OHSRP) and has been approved by the University of Liberia-Pacific Institute for Research and Evaluation (UL-PIRE) IRB.

RESULTS

Dataset

727 unique samples from 528 unique individuals met the selection criteria and were processed in the laboratory. 463 of these samples were first-draw samples obtained at admission; the remainder were repeat samples obtained from survivors during convalescence to assess for viral clearance (de Wit et al., 2016b). The 463 first-draw admission samples included 307 EBOV-positive patients and comprised the study population reported here. The overall case fatality rate (CFR) was 58.6% (180/307), 50% (155/307) were female, and the median age was 29 (range, 2-80) (Table 2.1).

Table 2.1. Study cohort demographics and outcome by viral load

Variable	Count	Mean ddPCR $\log_{10}$ EBOV genome copies/mL (95% Poisson CI)
Sex		
Female	155	6.3 (6.1-6.6)
Male	145	6.6 (6.3-6.9)
Unknown	7	7.2 (5.8-8.5)
Age		
< 5	3	6.0 (1.9-10.1)
5-14	36	6.5 (5.9-7.1)
15-40	181	6.4 (6.2-6.6)
> 40	78	6.7 (6.4-7.1)
Unknown	9	5.8 (4.2-7.4)
Onset to admission (days)*		
< 3	29	6.3 (5.6-6.9)
3-4	100	6.6 (6.3-6.9)
5-6	45	6.9 (6.5-7.4)
7-14	75	6.5 (6.1-6.9)
> 15	12	6.1 (4.9-7.3)
Unknown	46	6.0 (5.6-6.5)
Outcome		
Survivor	127	5.5 (5.3-5.8)
Non-survivor	180	7.1 (6.9-7.3)

*Self-reported

Absolute viral load

Diagnostic C_t values obtained by qRT-PCR at admission demonstrated a strong correlation with ddPCR $\log_{10}$ EBOV genome copies/mL in surviving patients obtained by ddPCR upon laboratory reanalysis (Spearman $\rho = 0.70$, $p < 0.001$, line of best fit $R^2 = 0.51$)

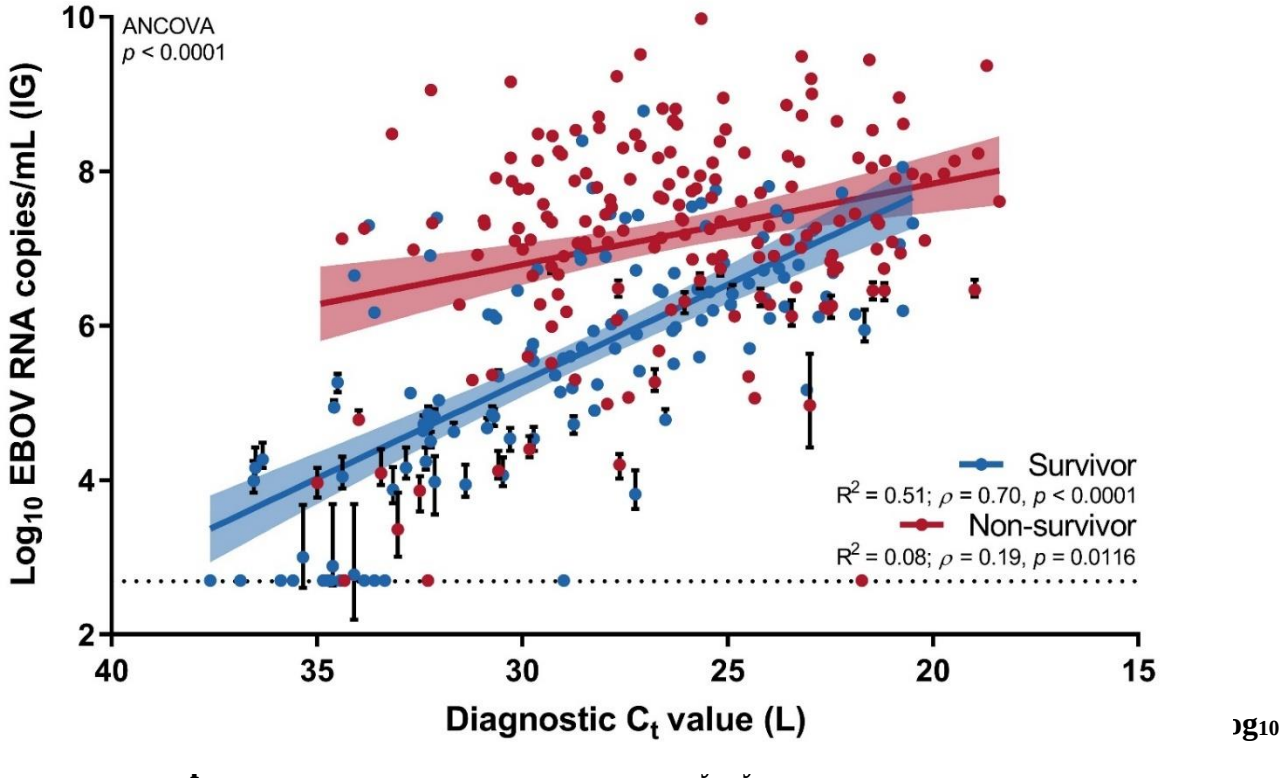
(Figure 2.5). For non-surviving patients, the correlation was considerably weaker (Spearman ρ = 0.19, p = 0.01, line of best fit R^2 = 0.083), with increasing deviation as C_t values increased. The slopes of the lines of best fit for the qRT-PCR C_t values and the ddPCR $\log_{10}$ EBOV genome copies/mL for survivors (-0.25, 95% CI -0.29 to -0.21) and non-survivors (-0.10, 95% CI -0.16 to -0.053) also differed significantly from one another ($p < 0.0001$). The mean ddPCR $\log_{10}$ EBOV genome copies/mL in whole blood samples obtained at admission for survivors (5.54 [95% CI 5.28 to 5.81]) was significantly lower ($p < 0.001$) than in non-survivors (7.13 [95% CI 6.92 to 7.33]) (Figure 2.6), even when accounting for patient age, sex, and days since symptom onset. The maximum ddPCR $\log_{10}$ EBOV genome copies/mL observed in a surviving patient and non-surviving patients was 8.78 (95% Poisson CI 8.77 to 8.80) and 9.98 (95% Poisson CI 9.97 to 10.00), respectively.

Viral load at admission as a prognostic indicator

When comparing the utility of using admission sample viral load versus diagnostic C_t to predict patient outcome, the ddPCR $\log_{10}$ EBOV genome copies/mL provided greater predictive capability (area under the curve (AUC) 0.799, 95% CI 0.75 to 0.849) than the diagnostic C_t values (AUC 0.656, 95% CI 0.593 to 0.718) (Figure 2.6).

Using ddPCR, the optimal cutoff was 6.71 ddPCR $\log_{10}$ EBOV genome copies/ml (Figure 2.5). Patients that presented with a viral load above this had a substantially increased likelihood of death occurring in the ETU (odds ratio 8.06, 95% CI 4.81 to 13.53, $p < 0.0001$) and the CFR for the group was 80.1%, compared to a 33.3% CFR for the group of patients presenting with viral loads below 6.71 ddPCR $\log_{10}$ EBOV genome copies/mL. The optimal cutoff for the admission sample using qRT-PCR was a diagnostic C_t value of 27.37 (Figure 2.6). While less discriminatory than ddPCR, patients with C_t values below 27.37 had an increased risk of death in

the ETU (odds ratio 2.02, 95% CI 1.27 to 3.20, p = 0.0028). The CFR for the group of patients with a C_t < 27.37 was 66.9%; the group of patients with a C_t > 27.37 had a CFR of 50%.



Panel A: correlation of diagnostic C_t value as measured by qRT-PCR using the assay targeting the polymerase (L) gene in the field at the ETU with absolute viral load in ddPCR $\log_{10}$ EBOV genome copies/mL as measured by droplet digital PCR (ddPCR) in the laboratory using the assay targeting an intergenic region (IG), separated by patient outcome. Differences beyond the instrumentation used for measurement are outlined in Table 2.2. Lines of best fit included 95% confidence intervals (shaded areas). Error bars indicate 95% Poisson confidence interval. Most confidence intervals are too narrow to be shown. Note that the x-axis is reversed.

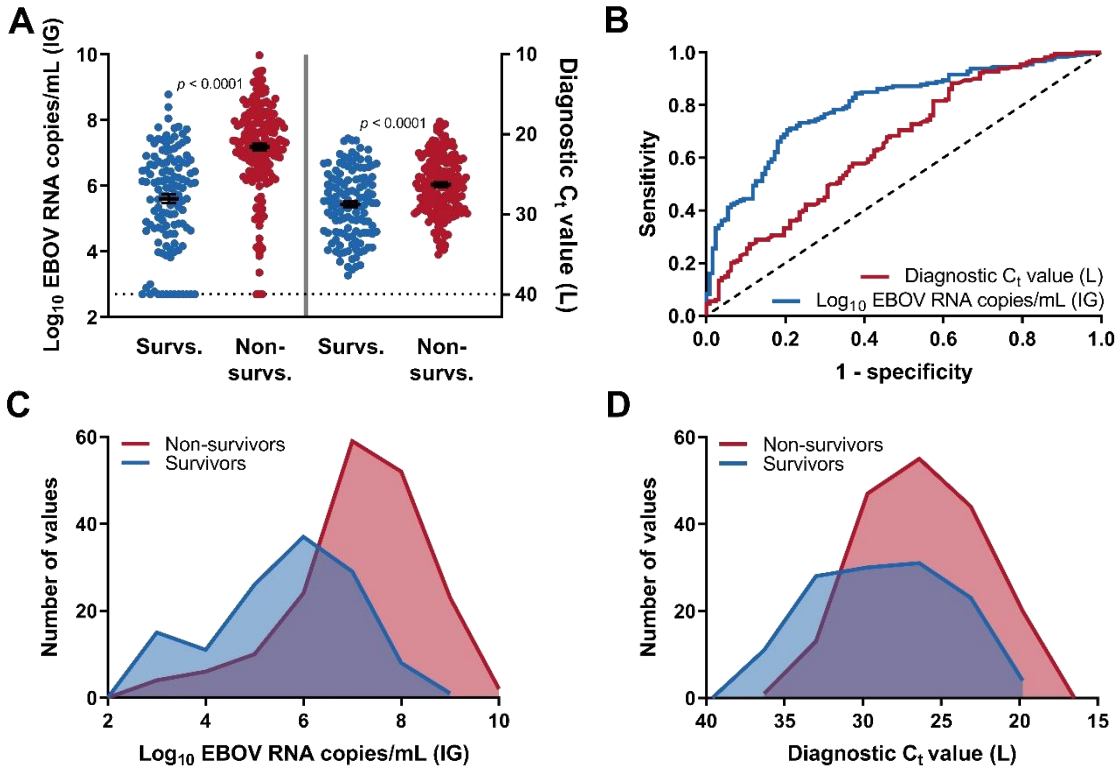


Figure 2.6. Correlation of Ebola virus RNA copies/mL and C_t values at triage with outcome and their use as prognostic indicators.

The two approximate measurements of viral load at triage – $\log_{10}$ EBOV RNA copies/mL measured by ddPCR in the laboratory and C_t value measured by qRT-PCR in the field – compared by patient outcome. Note that the right y-axis is reversed in A and the x-axis is reversed in D. Panel A: Log_{10} EBOV RNA copies/mL as measured by ddPCR using the assay targeting the intergenic region (IG) on triage blood samples upon reevaluation in the laboratory (left y-axis) compared with diagnostic C_t value as measured by qRT-PCR using the assay targeting the polymerase (L) gene in the field (right y-axis), both by outcome. Bars indicate mean and standard error of the mean. Group means were compared with an unpaired *t*-test with Welch's correction, and two-tailed *p*-values are shown. Panel B: Receiver operating characteristic (ROC) curve analysis of ddPCR $\log_{10}$ EBOV RNA copies/mL and diagnostic C_t value based on patient outcome. Panel C: number of patients by ddPCR $\log_{10}$ EBOV RNA copies/mL, separated into survivors and non-survivors. Panel D: number of patients by diagnostic C_t value, separated into survivors and non-survivors.

Changes in viral load at presentation over time

The mean ddPCR $\log_{10}$ EBOV genome copies/mL in non-survivors at admission demonstrated a significant downward trend (Spearman ρ = -0.31, $p < 0.0001$) and decreased by 0.016 ddPCR $\log_{10}$ EBOV genome copies/mL per day (95% CI -0.023 to -0.008) as the outbreak progressed, from 7.33 ± 0.22 $\log_{10}$ EBOV genome copies/mL during the first half of the sampling period to 6.68 ± 0.37 $\log_{10}$ EBOV genome copies/mL during the second half (p = 0.0043). No similar significant trend (Spearman ρ = 0.034, p = 0.7028) or daily decrease was observed in survivors (β = 0.001, 95% CI -0.010 to 0.012), even when controlling for patient age, sex, and days from symptom onset to admission (first half of sampling period = 5.54 ± 0.30 $\log_{10}$ EBOV genome copies/mL, second half of sampling period = 5.84 ± 0.53 $\log_{10}$ EBOV genome copies/mL; p = 0.3490) (Figure 2.7).

There was also a significant downward trend in the time from symptom onset to ETU admission observed for both non-survivors (Spearman ρ = -0.20, p = 0.0098) and survivors (Spearman ρ = -0.21, p = 0.0498), although the mean time to presentation was significantly shorter only for non-survivors when comparing the first half (< 59 days after August 22, 2014) of the sampling period to the second half of the (≥ 59 days after August 22, 2014) and decreased from 6.2 ± 0.70 days to 4.7 ± 1.1 days (p = 0.0301) (Figure 2.7). Survivors presented 6.5 ± 0.99 days after symptom onset in the first half of the sampling period, and 5.8 ± 2.8 days after in the second half (p = 0.6510). The case fatality rate did not significantly change (slope = 0.26, p = 0.91) as the outbreak progressed when averaged over 3-week intervals from August 28, 2014 to December 11, 2014 according to date of ETU admission (Figure 2.7).

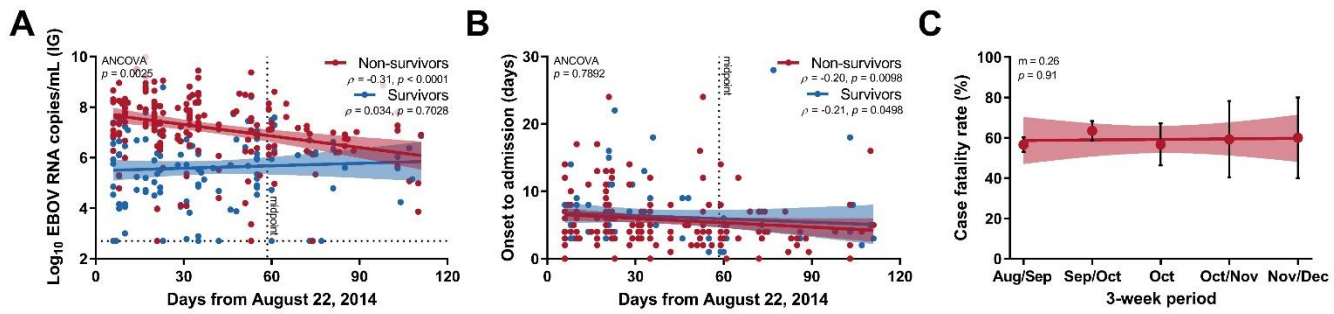


Figure 2.7. Changes in log_{10} EBOV RNA copies/mL, time from symptom onset to presentation, and case fatality rate over the observed period.

Changes in log_{10} EBOV RNA copies/mL over time, compared with changes in the self-reported time from symptom onset to admission over time and changes in the mean case fatality rate over time for the sampling period. Panel A: ddPCR log_{10} EBOV RNA copies/mL by days since the beginning of the sampling period, separated into survivors and non-survivors. Midpoint shown is at day 59, halfway through the sampling period. Linear regression lines of best fit include 95% confidence intervals (shaded area). ANCOVA compares the slopes of the lines of best fit with a two-tailed *p*-value. Spearman ρ value shown indicates the correlation between the variables for non-survivors and survivors with associated two-tailed *p*-value. Panel B: Days from self-reported symptom onset to presentation by outbreak period, separated into survivors and non-survivors. Midpoint shown is at day 59, halfway through the sampling period. Linear regression lines of best fit include 95% confidence intervals (shaded area). ANCOVA compares the slopes of the lines of best fit with a two-tailed *p*-value. Spearman ρ value shown indicates the correlation between the variables for non-survivors and survivors with associated two-tailed *p*-value. Panel C: case fatality rate by admission date averaged over three-week intervals, starting with the date of admission for the first patient in the dataset (August 27, 2014). Points indicate mean and error bars indicate standard deviation. Linear regression line of best fit includes 95% confidence interval (shaded area).

ETU admission dynamics

The median length of stay in the ETU by survivors was 11 days (interquartile range, 6) and by non-survivors was 4 days (interquartile range, 5) (Figure 2.8). In survivors, time spent in the ETU trended upward (Spearman $\rho = 0.54$, $p < 0.0001$), increasing by 11.4% (95% CI 6.33 to 16.73%) as viral load increased, when controlling for patient age, sex, and days from onset to admission (Figure 2.8). Time spent in the ETU trended downward (Spearman $\rho = -0.20$, $p = 0.0074$) for non-survivors as viral load increased, but was found not to be significant when controlling for patient age, sex, and days from onset to admission (-6.1%, 95% CI -13.81 to 2.31%). As the outbreak progressed, the length of stay significantly increased for survivors

(0.43%, 95% CI 0.20 to 0.66%) and decreased for non-survivors (-0.56%, 95% CI -0.99 to -0.12%) (Figure 2.8). Based on self-reported time of symptom onset, non-survivors died slightly but significantly sooner (-0.46%, 95% CI -0.79 to -0.12%) as the outbreak progressed, while no significant change was observed in survivors (-0.40%, 95% CI -0.84 to 0.04%) (Figure 2.8). Non-survivors died in a median of 9 days (range, 0-25 days) following self-reported symptom onset, and survivors took a median of 17 days (range, 7-35 days) to clear virus following self-reported symptom onset.

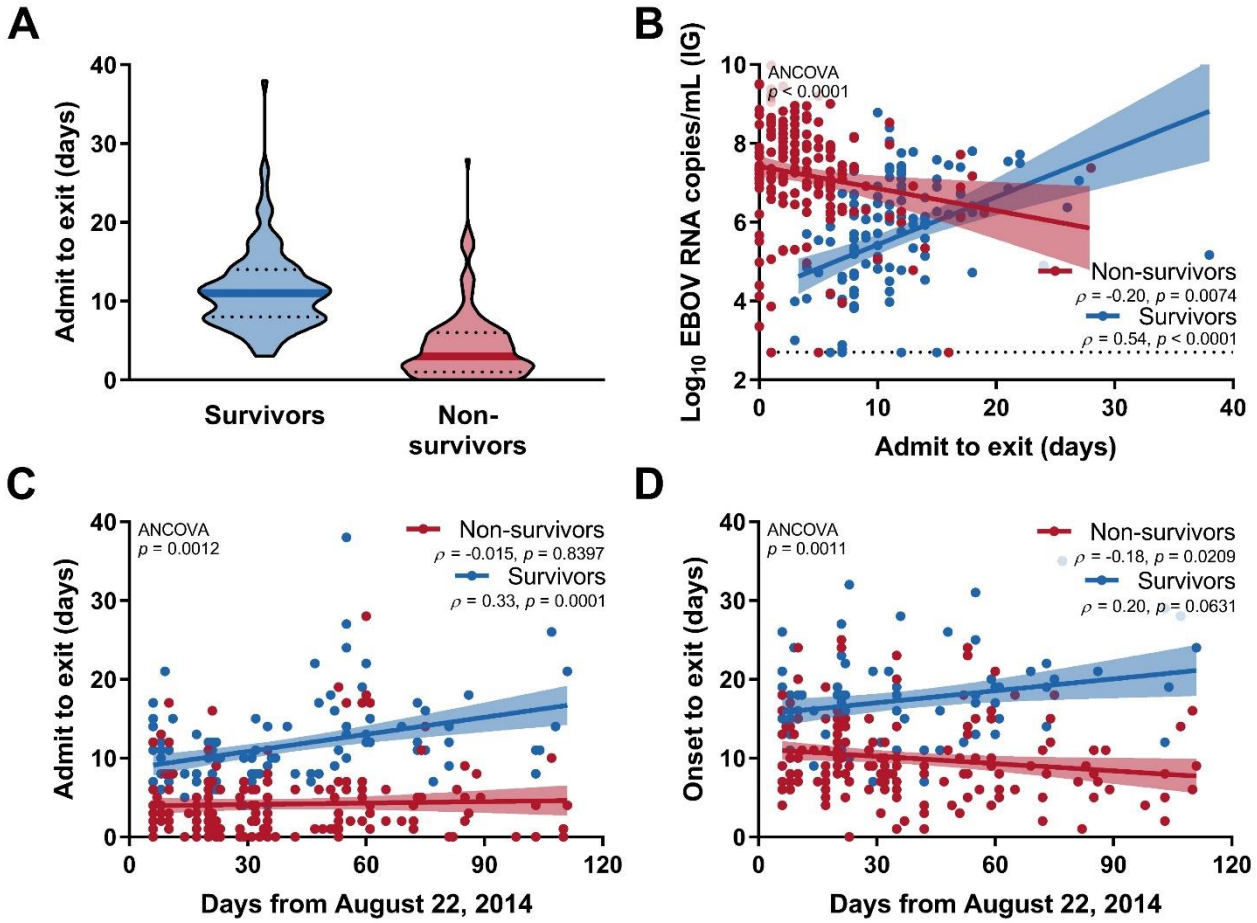


Figure 2.8. ETU admission dynamics for survivors and non-survivors.

Panel A: Violin plot of admission time for survivors and non-survivors. Solid line represents median and dotted lines show quartiles. Panel B: ddPCR $\log_{10}$ EBOV genome copies/mL by days from ETU admission to death or discharge, separated into survivors and non-survivors. Linear regression lines of best fit include 95% confidence intervals (shaded area). ANCOVA compares the slopes of the lines of best fit with a two-tailed *p*-value. Spearman ρ value shown indicates the correlation between the variables for non-survivors and survivors with associated two-tailed *p*-value. Panel C: Days from ETU admission to death or discharge by outbreak period, separated into survivors and non-survivors. Linear regression lines of best fit include 95% confidence intervals (shaded area). ANCOVA compares the slopes of the lines of best fit with a two-tailed *p*-value. Spearman ρ value shown indicates the correlation between the variables for non-survivors and survivors with associated two-tailed *p*-value. Panel D: days from self-reported symptom onset to death or discharge by outbreak period, separated into survivors and non-survivors. Linear regression lines of best fit include 95% confidence intervals (shaded area). ANCOVA compares the slopes of the lines of best fit with a two-tailed *p*-value. Spearman ρ value shown indicates the correlation between the variables for non-survivors and survivors with associated two-tailed *p*-value.

Diagnostic C_t values, absolute viral load, and laboratory C_t values

The subset of 75 samples that were reanalyzed by the qRT-PCR assay and instruments used in the field were first compared with the ddPCR $\log_{10}$ EBOV genome copies/mL (Table 2.2, Figure 2.9). Diagnostic C_t values from both instruments fit similarly (R^2 LightCycler = 0.61, R^2 SmartCycler = 0.50) to the ddPCR $\log_{10}$ EBOV genome copies/mL, with no significant differences in the slopes of the lines of best fit ($p = 0.8334$). Upon comparison of the C_t values obtained in the lab from the reextracted samples with the ddPCR $\log_{10}$ EBOV genome copies/mL, the fit was considerably better (R^2 LightCycler = 0.84, R^2 SmartCycler = 0.91), with no significant differences between the slopes ($p = 0.2789$) (Figure 2.9). Comparison of the diagnostic C_t values with the lab C_t values yielded similar results (R^2 LightCycler = 0.52, R^2 SmartCycler = 0.47), as expected, again with no significant differences in the slopes of the lines (LightCycler m = 1.0, SmartCycler m = 0.94; $p = 0.7630$) (Figure 2.9). When the diagnostic C_t and lab C_t values were compared by patient outcome, rather than instrument, a similar trend was apparent to that obtained by comparing diagnostic C_t values with ddPCR $\log_{10}$ EBOV genome copies/mL as in Figure 2.8, with deviation only in non-survivors as C_t values increased and significant differences in the slopes (survivors m = 0.96, non-survivors m = 0.47; $p = 0.0368$).

Table 2.2. Comparison of datasets analyzed

Dataset	Sample type	Extraction method	Assay type	PCR target	Instrument
Diagnostic C_t values (n = 307)	Fresh whole blood	QIAGEN QIAamp Viral RNA Mini Kit	One-step	Polymerase gene (L) – detects vRNA, cRNA, and mRNA	Cepheid SmartCycler and Roche LightCycler
Lab C_t values (n = 75, subset of the 307 total)	Stored whole blood (freeze/thaw)	TRIzol, Phasemaker tubes, PureLink columns	One-step	Polymerase gene (L) – detects vRNA, cRNA, and mRNA	Cepheid SmartCycler and Roche LightCycler
Lab ddPCR $\log_{10}$ EBOV RNA copies/mL (n = 307)	Stored whole blood (freeze/thaw)	TRIzol, Phasemaker tubes, PureLink columns	Two-step (cDNA generated with SuperScript IV)	Intergenic (between VP30-VP24) – detects only vRNA and cRNA*	Bio Rad QX200 Droplet Digital PCR

*The intergenic (IG) assay excludes viral mRNA as it targets a region of the genome that is never transcribed. vRNA = viral genomic RNA; cRNA = viral complementary RNA (positive-strand intermediate used as a template for genome replication.

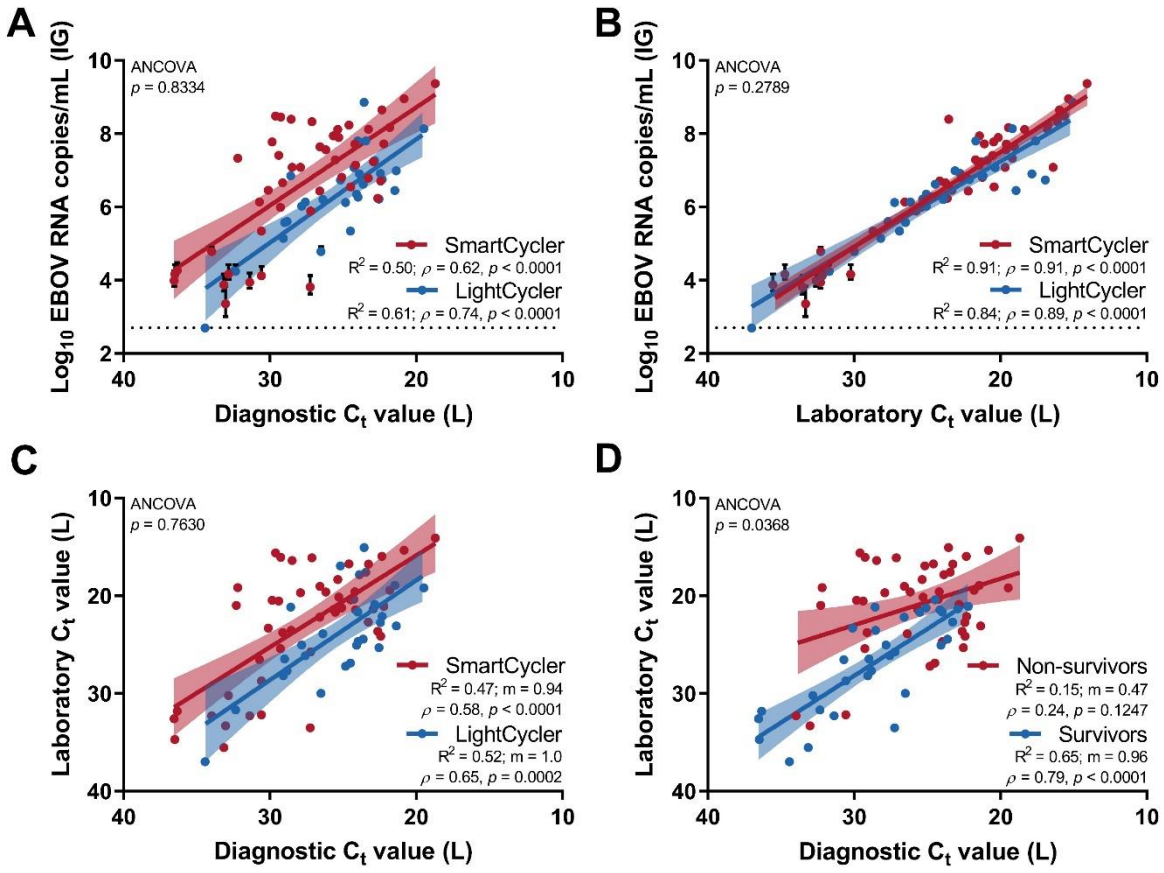


Figure 2.9. Comparisons of log_{10} EBOV RNA copies/mL as measured in the laboratory by ddPCR with diagnostic C_t values as measured in the field by qRT-PCR and with laboratory C_t values upon reevaluation by qRT-PCR.

The diagnostic C_t values, obtained using either SmartCycler or LightCycler instruments, were compared with the laboratory reanalyzed data, both the ddPCR log_{10} EBOV RNA copies/mL and the C_t values from the same SmartCycler and LightCycler instruments used at the ETU. The log_{10} EBOV RNA copies/mL from ddPCR are always generated using the assay that targets the intergenic (IG) region, while the C_t values from qRT-PCR are always generated using the assay that targets the polymerase (L) gene. Note the reversal of x-axes in A and B, and both axes in C and D. Panel A: Correlation of diagnostic C_t value and ddPCR log_{10} EBOV genome copies/mL, separated by instrument used for qRT-PCR analysis in the field. Panel B: correlation of lab C_t values and ddPCR log_{10} EBOV genome copies/mL, separated by instrument used for qRT-PCR analysis in the lab. Panel C: correlation of diagnostic C_t value and lab C_t value, separated by instrument used for qRT-PCR analysis in the field and in the lab. Panel D: correlation of diagnostic C_t value and lab C_t value, separated by patient outcome.

DISCUSSION

In this study we quantified EBOV viral load using ddPCR from a large group of Liberian EVD patients to determine its association with several epidemiological parameters. The use of ddPCR is becoming increasingly preferred for viral load measurements due to increased precision and replicability and the lack of a need for a standard curve, amongst other benefits (Huang et al., 2015; Kuypers & Jerome, 2017; Sedlak et al., 2014; Suo et al., 2020; Yu et al., 2020). In addition, the semi-strand specific assay that we developed excludes EBOV mRNA from measurement which allows for comparable viral load estimations, rather than relying on other commonly employed assays that target different intragenic regions (e.g. L, NP, etc.) and do not discriminate amongst viral RNA species (Muhlberger, 2007). Furthermore, the optimized extraction protocol utilized here ensured efficient extraction from the whole blood samples and minimization of PCR inhibitors and RNases.

The deviation of the ddPCR viral load data from what was expected based on the diagnostic C_t values only amongst the non-survivors was strikingly apparent. The differences in the non-survivor mean ddPCR $\log_{10}$ EBOV genome copies/mL and diagnostic C_t values further highlighted the discrepancy. Given the numerous differences between the extractions, assays, and platforms used, further analyses clarified that the deviation in the viral load of non-survivors was not merely due to the use of ddPCR. First, while the diagnostic C_t values plotted against the ddPCR $\log_{10}$ EBOV genome copies/mL produced relatively poor R^2 values, the subset of rerun lab C_t values and the ddPCR $\log_{10}$ EBOV genome copies/mL aligned extremely well. Furthermore, comparison of the diagnostic C_t values with the lab C_t values yielded slopes of approximately 1 – exactly what would be expected – although the R^2 values were relatively poor. The reason for this is apparent when comparing the diagnostic C_t values with the lab C_t values by

outcome, rather than by instrument. It is the C_t values of only the non-survivors again that deviate significantly from one another and, given the roughly equal split of survivors and non-survivors in the cohort overall, the differences averaged out for each instrument and produced expected slopes close to 1 with poor R^2 values. Thus, the deviation most likely arose from the re-extraction of the samples, rather than from differences in the assay design (one-step or two-step), target (L or IG), or platform (ddPCR or qRT-PCR).

The reasons for this deviation, observed from the reextracted samples when analyzed both with ddPCR (Figure 1A) and qRT-PCR, are not entirely clear, but a number of explanations are possible. Other reports have shown qRT-PCR analysis of samples taken from patients with acute viral hemorrhagic fevers, including EVD, to be particularly susceptible to PCR inhibitors which were present at unusually high concentrations, possibly due to cell and tissue death (Drosten et al., 2002). In these scenarios, test results may yield incorrectly high C_t values or even false negatives, despite the presence of extremely high concentrations of viral RNA. That our reanalysis very preferentially demonstrated increased viral loads for non-survivors only lends credence to this theory. Alternatively or additionally, the diagnostic extraction protocol used in the field, which is a broadly implemented standard, is nevertheless intended for cell-free media and may have been less efficient than the optimized protocol used here at extracting EBOV RNA from virus-laden circulating macrophages and dendritic cells, which are well-established as primary EBOV targets (Bray & Geisbert, 2005). Diagnostic and viral load studies on other viruses with a white blood cell (WBC) tropism have demonstrated a marked benefit for the use of whole blood over plasma samples given efficient lysis and extraction (Cook et al., 2000; Hakim et al., 2007; Lazzarotto et al., 2018; Stapleton et al., 1999), and thus may similarly be an important consideration for EVD. Whatever the explanation, the reanalysis here provided vastly

improved discrimination between EVD survivors and non-survivors based on viral load, which would be of great prognostic value at admission as that was generally the only time of sample collection prior to convalescence or death at ELWA-3. Furthermore, reliable stratification of disease severity by viral load is a decisive component of well-designed randomized controlled trials evaluating therapeutics and treatments for EVD (Group et al., 2016; Mulangu et al., 2019).

The significant decrease in admission sample viral load observed in non-survivors as the outbreak progressed is most likely explained by the earlier presentation that was concurrently observed. Previous studies have shown that EBOV viremia tends to peak in survivors around day 5 and in non-survivors around day 7 after symptom onset, and prior to peaking rises rapidly with daily log-fold or greater increases often observed (Chertow et al., 2016; Lanini et al., 2015; Malvy et al., 2019; Towner et al., 2004; Uyeki et al., 2016; Vernet et al., 2017; Vetter et al., 2016). The non-survivors in our cohort were presenting to the ETU 6.2 days after symptom onset, on average, in the first half of the sampling period, but this decreased to an average of 4.7 days during the second half of the sampling period. Thus, presenting even a day-and-a-half earlier, prior to peak viremia, can have a profound impact on the viral load at triage. For the survivors, presentation was not significantly earlier later in the outbreak, and even in the second half of our sample collection period, our survivor cohort was presenting on average 5.8 days after symptom onset, still within the plateau phase of viremia. These data imply that even with earlier initiation of the supportive care available at ELWA-3 (which consisted of oral rehydration, anti-diarrheal and antiemetic medications, and antibiotic and antimalarial treatment), prior to peak viremia, patient outcomes did not improve, as the CFR remained stable throughout the observed period. This is in contrast to some studies that reported an increase in viral load at admission together with an increase in CFR as the outbreak progressed, as well as to other

studies that reported a decrease in viral load at admission together with a decrease in CFR (de La Vega et al., 2015; Faye et al., 2015; Furuse et al., 2017; Jiang et al., 2017; Lanini et al., 2015; Wang et al., 2015).

The correlation of viral load at admission with time spent in the ETU is not surprising, yet could be a useful metric for gauging resource and bed availability in an outbreak setting. While the median stay for survivors was approximately 1.5 weeks, those with higher viral loads often required substantially more time for viral clearance and thus remained admitted for a longer period, while the converse was true for those presenting with lower viral loads. Additionally, given that survivor and non-survivor viral loads at admission had begun to converge by the end of the observed period as patients presented earlier, an additional blood draw a few days after admission to assess changes in viral load could offer valuable prognostic information, as viremia shortly after symptom onset would be unlikely to allow for stratification into high-risk and low-risk groups. By the end of the observed period, survivors were spending significantly more time in the ETU than earlier in the outbreak, likely due to presenting earlier after symptom onset, and possibly because of less pressure for discharge as the outbreak waned. For non-survivors, it was surprising that the time from admission to death did not change as the outbreak progressed, but the time from self-reported symptom onset to death decreased, despite their earlier presentation with lower viral loads. This may have simply been due to the smaller sample sizes in December versus August. Alternatively, changes in comorbidities, coinfections, or patient demographics could be responsible, but data were not available to address these questions.

Determination of viral load in EVD is crucial as it correlates significantly with outcome and various epidemiological parameters and offers critical prognostic information. Our findings

here support prioritizing further standardization of EVD diagnostic assays and methods for reliably quantifying viral loads. This will allow for larger, multi-site comparisons to be made and will yield valuable insights that will further EVD patient care. Furthermore, these data suggest that earlier initialization of supportive care at the level available at ELWA-3 did not significantly reduce mortality. Whether this would remain the case given even earlier presentation, perhaps one to two days following symptom onset, is unknown. This finding underpins the need for continued research into the best treatment protocols for EVD patients that present at various times during the course of disease.

CHAPTER 3: EBOLA VIRUS DISEASE AND BACTEREMIA

§ 1 – Development and Optimization of Protocols

Blood culture remains the gold standard for diagnosing bacterial bloodstream infections (Lamy et al., 2016). Despite its limitations – slow, time-consuming, requiring microbiological expertise, etc. – it remains superior to current sequencing-based approaches that are in development due to being less prone to contamination (although contamination remains a major issue for culture-based approaches as well) and its ability to report viable organisms. A typical clinical diagnostic blood culture protocol for an adult patient is as follows. The skin at the site for venipuncture is first thoroughly cleaned with a chlorhexidine solution (or, less ideally, a povidone-iodine solution; alcohol swabs alone are insufficient) to reduce the chance of skin contaminants entering the culture, then an aerobic and anaerobic culture bottle are each inoculated with at least 10 mL blood. This process may then be repeated immediately at a different venipuncture site to produce a total of four culture bottles (two aerobic, two anaerobic) from a single timepoint. The bottles are then incubated in automated continuously-monitoring instruments, typically for up to five days. Bottles in which growth is detected by the instrument are sub-cultured on agar plates to produce pure colonies for identification. Most often, this identification is performed utilized a biochemical algorithm. Alternatively, more modern techniques such as MALDI-TOF mass spectrometry may be utilized. In some instances, serial blood cultures are needed to effectively rule-in or rule-out a true, clinically relevant bloodstream infection. The protocol for pediatric patients is essentially identical, with the collection of smaller volumes of blood as the only difference.

To our knowledge, there are only three instances in the literature of a bacterium being successfully cultured from the blood of an EVD patient: the first of which was an unidentified

gram-negative organism thought to be clinically relevant (but very likely iatrogenic) (Kreuels et al., 2014), and the other two were both coagulase-negative staphylococci that were presumed to be skin contaminants and not clinically relevant (Lamb et al., 2015; Wolf et al., 2015). Other attempts to culture bacteria from EVD patients either during the course of treatment (which is rarely performed in ETUs) or from stored samples have been unsuccessful (Carroll et al., 2017). The stored whole blood EVD patient samples which were to be analyzed for our study were not ideally suited for blood culture. They were collected using only an alcohol swab at the site of venipuncture, the blood was anticoagulated in EDTA tubes, they had been stored under suboptimal conditions in the field, and only very small volumes were available for analysis that were below recommended volumes for blood culture. Nevertheless, given the importance of blood culture for establishment of bacteremia, we decided to proceed with culturing our full sample set with a full awareness of the limitations at hand. Furthermore, as we would be undertaking a targeted deep-sequencing approach on a matched subset of 90 of the samples (see § 2 of this chapter), the combination of culture and sequencing results would provide the most thorough assessment possible.

To address the concern of low sample volume, we chose to use a BD BACTEC FX40 automated culture instrument with BD BACTEC PEDS Plus /F culture bottles. These culture bottles are optimized for low inoculum volumes, with a minimum recommended volume of 0.5 mL. We established that we could use 0.2 mL from each of our samples, so although this was below the recommended minimum volume, it was the best possible solution. We also chose to add a supplement – BD BACTEC Fastidious Organism Supplement – to all of the blood culture bottles, as this was recommended by the manufacturer to improve the recovery of certain organisms (e.g. *Hemophilus* and *Neisseria* spp.), but we did not verify this independently.

Next, we sought to determine how much of a negative impact EDTA may have on the culture sensitivity, if any. Blood for culture is typically inoculated into the culture bottles directly from the patient prior to coagulation occurring – there is no step where anticoagulants (e.g. EDTA) are introduced. We used 0.2 mL (to mimic the volume of our study protocol) non-human primate blood that was either inoculated directly into the culture bottles, or that was first placed in an EDTA tube and then inoculated into the bottles. The bottles were then inoculated with either *S. aureus* or *E. coli*, as model Gram-positive and Gram-negative organisms respectively, at known approximate concentrations. The endpoints to be measured was whether or not growth was detected and, if so, the time to detection. We found no significant impact of the EDTA on the growth or time to detection for either bacterial species (Figure 3.1).

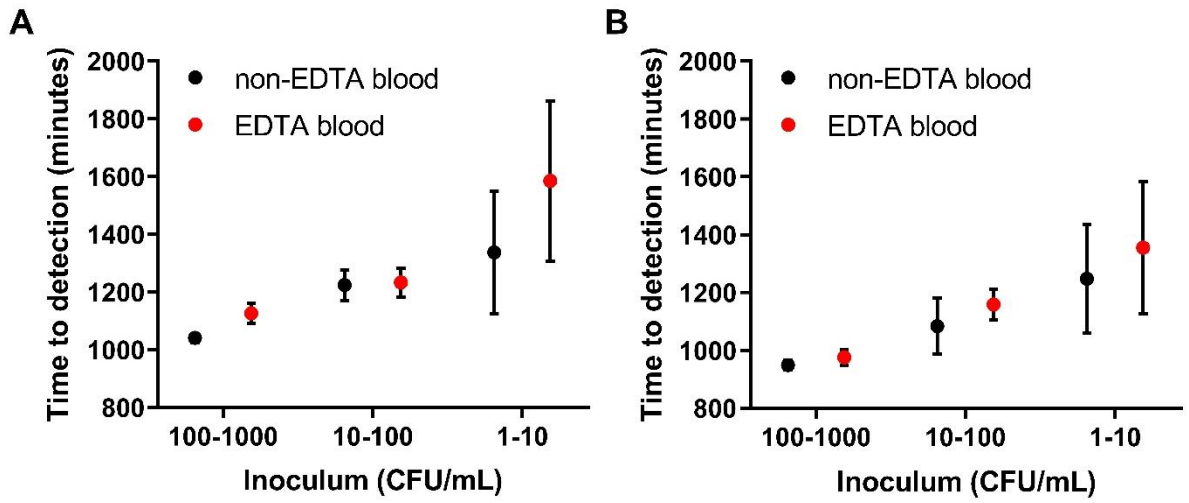


Figure 3.1. Effect of EDTA in whole blood on the culture time-to-detection for *Staphylococcus aureus* (A) and *Escherichia coli* (B).

Each point is the mean time-to-detection in minutes of 3 independent replicates, and error bars represent the standard deviation. Non-human primate blood was utilized with or without EDTA and dilutions of bacteria were added to the culture broth. No changes between paired non-EDTA vs. EDTA samples were significant ($p < 0.05$) using a paired *t*-test.

Another significant concern was the potential impact of suboptimal storage conditions in the field, which included freeze/thaw cycles due to power outages at the ETU. To assess this, we made a dilution series of *S. aureus* and *E. coli*, again as model Gram-positive and Gram-negative

organisms respectively, and inoculated non-human primate blood with the dilutions. Each was then subjected to four freeze/thaw cycles that slowly went from -80 °C to room temperature (approximately 21 °C), and then remained at room temperature overnight before being place back at -80 °C. This was meant to roughly simulate the freeze/thaw cycles as they would have occurred in the ETU. Prior to the first freezing of the samples, and each time the samples were thawed, an aliquot from each dilution was obtained and inoculated into the culture bottles. The endpoints to be measured were again whether or not growth was detected and the time to detection, if so, as well as a calculation of the CFU/mL for the last dilution and freeze/thaw for which growth still occurred. We found that freeze/thaw cycles had a much more profound impact on the viability of Gram-negative bacteria than Gram-positive, and some similar results had been previously published (Miyamoto-Shinohara et al., 2000; Rice et al., 2015). The time to detection was consistently prolonged following subsequent freeze/thaw cycles for Gram-negative bacteria, whereas little effect was observed in Gram-positive bacteria (Figure 3.2). These results were further confirmed by calculating the CFU/mL of the final dilution (1:100,000) for each species: *S. aureus* had not appreciably changed following 3 freeze/thaw cycles, but *E. coli* had dropped almost below the limit of detection.

This was a somewhat concerning finding, as it suggested that Gram-negative bacteria may be much less likely to grow from the samples. The only solution to this was the targeted deep-sequencing follow-up study which is planned, as it would not suffer in sensitivity due to a lack of bacterial viability,

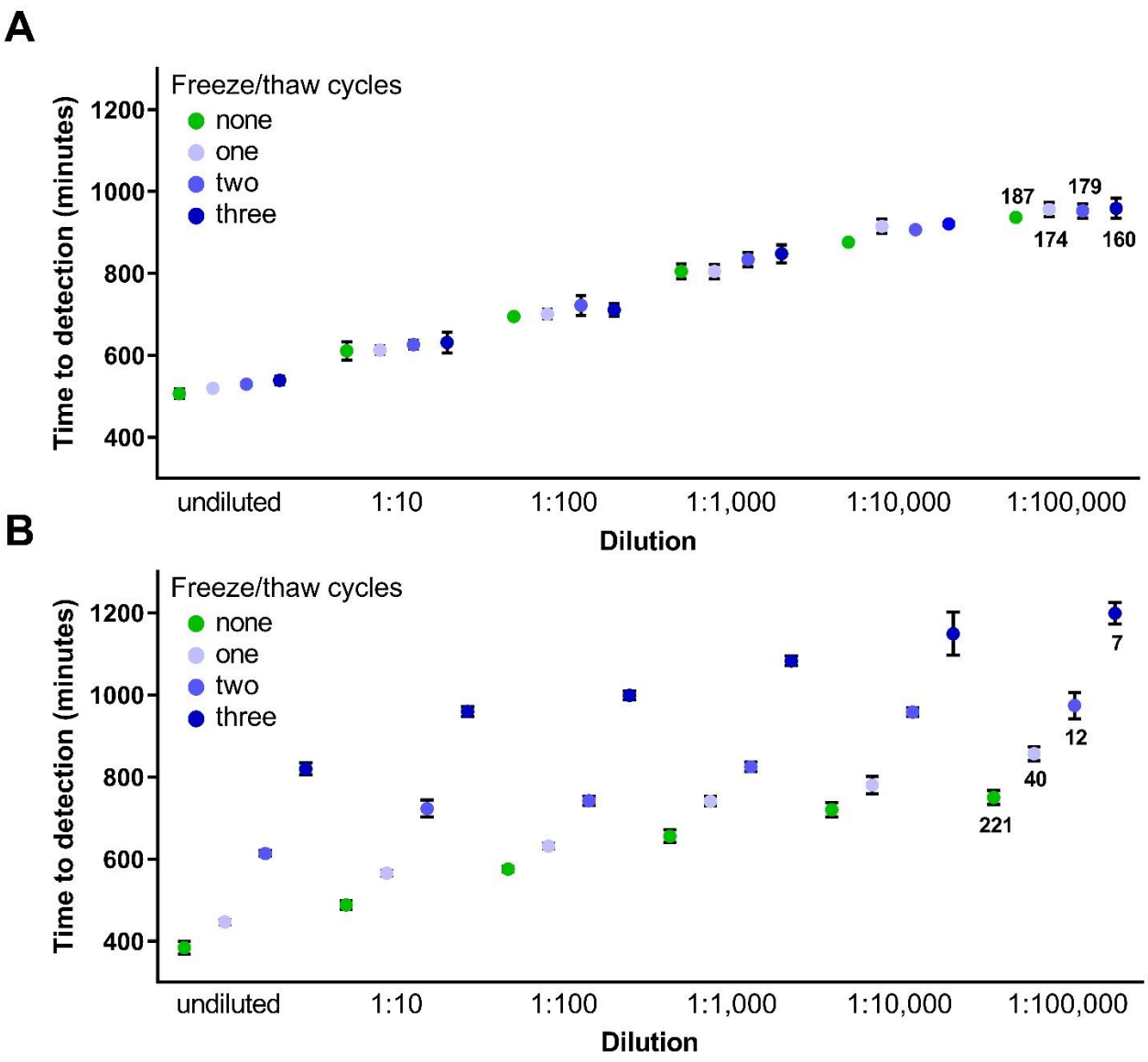


Figure 3.2. Effect of freeze/thaw cycles on *Staphylococcus aureus* (A) and *Escherichia coli* (B).

Each point represents 3 independent replicates. For A, no changes in time-to-detection (TTD) between adjacent samples for a given dilution were significant by a paired *t*-test. For B, all changes in TTD between adjacent samples for a given dilution were significant ($p < 0.05$) by a paired *t*-test. The numbers next to the symbols for the final series of points (1:100,000 dilution) correspond to the mean CFU/mL present in the three replicates.

The issue of skin contamination could not really be addressed with any prospective studies, and it was decided that all organisms cultured would be assessed on a case-by-case basis as to their pathogenic potential and clinical relevance. Significant literature is available on this topic, and many bacterial species are generally considered to be contaminants or true pathogens under most circumstances (Hall & Lyman, 2006).

For any samples positive for bacterial growth, a non-selective sub-culturing approach was chosen (Figure 3.3). The vast majority of clinically relevant isolates will grow on either sheep blood agar (SBA) or chocolate agar (made from lysed blood), or both. The BSL-4 facilities at RML, where the blood cultures would be performed, are not set up for clinical microbiology (e.g. Gram stains, bacterial microscopy, etc.), and the personnel available to conduct the study were not trained or experienced in identifying bacterial isolates through standard biochemical algorithms. Thus, following agar sub-culturing, pure colonies were to be selected for inactivation and removal from BSL-4 for identification in BSL-2 by 16S sequencing and by MALDI-TOF MS, for which the optimization and validation is described below. Utilization of both modalities would ideally provide reliable species-level identification with a high degree of confidence.

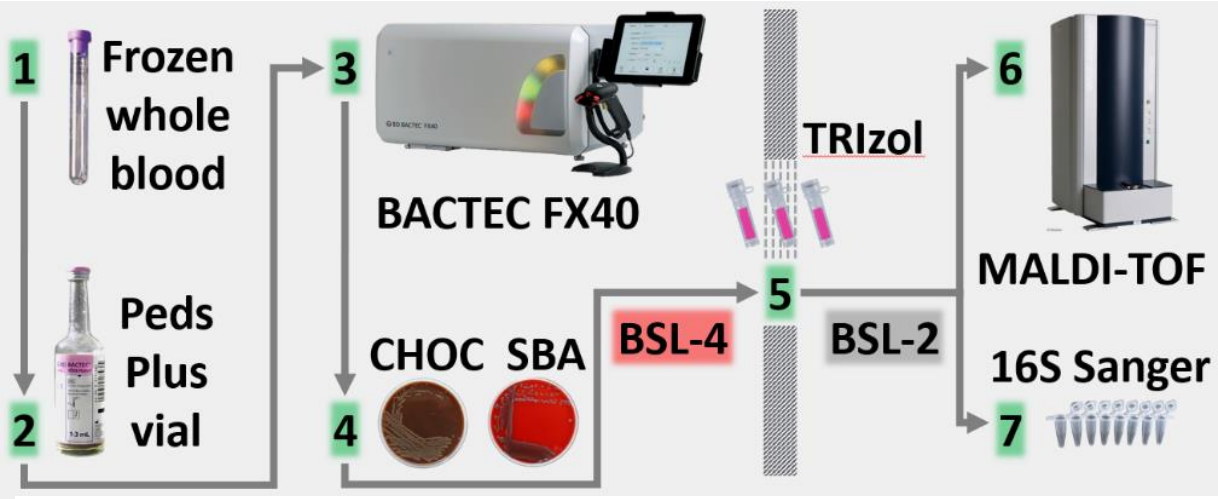


Figure 3.3. Workflow for culturing and identifying the ELWA-3 patient samples.

Compatibility of maximum-containment virus-inactivation protocols with identification of bacterial co-infections by matrix-assisted laser desorption/ionization time-of-flight mass spectrometry

Adapted from an article published in *The Journal of Infectious Diseases*

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Abstract

Diagnostics and research analyses involving samples containing maximum-containment viruses present unique challenges, and inactivation protocols compatible with downstream testing are needed. Our aim was to identify a validated viral inactivation protocol compatible with bacterial identification by matrix-assisted laser desorption/ionization – time-of-flight mass spectrometry (MALDI-TOF MS). We assessed a panel of bacteria with six validated maximum-containment virus inactivation protocols and report that inactivation with TRIzol or gamma irradiation is compatible with MALDI-TOF MS. The TRIzol-based inactivation protocol is widely applicable for speciation of bacteria from samples containing maximum-containment viruses.

BACKGROUND

The importance of developing maximum-containment virus inactivation protocols that are compatible with diagnostics was demonstrated during the 2013-2016 West Africa Ebola epidemic as 27 patients infected with Ebola virus (EBOV) were treated in advanced healthcare facilities in Europe and the United States. These facilities and their associated laboratories had limited capacity to isolate and identify bacterial co-pathogens from clinical specimens of Ebola virus disease (EVD) patients. An EVD patient treated in Hamburg, Germany developed severe gram-negative sepsis, but the etiological agent was not determined as 'more advanced tools for full identification of the organism and assessment of speciation were not accessible' (Uyeki et al., 2016). A recent study utilizing an unbiased sequencing approach applied to blood specimens of EVD patients cared for in Guinea suggests bacterial sepsis might frequently complicate EVD (Carroll et al., 2017), further emphasizing the need for improved diagnostics to detect bacterial co-infection among EVD patients. Current EVD treatment protocols recommend empirical use of broad-spectrum antibiotics for patients treated in African Ebola treatment units (WHO, 2014b), and 81% of the EVD patients treated in Europe or the United States during the 2013-2016 epidemic also received intravenous broad-spectrum antibiotics (Uyeki et al., 2016). Improved diagnostics and continued research may allow for more targeted use of antibiotics in EVD or other viral hemorrhagic fevers.

Laboratory handling of EVD patient specimens requires effective virus inactivation and maintenance of specimen integrity for downstream analyses (Haddock et al., 2016). Here we report testing of six validated virus inactivation protocols (Haddock et al., 2016; Hume et al., 2016; van Kampen et al., 2017) for their downstream compatibility with bacterial identification by matrix-assisted laser desorption/ionization – time-of-flight mass spectrometry (MALDI-TOF

MS). Each inactivation protocol has been validated with EBOV (Hume et al., 2016; van Kampen et al., 2017) or vesicular stomatitis virus expressing EBOV glycoprotein (rVSV-EBOVgp-GFP) (Haddock et al., 2016) as surrogates for a range of maximum-containment enveloped single-strand negative-sense RNA viruses, including other filoviruses, arenaviruses, bunyaviruses, orthomyxoviruses, and paramyxoviruses.

MATERIALS AND METHODS

Bacteria Species

Six representative bacteria commonly associated with bloodstream infections in human were selected for evaluation in this study, including gram-negative and gram-positive species: *Escherichia coli* (DH10B), *Pseudomonas aeruginosa* (PRD-10), *Klebsiella pneumoniae* (ATCC® 13883™), *Acinetobacter baumannii* (ATCC® BAA-1605™), *Staphylococcus aureus* (ATCC® 29213™), and *Streptococcus pyogenes* (ATCC® BAA-947™). To simulate protocols for bacterial isolation in clinical microbiology laboratories, aliquots of each bacteria were first inoculated into BACTEC™ Peds Plus™ aerobic media culture bottles (Becton, Dickinson and Company, Franklin Lakes, NJ, USA) and incubated in a BACTEC™ FX instrument (Becton, Dickinson and Company) until positive. Positive media were sub-cultured by plating on non-selective sheep blood agar (SBA) and incubated overnight (35°C, 5% CO_2).

Inactivation Protocols

Six virus inactivation protocols were assessed. The first (1) is based on treatment with 8 megarads of gamma radiation. The remaining protocols are based on chemical lysis. Three rely on guanidinium thiocyanate-based lysis reagents: (2) TRIzol (Life Technologies, Grand Island, NY, USA); and (3) Buffer AVL (QIAGEN, Hilden, Germany) and (4) Buffer RLT (QIAGEN). The final two protocols involve: (5) incubation in 10% neutral buffered formalin (NBF); and (6)

lysis with an SDS buffer consisting of 4% SDS, 35% glycerol, 0.05% bromophenol blue, and 20% β-mercaptoethanol, buffered to pH 6.8 with 200 mM Tris followed by heating for 10 minutes at 100°C.

In each inactivation protocol, approximately one-half loopful of a 10 µL loop of each bacterial species was collected in triplicate from the SBA as starting material. For gamma-irradiation, the bacteria were suspended in tryptic soy broth containing 10% glycerol, vortexed, and then irradiated with a ^{60}Co source in a Model 484 irradiator (J. L. Shepherd and Associates, San Fernando, CA, USA) frozen on dry ice until an 8 megarad dose was achieved. For TRIzol, the bacteria were placed directly in 1 mL of TRIzol, vortexed, and incubated at room temperature for 10 minutes. Proteins were then purified and pelleted according to the manufacturer's instructions; however, the pellet was not resuspended in 1% SDS buffer and was instead frozen in pellet form. For AVL, the bacteria were first suspended in 250 µL of PBS. 140 µL of this suspension was then transferred to 560 µL of AVL, vortexed, and incubated at room temperature for 10 minutes. The entire sample was transferred to a 2-mL tube containing 560 µL of 100% ethanol. For RLT, the bacteria were placed directly in 600 µL of the RLT, vortexed, and incubated at room temperature for 10 minutes. The entire sample was then transferred to a 2-mL tube containing 600 µL of 70% ethanol. For 10% NBF and SDS buffer, the bacteria were placed directly in 1 mL of the respective reagent, vortexed, and incubated at room temperature for 10 minutes. The SDS samples were further heated at 100°C for 10 minutes.

MALDI-TOF MS

The MALDI-TOF MS testing was performed on a MALDI Biotyper Microflex LT system (Bruker Daltonik GmbH, Bremen, Germany). For TRIzol, the protein pellet was resuspended in 50 µL of 70% formic acid, mixed by pipetting, followed by the addition of 50 µL

of 100% acetonitrile and vortexed to mix. For gamma-irradiation, AVL, RLT, 10% NBF, and SDS buffer, the samples were centrifuged at 15,000 x *g* for 2 minutes, and the supernatant was removed. 50 µL 70% formic acid was added to each pellet, mixed by pipetting, followed by the addition of 50 µL of 100% acetonitrile and vortexed to mix. Standards were prepared according to a routine MALDI-TOF protocol by suspension of half loopfuls of bacteria in 1 mL of 70% ethanol, centrifugation at 15,000 x *g* for 2 minutes to pellet, discarding of supernatant, and resuspension of pellets in formic acid and acetonitrile as above. Controls consisting of commercially prepared bacterial peptides were included on each run. 1 µL aliquots of each of these samples were spotted onto a microScout™ plate (Bruker), allowed to dry, and overlaid with Bruker Matrix HCCA solution (α-cyano-4-hydroxycinnamic acid in a standard solvent containing 50% acetonitrile, 47.5% water, and 2.5% trifluoroacetic acid). A $\log_{(score)}$ of ≥ 2.0 is recommended for reliable identification at the species level, and a $\log_{(score)}$ of $1.7 \leq x < 2.0$ is recommended by the manufacturer for confident identification at the genus level. A $\log_{(score)}$ of < 1.7 does not provide any confident identification.

Statistical Analysis

It was required that all identifications within 10% of the top $\log_{(score)}$ value concorded with the reported genus (for genus-level identification) or species (for species-level identification) (Haddock & Feldmann, 2017; Khot et al., 2012; Saffert et al., 2011). A two-tailed *t*-test was used to assess significant differences in $\log_{(score)}$ values across inactivation methods. A p-value < 0.05 was considered significant.

RESULTS

Sample Inactivation by TRIzol or Gamma Irradiation is Compatible with MALDI-TOF MS

Inactivation of bacterial samples from culture by gamma irradiation or TRIzol prior to analysis by MALDI-TOF MS allowed for confident and accurate bacterial speciation in all 18 samples tested.

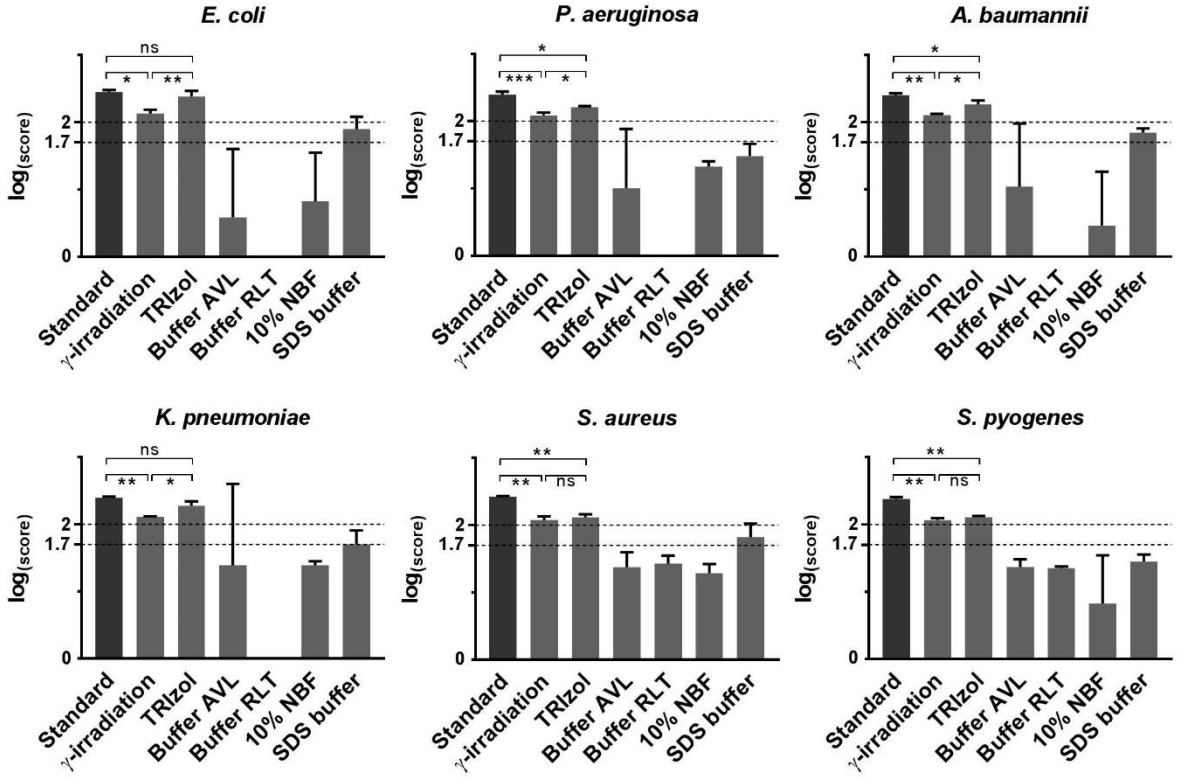

Figure 3.4 – Bacterial identification $\log_{(score)}$ values for the six viral inactivation protocols and a standard protocol tested with six bacterial species

All samples were analyzed in triplicate, and data are presented as mean values with standard deviations. Manufacturer $\log_{(score)}$ recommendations are as follows: ≥ 2.0 indicates confident species-level identification, $1.7 \leq x < 2.0$ indicates confident genus-level identification, and < 1.7 does not indicate confident identification. Statistical significance was calculated with a 2-tailed *t*-test. *A. baumannii*, *Acinetobacter baumannii*; *E. coli*, *Escherichia coli*; *K. pneumoniae*, *Klebsiella pneumoniae*; NBF, neutral buffered formalin; NS, not significant; *P. aeruginosa*; *Pseudomonas aeruginosa*; *S. aureus*, *Staphylococcus aureus*; SDS, sodium dodecyl sulfate; *S. pyogenes*, *Streptococcus pyogenes*.

Although significant differences did exist in some $\log_{(score)}$ values between the standard MALDI-TOF MS protocol and the gamma radiation- or TRIzol-inactivated samples (Figure 3.4), all inactivated samples provided species-level confident $\log_{(score)}$ values of ≥ 2.0 that were also accurate. For *E. coli* and *K. pneumoniae,* the $\log_{(score)}$ values obtained after TRIzol inactivation were not significantly different than the standard protocol. Additionally, TRIzol inactivation provided significantly higher $\log_{(score)}$ values when compared to gamma irradiation for all gram-negative species tested. The other four inactivation protocols failed to consistently provide confident species-level or genus-level results, and often produced no identifiable spectra (Table 3.1). Results from all inactivation protocols with a $\log_{(score)}$ value of ≥ 1.7 were accurate to the respective genus- or species-level and met the 10% rule.

Table 3.1 – Bacterial species and genus identification results by inactivation protocol

	Inactivation method					
	TRIzol	Buffer AVL	Buffer RLT	10% NBF	SDS buffer	γ-irradiation
Confident species identification[a] ($\log_{(score)} \geq 2.0$)	18/18	1/18	0/18	0/18	2/18	18/18
Confident genus identification[b] ($\log_{(score)}$ $1.7 \leq x < 2.0$)	n/a	3/18	0/18	3/18	9/18	n/a
No confident identification ($\log_{(score)} < 1.7$)	n/a	14/18	18/18	15/18	7/18	n/a

[a]All $\log_{(score)}$ values ≥ 2.0 produced accurate species level identification and met the 10% rule.
[b]All $\log_{(score)}$ values $1.7 \leq x < 2.0$ produced accurate genus level identification and met the 10% rule.

DISCUSSION

Each of the inactivation protocols described here is effective for the inactivation of maximum-containment enveloped single-strand negative-sense RNA viruses such as EBOV. However, the suitability of each protocol for maintaining bacterial proteins that are identifiable by MALDI-TOF MS was unknown. Our hypothesis was that the gamma irradiation protocol would allow for the greatest preservation of protein integrity and thus produce the most robust MALDI-TOF MS results. The effect of gamma radiation on proteins in solution has been shown to be mediated primarily through an indirect mechanism involving radiolytic H^+ and OH^- formed from water and can be mitigated 1,000–10,000 fold if the proteins are irradiated while in a frozen solution (Kempner, 2001), as was the case in our study. While the gamma irradiation protocol ultimately did produce acceptable results, some damage to the proteins likely still occurred as evidenced by the significantly decreased $\log_{(score)}$ values when compared to the standard and the TRIzol protocol. The wash steps during purification of the protein pellet after TRIzol inactivation were adequate for removal of excess salts and other contaminants known to interfere with MALDI-TOF MS analysis by formation of adducts and signal suppression if present in sufficient concentrations (Schaiberger & Moss, 2008; Xu et al., 2006). As the other inactivation protocols involve no such purification and washing steps, it is reasonable to posit that excess salt and other impurities remaining in solution were responsible for poor performance. Consistent with this hypothesis is that noticeable crystalline residue formed on the microScout™ plate from many of the sample aliquots suggesting further optimization of these methods might be possible. The gamma-irradiation protocol is likely of limited utility due to its lack of accessibility, cumbersome infrastructure required, and slow processing time. The TRIzol-based viral inactivation protocol is simple, economical, easily accessible, and rapid, and is widely applicable

in both clinical and research settings for speciation of bacteria in samples containing maximum-containment viruses such as EBOV. The method described here requires a sample culturing step for bacteria, as is the typical protocol for other bacterial diagnostics. Samples must be handled appropriately within containment during culturing as this step must take place prior to viral inactivation. MALDI-TOF MS is a robust, high-throughput alternative to traditional biochemical analysis or 16S sequencing and is an emerging microbiological diagnostic platform of choice. Further studies with an expanded panel of bacteria and pathogenic yeast could further validate this promising method, and ultimately improve diagnostics and research analyses involving bacterial co-pathogens in maximum-containment viral infections. The burgeoning threat of emerging viruses necessitates development of viral inactivation protocols that are compatible with a full range of downstream diagnostic analyses, such as that reported here, to maximize preparedness for inevitable future outbreaks.

§ 2 – The Study

Ebola virus disease is not routinely complicated by bacteremia: a cross-sectional study
A manuscript in preparation

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Introduction

Ebola virus disease (EVD) is caused by Ebola virus (EBOV), a single-stranded negative-sense RNA virus of species *Zaire ebolavirus* that is thought to be enzootic in central and western Africa.(Feldmann & Geisbert, 2011; Groseth et al., 2007) Human outbreaks tend to be relatively short and small and are likely initiated by rare, sporadic zoonotic spillover events following contact with an unidentified animal reservoir or an intermediate host.(Matson et al., 2020b) EVD is clinically characterized by early constitutional signs and symptoms, followed by voluminous watery diarrhea, vomiting, abdominal pain, rash, and occasionally hemorrhagic features, meningoencephalitis, and respiratory distress.(Chertow et al., 2014; Feldmann & Geisbert, 2011; Malvy et al., 2019)

Recently, particularly during and following the 2013-2016 West Africa epidemic, concern has arisen that bacteremia may be a routine complication secondary to EVD, possibly via gut translocation of bacteria following disruption of intestinal architecture.(Carroll et al., 2017; Kreuels et al., 2014; Lamontagne et al., 2018; Reisler et al., 2018; Uyeki et al., 2016; WHO, 2019d; Wolf et al., 2015) However, evidence in support of this is limited, consisting of a single case report and various other indirect observations (e.g. clinical suspicion, unbiased deep sequencing of patient samples).(Carroll et al., 2017; Kreuels et al., 2014; Wolf et al., 2015) Microbiological investigation is not routinely performed for EVD patients in resource-limited settings due to containment concerns and other logistical considerations. Nevertheless, current guidelines, including from WHO, for EVD supportive care include the recommendation for empiric administration of broad-spectrum antibiotics, and this was established as part of the standard of care for EVD patients during the 2013-2016 West Africa epidemic.(Ansumana et al., 2015; Bah et al., 2015; Chertow et al., 2014; Kreuels et al., 2014; Lamontagne et al., 2018;

Langer et al., 2018; Mulangu et al., 2019; Schieffelin et al., 2014; Sueblinvong et al., 2015; Uyeki et al., 2016; WHO, 2019d)

Here our aim was to determine the prevalence of bacteremia amongst a large cohort of EBOV-positive patients, with a large cohort of EBOV-negative patients that presented to the same ETU as a control group. We employed both a classical microbiology approach, which entailed the culturing of over 700 EVD patient whole blood samples, and also a targeted deep sequencing approach – the bacterial capture sequencing (BacCapSeq) platform(Allicock et al., 2018) – on a matched selection of 45 EBOV-positive and 45 EBOV-negative samples.

Materials and Methods

Whole blood samples were collected in EDTA tubes from patients at the Eternal Love Winning Africa 3 (ELWA-3) Ebola treatment unit (ETU) in Monrovia, Liberia between August and December 2014 for diagnostic use.(de Wit et al., 2016b) Samples were obtained following routine skin cleaning with an alcohol swab; as blood culture was not the intended use, more thorough surface preparation (e.g. chlorhexidine) was not performed. At the ETU, aliquots of the samples were inactivated and extracted either in a mobile biosafety level 3 (BSL3) laboratory or under negative pressure in a mobile glove box unit using the Qiagen Viral RNA Mini Kit.(de Wit et al., 2016b) Diagnostic testing for EBOV and for *Plasmodium* spp. parasitemia (de Wit et al., 2016a) was performed in the field using quantitative reverse-transcription polymerase chain reaction (qRT-PCR, for EBOV) and qPCR (for *Plasmodium* spp. parasitemia). Unused portions of the samples that were not inactivated were frozen and transported to the BSL-4 facility at the NIH/NIAID Rocky Mountain Laboratories.

A deidentified patient database was generated and cleaned, and included variables of age, sex, time from symptom onset to presentation, and outcome, amongst others (Table 3.2). A total

of 720 unique samples were cultured (Figure 3.5). Of these, 456 were first-draw samples obtained at triage, and consisted of 307 EBOV-positive samples and 149 EBOV-negative samples. The remaining 264 samples were re-draws, obtained following either an equivocal test at triage or upon convalescence in surviving patients to assess for viral clearance.

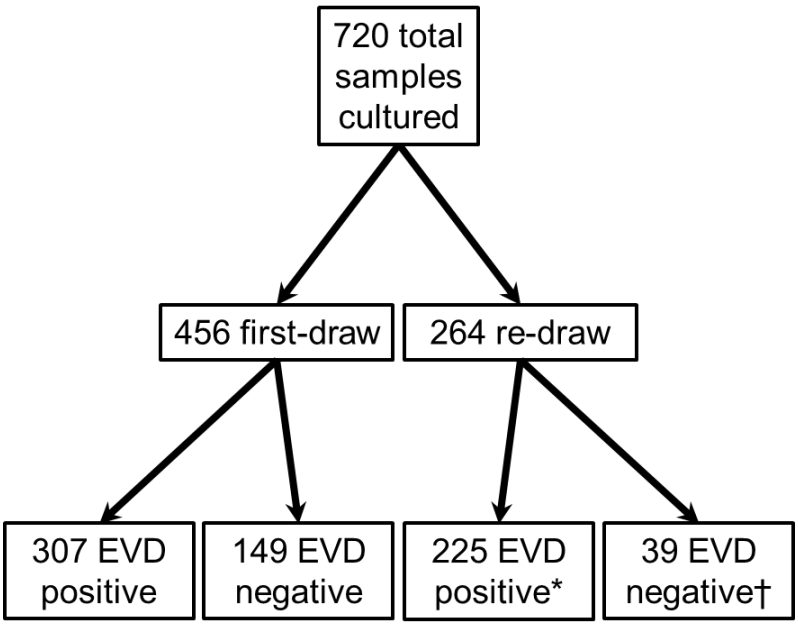

Figure 3.5. Sample selection for blood culture.

The 264 re-draw samples are sometimes, but not always, from the same patients as 456 first-draw samples. This was solely determined by sample availability. *For the EVD-positive re-draws, samples were collected from convalescing patients to assess viral clearance. The sample itself is not necessarily EBOV-positive. †EVD-negative re-draws were obtained to clarify an equivocal initial diagnostic test or upon continued clinical suspicion.

Samples remained sealed and frozen until processing in a class II biosafety cabinet in BSL-4 using sterile technique. Culturing was performed within BSL-4 containment using BD BACTEC Peds Plus /F culture vials containing BD fastidious organism supplement and a BD BACTEC FX40 automated blood culture instrument. 200 µL aliquots of each sample were inoculated aseptically into separate vials and incubated for a standard five-day protocol. Positive vials were sub-cultured on non-selective sheep blood agar and chocolate agar, and pure colonies were inactivated in TRIzol reagent for removal from BSL-4. 16S Sanger sequencing and

MALDI-TOF MS were performed on the nucleic acid extracts and protein extracts, respectively, for species-level identification of the isolates. For 16S Sanger sequencing, separate amplifications were performed with a panel of commonly used primers to ensure sufficient high-quality sequence was obtained: 8F (5'-AGAGTTTGATCCTGGCTCAG-3'),(Edwards et al., 1989) 27F (5'-AGAGTTTGATCMTGGCTCAG-3'),(Lane, 1991) 357F (5'-CCTACGGGAGGCAGCAG-3'),(Muyzer et al., 1993) 1100R (5'-AGGGTTGCGCTCGTTG-3'),(Turner et al., 1999) 1391R (5'-GACGGGCGGTGTGTRCA-3'),(Turner et al., 1999) and 1492R (5'-GGTTACCTTGTTACGACTT-3')(Lane, 1991). Contigs were assembled in Geneious Prime 2020.1 and results were analyzed with BLAST for identification. Assembled 16S contigs used for identification were a minimum of 800 bp in length. For MALDI-TOF MS, purified protein pellets were analyzed with a Bruker MALDI Biotyper Microflex LT system as previously described.(Matson et al., 2018)

This study received a determination of 'not human subjects research' by the NIH Office of Human Subjects Research Protection (OHSRP) and has been approved by the University of Liberia-Pacific Institute for Research and Evaluation (UL-PIRE) IRB. All procedures involving viable EBOV were carried out in BSL-4 containment according to standard operating procedures.

Table 3.2. Demographic data for the 456 first-draw samples assessed by blood culture

Characteristic	All samples (n = 456)	EVD-positive (n = 307)	EVD-negative (n = 149)
Age – median (range)	28 (0-90)	29 (2-80)	27 (0-90)
Female sex – no. (%)	219 (48)	155 (50)	64 (43)
Days since symptom onset – median (range)	4 (0-28)	5 (0-28)	4 (0-21)*

*EVD-negative patients had self-reported symptoms that were consistent with EVD at triage

Results

Bacterial cultures were positive for 4.6% (14/307) of the first-draw EBOV-positive patient samples and 4.7% (7/149) of the first-draw EBOV-negative patient samples (Table 3.3). Comparison of this bacterial culture-positive rate between EBOV-positive and EBOV-negative patients, without consideration of the pathogenic potential of the species isolated, did not yield significant differences by Fisher's exact test (two-tailed *p*-value = 1.0000). Repeat samples which were bacterial culture positive after the first draw, obtained either to clarify an indeterminate initial test or to assess viral clearance in a convalescing patient, were excluded from this analysis due to the possibility of iatrogenic infection and because antibiotics were administered to all admitted patients prior to re-draws. They are included in Table 3.4. The presence of EDTA in the whole blood samples did not have a significant effect on sensitivity or time to detection (TTD) when tested using non-human primate blood samples with and without EDTA that were artificially inoculated with *E. coli* and *S. aureus* as model Gram-negative and Gram-positive organisms, respectively (Supplementary).

Table 3.3. First-draw blood culture results by Ebola virus disease status

	Bacterial culture-positive	**Bacterial culture-negative**
EBOV-positive	14	293
EBOV-negative	7*	142

*one sample grew a fungal isolate; see Table 3.4

Table 3.4. All blood culture-positive results by individual patient, including re-draw samples

ID, age, gender, date, first- (F) or re-draw (R)	EVD status, diagnostic C_t value, symptom onset	Isolate species, time to detection (hours), pathogen (P), contaminant (C), or equivocal (E)	Outcome
28 F 08/28/14, F	**Pos**, C_t = 30.3, S_x 14d	***Staphylococcus epidermidis***†, 24, C	**Survived**
24 F 08/30/14, F	**Pos**, C_t = 30.6, S_x ?d	***Micrococcus luteus****, 49, C	**Survived**
19 F 08/30/14, F	**Pos**, C_t = 32.1, S_x 4d	***Staphylococcus epidermidis***†, 23, C	**Survived**
40 M 08/31/14, R	**Convalescing**, C_t = 40, S_x 15d	***Dermococcus nishinomiyaensis***‡, 21, C	**Survived**
43 F 09/11/14, F	**Pos**, C_t = 25.9, S_x 9d	***Staphylococcus epidermidis***‡, 41, C	**Died** 09/14/2014
49 F 09/11/14, F	**Pos**, C_t = 24.1, S_x 7d	***Staphylococcus hominis***‡, 28, C	**Survived**
21 F 09/11/14, F	**Pos**, C_t = 29, S_x 13d	***Staphylococcus hominis***‡, 24, C	**Died** 09/12/2014
28 M 09/20/14, F	**Pos**, C_t = 33.2, S_x 3d	***Staphylococcus pettenkorferi***§, 19, C	**Survived**
51 M 09/22/14, F	**Pos**, C_t = 24.1, S_x 8d	***Staphylococcus epidermidis***‡, 22, C	**Survived**
28 F 09/26/14, F	**Pos**, C_t = 28.12, S_x 4d	***Paenibacillus cineris****, 42, E	**Died** 09/28/2014
13 F 09/26/14, F	**Pos**, C_t = 30.3, S_x ?d	***Staphylococcus aureus****, 21, P	**Died** 09/27/2014
25 F 09/28/14, F	**Pos**, C_t = 26.66, S_x 2d	***Staphylococcus warneri*#,** 31	**Died** 09/28/2014
56 M 10/09/14, F	**Pos**, C_t = 26.43, S_x 5d	***Micrococcus luteus****, 54, C	**Died** 10/15/2014
31 M 10/22/14, F	**Pos**, C_t = 24.92, S_x 3d	***Corynebacterium ulcerans***¶, 30, E	**Survived**
?? F 11/02/14, F	**Pos**, C_t = 23.6, S_x 1d	***Bacillus paranthracis***#, 12, E	**Died** 11/04/2014
16 F 09/08/14, F	**Neg**, S_x 7d	***Enterobacter cloacae***‡, 14, P	**Died** 09/08/2014
0 M 10/22/14, F	**Neg**, S_x 2d	***Staphylococcus cohnii***§, 27, C	**n/a**
6 M 11/04/14, F	**Neg**, S_x 4d	***Scopulariopsis brevicaulis***, 117, P	**Died** 11/05/2014
22 F 11/17/14, F	**Neg**, S_x 21d	***Paenibacillus barengoltzii****, 55, E	**n/a**
39 M 12/05/14, R	**Neg**, S_x 4d	***Micrococcus luteus***†, 63, C	**n/a**
19 F 12/05/14, F	**Neg**, S_x 9d	***Staphylococcus lugdunensis****, 25, C	**n/a**
43 M 12/04/14, R	**Neg**, S_x 2d	***Staphylococcus epidermidis***†, 26, C	**n/a**
58 M 12/10/14, F	**Neg,** S_x 8d	***Staphylococcus hominis***†, 114, C	**n/a**
29 M 12/10/14, F	**Neg**, S_x 3d	***Salmonella enterica****, 15, P	**n/a**

*16S sequencing and MALDI-TOF MS provided identical species-level identification (MALDI-TOF MS log(score) ≥ 2.0)
†16S sequencing and MALDI-TOF MS provided identical species-level identification; MALDI-TOF MS reliability scores were confident only to the genus level (MALDI-TOF MS log(score) $1.7 \leq x < 2.0$)
‡16S sequencing and MALDI-TOF MS provided identical species-level identification; MALDI-TOF MS reliability scores were below the threshold for confident identification (MALDI-TOF MS log(score) < 1.7)
§16S sequencing provided species-level identification; MALDI-TOF MS log(score) < 1.7 and agreed with 16S at the genus level
¶16S sequencing provided species-level identification; MALDI-TOF MS log(score) $1.7 \leq x < 2.0$ and agreed with 16S at the genus level
#16S sequencing and MALDI-TOF MS provided genus-level identification only; species determined by deep sequencing (Matson et al., 2020a)

For all the isolates, 16S sequencing and MALDI-TOF MS agreed to the genus level, and for most isolates, there was agreement to the species level (Table 3.4). In some instances, the MALDI-TOF MS log(score) was below recommended confidence thresholds, but nevertheless agreed with the 16S sequencing results. Coagulase-negative staphylococci (CoNS), which are generally considered to be skin contaminants introduced at the time of blood draw, accounted for many of the positive cultures, comprising 46.7% (7/15) (pending identification of 1677) of isolates from EBOV-positive samples and 44.4% (4/9) of isolates from EBOV-negative samples

(Table 3). Species which are universally considered pathogenic upon isolation from blood culture accounted for 7.1% (1/14) (pending identification of 1677) isolates from EBOV-positive samples (*S. aureus*, sample #1588) and for 30% (3/10) isolates from EBOV-negative samples (*E. cloacae*, sample #745; fungal, sample #3372; *S. enterica*, sample #4253). Three samples suspected of producing polymicrobial growth based on colony morphology were found to be monomicrobial upon identification by 16S sequencing and MALDI-TOF MS. Amongst the samples that were not first-draws (Figure 1, Table 3), 0.44% (1/225) of re-draw samples obtained from convalescing EVD patients were positive, and 5.1% (2/39) of re-draw samples obtained to confirm an equivocal first-draw sample were positive.

Discussion

Here we report the most extensive microbiological examination for the presence of bacteremia in human EVD blood samples to date, with an EVD-negative cohort triaged at the same ETU as a control group. We cultured the entirety of stored blood samples with sufficient volume which were available to us (n = 720) using a gold-standard diagnostic microbiology approach, with minor modifications for the unique needs of this study. Blood cultures are rarely obtained in the field at ETUs, although one small study reported only a single CoNS isolate from 18 EVD patients at a military Ebola treatment unit (ETU) in Sierra Leone.(Lamb et al., 2015) Very limited blood culturing was reported amongst the 27 EVD patients treated in Europe and the United States, and yielded only a single, unidentified Gram-negative isolate from a patient following extensive invasive supportive care.(Kreuels et al., 2014) Another study that utilized stored EVD patient blood samples failed to produce any bacterial isolates upon culturing, although a modern, diagnostic-standard automated culture system (e.g. BACTEC) was not utilized.(Carroll et al., 2017)

In this study, the prevalence of what was likely clinically relevant bacteremia from species generally considered to be pathogenic was very low for both the first-draw EVD-positive (1/307, 0.33%) (pending identification of 1677) and first-draw EVD-negative (3/149, 2.0%) (pending fungal identification) samples (Table 2). This may have been partly due to the inherent limitations of these stored samples. However, the percentages of samples from which a CoNS species was obtained (7/307, 2.3% from EVD-positive first-draw samples; 4/149, 2.7%, from EVD-negative first-draw samples), which are almost universally considered to be false-positives, fell well within the positive-culture percentage range to be expected from skin contaminants,(Hall & Lyman, 2006) suggesting that the viability of bacteria in the samples was not overtly compromised and that the sensitivity of our culture protocol was acceptable. The predominance of Gram-positive bacteria amongst the cultured isolates may be explained by their increased tolerance of freeze/thaw cycles compared to Gram-negative bacteria,(Miyamoto-Shinohara et al., 2000; Rice et al., 2015) a phenomenon we also observed in our preliminary optimization of the culture protocol implemented here (Supplemental). The pathogenic potential of some of the isolates (e.g. *Paenibacillus* spp. and *Bacillus paranthracis*(Matson et al., 2020a)) is less certain, particularly considering the immunocompromised state of an EVD patient, and could not be reliably determined without numerous other clinical and laboratory observations. The lower positive rate (1/225, 0.44%) amongst re-draws from convalescing EVD patients may be due to the empiric administration of antibiotics. For samples #745 and #3372, obtained from EVD-negative patients, the cultured organisms provide a likely explanation both for presentation to the ETU and for the fatal outcomes (which were not typically known for EVD-negative patients).

It has long been recognized that the clinical features and laboratory findings of advanced EVD overlap significantly with bacterial sepsis (e.g. systemic inflammation, coagulopathy, vascular leakage, multi-organ failure, etc.),(Bray & Mahanty, 2003; Hellman, 2015) and more recent studies utilizing an array of 'omics approaches have characterized numerous parallels on a molecular level.(Eisfeld et al., 2017) Given these similarities, clinical suspicion and other non-specific biochemical findings, in the absence of culture confirmation, should not be relied upon for a diagnosis of bacterial sepsis in an EVD patient. Prior to the West Africa epidemic, systematic empiric antibiotic administration to EVD patients was rarely reported.(Bwaka et al., 1999; Georges et al., 1999; Guimard et al., 1999; Kratz et al., 2015) Médecins Sans Frontières 2008 Filovirus Haemorrhagic Fever Guideline recommended empiric antibiotics be administered to all suspect patients at ETUs explicitly to provide coverage for patients presenting with non-EBOV bacterial etiologies (e.g. typhoid). This was a rational protocol considering that it could take days to receive the results from EVD diagnostic testing in the Democratic Republic of the Congo at the time (on-site diagnostic testing with same-day result reporting was only broadly implemented for the first time in West Africa). Many bacterial illnesses (including bacterial sepsis, as was present in two of the EBOV-negative patients reported here) are clinically indistinguishable from EVD, and initiation of early treatment can be critical. Moreover, EVD patients may indeed present with bacterial co-infections, many of which do not typically result in bacteremia and so would not have been detected in this study, that must be concomitantly treated with antibiotics. Adjustments in treatment could be made as necessary when testing results became available, and antibiotics could be continued in EVD patients as well depending on the physician discretion.(Kratz et al., 2015; Sterk, 2008)

More recently though, the focus has shifted towards a concern for the development of bacterial coinfections secondary to EVD, as patients are in a susceptible immunocompromised state. This may occur either via an exogenous source (e.g. iatrogenic infection via invasive medical procedures), or an autogenous source (e.g. translocation of gut bacteria from disrupted intestinal architecture), with the latter of these receiving much attention in the literature as a working hypothesis, despite a paucity of evidence.(Carroll et al., 2017; Kreuels et al., 2014; Lamontagne et al., 2018; Reisler et al., 2018; WHO, 2019d) However, our findings reported here suggest that bacteremia is rare in EVD, regardless of the source, and imply that impact of systematic empiric antibiotic administration in EVD should continue to be evaluated. Significantly, the contributions of gut microbiota to immune function and the complexities of within-host interactions between commensal microbes and invasive viruses are increasingly being appreciated,(Ahern & Maloy, 2020; Dominguez-Diaz et al., 2019; Li et al., 2019; Zheng et al., 2020) and perturbations to the gut microbiome from antibiotic administration have recently been shown to negatively impact the immune response and outcome for various viral diseases in animal models.(Brown et al., 2019; Ichinohe et al., 2011; Thackray et al., 2018; Yaron et al., 2020) Furthermore, some antibiotics recommended for empiric administration to EVD patients, such as ciprofloxacin,(WHO, 2019d) can occasionally illicit dangerous cardiac arrythmias, particularly as electrolyte disturbances are commonly observed.

The initially reported CFR during the West Africa epidemic was markedly lower than historical outbreaks, with a naïve calculated value of approximately 40% often cited. Optimism arising from this figure has led to speculation that the new standard of care provided to EVD patients during the epidemic, including the empiric administration of antibiotics, may have improved patient outcomes.(Aluisio et al., 2020; Ansumana et al., 2015; Bah et al., 2015; Fischer

et al., 2019; Kreuels et al., 2014; Lamontagne et al., 2018; Langer et al., 2018; Schieffelin et al., 2014; Uyeki et al., 2016; WHO, 2019d) One retrospective cohort study demonstrated that empiric oral administration of cefixime may have reduced mortality, although the significance of the findings was equivocal.(Aluisio et al., 2020) However, the mean CFR amongst confirmed EVD patients with recorded clinical outcomes was approximately 63%, well within historical norms, and recent analyses have convincingly suggested that the CFR was likely even higher,(Forna et al., 2020; Garske et al., 2017) calling in to question the impact of the level of supportive care that was broadly provided in West Africa. Developing the capacity to safely and efficiently carry out diagnostic microbiology, including blood culture, in ETUs is thus a crucial component of improving the standard of care for EVD patients and informing the rational use of antibiotics as part of the treatment protocol. Animal studies addressing this topic are also extremely limited,(Reisler et al., 2018) and none that we are aware of have assessed the effect of empiric antibiotics in EVD. There is little doubt, however, that EVD is a treatable illness given an appropriate combination of advanced supportive care and drugs, as the overall CFR for the 27 EVD patients treated in Europe and the United States during the 2013-2016 epidemic was 18.5%.(Fischer et al., 2019; Uyeki et al., 2016) Continuing to optimize the supportive care protocols for EVD patients, particularly in combination with EVD-specific drugs in development,(Group et al., 2016; Mulangu et al., 2019) will improve patient outcomes and facilitate appropriate antibiotic stewardship.

***Bacillus paranthracis* isolate from blood of fatal Ebola virus disease case**
Adapted from an article published in *Pathogens*

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Abstract

A *Bacillus paranthracis* isolate was cultured from the blood of a fatal Ebola virus disease (EVD) case in Liberia and was identified by whole genome sequencing. Although *B. paranthracis* has only recently been described and is poorly characterized, this case may represent the bacterial co-infection of an EVD patient.

Bacteremia, possibly via gut translocation, may complicate Ebola virus disease (EVD) (WHO, 2019c) (Kreuels et al., 2014) (Wolf et al., 2015) (Carroll et al., 2017). However, supporting data are limited as microbiological investigations are not routinely performed in Ebola treatment units (ETUs) during outbreaks. A small cohort of 18 EVD-positive patients for which blood cultures were obtained in Sierra Leone yielded one isolate of a coagulase-negative *Staphylococci* skin contaminant (Lamb et al., 2015). A single case of gram-negative sepsis was reported in an EVD patient treated in Europe, although the organism was not fully identified, and iatrogenic infection could not be excluded (Kreuels et al., 2014). Other, indirect evidence of bacteremia in EVD patients hinges on clinical observation (WHO, 2019c) (Wolf et al., 2015), and retrospective unbiased deep sequencing (Carroll et al., 2017).

The study

We evaluated stored de-identified first-draw blood specimens from patients admitted to the ELWA-3 ETU in Monrovia, Liberia between August 28, 2014 and December 18, 2014 for the presence of bacterial co-infection (de Wit et al., 2016b). Here we report the microbiological and molecular characterization of a bacterial isolate from a single female patient who tested positive for Ebola virus by quantitative reverse-transcriptase polymerase chain reaction (cycle threshold = 23.58) four days following symptom onset. This patient tested negative for malaria and died two days after admission with no further samples collected. This study was designated 'not human subjects research' by the NIH Office of Human Subjects Research Protection (OHSRP) and approved by the University of Liberia-Pacific Institute for Research and Evaluation (UL-PIRE) institutional review board.

Upon triage, whole blood specimens were collected in EDTA tubes and frozen at -80 °C for long-term storage at biosafety level 4 (BSL-4). A 200 µL aliquot of whole blood from each

sample was inoculated into BD BACTEC Peds Plus /F culture vials with BD fastidious organism supplement and incubated in a BD BACTEC FX40 (Becton Dickinson, https://www.bd.com/). Logarithmic growth by fluorescence was detected in this patient's sample 11.5 hours after inoculation. Samples that flagged positive were sub-cultured on non-selective sheep blood agar (SBA) and chocolate agar at 35 °C without supplemental CO_2. This sample produced heavy monomicrobial growth of dull, gray-white, opaque colonies at 24 hours, with weak β-hemolysis evident directly beneath the colonies at 48 hours. The isolate was designated RML14492_ELWA-3_3298 (ELWA 3298).

For identification, colonies were removed from BSL-4 after inactivation in TRIzol (Haddock et al., 2016) (Thermo Fisher, https://www.thermofisher.com) according to standard operating procedures approved by the Institutional Biosafety Committee. Analysis of nucleic acids by 16S rRNA sequencing and purified protein extract by matrix-assisted laser desorption ionization–time of flight mass spectrometry (MALDI-TOF MS) (Matson et al., 2018) provided only confident genus-level identification within the *Bacillus cereus sensu lato* group (Pauker et al., 2018). The lack of strong β-hemolysis on SBA and the geographical proximity of the patient both to human infections of *B. anthracis* and zoonotic infections of *B. cereus* biovar *anthracis* (Klee et al., 2006) prompted full-genome sequencing for species-level identification, as conventional microbiological testing and other diagnostic assays were not immediately available in BSL-4 (Table 3.5). A TruSeq DNA Nano kit was used for library preparation and 250 bp paired-end sequencing was performed on a MiSeq sequencer (Illumina, https://www.illumina.com). Raw reads were trimmed, filtered, and coordinate-order sorted using Cutadapt v1.12, FASTX Toolkit v0.0.14, and a custom Perl script. Mira v4.0.2, Velvet v1.2.10, Sequencher (Genecode 5.4.6), and Pilon v1.22 were used for assembly and final polishing. The

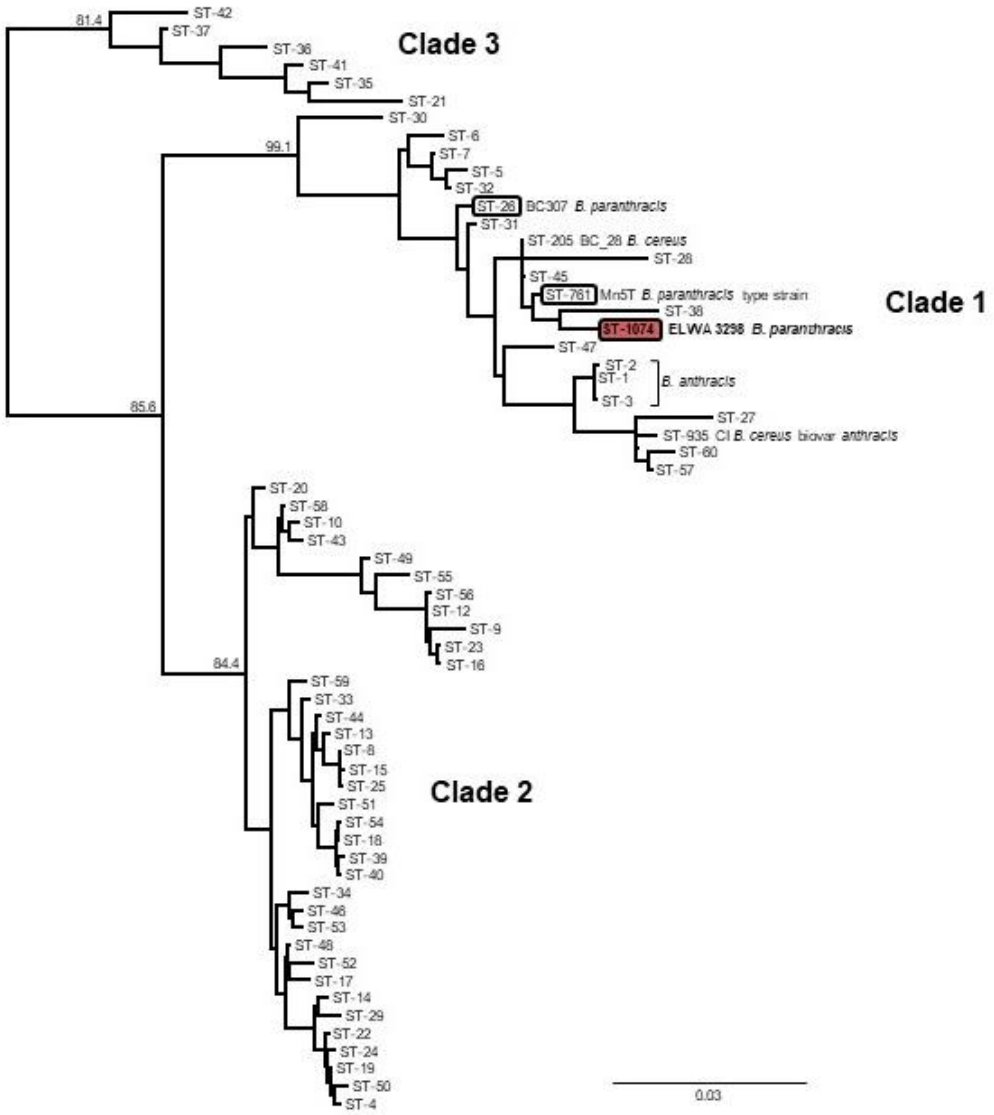

Figure 3.6 - Maximum-likelihood multilocus sequence typing (MLST) phylogenetic tree for the *B. cereus sensu lato* group, including isolate ELWA 3298

Concatenated sequences for seven genes (*glp*, *gmk*, *ilv*, *pta*, *pur*, *pyc*, and *tpi*) were downloaded from the publicly available *B. cereus* database at pubmlst.org. Representative sequence types (STs) for the *B. cereus sensu lato* group were chosen following Priest et al. (Priest et al., 2004), with additional STs relevant to this report included (1074, 761, 26, 935). The tree was constructed with PhyML using a GTR substitution model and 1,000 bootstrap replicates. Support values for the main branches are shown. STs with *B. paranthracis* are indicated with the ST-## enclosed, and the ST for the ELWA 3298 *B. paranthracis* isolate in this report is highlighted red. The tree was rooted with ST-83 *B. pseudomycoides* (not shown). Scale bar represents nucleotide substitutions per site. Bootstrap values are reported as percentage out of 1,000 replicates.

draft genome *de novo* assembly resulted in 71 contigs with a total length of 5,681,902 bp, an N50 value of 182,992 bp, and a GC content of 35.2%. This whole genome shotgun project has been deposited at DDBJ/ENA/GenBank under the accession WQMW0000000. Following annotation with the NCBI Prokaryotic Genome Annotation Pipeline and average nucleotide identify analysis, the isolate was identified as *B. paranthracis* as it demonstrated a 97.6% identity with 83% coverage to the type strain of *B. paranthracis*, Mn5T. *B. paranthracis* is poorly characterized and was first described in 2017 (Liu et al., 2017), although current reports describe its isolation from human feces (Bukharin et al., 2019) and it may have been responsible for a small emetic and diarrheal outbreak (Carroll et al., 2019).

Table 3.5 – Phenotypic characterization of isolate ELWA 3298 compared with other *Bacillus* spp. isolates

Bacillus species/strain	Hemolysis	Motility	Penicillin	Catalase	PEA[§] growth
ELWA 3298, *B. paranthracis*	weak β*	+[†]	R[‡]	+	+
B. cereus sensu stricto	strong β	+	R	+	+
B. cereus biovar *anthracis*	γ	+/-	S/R	+	unknown
B. anthracis	γ	-	S	+	-

Table adapted from American Society for Microbiology's *Sentinel Level Clinical Laboratory Guidelines for Suspected Agents of Bioterrorism and Emerging Infectious Diseases, 2017*

*β-hemolysis on sheep blood agar was most evident at 48 hours and directly beneath colonies
[†]Motility testing was performed with 2,3,5-triphenyltetrazolium chloride (TTC) motility agar using *Escherichia coli* DH10B as a positive control and *Klebsiella pneumoniae* (ATCC 13883) as a negative control
[‡]Antimicrobial susceptibility testing was performed with Sensititre broth microdilution (Thermo Fisher, https://www.thermofisher.com) and interpreted according to CLSI M45 MIC criteria; the isolate was also resistant to oxacillin, ampicillin, and cephalosporins
[§]Phenylethyl alcohol agar

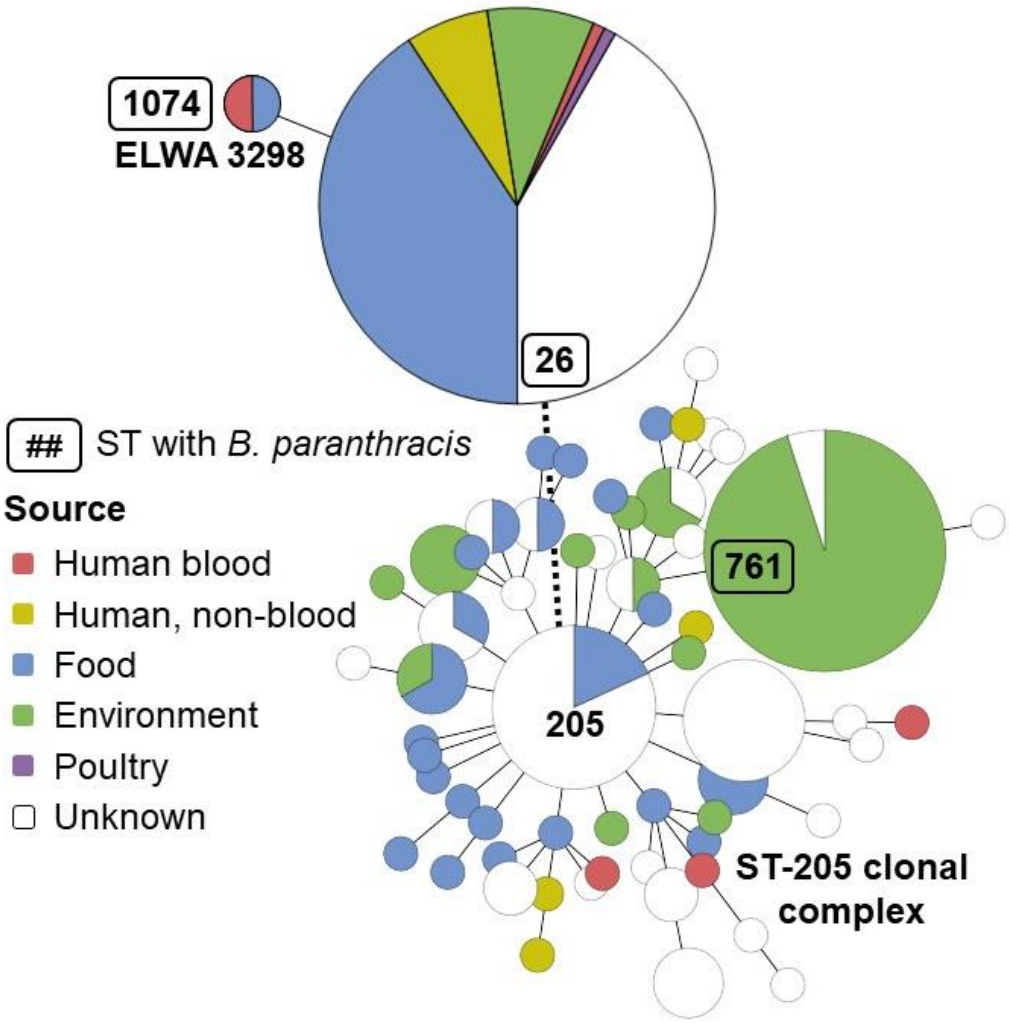

Figure 3.7 – Multilocus sequence typing (MLST) minimum-spanning tree for *B. paranthracis* isolates and related sequence types (STs)

STs for *B. cereus sensu lato* isolates were accessed at pubmlst.org and analyzed with GrapeTree. Each node represents a unique ST, and node size is proportional to the number of isolates with that ST. Selected nodes are labelled with their respective STs. Node color indicates the source from which the isolate was obtained. 'Human, non-blood' includes vomit, diarrhea, wound swabs, lung tissue, and lung aspirate; 'food' includes rice, pasta, food packaging, and other unspecified food; 'environment' includes soil, mud, leaves, and rivers. Branch length is logarithmically proportional to the number of allelic differences between the connected STs. The branch with a broken line indicates that ST-26 is not part of the ST-205 clonal complex but still shares multiple alleles.

BTyper predicted the ELWA 3298 sequence type (ST) as 1074 based on seven housekeeping genes (*glp*, *gmk*, *ilv*, *pta*, *pur*, *pyc*, and *tpi*) commonly used for *Bacillus cereus sensu lato* multilocus sequence typing (MSLT) (Priest et al., 2004). The other currently described

isolates of *B. paranthracis* have STs of 26 and 761. MLST phylogenetic analysis utilizing concatenated sequences of the seven housekeeping genes was performed. Sequences were aligned with MUSCLE and a maximum-likelihood tree with 1,000 bootstraps was generated with PhyML using a general time reversible nucleotide substitution model. The ELWA 3298 isolate fell into clade 1 of the *B. cereus sensu lato* group (Priest et al., 2004) and a lineage was formed with the other *B. paranthracis* STs (Figure 3.6). An MLST minimum spanning tree containing the STs for the known *B. paranthracis* isolates placed ELWA 3298 (ST-1074) closest to the ST-26 *B. paranthracis* isolate, as they share four of seven alleles (Figure 3.7). The Mn5T *B. paranthracis* type strain isolate, which has an ST of 761, is more distantly related and is part of the ST-205 clonal complex. Isolates across these STs have been cultured from numerous sources and have been the etiological agents of various human diseases, including sepsis (Didelot et al., 2009).

Conclusions

This is the first report, to our knowledge, that provides species-level identification and phenotypic characterization of a bacterium isolated from the blood of an EVD patient. However, although iatrogenic infection can be ruled out in this patient as the sample was collected at triage, non-anthracis *Bacillus* spp. are typically regarded as environmental contaminants when isolated from blood (Bottone, 2010). Nevertheless, invasive infections, including sepsis, are increasingly attributed to some non-anthracis *Bacillus* spp., particularly in immunocompromised patients (Bottone, 2010). Given the ability of *Bacillus* spp. to readily colonize the gut, both as transient flora and pathogenically (Bottone, 2010), a gastrointestinal source in this patient is plausible if true bacteremia was present (Kreuels et al., 2014) (Wolf et al., 2015). With the present limitations, however, caution must be taken with such interpretation and a determination of the

clinical significance of this isolate cannot be definitively made. This finding underpins the need for the continued study of possible bacterial co-infections with EVD.

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CHAPTER 4: ISOLATION AND SEQUENCING OF EBOLA VIRUS FROM CLINICAL SAMPLES

Ebola virus maintains consensus sequence but loses quasispecies diversity upon isolation from clinical samples in Huh7 and Vero E6 cells

A manuscript in preparation

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INTRODUCTION

Ebola virus (EBOV), species *Zaire ebolavirus*, is a member of the family *Filoviridae* and is an enveloped virus with a single-stranded, negative-sense, non-segmented RNA genome. It is the etiological agent of Ebola virus disease (EVD), a severe viral hemorrhagic fever that occurs in sporadic outbreaks primarily in central Africa following zoonotic spillover events from an unidentified animal reservoir. From 2013-2016, the largest and longest EVD outbreak on record occurred in the West African countries of Guinea, Liberia, and Sierra Leone, and the international medical and scientific community had significant involvement in the outbreak response effort.

Here we report on the isolation sensitivity for EBOV in Huh7 and Vero E6 cells from 89 clinical samples that were collected for diagnostic purposes from separate EVD patients in 2014 at the ELWA-3 Ebola treatment unit in Monrovia, Liberia. Furthermore, we evaluate the EBOV sequences obtained from the isolates in comparison with the EBOV sequences obtained directly from the blood samples on both consensus sequence and quasispecies levels.

Studies have previously reported EBOV isolation sensitivity in various cell lines using different sample types (e.g. whole blood, breast milk, etc.) spiked with EBOV, but have not assessed isolation sensitivity from clinical samples. Adaptations during *in vitro* cell culture, mostly in regards to changes in the glycoprotein (GP) RNA editing site following rescue of recombinant EBOV or serial passaging of EBOV stocks, have also been reported (Volchkova et al., 2011) (Alfson et al., 2015) (Kugelman et al., 2012) (Tsuda et al., 2015). To our knowledge, however, sequences obtained directly from human clinical samples has not been compared to sequence obtained from EBOV isolates obtained upon culture of the samples.

Quasispecies diversity has been shown to play an important role in disease pathogenesis. For example, mutant poliovirus (an RNA virus) with a higher fidelity RNA-dependent RNA polymerase (RdRp) is attenuated and lacks neurotropism due to loss of quasispecies diversity (Vignuzzi et al., 2006), whereas Marek's disease virus (a DNA virus) with impaired exonuclease activity becomes more pathogenic as a result of its acquired quasispecies-like behavior (Trimpert et al., 2019). As an RNA virus with a high mutation rate, which is likely similar to other -ssRNA viruses and between 1×10^{-6} and 1×10^{-4} substitutions per nucleotide per cell infection (Sanjuan et al., 2010), EBOV exists as a 'cloud' of closely-related mutants termed a quasispecies (Domingo et al., 2012; Lauring & Andino, 2010). Changes to this quasispecies diversity, along with consensus sequence changes, may have important phenotypic impacts.

RESULTS

Culture sensitivity and growth kinetics

Isolation of EBOV from clinical EVD whole blood was successful on both Huh7 and Vero E6 cells from 43 out of 89 samples (Figure 4.1). The median EBOV RNA copies/mL of these samples was 3.9×10^7 (interquartile range, 8.3×10^6 to 1.4×10^8) as determined by droplet digital PCR (ddPCR). 38 samples failed to produce an isolate on either Huh7 or Vero E6 cells and had a median of 1.7×10^5 EBOV RNA copies/mL (interquartile range, 8.1×10^3 to 2.4×10^6). For 8 samples, an isolate was obtained only from culture in Vero E6 cells and not from Huh7 cells. These samples had a median of 3.6×10^6 EBOV RNA copies/mL (interquartile range, 5.6×10^5 to 2.6×10^8). The maximum field diagnostic C_t for which an isolate was obtained was 31.09, although the samples had undergone freeze/thaw cycles during field storage and attempts were not made to isolate samples with a diagnostic $C_t > 35$ based on previous findings

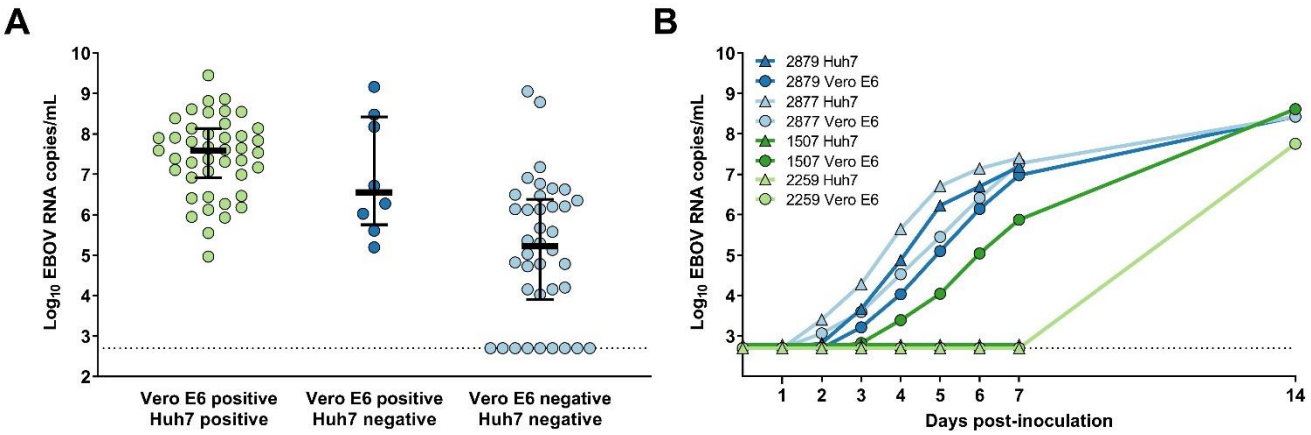

Figure 4.1. Sensitivity of Vero E6 cells and Huh7 cells for the isolation of Ebola virus (EBOV) from clinical samples and growth curve comparisons for selected samples.

EBOV isolation sensitivity was assessed by parallel isolation attempts on Vero E6 cells and Huh7 cells from 89 clinical samples (A). For 43 of the clinical samples, an isolate was obtained on both cell types (green); 8 samples produced an isolate only on Vero E6 cells (light blue); 38 samples did not produce an isolate on either cell type (dark blue). Circles correspond to the left y-axis and indicate the qRT-PCR C_t value recorded at the time of diagnosis in the field at the Ebola treatment unit. Lines and error bars represent the median with interquartile range.

Growth curves were analyzed for four clinical samples on both Vero E6 (circles) and Huh7 cells (triangles) (B). Samples #2877 (light blue) and #2879 (dark blue) produced an EBOV isolate on both cell types. Samples #1507 (dark green) and #2259 (light green) produced an EBOV isolate only on Vero E6 cells. Aliquots of supernatant were obtained every 24 hours for 7 days, and a final collection was obtained at 14 days for cultures in Vero E6 cells. Cultures in Huh7 cells were not grown beyond 7 days due to general loss of viability. There were no instances of successful EBOV isolation in Huh7 cells but not in Vero cells.

(Spengler et al., 2015). There were no instances of an isolate obtained from culture in Huh7 cells but not in Vero E6 cells, thus Vero E6 cells were slightly more sensitive for the isolation of EBOV from clinical whole blood samples.

During initial isolation from clinical whole blood samples, EBOV grew more rapidly on Huh7 cells than on Vero E6 cells (Figure 4.1). Huh7 cells produced approximately one $\log_{10}$ EBOV RNA copies/mL greater until day 7, at which point the difference became negligible. Cytopathic effect (CPE) from EBOV infection was easily distinguishable from mock-infected

flasks in Vero E6 cells than in Huh7 cells at end point (Figure 4.2). Although CPE consistently developed more quickly in Huh7 cells (typically around day 4 post-inoculation) than in Vero E6 cells (typically around day 10 post-inoculation), it was often more difficult to distinguish from background degradation of the monolayer, rendering determination of CPE due to EBOV infection beyond approximately 7 days in Huh7 cells unreliable (Figure 4.2). Vero E6 cells maintained a consistent monolayer throughout our 14-day protocol, and in one observed instance an isolate was obtained at day 14 that was not detectable by droplet digital PCR (ddPCR) at or before day 7 (Figure 4.1). Supernatant from all isolation attempts was analyzed by PCR to verify culture results.

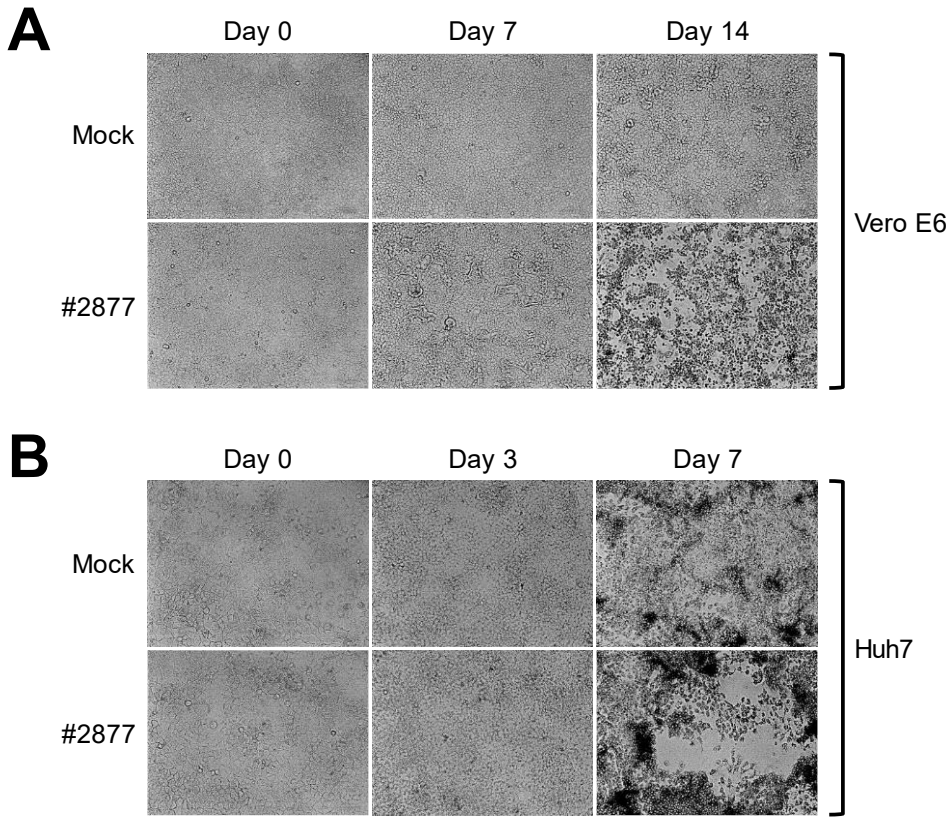

Figure 4.2. Cytopathic effect (CPE) of Ebola virus (EBOV) in Vero E6 and Huh7 cells.

Vero E6 cells demonstrate CPE in EBOV infected cells that is clearly distinguishable from control cells at the endpoint of the assay (A). CPE in EBOV infected Huh7 cells is less clearly distinguishable from uninfected cell death in control cells at the endpoint of the assay (B).

Table 4.1. Ebola virus sequencing depth, coverage, quality, and consensus similarity for all samples

Sample	Source	Mean depth	Genome coverage (%)*	Phred score ≥ 30 (%)	Consensus†
#2476	Blood	388	96.8	99.6	Identical
	Huh7	1,743	99.9	99.2	
	Vero E6	1,693	99.9	99.3	
#2483	Blood	47	90.8	99.6	Identical
	Huh7	1,466	99.96	99.2	
	Vero E6	1,100	99.9	99.3	
#2507	Blood	1,559	97.5	99.5	Identical
	Huh7	1,889	99.96	99.3	
	Vero E6	2,468	99.96	99.2	
#2805	Blood	845	96.4	99.3	Identical
	Huh7	3,097	100	99.2	
	Vero E6	1,760	99.96	99.2	
#2841	Blood	124	93.8	99.3	Identical
	Huh7	4,017	99.97	99.1	
	Vero E6	2,026	99.96	99.1	
#2845	Blood	202	98.1	98.9	Different
	Huh7	1,657	99.9	99.0	
	Vero E6	1,795	99.9	99.2	
#2849	Blood	6,236	99.7	97.4	Identical
	Huh7	2,630	99.96	99.1	
	Vero E6	2,269	99.97	99.2	
#2877	Blood	223	98.2	98.8	Identical
	Huh7	3,797	100	99.1	
	Vero E6	2,587	99.96	99.2	
#2879	Blood	246	96.7	98.9	Different
	Huh7	3,130	99.99	99.2	
	Vero E6	1,992	99.97	99.1	
#2882	Blood	220	98.1	98.7	Identical
	Huh7	1,500	99.97	99.1	
	Vero E6	2,590	99.99	99.0	
#2935	Blood	217	98.2	98.8	Different
	Huh7	3,330	100	99.2	
	Vero E6	1,936	99.95	99.1	
#2939	Blood	837	99.8	99.0	Identical
	Huh7	1,656	99.96	99.1	
	Vero E6	2,008	99.96	99.1	
#2942	Blood	27	86.3	98.7	Identical
	Huh7	3,603	100	99.1	
	Vero E6	1,905	99.97	99.2	

*The sequencing approach used for blood samples (VirCapSeq) primarily targets coding regions; gaps were sometimes present in 3' leader, 5' trailer, and intergenic regions, with the majority occurring between the VP30 and VP24 genes.

†Comparison of consensus sequences was made across all coding regions and across non-coding regions where coverage from the blood-derived sequence was sufficient. There were no consensus differences in Huh7- and Vero E6-derived sequences in regions with gaps in coverage from the matching blood-derived Ebola virus sequences.

Sequencing depth, coverage, and quality

Compared to EBOV sequences obtained directly from patient blood samples, sequence depth, coverage, and quality was generally greater from EBOV isolated on Huh7 and Vero E6 cells due to the high viral titer and reduced host background of the samples (Table 4.1). The Huh7- and Vero E6-derived sequences all had greater than 1,000x mean depth and genome coverage ≥ 99.9%. The results from the blood-derived sequences were more variable, but mean depth was above 200x in all but three instances and the genome coverage was greater than 96% in all but one instance.

Consensus sequence comparison

The consensus sequences obtained directly from blood samples and their respective cultured viruses were very similar. In 13 sets that produced full-genome or near full-genome EBOV sequence from each of the three sample types, only three sets demonstrated any consensus differences (Table 4.2). In two of the sets, mutations arose in EBOV isolated in Huh7 cells, but not in Vero E6 cells, as compared to the sequences derived directly from blood. These Huh7 mutations likely did not result from selection of low frequency single nucleotide variants (SNVs) in the blood sample, which demonstrated no variation at the respective sites at the depth obtained. In the first set (#2845), an A → C mutation occurred in the VP40 gene and resulted in a non-synonymous coding change (N80H). In the second set, another A → C mutation occurred in the polymerase gene but was synonymous in this instance.

Table 4.2. Ebola virus consensus differences in sequences obtained from 13 matched sets of sequences derived from patient blood, Vero E6 cultured virus, and Huh7 cultured virus

Sample	Position(s), gene(s)	Blood	Vero E6	Huh7	Effect
#2845	4,716, VP40	100% A	100% A	A (17%) → C (82%)*	N80H
#2879	11,790, L	100% A	100% A	A (41%) → C (59%)	synonymous
#2935	5,593, intergenic 7,561, GP	50% C, 50% T 80% A, 20% G	83% C, 17% T A (34%) → G (66%)	51% C, 49% T 76% A, 24% G	n/a synonymous

*the remaining 1% was accounted for by G and T

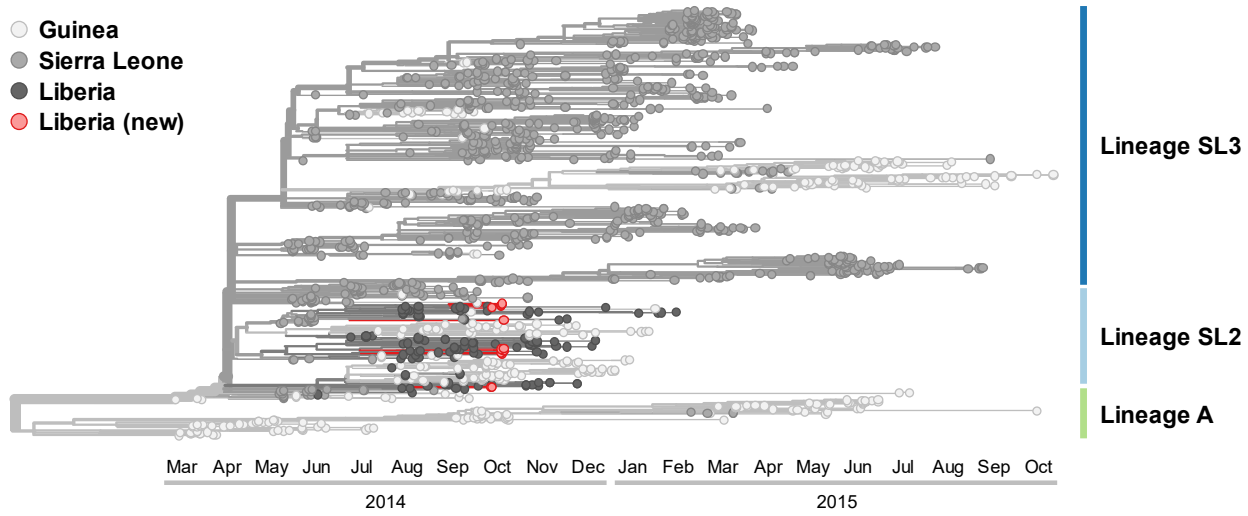

Figure 4.3. Maximum-likelihood time tree for 1,610 existing Ebola virus (EBOV) Makona sequences with 13 new Liberian sequences from this study added.

To place the 13 new Liberian sequences into the greater context of the 2013-2016 Ebola virus disease epidemic, existing sequences were downloaded from the Nextstrain EBOV repository, the 13 new Liberian sequences were added, alignment was performed with MAAFT, a maximum-likelihood tree was generated with IQ-TREE, and a time tree was inferred with TreeTime. The new Liberian sequences (red), which were generated from samples that were all collected in October, 2014, cluster together as expected with currently published Liberian sequences in the SL2 lineage. The minor consensus differences in the three new Liberian sequences (Table 4.2) did not result in any topological changes.

In the third set, there were two sites of difference in the genomes. The first site was in an intergenic region between the VP40 and glycoprotein genes and was evenly split between C and T in the blood sample. In EBOV isolated in Huh7 cells, this ratio was maintained, whereas EBOV isolated in Vero E6 cells, C had emerged as a strong consensus. The second site of difference was in the glycoprotein gene. The sequence from the blood sample was split here as well, but A was a clear consensus. EBOV isolated in Huh7 cells again closely maintained the ratio seen from the blood-derived sequence, whereas the minor variant (G) was selected for in the Vero E6 cells, resulting in a synonymous mutation.

Maximum-likelihood phylogenetic analysis of the 13 new sequences within the greater context of the 2013-2016 EVD epidemic clustered the new sequences in the SL2 lineage along with the other published Liberian sequences, as expected (Figure 4.3). The consensus differences

present in the three samples (Table 4.2) did not result in any changes in tree topology, with Vero E6- and Huh7-derived isolate sequences placed on immediately adjacent tips along with their respective blood-derived sequences in all cases.

Single nucleotide variant analysis and quasispecies diversity

Three sets of sequences (one sample each of sequences direct from blood, Vero E6-cultured virus, and Huh7-cultured virus) that had comparable and sufficient mean depth of coverage and genome coverage were analyzed for single nucleotide variants (SNVs) in coding regions: #2507, #2805, and #2939 (Table 1). A similar pattern in the abundance of intra-host single nucleotide variants (iSNVs) emerged across all three sets of samples: blood- and Vero E6-derived sequences were generally similar in abundance, while Huh7-derived sequences had the lowest number of iSNVs in all three comparisons (Figure 4.4).

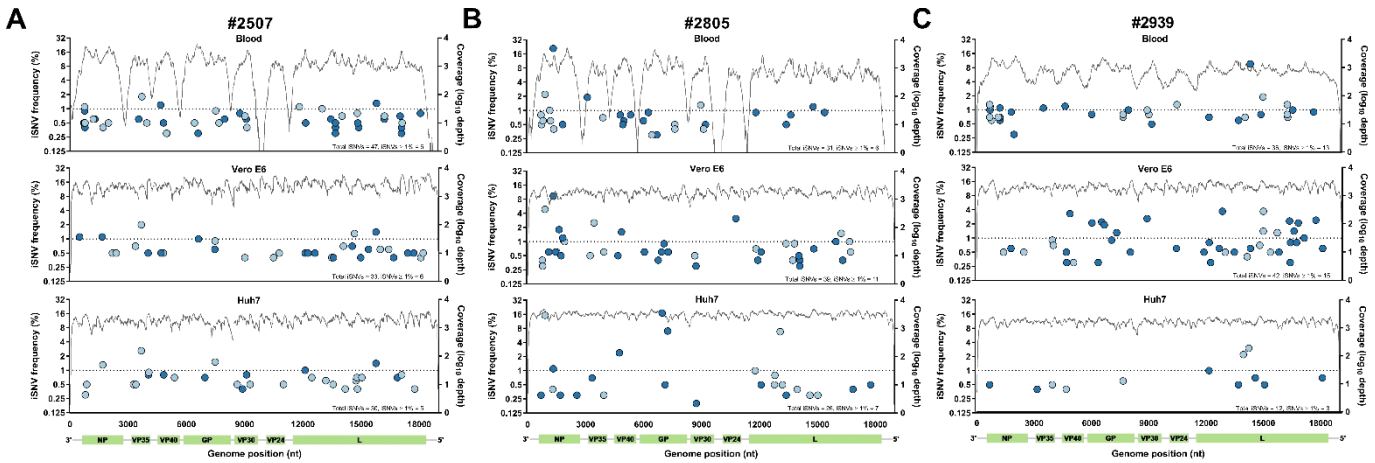

Figure 4.4. Comparison of Ebola virus (EBOV) low-frequency intra-host single nucleotide variants (iSNVs) in sequences obtained directly from blood, from Vero E6-cultured virus, and from Huh7-cultured virus from three separate clinical samples.

The points (dark and light blue circles) indicate the nucleotide position where the iSNV was detected, and their frequency corresponds to the left y-axis. Dark blue circles represent non-synonymous SNVs, and light blue circles represent synonymous iSNVs. iSNV analysis was only performed on coding regions of the EBOV genome. The line graph indicates the genome coverage by position and corresponds to the right y-axis.

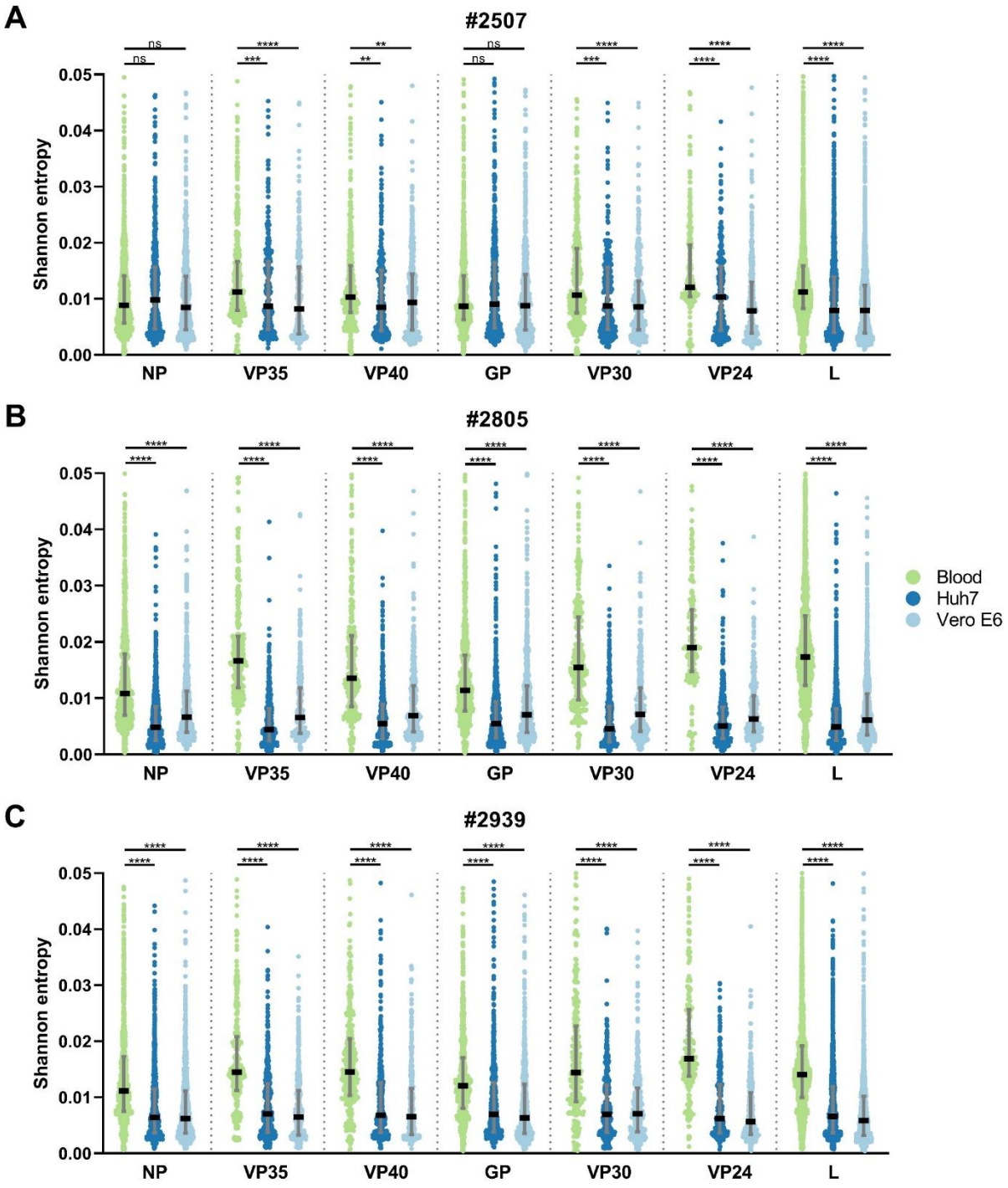

Figure 4.5. Shannon entropy per genome position from sequences obtained directly from blood, from Vero E6-cultured virus, and from Huh7-cultured virus from three separate clinical samples.

Shannon entropy values are shown in bits, and lines and error bars represent the median and interquartile range, respectively.

Shannon entropy was also calculated across the coding sequences (CDS) for each gene for the same three sequences: #2507, #2805, and #2939 (Figure 4.5). In this comparison, the Shannon entropy was significantly greater in the blood-derived CDS regions than both the Huh7- and Vero E6-derived sequences in all but two instances (NP and GP CDS in #2507).

DISCUSSION

Isolation of EBOV from clinical samples is an important undertaking that both establishes the infectiousness of the samples and provides isolates that can be used for in vitro and in vivo studies. The sensitivity of the cell lines used for this process is thus a crucial first parameter for consideration. Here we have shown that Vero E6 cells are more sensitive than Huh7 cells for isolating EBOV from clinical whole blood samples. While Vero E6 cells have long been the standard for EBOV isolation, some recent studies suggested that Huh7 cells were similarly sensitive to Vero E6 cells for isolation, and that Huh7 cells may have offered other advantages (Logue et al., 2019). This study did not utilize clinical EVD samples, however, and thus is difficult to compare directly to what we report here. Nevertheless, approximately 10% of the clinical whole blood samples cultured for EBOV in this study (8 of 89) produced an isolate only in Vero E6 cells, suggesting substantially greater isolation sensitivity in Vero E6 cells. As we did not have access to other clinical EVD samples (e.g. semen, breastmilk, etc.), only whole blood could be assessed. An important caveat to note, however, is that the samples that were used for these isolation attempts had been stored for some time and had undergone freeze/thaw cycles, so the EBOV RNA/copies per mL and C_t values referenced here should not be interpreted to reflect cutoffs for infectivity in fresh samples.

The consensus sequence of EBOV was generally maintained upon isolation from clinical samples in both Huh7 and Vero E6 cell culture-derived isolates, but again Vero E6 cells may

offer a slight advantage. This is in contrast to a previous study which found that recovery of recombinant EBOV in Huh7 cells elicited fewer mutations than in Vero E7 cells (Tsuda et al., 2015). Maintenance of consensus sequence in EBOV following isolation has obvious important implications for maintaining phenotype, and also is critical for reliable phylogenetic analyses. Prior to 2014, nearly all the full-genome EBOV sequences in GenBank were derived from cultured virus, rather than directly from patient samples.

A final important observation is that potentially impactful differences arise in the quasispecies diversity upon isolation of EBOV from clinical samples, with significant reductions present in the sequences of both the Huh7 and Vero E6 isolates. Bovine viral diarrhea virus has recently been reported to lose quasispecies diversity upon isolation (Russell et al., 2020), and similar host-dependent changes in quasispecies diversity have been shown to occur in Alpha-like plant RNA viruses, often used as models for the study of viral evolution. Evidence from experiments with these viruses further reinforces the importance of the host in shaping quasispecies diversity, as serial passages elicited little effect, while inoculation into a different host immediately incurred substantial change (Schneider & Roossinck, 2000) (Schneider & Roossinck, 2001). It is crucial to recognize that while quasispecies diversity is dependent upon the error prone RdRp, it is not merely a function of it alone. The entire quasispecies cloud is the subject of selective pressures, and as such is a dynamic, host-dependent entity. Within the human host, EBOV has an extremely broad tropism, initially targeting cells of the mononuclear phagocytic system, endothelial cells, and hepatocytes, but ultimately capable of infecting almost any endothelial cell or fibroblast (Ito et al., 2001; Martines et al., 2015; Takada, 2012). Thus, different selective pressures throughout the range of host cells may contribute to shaping the

overall quasispecies diversity of EBOV during human infection. This scenario is very different from that found in cell culture.

EBOV stocks for in vivo and in vitro experimental use have been generated from a limited number of clinical samples. Typically, large stocks are created from the lowest possible passage number in an effort to avoid culture adaptations as determined by consensus sequence. However, quasispecies diversity has been shown to play a significant role in the outcomes of both in vitro and in vivo experiments with other RNA viruses (Domingo et al., 1998; Farci et al., 2000; Vignuzzi et al., 2006), and intra-quasispecies interactions can play defining phenotypic roles (Domingo et al., 2012), thus consideration to this metric is warranted with EBOV. Historically, most in vivo EBOV studies have very effectively employed a lethal challenge that facilitates vaccine and therapeutic development. Thus, there is no questioning the virulence of cultured EBOV stocks. However, given the successful development and licensing of highly protective EVD vaccines (Ehrhardt et al., 2019), a new focus on development of animal models to further understand the nuances of EBOV pathogenesis. Furthermore, in vivo examination of nuanced differences in pathogenesis, particularly in the context of persistent infections, has not been broadly undertaken. Following the 2013-2016 West Africa Ebola epidemic, important questions have arisen regarding persistent EBOV infections however, and quasispecies diversity is known to be necessary for the initiation and maintenance of persistence in other RNA virus infections (Domingo et al., 1998; Farci et al., 2000). Successful laboratory study of this phenomenon will depend upon in vivo modeling that can accurately recapitulate the full range of human EVD, including chronicity, and may require utilization of EBOV stocks with sufficient quasispecies diversity.

MATERIALS AND METHODS

Cells

African green monkey kidney cells (Vero E6) and human hepatocellular carcinoma cells (Huh7) were cultured in Dulbecco's modified Eagle's medium (DMEM) containing 2mM L-glutamine and 10% fetal bovine serum (FBS) in T25 flasks at 37 °C with 5% CO_2. Cells were grown to approximately 90% confluency prior to inoculation.

EVD patient blood samples

Samples were collected at the Eternal Love Winning Africa 3 (ELWA-3) Ebola Treatment Unit in Monrovia, Liberia between August and December of 2014, during the 2013-2016 West Africa EVD outbreak. Samples were transported to the biosafety level 4 (BSL-4) laboratories of the Integrated Research Facility at Rocky Mountain Laboratories (RML), Division of Intramural Research, National Institute of Allergic and Infectious Diseases, National Institutes of Health. Use of these samples for research purposes was designated 'not human subjects research' by the NIH Office of Human Subjects Research Protection (OHSRP) and approved by the University of Liberia-Pacific Institute for Research and Evaluation (UL-PIRE) institutional review board.

Virus culture and sampling

10 μL aliquots of whole EDTA blood were diluted in 1 mL plain DMEM and placed on the 90% confluent Vero and Huh7 cells in T25 flasks and incubated for 1 hour at 37 °C with 5% CO_2. The cells were then rinsed twice with 3 mL PBS and 6 mL DMEM containing 2mM L-glutamine and 2% FBS was added to each flask. Incubation proceeded for 7 days (Huh7 cells) or 14 days (Vero E6 cells) at 37 °C and 5% CO_2. A random subset of the samples was selected for growth curve analysis, and 1 mL aliquots of supernatant were harvested daily, and an equal

volume of media was replaced. Aliquots were removed from all samples at the end of the incubation periods for validation by qRT-PCR of tentative culture results as determined by the presence or absence of CPE.

Virus inactivation, RNA extraction, and qRT-PCR

Viral RNA was inactivated and extracted from whole blood in EDTA both in the field at the ELWA-3 ETU in Monrovia, Liberia and in the BSL-4/BSL-2 laboratories at RML in Hamilton, MT. At the ELWA-3 ETU, viral RNA extractions from whole blood in EDTA were performed with the QIAGEN QIAamp Viral RNA Mini Kit, as previously described (de Wit et al., 2016b). In the BSL-4/BSL-2 laboratory, 140 μL whole blood in EDTA was placed in 1.26 mL TRIzol reagent and extraction proceeded using a column-based TRIzol Plus RNA Purification Kit and Phasemaker Tubes. Viral RNA was extracted from viral culture supernatant with the QIAGEN QIAamp Viral RNA Mini Kit. All samples processed at RML were inactivated according to standard operating protocols prior to removal from BSL-4.

PCR analyses

The C_t values were generated at the time of patient triage at the ELWA-3 ETU as previously described (de Wit et al., 2016b). EBOV RNA copies/mL in the TRIzol-reextracted whole-blood patient samples were measured using a droplet digital PCR (ddPCR) targeting an intergenic region between VP30 and VP24 as follows: forward, 5'-TGACGGAACATAAATTCTTTCTGC-3'; reverse, 5'-CGGTCACAATATACCTCCTGAAA-3'; probe, 5'-FAM-TGTGGAGGAGGTCTATGGTATTCGCT-3'. This region is never transcribed, thus the quantification excludes viral mRNA and more closely approximates a true genome copy number. Supernatant from all isolation attempts was analyzed by qRT-PCR assay

as used in the field (targeting an intragenic, transcribed region of L) to confirm culture CPE results.

Sequencing and bioinformatics

For the unbiased sequencing of the viral culture supernatants, NGS libraries were prepared following the TruSeq Total RNA-Seq (Illumina Inc., San Diego, CA) protocol with the only modification of starting at the fragmentation step by combining 5uL of viral supernatant with 13 uL of Fragment, Prime, Finish Mix. The NGS libraries were assessed on a BioAnalyzer DNA1000 chip for sizing (Agilent Technologies, Inc., Santa Clara, CA) and quantified using the Kapa Quantification Kit for Illumina Sequencing (Kapa Biosystems Inc, Wilmington, MA). Paired-end sequencing was completed on a MiSeq (Illumina, Inc.) using v2 300 cycle chemistry.

Prior to NGS library preparation, the blood extracted RNA was rRNA and globin transcript depleted using the Globin-Zero Gold rRNA removal kit (Illumina) using a low-input approach by adjusting the Ribo-Zero procedure to a 20 uL reaction using 16 uL RNA, 2 uL reaction buffer and 2 uL Globin-Zero probes. Ninety microliters of magnetic capture beads were washed and resuspended in 35 uL. The depleted RNA was purified with Agencourt RNAClean beads (Beckman Coulter, Inc., Brea, CA) and eluted in 9 uL water. NGS libraries were prepared using the Takara SMARTer Stranded Total RNA-Seq Kit v2 (Takara Bio, Inc., Kusatsu, Shiga, Japan), reducing the fragmentation time to 1.5 seconds and omitting the rRNA depletion/ZapR step. The libraries were amplified for 16 cycles. The NGS libraries were assessed on a BioAnalyzer High Sensitivity chip (Agilent Technologies, Inc.) for sizing and quantified using the Kapa Quantification Kit for Illumina Sequencing (Kapa Biosystems, Inc.). Paired-end sequencing was completed on a MiSeq (Illumina, Inc.) using v2 300 cycle chemistry. Raw reads were trimmed of adapter sequence using cutadapt (https://cutadapt.readthedocs.io/en/stable/).

The remaining reads were then filtered for low quality bases and low quality reads using the FASTX-Toolkit (http://hannonlab.cshl.edu/fastx_toolkit/). Remaining reads were mapped to the Ebola (KJ660347.2) genome, using Bowtie 2 (Langmead & Salzberg, 2012). Variants were detected across all samples using GATK to generate genomic variant call format (VCF) files (McKenna et al., 2010).

VirCapSeq-VERT sequencing

For VirCapSeq-VERT, first-strand cDNA synthesis was performed with SuperScript IV using 11 µL of TRIzol-extracted RNA and included a final RNase H step. Second-strand synthesis was performed by incubating 21 µL first-strand cDNA with 4 µL Klenow exo- (5 unit/µL), 10 µL 10x NEBuffer 2, 2 µL dNTPs (25 mM), 1 µL random hexamers (600 ng/µL) and 62 µL water for 45 minutes at 37 °C without a heated lid. The product was then bead-purified with the AMPure XP kit following the standard protocol and eluted with 36 µL water.

Purified, double-stranded cDNAs were used as template to prepare individual libraries using the Kapa HyperPrep Library kit and following the SeqCap EZ HyperCap Worflow User's Guide, version 2.3 (Roche Sequencing Solutions, Inc., Pleasanton, CA). Fifteen to twenty-five microliters ds-cDNA was brought to a final volume of 53 µL and sheared on the Covaris LE220 instrument (Covaris, Woburn, MA) to generate an average size of 180-220 bp. The following settings were used: peak incident power, 450 watts; duty factor, 15%; cycles per burst, 100; and time, 300 seconds. End repair, A-tailing, and adapter ligation was performed as instructed in the HyperPrep kit procedure described within the SeqCap User's guide. KAPA unique dual-index (UDI) adapters from Roche were used for library preparation to facilitate pre-capture multiplexing. Adapter-modified libraries were enriched with 12-15 PCR amplification cycles and then purified with Ampure XP beads, according to the manufacturer's protocol (Beckman

Coulter, Brea, CA). Following purification, library distributions were assessed on the 2100 Bioanalyzer using High Sensitivity DNA kit (Agilent Technologies, Santa Clara, CA) and quantified using Kapa Library Quantification kit (Roche Sequencing Solutions, Inc., Pleasanton, CA).

The VirCapSeq-VERT capture panel (Roche Sequencing Solutions, Inc., Pleasanton, CA) was used for viral enrichment. Each capture reaction consisted of 20 libraries, with ~50 ng pooled for a combined mass of 1.0 µg. The library pool was hybridized to the VirCapSeq-VERT capture panel at 47^0C for 17 hours and then virus-enriched libraries were recovered, washed, and PCR amplified 12 cycles. Final, virus-enriched library pools were quantified with the Kapa Quantification Kit (Roche), pooled in equimolar amounts, and sequenced as 2 X 75 bp reads on the NextSeq 550 platform using the Mid Output v2.5 Reagent Kit (Illumina, San Diego, CA).

Raw image files were converted to fastq files using bcl2fastq (v2.20.0.422, Illumina Inc. San Diego, CA), trimmed of adapter sequences using cutadapt version 1.12 (Martin, 2011), and quality trimmed and filtered using the FASTX Toolkit, v 0.0.14 (Hannon Lab, CSHL). Singletons were removed and trimmed reads were sorted in coordinate order using a custom Perl script. As an initial QC on the sequence data, adapter- and quality-trimmed reads were processed through FastQC (Babraham Institute) as well as sequence classification programs Kraken and Kaiju (Wood & Salzberg, 2014) (Menzel et al., 2016). Quality trimmed and sorted reads were first aligned with Bowtie 2 version 2.2.9 to the human ribosomal RNA sequences and then to the whole genome reference build GRCh38 with options --no-mixed and -X 1000 (Langmead & Salzberg, 2012). Read pairs that did not align to human sequences were aligned to Zaire ebolavirus (EBOV) isolate Makona-Gueckedou-C07 (accession KJ660347.2) or to a reference containing various viruses identified in the Kraken/Kaiju sequence classification results. Aligned

Sequence Alignment/MAP (SAM) files were converted to Binary Sequence Alignment/Map (BAM) format and sorted and indexed using SAMTOOLS version 1.8 (Li et al., 2009) (Anders et al., 2015). PCR duplicates were removed using SAMTOOLS and EBOV coverage was calculated using GATK, version 3.8, DepthOfCoverage Tool (Broad Institute).

For iSNV analysis, three samples that had sufficient depth of coverage for all sample types (blood, Vero E6, and Huh7) were chosen for analysis, and only CDS were analyzed. Variants were called with LoFreq version 2.1.0 and verified visually. Shannon entropy (H) was calculated for all genomic positions within the coding sequences (CDS), since haplotypes were unknown. The equation for Shannon entropy is

$$\mathrm{H} = -\sum_{n=1}^{N} p_n \log_2(p_n)$$

where p_n is the frequency of a nucleotide of symbol $_n$ at a given position and N is the number of distinct symbols possible. As $\log_2$ is used, Shannon entropy is reported here in units of 'bits', with a maximum value of 2 possible ($\log_2 4 = 2$). Statistical analysis of the Shannon entropy values was performed with a non-parametric Kruskal-Wallis H-test with Dunn's multiple comparison test. Differences were considered statistically significant at $p < 0.05$.

Biosafety

All aspects of the study involving infectious EBOV were performed in the RML BSL-4 laboratories in accordance with regulations set forth by the Division of Select Agents and Toxins.

CHAPTER 5: THE WEST AFRICA EBOLA VIRUS DISEASE OUTBREAK IN PERSPECTIVE

Delayed recognition of Ebola virus disease is associated with longer and larger outbreaks

Adapted from an article published in *Emerging Microbes and Infections*

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Abstract

The average time required to detect an Ebola virus disease (EVD) outbreak following spillover of Ebola virus (EBOV) to a primary human case has remained essentially unchanged for over 40 years, with some of the longest delays in detection occurring in recent decades. In this review, our aim was to examine the relationship between delays in detection of EVD and the duration and size of outbreaks, and we report that longer delays are associated with longer and larger EVD outbreaks. Historically, EVD outbreaks have typically been comprised of less than 100 cases (median = 60) and have lasted less than 4 months (median = 118 days). The ongoing outbreak in Democratic Republic of the Congo, together with the 2013–2016 West Africa outbreak, are stark outliers amidst these trends and had two of the longest delays in detection on record. While significant progress has been made in the development of EVD countermeasures, implementation during EVD outbreaks is problematic. Thus, EVD surveillance must be improved by the broad deployment of modern diagnostic tools, as prompt recognition of EVD has the potential to stem early transmission and ultimately limit the duration and size of outbreaks.

Introduction

Ebolaviruses are non-segmented, negative-sense, single-stranded RNA viruses in the family *Filoviridae* and the genus *Ebolavirus*. Six closely related viruses, each a member of a separate species, are currently known: Ebola virus (EBOV), species *Zaire ebolavirus*; Sudan virus (SUDV), species *Sudan ebolavirus*; Bundibugyo virus (BDBV), species *Bundibugyo ebolavirus*; *Taï* Forest virus (TAFV), species *Taï Forest ebolavirus*; Reston virus (RESTV), species *Reston ebolavirus*; and tentatively Bombali virus (BOMV), species *Bombali ebolavirus* (Goldstein et al., 2018). Amongst these, EBOV is currently responsible for the majority of human infections and is the etiological agent of Ebola virus disease (EVD) (Kuhn et al., 2019). EVD is characterized by acute onset of constitutional signs and symptoms, typically after an incubation period of 6-12 days, followed by emesis, diarrhea, multiorgan system dysfunction or failure, and occasionally hemorrhage. Fulminant cases often prove fatal within 10-14 days of symptom onset, and the EVD case fatality rate (CFR) may approach 90% (Daniel S Chertow, 2018). For reasons which are unclear, EVD outbreaks have been occurring with increasing frequency over the past two decades in Democratic Republic of the Congo (DRC), and since mid-2018, DRC has been experiencing its largest and longest outbreak, second overall only to that which occurred in West Africa from 2013-2016 (Figure 5.1). The ongoing outbreak is the ninth of EVD in DRC; additionally, DRC previously experienced a single outbreak of Bundibugyo virus disease (BVD) in 2012, which is caused by BDBV (Kratz et al., 2015).

EVD outbreaks are zoonotic in origin and all EBOV spillovers, including those that resulted in the West Africa and ongoing DRC outbreaks, have occurred at similar latitudes less than 10° north or south of the equator (Groseth et al., 2007) and within the Guineo-Congolian rainforest terrestrial ecosystem (Geographers, 2013) (Figure 5.2).

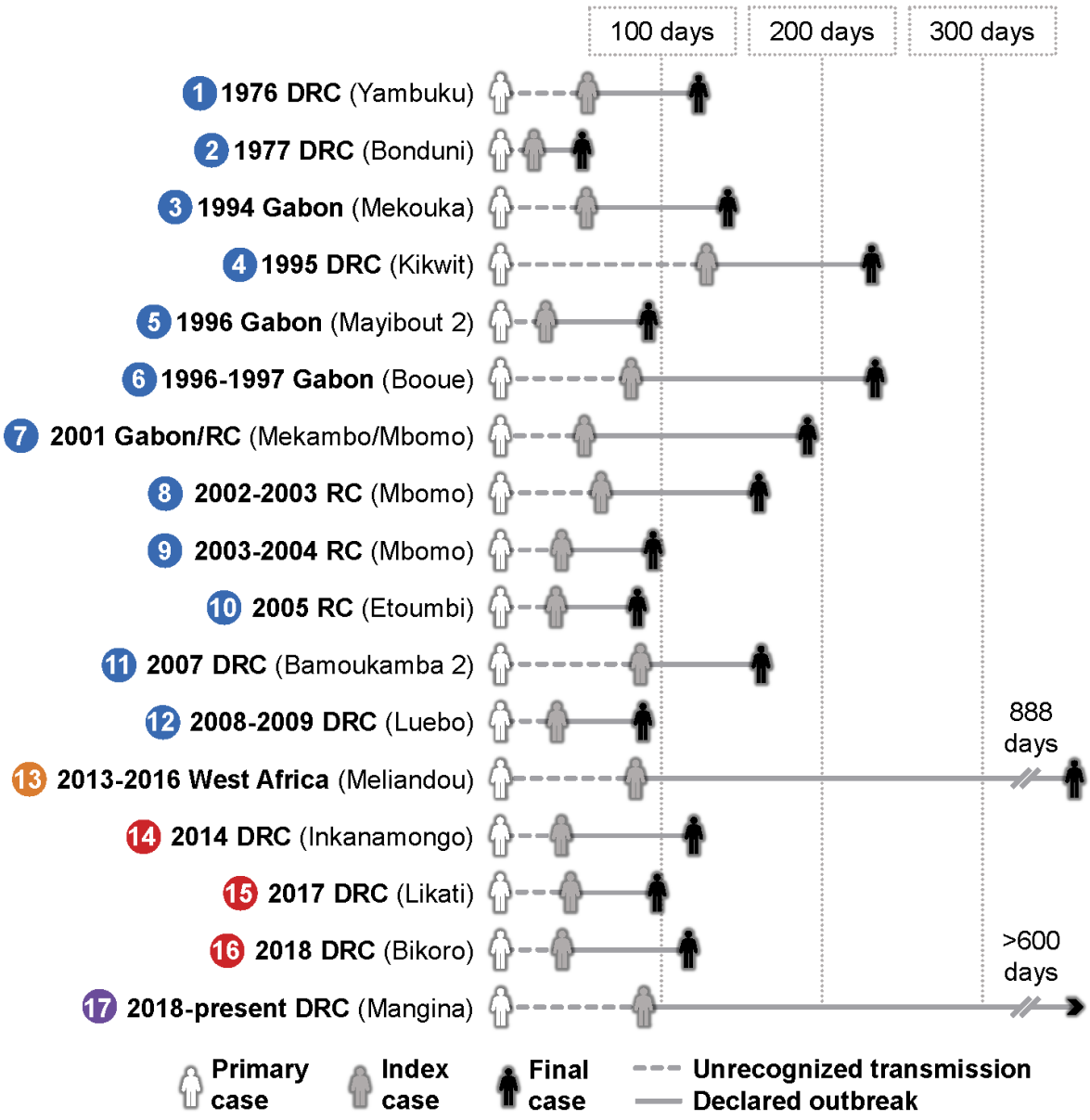

Figure 5.1 – Chronology for all Ebola virus disease outbreaks

The primary case results from zoonotic spillover and leads to a period of undetected transmission. It is typically determined retrospectively with epidemiological investigations. The index case is the first case to be recognized and marks the official beginning of an outbreak. The final case includes the 42-day observation period. Colors around numbers indicate the following groupings of outbreaks by spillover date: 1976-2012, West Africa (2013), 2014-2018, and ongoing DRC (2018). Locations in parentheses are reported outbreak spillover locations. *For the 1977 DRC (Bonduni) outbreak, the primary case, index case, and final case are the same.

Frugivorous and insectivorous bats, including *Hypsignathus monstrosus* (hammer-headed fruit bat), *Eidolon helvum* (straw-colored fruit bat), *Epomops franqueti* (Franquet's epauletted fruit bat), *Mops condylurus* (Angolan free-tailed bat), and *Miniopterus inflatus* (greater long-fingered bat), are implicated as potential natural reservoirs for EBOV (De Nys et al., 2018), and other mammals including gorillas, chimpanzees, and duikers (Leroy et al., 2004) (Groseth et al., 2007) likely act as intermediate, amplifying, dead-end hosts. Several EVD outbreaks have reported contact of the primary case with these animals, suggesting that humans may be infected by handling EBOV-infected bushmeat (Judson et al., 2016). Despite this, EBOV has never been isolated from any naturally-infected host other than humans, hence its enzootic and epizootic

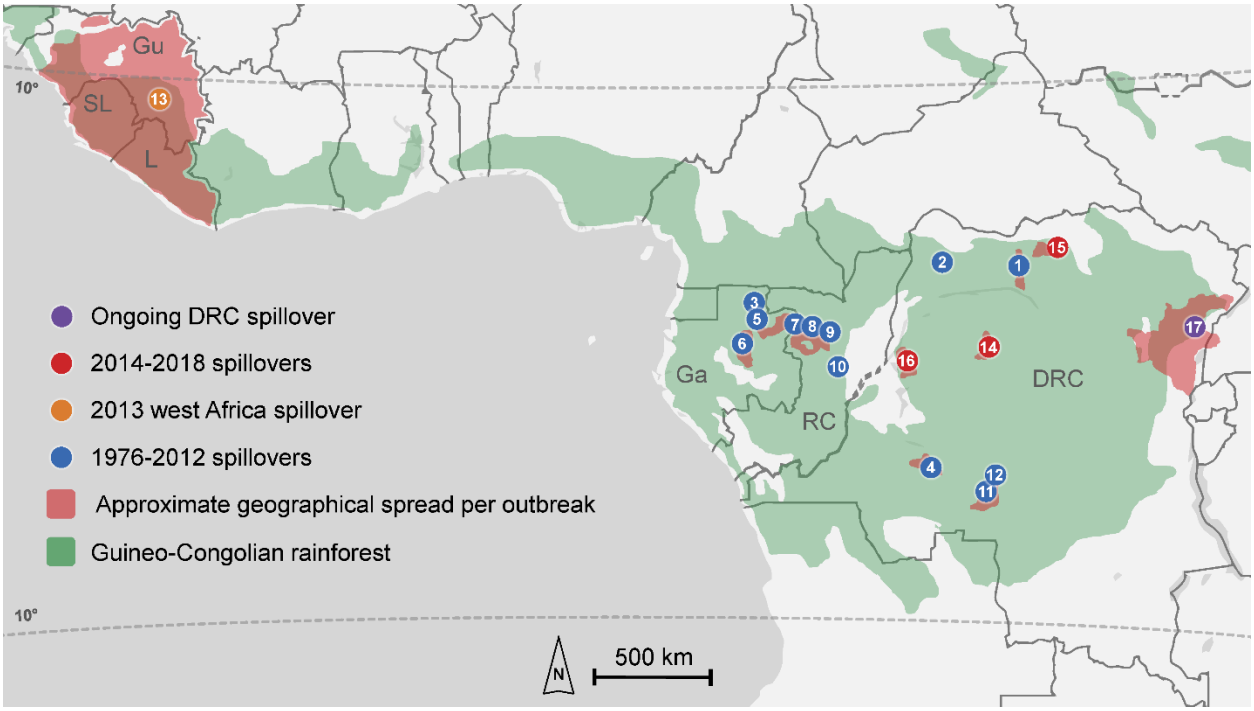

Figure 5.2 – Location and spread of all currently described Ebola virus disease outbreaks

Zoonotic spillover location per outbreak is indicated by circles with numbers. Numbers inside circles represent the order of the 17 spillovers from 1976-present correspond to Figure 5.1. For the 2001-2002 Gabon/Republic of Congo outbreak, which had multiple spillovers suspected, only the first spillover location is indicated. *Primary transmission zone; outbreaks 1, 6, 7, and 13 had distant case spread that is not shown in this figure; see Table 5.1.

transmission cycles are yet to be elucidated (Groseth et al., 2007). Furthermore, the circumstances that precipitate EBOV spillover are largely unknown and are likely a complex interplay of anthropogenic and environmental factors (Plowright et al., 2017). Although studies have reported spatiotemporal patterns in EBOV spillover (Schmidt et al., 2017), analyses are hampered by a relative paucity of data and the infrequent occurrence of such events. In addition, biotic and abiotic heterogeneity within the vast region of the Guineo-Congolian rainforest terrestrial ecosystem necessitates that generalizations regarding drivers of spillover be treated cautiously. Thus, definitive patterns in the ecology of EBOV that could inform public health efforts remain elusive.

However, once EBOV spillover has occurred, the dynamic and shape of most EVD outbreaks are relatively less obscure and often follow similar patterns. The aim of this review is to revisit the 17 known EVD outbreaks and examine these patterns, specifically regarding the association between the length of initial delays in detecting EVD and the subsequent duration and size of outbreaks. This association underscores the importance of early EVD detection following a spillover event and provides a strong rationale for significantly bolstering EVD diagnostic capabilities and surveillance throughout at-risk regions.

Historical EVD outbreak patterns

Typically, as with the other filoviruses, EBOV is transmitted from an animal reservoir or intermediate host to a primary human case in a single zoonotic spillover event. The primary case subsequently initiates all human-to-human transmission. This is a unique pattern in marked contrast to outbreaks of other zoonotic viral hemorrhagic fevers (e.g. Lassa fever), which are characterized by sustained spillover and very limited human-to-human transmission (Figure 5.3).

In EVD, once human-to-human transmission has begun it generally proceeds undetected for a period until an index case is diagnosed and recognized by health authorities, marking the official beginning and declaration of an outbreak. In most instances, the suspected primary case is then established retrospectively based on epidemiological evidence and is usually not diagnostically confirmed, thus some uncertainty is inherently present in outbreak timelines. Outbreaks continue until 42 days have elapsed after the last EVD case, which is twice the longest known EBOV incubation period of 21 days. All EVD outbreaks which began between 1976-2012 and 2014-2018 (excluding the ongoing DRC outbreak) share a generally similar dynamic and shape, while the West Africa outbreak, which began in 2013, and the ongoing DRC outbreak stand out as stark outliers.

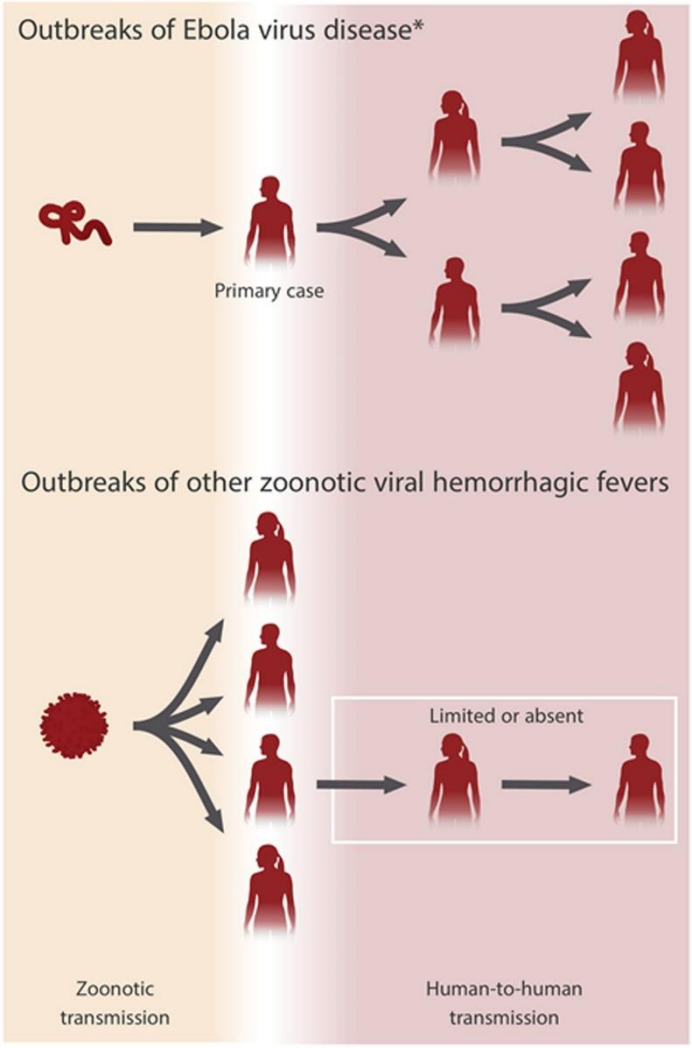

Figure 5.3 – Transmission of Ebola virus (EBOV) in Ebola virus disease (EVD) outbreaks compared to virus transmission in other zoonotic viral hemorrhagic fevers

EVD outbreaks usually result from exclusively human-to-human transmission following a single zoonotic spillover to a primary EVD case. Outbreaks of other zoonotic viral hemorrhagic fevers are often characterized by sustained spillover from a reservoir/intermediate host and limited human-to-human transmission. *Other filovirus diseases (e.g. Marburg virus disease) follow a similar pattern to EVD.

EVD outbreaks, 1976-2012

From 1976-2012, 12 EVD outbreaks occurred, the largest of which was the first known occurrence of EVD in 1976 in Yambuku, DRC (318 cases). EBOV was identified as the novel etiological agent from the index patient 48 days after the symptomatic onset of the suspected primary case, and the public health measures implemented, including isolation of cases, rapid burial, and quarantine of the entire health zone proved to be effective (Breman et al., 2016). The outbreak was declared over after 112 total days – below the median of 118 days calculated for all outbreaks (Figure 1.4A). In 1977, a single case of EVD was then identified in Tandala, DRC when a young girl became infected in her neighboring village of Bonduni. Subsequently, no outbreaks were reported for nearly 20 years until EBOV reemerged in 1994 in Gabon. From 1994-2012, EVD outbreaks occurred with gaps of no more than a few years, and each was confined to generally remote, sparsely populated areas in Gabon (Georges et al., 1999), the Republic of Congo (Leroy et al., 2004), and DRC (Grard et al., 2011). The 1995 Kikwit, DRC outbreak was the only exception to this, with its occurrence in an urban setting amidst a substantial population (~400,000 circa 1995) (Muyembe-Tamfum et al., 1999). All the outbreaks during this time were ultimately successfully contained and ended with public health measures comparable to those utilized during the first outbreak in 1976.

The West Africa EVD outbreak, 2013-2016

The West Africa outbreak marked a significant paradigm shift in the public health perception of EVD. Although some experts had maintained that EVD was a perennial threat of significant public health concern (Feldmann & Geisbert, 2011), it had largely come to be regarded as a minimal threat that was confined to remote populations and of very limited outbreak potential, insignificant in comparison to other infectious diseases (Leroy et al., 2011)

(Leroy et al., 2014). Ultimately, however, approximately 30,000 cases were reported during the West Africa outbreak (Garske et al., 2017) (Figure 5.4) – two orders of magnitude greater than any preceding outbreak and over 20 times the total of all previously known cases – and at 888 days in duration it was nearly four times longer than any previous outbreak (Figures 5.1, 5.4). EVD patients from the outbreak eventually reached 15 different countries spanning three continents as part of transmission chains or for medical treatment (Table 5.1), with Guinea, Liberia, and Sierra Leone at the epicenter. The World Health Organization (WHO) declared the outbreak a Public Health Emergency of International Concern (PHEIC) from August 8, 2014 until March 29, 2016, marking only the third time that such a declaration had been made. In addition to the direct public health impact of this EVD outbreak, the societal burden and economic cost were enormous, with estimates exceeding $50 billion in West Africa alone (Huber et al., 2018).

The location of the West Africa outbreak was of principal consequence: it originated 2,400 km further West than any previous EVD outbreak in a region where the only warnings that filoviruses might pose a threat were a single human infection with TAFV in Côte d'Ivoire in 1994 (Le Guenno et al., 1995) and a small outbreak of hemorrhagic fever in Pleebo, Liberia in 1995 that was suspected to be caused by an ebolavirus based on retrospective clinical and serological evidence (Le Guenno et al., 1999). Thus, the regional unfamiliarity with EVD in West Africa provides a rational explanation for the lengthy span of 86 days between the suspected primary case and the index case (Figure 5.1). Cholera was initially suspected and eventually diagnostically confirmed in a group of seven patients at a hospital in Gueckedou, although retrospectively it is likely that these patients were co-infected with EBOV based on

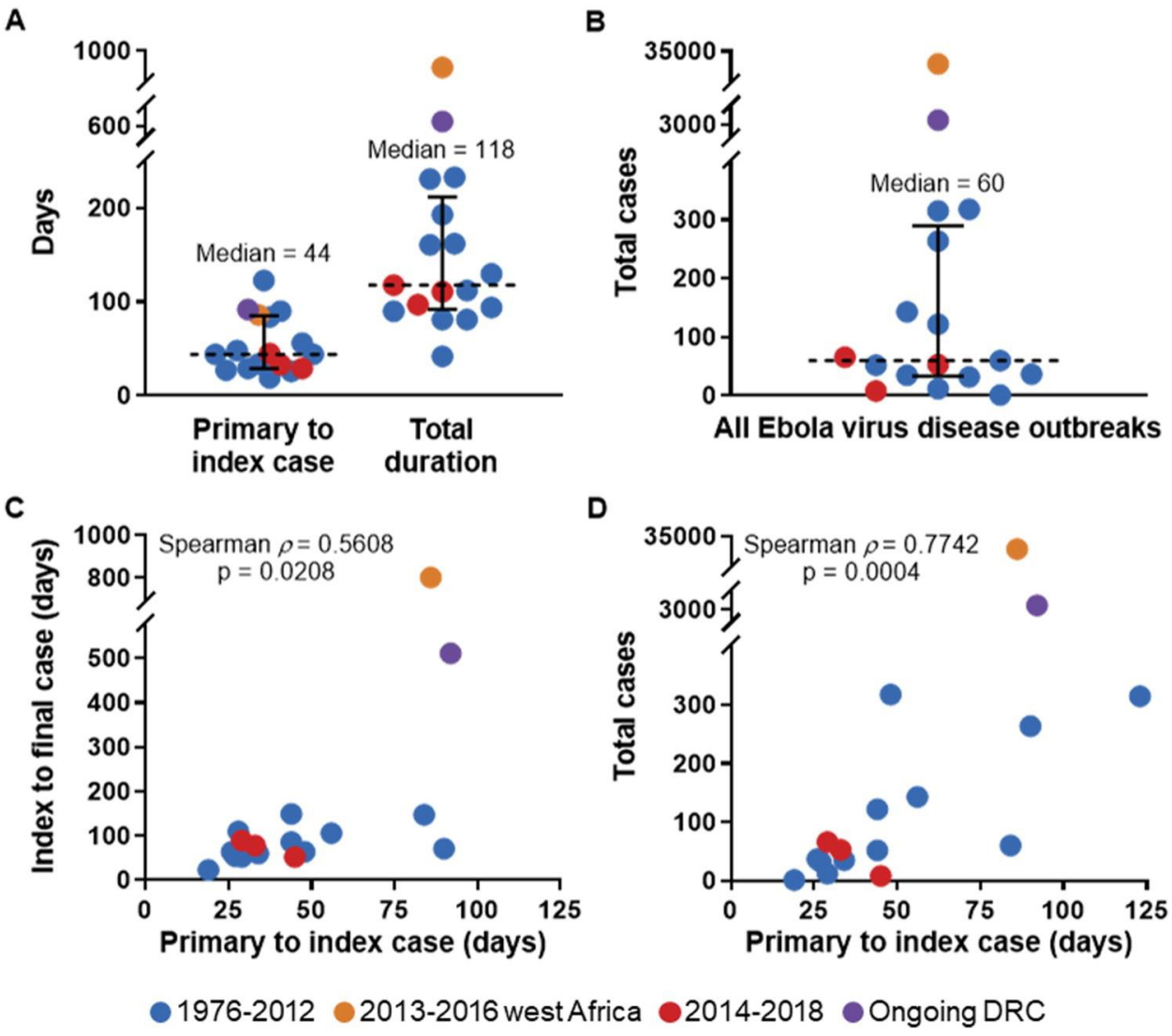

Figure 5.4 – Median Ebola virus disease (EVD) outbreak metrics and correlations of outbreak duration and size to the initial period of undetected transmission

A) Median time elapsed (dashed line) from primary case to index case and total days with interquartile range (bars) for all EVD outbreaks. B) Median cases (dashed line) with interquartile range (bars) for all EVD outbreaks. C) Correlation of time elapsed from suspected primary case to index case to outbreak duration for all EVD outbreaks. D) Correlation of time elapsed from suspected primary case to index case to total cases for all EVD outbreaks.

epidemiological evidence (WHO, 2014a). For other early cases, a presumptive diagnosis of Lassa fever was maintained by the WHO as late as March 18, 2014 (Goba et al., 2016), less than a week before EBOV was identified. These delays in identification were key in allowing the

early dissemination of EVD in southern Guinea and into the bordering regions of Sierra Leone and Liberia. Together with this undetected initial spread, numerous other factors converged to produce an exponential surge in cases (Figure 5.5), including the dense and mobile population structure (Munster et al., 2018) (Dudas et al., 2017), delays in utilization of investigational vaccines, and community resistance to public health efforts, which sometimes turned violent (Coltart et al., 2017).

EVD outbreaks, 2014-2018

Excluding the ongoing DRC outbreak, there were three EVD outbreaks between 2014-2018, all largely reminiscent of those which occurred from 1976-2012. Each was in DRC in general proximity to areas that had experienced previous EVD outbreaks: Inkanamongo in 2014 (Maganga et al., 2014), Likati in 2017 (Nsio et al., 2020), and Bikoro in 2018 (Ebola Outbreak Epidemiology, 2018) (Figures 5.1, 5.2). Each also adhered to historical norms for EVD outbreaks in regards to time elapsed from primary case to index case (29, 45, and 33 days, respectively) (Figures 5.2, 5.4), overall duration (118, 71, and 111 days, respectively) (Figures 5.2, 5.4), and total cases (66, 8, and 53 cases, respectively) (Figure 5.4). The overarching similarity of these three outbreaks to those prior to 2013 arguably strengthened the notion that the West Africa outbreak would endure as a sole outlier.

The 2014 Inkanamongo outbreak, which took place concurrently with the West Africa outbreak, provided a particularly striking juxtaposition: it began when the West Africa outbreak had resulted in less than 2,000 cases, but by the time it was declared over only four months later (Figure 5.2), the cases in West Africa had reached over 15,000, whereas the Inkanamongo outbreak totaled 66 cases (Maganga et al., 2014). The relatively prompt recognition of the Inkanamongo index case and the outbreak's occurrence in an area that was both comparatively

remote and familiar with EVD no doubt played roles in this sharp divergence. Similarly, the 2017 Likati outbreak unfolded in an isolated area of the Bas Uele province in DRC and was one of the smallest and shortest outbreaks on record (Figure 5.2) thanks to timely identification of the index case, a prompt domestic response (Nsio et al., 2020), and the rapid deployment of 50 WHO responders through the newly created Health Emergencies Programme, an initiative born out of the inadequacies realized in West Africa (Wenham, 2017). However, in April of 2018, the outbreak in Bikoro presented a significant test for EVD response efforts. It was concerning for its proximity to international borders and the potential to spread into the major urban center of Mbandaka (population 1.2 million) (Ebola Outbreak Epidemiology, 2018). Nevertheless, the outbreak was efficiently contained and ended thanks to a relatively speedy identification of the index case and decisive public health efforts. Significantly, the Bikoro outbreak also marked the first time that a vaccination campaign was utilized as an EVD outbreak countermeasure with the use of Merck's V920 vaccine (rVSVΔG-ZEBOV-GP) – a recombinant vesicular stomatitis virus vector expressing EBOV Kikwit glycoprotein (GP) that has been shown to be highly protective after a single dose, and may also be administered as a homologous prime-boost regimen (Wells et al., 2019).

The ongoing EVD outbreak in eastern DRC, 2018-present

The ongoing DRC outbreak is thought to have begun on April 30, 2018, less than 4 weeks after the Bikoro, DRC outbreak began (WHO, 2018b). Despite the similar timing in their beginnings, the Bikoro outbreak came to an end one week before the index case for the ongoing eastern DRC outbreak even came to be recognized on July 31, 2018 – 92 days after the putative primary case fell ill. With this, the eastern DRC outbreak went on record with one of the longest

delays in identification of the index case, despite the domestic familiarity with the disease and the tacitly heightened awareness following West Africa. It was not until a 65-year-old woman

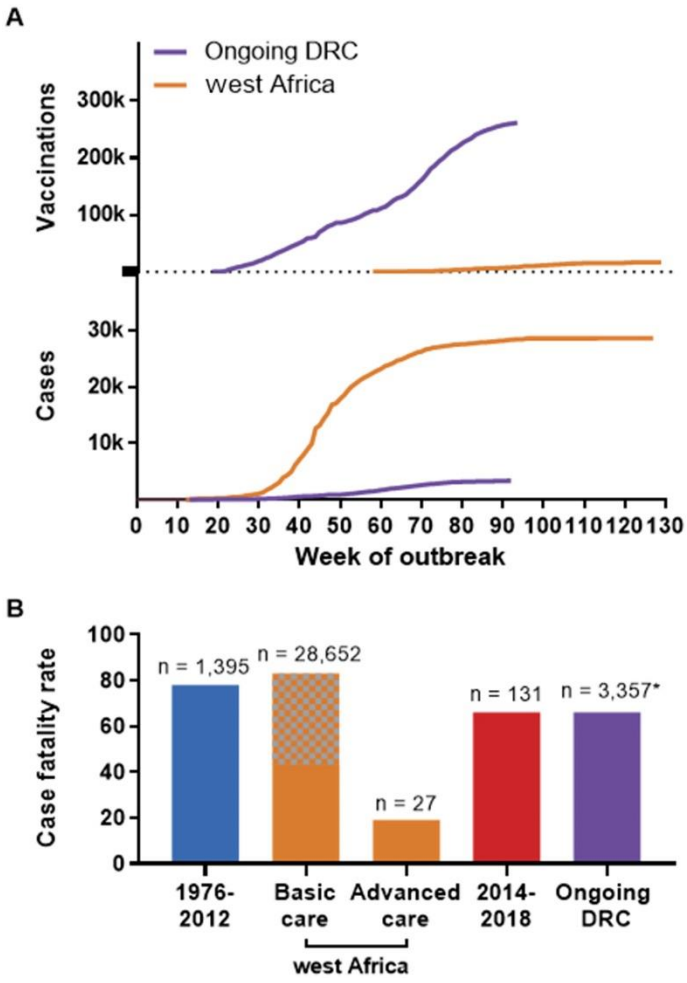

Figure 5.5 – Cases and vaccinations for West Africa and the ongoing DRC Ebola virus disease outbreaks, and overall case fatality rate (CFR) for all EVD outbreaks

A) Total cases (suspected, probable, confirmed) are included for the 2013-2016 West Africa Ebola virus disease (EVD) outbreak and the ongoing EVD outbreak in the Democratic Republic of the Congo and plotted by week. For vaccinations in West Africa, all vaccine platforms that were utilized are included; in the current DRC outbreak, Merck's V920 (rVSVΔG-ZEBOV-GP) accounts for most vaccinations; as of December 24, 2019, 2,938 doses of the Johnson & Johnson Ad26.ZEBOV/MVA-BN-Filo have been administered. Beginning dates represented are December 26, 2013 and April 30, 2018 for the West Africa outbreak and the ongoing DRC outbreak, respectively. B) Comparison of CFR for EVD cases during the following periods: 1976-2012, West Africa (2013-2016), 2014-2018, and the ongoing outbreak in the Democratic Republic of the Congo (2018-present). For West Africa, basic care is that which patients received in Ebola treatment units; advanced care is that which patients received when treated in the US or Europe. Gray and orange checkered coloring indicate that the CFR for the West Africa outbreak range from the naïve calculated 40% to recent corrected estimates of 63% (Garske et al., 2017) and 83% (Forna et al., 2019). Numbers above bars indicate total number of cases represented. *As of December 24, 2019.

died on July 25, 2018 at Mangina referral health center (WHO, 2018c) and her unsafe burial resulted in seven secondary cases (Dyer, 2018) that EVD was suspected and confirmed. This delay is perhaps associated with the spillover occurring on the outermost boundaries of the Guineo-Congolian rainforest, more than 600 km further east than in any previous EVD outbreak (Figure 5.2). While this region had previously experienced two outbreaks of BVD – the first in the town of Bundibugyo, Uganda in 2007 (MacNeil et al., 2010) and the second in the Isiro Health Zone, DRC in 2012 (Kratz et al., 2015) – EVD had historically only been known much further West. Thus, the documented range of EBOV greatly expanded for the second time in only five years, following West Africa, with the easternmost and westernmost spillovers now spanning 4,500 km – approximately equal in size to the continental United States. The outbreak is occurring in a densely populated urban area proximal to international borders with Rwanda, Uganda and South Sudan and major transportation thoroughfares. Distrust of public health authorities (Vinck et al., 2019) and misconceptions regarding EVD (Claude et al., 2018) are proving extremely problematic given the longstanding presence of conflict and social unrest in the region. On July 17, 2019, the WHO declared the outbreak a PHEIC, making EVD responsible for two of the five times this designation has been used to date (WHO, 2019a).

Despite these challenging circumstances, domestic spread to distant localities within DRC has not yet been reported, and international spread thus far has been limited to three cases imported to Uganda (WHO, 2019b). On the contrary, in addition to the broad domestic dissemination of EVD throughout Guinea, Liberia, and Sierra Leone, cases began to spread internationally during the West Africa outbreak relatively much sooner, beginning with Nigeria in July 2014 at approximately week 30 of the outbreak, and ultimately to numerous other countries (Table 5.1). And whereas the West Africa outbreak went on to experience a prolonged

period of exponential case growth beginning around week 35, the ongoing DRC outbreak has maintained relatively slow, insidious growth (Figure 5.5). While it is difficult to attribute these differences with any great degree of certainty to specific underlying factors given the complexity of the circumstances and general lack of data, the extraordinary vaccination campaign – which was promptly initiated within one week of the outbreak being recognized – in eastern DRC appears to be a major demarcating factor between the dynamics of the two outbreaks. This observation is supported by modeling analyses which suggest that EBOV vaccination significantly reduced the risk of further geographical spread of the outbreak (Wells et al., 2019). At present, more than 250,000 doses of the rVSVΔG-ZEBOV-GP have been administered (Figure 5.5) following the guidelines of the WHO Strategic Advisory Group of Experts and under expanded access to investigational new drugs (Van Norman, 2018) (WHO, 2017). Significantly, in a set of landmark decisions, the rVSVΔG-ZEBOV-GP received approval from the European Medicines Agency on November 11, 2019 (Callaway, 2019) and the US Food and Drug Administration (FDA) followed suit with their own approval of the vaccine on December 19, 2019 (FDA, 2019b), thus potentially greatly expanding access to the vaccine, principally in at-risk African nations. Furthermore, a second EVD vaccine – the Johnson & Johnson Ad26.ZEBOV/MVA-BN-Filo, a heterologous prime-boost regimen consisting of a monovalent human adenovirus serotype 26 vector expressing EBOV Mayinga GP followed by a multivalent modified vaccinia virus Ankara vector expressing EBOV Mayinga GP, Sudan virus Gulu GP, Marburg virus Musoke GP, and TAFV nucleoprotein – has been under evaluation in Uganda since August 2019 as a phase 2 clinical trial, and in November 2019 was introduced to DRC to augment use of the rVSVΔG-ZEBOV-GP vaccine in the ongoing outbreak (Callaway, 2019).

Outbreak trends summary

In summary, the median overall duration for the 17 currently described outbreaks is approximately four months (median = 118 days; interquartile range, 92 to 212 days) (Figure 5.4). This includes the approximately one-and-a-half months (median = 44 days; interquartile range, 28 to 70 days) that typically elapses from the suspected time of spillover to a primary case until declaration of an outbreak following the identification of the index patient (Figure 5.4). The majority of EVD outbreaks have been comprised of less than 100 human cases (median = 60; interquartile range, 34 to 290 cases) (Figure 5.4) and have taken place in remote locations with relatively low population densities (Schmidt et al., 2017). The spread of cases during most outbreaks has been limited to 150 km or less from the initial origin (Figure 5.2), although in a few instances cases have travelled to distant locations domestically or internationally, with further transmission sometimes occurring in those sites (Table 5.1).

The length of the initial period of undetected transmission between the primary case and the index case significantly correlates both with the duration (Spearman $\rho = 0.5608$, $p = 0.0208$) (Figure 5.4) and total cases (Spearman $\rho = 0.7742$, $p = 0.0004$) of the ensuing outbreak (Figure 5.4). As EVD outbreaks are characterized by a single zoonotic spillover followed by exclusively human-to-human transmission, this correlation is not surprising. The longer that the initial, unrecognized transmission chains propagate undetected, the longer the outbreak stands to continue due to a delay in control measures, and ultimately more infections are likely to occur. Therefore, any delay in detection of initial EVD cases following a spillover can result in uncontrolled expansion and prolonged duration of the outbreak, particularly in regions with higher population density, greater spatial connectivity, and sociopolitical unrest.

Table 5.1 – EVD outbreaks with transmission and/or case(s) treated > 150 km beyond primary transmission zone

Outbreak origin	Location(s) of cases	Comments
1976 Democratic Republic of the Congo (Yambuku)	Democratic Republic of the Congo (WHO, 1978) (Breman et al., 2016) – 3 cases: Kinshasa	Healthcare workers transported from Yambuku
1996-1997 Gabon (Booue)	Gabon (Georges et al., 1999) – 15 cases: Libreville	Patients from Booue; subsequent transmission
	South Africa (Georges et al., 1999) – 2 cases: Johannesburg	Healthcare workers from Libreville; one nosocomial case
2001-2002 Gabon/Republic of Congo (Mekambo/Mbomo)	Gabon (Nkoghe et al., 2005) – 1 case: Franceville (transferred to Libreville for treatment)	No epidemiological links to concurrent outbreak in northern Gabon/Republic of Congo
2013-2016 Guinea (Meliandou)	Nigeria (Fasina et al., 2014) (Otu et al., 2017) – 20 cases: Lagos (18 cases), Port Harcourt (2 cases)	Traveler from Liberia, subsequent transmission
	United States (CDC, 2014) (Fernandez, 2014) (*Nbc*, 2014) – 11 cases: Dallas, TX (3 cases, one transferred to Atlanta and one transferred to Bethesda for treatment)*, Atlanta, GA (3 cases + 1 transfer)*, Omaha, NE (3 cases), New York City, NY (1 case), Bethesda, MD (1 case + 1 transfer)	Seven medical evacuations; first Dallas case was a traveler from Liberia that resulted in two nosocomial transmission twice; New York City case was a healthcare worker (HCW) returning from Guinea
	Mali (Diarra et al., 2016) – 8 cases: Kayes (1 case), Bamako (7 cases)	Two introductions by travelers from Guinea; subsequent transmission
	United Kingdom (WHO, 2014a) (BBC, 2015) – 3 cases: London (2 cases + 1 transfer), Glasgow (1 case, transferred to London for treatment)	Two medical evacuations; Glasgow case was a returning HCW
	Spain (Jose María Marimón, 2014) (Parra et al., 2014) – 3 cases: Madrid	Two medical evacuations; one nosocomial case
	Italy (Salce et al., 2015) (WHO, 2015) – 2 cases: Rome	One medical evacuation; one returning HCW
	Germany (Smale, 2014) – 3 cases: Leipzig (1 case), Hamburg (1 case), Frankfurt (1 case)	All medical evacuations
	France (Jeremy Ashkenas, 2015) – 2 cases: Paris	Medical evacuations
	Norway (Jeremy Ashkenas, 2015) – 1 case: Oslo	Medical evacuation
	Netherlands (Jeremy Ashkenas, 2015) – 1 case: Utrecht	Medical evacuation
	Switzerland (Jeremy Ashkenas, 2015) – 1 case: Geneva	Medical evacuation
	Senegal (Ka et al., 2017) – 1 case: Dakar	Traveler from Guinea

Discussion

Significant progress has been made since the West Africa outbreak. Most notably, multiple vaccines have been developed and evaluated and are being utilized as countermeasures against EVD. Until this recent implementation of vaccination as part of the outbreak response, the public health efforts to combat EVD had remained effectively unchanged for over 40 years. Additionally, evidence suggests improved survival with basic supportive care including fluid replacement and electrolyte management (Lamontagne et al., 2018). The dramatically decreased CFR observed for EVD patients who received treatment in Europe and the United States (Uyeki et al., 2016) during the West Africa outbreak (Figure 5.5) provides a strong argument for application of universal standards of care (Fischer et al., 2019), and some estimate that the CFR may be less than 10% given early presentation and access to high-level intensive care (Richardson & Fallah, 2019). Additionally, multiple promising targeted therapies (Rojek et al., 2017) are currently under evaluation in a multi-arm phase III trial in DRC (NIH, 2018) (Friedrich, 2019), with preliminary data indicating significantly improved outcomes for patients receiving either of two biologics: a single monoclonal antibody, MAb114, or a cocktail of three monoclonal antibodies, REGN-EB3 (Mulangu et al., 2019). On December 23, 2019, REGN-EB3 was granted orphan drug status by the FDA, potentially increasing its accessibility for use during outbreaks (FDA, 2019c).

Nevertheless, EVD continues to present new and sobering challenges, and the ongoing outbreak has demonstrated that containing EVD in settings with a complicated sociopolitical milieu and extensive urban infrastructure is extremely difficult. This is particularly concerning as several countries with a comparable setting, including the Central African Republic and South Sudan, are thought to be at risk for outbreaks of EVD and other filovirus diseases (Pigott et al.,

2016). Perhaps the single greatest shortfall in efforts to curtail EVD outbreaks is the delay in diagnosing the index case following spillover to the primary case: three of the four longest times required for this (ongoing DRC = 92 days; 2007 Bamoukamba 2, DRC = 90 days; 2013-2016 West Africa = 86 days) have occurred approximately within the last decade (Figure 5.2). During the first EVD outbreak in 1976, EBOV was characterized as a novel filovirus only 48 days after the symptomatic onset of the primary case, a time approximately equal to the median for all EVD outbreaks. This required slow, labor-intensive analyses, including immunofluorescent and serological assays and electron microscopy, and the necessity for the international transportation of patient samples (WHO, 1978) (Breman et al., 2016). In contrast, the current gold-standard for EVD diagnostics – quantitative reverse-transcription polymerase chain reaction (qRT-PCR) – can be safely performed on location in a matter of hours with technology that has been utilized during EVD outbreaks since 1995 in Kikwit, DRC (Muyembe-Tamfum et al., 1999).

Timely recognition of an index EVD case is hampered by a lack of pathognomonic signs or symptoms at presentation, and the differential diagnosis includes malaria, typhoid fever, cholera, yellow fever, dysentery, Lassa fever, and other endemic febrile infectious diseases (WHO, 1978) (Muyembe-Tamfum et al., 1999) (Breman et al., 2016) (WHO, 2014a). However, while prompt, accurate diagnoses are important for any disease outbreak response, the stakes for EVD are markedly higher. EVD outbreaks result from exclusively human-to-human transmission following a single zoonotic spillover, and EBOV's intrinsic capacity for relatively efficient human-to-human transmission without prior adaptation is markedly different from many other emerging viruses (Figure 1.3). With EVD, rapid identification and isolation of the primary case and case contacts would likely prevent further human-to-human EBOV transmission and stem

the development of an EVD outbreak. Accomplishing this hinges upon incisive clinical suspicion and readily accessible diagnostics.

As even short delays in the recognition of an index EVD case may result in development of an outbreak, expansion and strengthening of basic diagnostics in western and central Africa is critical. In 2018, WHO published the first edition of its Model List of Essential In Vitro Diagnostics (EDL) as a complement to their perennial Model List of Essential Medicines (WHO, 2018e). The EDL recognizes the increasingly essential role that in vitro diagnostics (IVDs) have in providing accurate diagnoses and enabling public health efforts. For the primary health care level (e.g. doctor's offices, community health centers, etc.), recommended testing includes the use of rapid diagnostic tests (RDTs) utilizing capillary whole blood for endemic infectious diseases such as HIV, tuberculosis, and syphilis. More complex and confirmatory testing is recommended to then be undertaken at district hospitals and regional or national laboratories. A similar tiered diagnostic approach for EVD in countries known to be at-risk would allow for prompt detection of index EVD cases. RDTs for EVD are already available including the ReEBOV Antigen Rapid Test (Broadhurst et al., 2015) and the OraQuick Rapid Antigen Test (Jean Louis et al., 2017), the latter of which received crucial FDA approval on October 10, 2019 (FDA, 2019a). While less sensitive and/or specific than qRT-PCR based diagnostics, these antigen-based tests can provide results almost immediately at the point-of-care and should be made available at a primary health care level to enable routine screening of any suspected cases, with results confirmed with other IVDs in regional laboratories. Moreover, modern qRT-PCR platforms, such as Cepheid's cartridge-based GeneXpert, require minimal additional infrastructure and training and should be readily available for confirmation of RDT testing results. Utilization of the GeneXpert platform for EVD diagnostics is an example of a value-

added approach, as a large network of these machines has been deployed through a WHO-coordinated effort for detection of multidrug-resistant TB, and is therefore also accessible for EVD diagnostics (Albert et al., 2016). DRC began implementing GeneXperts for tuberculosis diagnostics at regional facilities in 2013, and hundreds of these instruments are already available and in use in numerous provinces across the country (Bulabula et al., 2019) (Kayomo et al., 2018) (Cazabon et al., 2018). Furthermore, diagnosis of the first patients in the ongoing outbreak was accomplished with a GeneXpert, although this required shipment of the samples to the Institut National de Recherche Biomédicale in Kinshasha, DRC (WHO, 2018a). Given the enormous medical, economic, and political costs of EVD outbreaks, strengthening EVD diagnostic capacity to facilitate early detection of spillovers and rapid response to contain further transmission should be the highest priority.

The geographic footprint of EBOV has increased dramatically over the past five years. While EBOV spillover nevertheless remains rare, our ability to promptly detect EVD is of utmost importance. In the wake of the West Africa outbreak, nearly every state in the US established EVD diagnostics in local public health laboratories (Spengler et al., 2016). However, EVD likely only poses an indirect threat to the US; therefore, the emphasis should be focused on preparedness and outbreak prevention by bolstering front-line diagnostic capacities in the regions at direct risk of EVD outbreaks (Munster et al., 2018). If a strong case can be made that a universal standard of care should be provided for all EVD patients (Fischer et al., 2019) – which will require significant investments and capacity-building and is not preventative in nature – then certainly an equally strong or stronger case can be made for vastly increasing surveillance and diagnostic capacity for EVD throughout central Africa. Such an approach is the most efficient and effective public health strategy and should be the first-line defense in the fight against EVD.

CHAPTER 6: SUMMARIZING DISCUSSION

The studies undertaken here have attempted to address unanswered questions regarding EBOV and EVD.

In Chapter 2, two crucial observations were made. First, the sickest EVD patients – those that would go on to not survive the disease – were arriving at the ELWA-3 ETU over 1.5 days earlier in the course of their illness, on average, in the second half of the observation period versus the first. This is an extremely encouraging finding as it speaks to the effectiveness of the public health outreach efforts and should be viewed as a profound success. Early initiation of treatment has been shown to play a profoundly important role in improving outcomes in EVD, as indicated by the recent PALM trial results: even with the most effective experimental monoclonal antibody drugs, each day of symptoms prior to treatment was associated with an increase of 11% in the odds of death (Mulangu et al., 2019). The sobering finding in our data, however, was that the likelihood of survival did not concomitantly increase with earlier presentation. This suggests that more rapid initiation of the level of supportive care that was available at ELWA-3 likely did not have a significant impact, at least for the change in presentation time that we observed. Would it have made a difference if intravenous fluids were provided at ELWA-3? What if the patients came in, on average, at day two of their illness, rather than at four or five days into their illness? These are questions which we cannot answer, but which underpin the necessity for ongoing research into how EVD patients can best be cared for.

Second, the reanalyzed sample set provided data that was much more capable of discriminating between survivors and non-survivors based on measurements of viral RNA in the blood at triage, with an odds ratio for a fatal outcome 4x greater (approximately 8 vs. 2) in the high-risk (meaning higher EBOV RNA copies/mL or lower C_t value) using the reanalyzed data vs. the field data. Sample degradation is not responsible for this phenomenon. For the 75-sample

subset that was reanalyzed on the same exact SmartCycler and LightCycler qRT-PCR instruments as used in the field, the reanalyzed C_t values were lower in 59 out of the 75 samples (79%). The only differences between these analyses is the fact that the samples had been in storage and extraction method performed: in the field, RNA was extracted from fresh whole blood using the Qiagen Viral RNA Mini Kit, while the laboratory reanalysis utilized a TRIzol-based approach on the stored samples. If sample degradation was responsible for a change in C_t value, that change should be an increase in value, not a decrease. Furthermore, for the 59 samples which had a lower C_t value upon reanalysis, the difference was on average 4.98 C_t values lower; the 16 samples which had a higher C_t upon reanalysis had on average an increase of 1.9 C_t values. Thus, the samples for which the reanalyzed C_t value was higher had a minimal increase, while the reanalyzed samples with lower C_t values decreased substantially more in comparison. When the C_t values of these same 75 samples are analyzed according to outcome (survivors = 32; non-survivors = 43), the reanalyzed survivor samples were on average 1.75 C_t values lower than those obtained in the field ($p = 0.0019$), while the non-survivors were on average 4.67 C_t values lower ($p < 0.0001$). This again reinforces two notions: (1) the samples overall had essentially no signs of degradation by qRT-PCR analysis, and (2) the decrease in C_t values amongst the reanalyzed samples was much more prominent in the non-surviving cohort. This leads to the conclusion that some combination of the samples being stored and the different extraction protocol led to a more efficient harvesting of RNA, or possibly a reduction in the amount of PCR inhibitors present (a point which is argued for in Chapter 2 based on previous publications). While the primary purpose of running qRT-PCR assays in the field at ETUs is for diagnostic, rather than prognostic, purposes, viral load as estimated by C_t value has emerged as

one of the only variables (along with age) that correlates with outcome. Therefore, accurate measurement of this variable will provide data from which reliable conclusions can be drawn.

The role of supportive care for EVD – and how to best optimize it – has also been a major focal point following the West Africa outbreak. As discussed in Chapter 3, prior to the West Africa EVD outbreak, antibiotics were primarily recommended for suspected EVD patients prior to laboratory-confirmed diagnosis. The rationale for this was simple: numerous bacterial diseases (e.g. typhoid) are clinically indistinguishable from EVD and relatively much more common, and EVD diagnostics were generally not performed on-site prior to West Africa and thus a laboratory confirmation could be delayed by days. Thus, presumptive treatment with antibiotics for bacterial etiologies was recommended, and following a definitive diagnosis of EVD may or may not be continued throughout the supportive care regimen for EVD, if confirmed as the diagnosis, at the clinician's discretion (MSF, 2008). The similarities between severe EVD and bacterial sepsis had long been noted, but were assumed to be just that – similarities (Bray & Mahanty, 2003). It was during the West Africa outbreak that bacterial sepsis began to be thought of as a comorbid condition that could arise secondary to EVD, and which required empiric treatment with broad-spectrum antibiotics.

In 2014, a single case report seemingly shifted this paradigm and initiated the concern that bacteremia may routinely complicate EVD, possibly via gut translocation. The case was published by Kreuels et al. on October 22, 2014 in the New England Journal of Medicine (Kreuels et al., 2014). An unidentified Gram-negative bacterium was cultured from the blood of an EVD patient that had been evacuated to Germany for treatment 10 days following symptom onset, and four days following a positive qRT-PCR for EBOV in Sierra Leone. The patient had received empiric antibiotic treatment prior to evacuation to Germany and was again placed on

antibiotics during treatment in Germany. The gram-negative organism that was cultured on day 12 of the patient's illness was resistant to ampicillin, ciprofloxacin, and third-generation cephalosporins – a finding that could certainly reasonably interpreted to indicate iatrogenic infection in the ICU as opposed to translocation of gut bacteria. Nevertheless, fewer than two months later, on December 18, 2014, another influential publication in The Lancet by Wolf et al. stated "Bacterial infection is an important complication in severe Ebola virus disease", with only the aforementioned NEJM article cited as evidence (Wolf et al., 2015). Bacterial sepsis was continuously suspected in the patient, and blood cultures were regularly obtained, but only one bottle was positive for any organism – a coagulase negative staphylococcus, most likely a skin contaminant. Despite this, the antibiotics administered during the course of treatment included imipenem-cilastatin, meropenem, metronidazole, colistin, and tigecycline (Wolf et al., 2015).

In the investigation here of the prevalence of bacteremia in EVD – which is the largest carried out to date – no evidence was found amongst the standard diagnostic microbiology data to suggest that bacteremia is a secondary complication of EVD. In fact, the only Gram-negative pathogens that were cultured were found in the smaller EVD-negative 'control' cohort – a finding that does support the empiric administration of antibiotics to patients during triage, as many could present to ETUs with EVD-like symptoms that, upon further investigation, turn out to have a bacterial etiology. The targeted deep sequencing approach – BacCapSeq – also failed to yield any significant differences between the groups in terms of prevalence of bacteremia. It must be acknowledged, however, that ideally our control population would have consisted of truly healthy controls, rather than individuals reasonably suspected of having EVD. Perhaps future clinical trials investigating EVD supportive care treatments can be designed to evaluate the effect of antibiotic administration. These questions could also be addressed, at least in part,

using animal models of EVD. Reliably parsing out the effects of different levels of supportive care provided for EVD patients based on currently available data is not possible.

As discussed in Chapters 2 and 3, much of the discussion regarding the purported benefits of early initiation of aggressive fluid resuscitation or empiric administration of antibiotics, for example, is based on extremely limited evidence. Furthermore, much of the optimism surrounding the purported benefits of these practices centered on the widely reported CFR of 39.5% amongst a case count of 28,616. This reported CFR is well below historical means and is still claimed in many official records by WHO and CDC and broadly cited in the literature (and which are based on the situation reports that were released on roughly a weekly basis by WHO throughout the outbreak). However, it is all but certainly incorrect. The WHO's own viral hemorrhagic fever database lists 33,338 confirmed, suspected and probable cases – with only 8,413 actually laboratory confirmed with a known outcome (Forna et al., 2020). Amongst these 8,413, the overall case fatality rate for the three countries was 62.9%. Other more recent studies have estimated this number to be even higher. This ambiguity both undercuts the narrative that the level of supportive care provided in West Africa significantly improved outcomes, and it also highlights the difficulties in obtaining high quality data under such extremely challenging outbreak conditions as were seen in West Africa.

In Chapter 4, the finding that Vero E6 cells are advantageous over Huh7 cells for the isolation and sequencing of EBOV has a number of implications. For ecological studies that sample animals in search of the zoonotic reservoir of EBOV, often only very small sample volumes are available for virus culture, and multiple attempts across various cell lines are generally therefore not feasible. While only two cell lines were tested in these studies, Vero E6 and Huh7 cells are certainly the most commonly used for these purposes. However, their

respective sensitivity for the isolation of EBOV from clinical whole blood samples had not been previously assessed, to our knowledge. Furthermore, Vero E6 cells preserved the consensus sequence of EBOV as it was prior to culture and isolation slightly better than Huh7 cells. Maintenance of the consensus sequence has generally been the primary concern when passaging virus and growing viral stocks. However, the studies here also demonstrated that the quasispecies diversity of EBOV is likely reduced significantly upon isolation. This finding may have implications for various studies that attempt to characterize more nuanced phenotypic aspects of EBOV and its pathogenesis. Future work could assess the effect of further passages of EBOV in cell culture on the quasispecies diversity and could evaluate the impact of any changes observed with in vitro and in vivo studies.

Recognizing the lack of progress in detecting EVD quickly, as we saw in Chapter 5, is both sobering and enlightening. It is sobering to realize that we have failed to progress, despite enormous leaps in the technology used for diagnostics. But it is also enlightening in that a relatively simple step is still readily addressable in the fight against EVD. Earlier detection of outbreaks does not require breakthroughs in antivirals or immense improvements in healthcare infrastructure in central and western Africa – it involves only the further deployment and utilization of existing technologies, and continued education for both the public and for healthcare providers. Prioritizing the capacity to identify primary EVD cases prior to their recovery or death is thus likely one of the single most efficient and important measures that can be implemented to reduce the size and duration of EVD outbreaks, as it allows for prompt contact tracing and quarantine prior to widespread dissemination of the virus throughout communities.

At the time of writing, the 18th known outbreak of EVD, which was officially recognized on June 1, 2020 in western DRC city of Mbandaka, continues to slowly grow. It is the 10th outbreak of EVD in the DRC on record. Only weeks later, on June 25, 2020, a concurrent EVD outbreak centered in the eastern DRC provinces of North Kivu and Ituri, was finally declared over 829 days after the suspected primary case became ill. Nearly 3,500 cases were recorded, which resulted in approximately 2,300 deaths. It was the world's second longest and largest outbreak of EVD, following that seen in West Africa from 2013-2016.

It seems clear, then, that EVD will likely continue to be a perennial problem. Our understanding of it has increased dramatically in recent years (a cursory PubMed search yields 1,878 results for the term 'Ebola' prior to 2013; from 2013-present, 7,509 results are produced), and substantial progress has been made in the development and deployment of countermeasures, including highly effective vaccines and antibody-based therapeutics. Yet outbreaks will continue to happen, and the particular severity and deadliness of EVD place it in a category almost all its own. It is a feared and stigmatized disease, and its repercussions reach far beyond the death tolls that it elicits. Much remains to be learned, but it is hoped that the studies undertaken here have provided an incremental advance in our understanding of EBOV and EVD.

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ABBREVIATIONS

BacCapSeq – bacterial capture sequence

BDBV – Bundibugyo virus

bp – base pairs

BSL-3 – biosafety level 3

BSL-4 – biosafety level 4

cDNA – complementary DNA

CFR – case fatality rate

CFU – colony-forming unit

CPE – cytopathic effect

C_t – cycle threshold (cf. qRT-PCR)

ddPCR – droplet digital polymerase chain reaction (cf. PCR, qRT-PCR)

DMEM – Dulbecco's modified Eagle's medium

EBOV – Ebola virus

EVD – Ebola virus disease

HCW – healthcare worker

MALDI-TOF – matrix-assisted laser desorption ionization, time-of-flight

moi – multiplicity of infection

mRNA – messenger RNA

NGS – next-generation sequencing

nt – nucleotide

PBS – phosphate buffered saline

PCR – polymerase chain reaction (cf. ddPCR, qRT-PCR)

qRT-PCR – quantitative reverse-transcription polymerase chain reaction (cf. ddPCR, PCR)

RESTV – Reston virus

RDT – rapid diagnostic test

TAFV – Taï Forest virus

$TCID_{50}$ – 50% tissue culture infectious dose

SUDV – Sudan virus

VirCapSeq-VERT – virome capture sequencing for vertebrate viruses

x *g* – relative centrifugal force in units of times gravity

APPENDICES

Appendix 1: MU Research Ethics

MARSHALL UNIVERSITY
www.marshall.edu

Office of Research Integrity

March 29, 2022

M. Jeremiah Matson
17667 State Route 7 S
Crown City, OH 45623

Dear Jeremiah:

This letter is in response to the submitted dissertation abstract entitled *"A Retrospective Analysis of Ebola Virus Disease in Liberia."* After assessing the abstract it has been deemed not to be human subject research and therefore exempt from oversight of the Marshall University Institutional Review Board (IRB). The Code of Federal Regulations (45CFR46) has set forth the criteria utilized in making this determination. Since the study does not involve human subjects as defined in DHHS regulation 45 CFR §46.102(e) it is not considered human subject research. If there are any changes to the abstract you provided then you would need to resubmit that information to the Office of Research Integrity for review and determination.

I appreciate your willingness to submit the abstract for determination. Please feel free to contact the Office of Research Integrity if you have any questions regarding future protocols that may require IRB review.

Sincerely,

Bruce F. Day, ThD, CIP
Director

Appendix 2: Table of the Hemorrhagic Fever Viruses

Order	Family	Species	Member virus(es)	Known reservoir(s)	Spillover mechanism	Non-human impacts
Amarillovirales	*Flaviviridae*	*Dengue virus*	dengue virus (DENV) (Gubler, 1998; Hanley et al., 2014; Huy et al., 2013; Ranjit & Kissoon, 2011; Rico-Hesse, 2010)	hard-body ticks (*Dermacentor* spp., OHFV; *Hemaphysalis spinigera*, KFDV; *Hyalomma dromedarii*, AHFV; *Ixodes* spp., TBEV, OHFV), soft-body *Ornithodoros savigyni* ticks (AHFV), small rodents (TBEV, OHFV), and non-human primates (DENV, YFV)	direct contact with infected animal (AHFV, KFDV, OHFV, TBEV) tick bites (AHFV, KFDV, OHFV, TBEV), and *Aedes aegypti* (and possibly other spp.) mosquito bites (DENV, YFV)	high-mortality epizootics amongst non-human primates (KFDV)
		Kyasanur Forest disease virus	Alkhumra hemorrhagic fever virus (AHFV) (Hotez et al., 2012; Memish et al., 2012; Shibl et al., 2012) and Kyasanur forest disease virus (KFDV) (Gould & Solomon, 2008; Yadav et al., 2020)			
		Omsk hemorrhagic fever virus	Omsk hemorrhagic fever virus (OHFV) (Ruzek et al., 2010)			
		Tick-borne encephalitis virus	tick-borne encephalitis virus (TBEV) (Madison-Antenucci et al., 2020; Ruzek et al., 2019)			
		Yellow fever virus	yellow fever virus (YFV) (Gardner & Ryman, 2010; Ndeffo-Mbah & Pandey, 2020)			
Bunyavirales (Abudurexiti et al., 2019)	*Arenaviridae*	*Chapare mammarenavirus*	Chapare virus (CHAPV) (Delgado et al., 2008; Shao et al., 2015)	small rodents (GTOV, JUNV, LASV, LUJV, MACV, WWAV); unknown for CHAPV and SBAV, but small rodents suspected	direct or indirect (e.g. secretions, excretions) contact with small rodents (confirmed for all but CHAPV and SBAV)	none reported
		Guanarito mammarenavirus	Guanarito virus (GTOV) (Hallam et al., 2018; Shao et al., 2015)			
		Argentinian mammarenavirus	Junín virus (JUNV) (Grant et al., 2012; Hallam et al., 2018; Shao et al., 2015)			
		Lassa mammarenavirus	Lassa virus (LASV) (Gunther & Lenz, 2004; Hallam et al., 2018; Richmond & Baglole, 2003; Shao et al., 2015)			
		Lujo mammarenavirus	Lujo virus (LUJV) (Briese et al., 2009; Sewlall et al., 2014; Shao et al., 2015)			
		Machupo mammarenavirus	Machupo virus (MACV) (Hallam et al., 2018; Patterson et al., 2014; Shao et al., 2015)			
		Brazilian mammarenavirus	Sabiá virus (SBAV) (Hallam et al., 2018; Lisieux et al., 1994; Shao et al., 2015)			
		Whitewater Arroyo mammarenavirus	Whitewater Arroyo virus (WWAV) (Fulhorst et al., 2001; Milazzo et al., 2011)			
	Hantaviridae	*Dobrava-Belgrade orthohantavirus*	Dobrava virus (DOBV) (Faber et al., 2019; Papa, 2012; Plyusnin et al., 2006), Kurkino virus (KURV) (Klempa et al., 2013; Tkachenko et al., 2019), Saaremaa virus (SAAV) (Plyusnin et al., 2006; Plyusnina et al., 2009; Sironen et al., 2005), and Sochi virus (SOCV)(Jonsson et al., 2010; Zelena et al., 2019)	small rodents (numerous species)	direct or indirect (e.g. secretions, excretions) contact with small rodents	none reported
		Dobrava-Belgrade orthohantavirus				

Bunyavirales (Abudurexiti et al., 2019)	*Hantaviridae*	*Hantaan orthohantavirus*	Amur virus (AMRV) (Lokugamage et al., 2004; Yao et al., 2012), Hantaan virus (HTNV) (Avsic-Zupanc et al., 2019; Jonsson et al., 2010; Kruger et al., 2011; Schmaljohn & Hjelle, 1997), and Soochong virus (SOOV) (Baek et al., 2006; Song et al., 2009)	small rodents (numerous species)	direct or indirect (e.g. secretions, excretions) contact with small rodents	none reported
		Puumala orthohantavirus	Puumala virus (PUUV) (Hjertqvist et al., 2010; Pettersson et al., 2008)			
		Seoul orthohantavirus	gōu virus (GOUV) (Jonsson et al., 2010; Wang et al., 2013) and Seoul virus (SEOV) (Clement et al., 2019; Swanink et al., 2018)			
		Tula orthohantavirus	Tula virus (TULV) (Plyusnin et al., 1994; Zelena et al., 2013)			
	Nairoviridae	*Rift Valley fever phlebovirus*	Rift Valley fever virus (RVFV) (Hotez et al., 2012; Nanyingi et al., 2015; Wright et al., 2019)	mosquitoes of genera *Aedes* and *Culex*	mosquito bites and/or contact with infected livestock	high-mortality epizootics amongst livestock
	Phenuiviridae	*Crimean-Congo hemorrhagic fever orthonairovirus*	Crimean-Congo hemorrhagic fever virus (CCHFV) (Bente et al., 2013; Flick & Whitehouse, 2005; Whitehouse, 2004)	hard-body ticks of genus *Hyalomma*	tick bites and/or contact with infected livestock	none reported
Mononegavirales	*Filoviridae*	*Bundibugyo ebolavirus*	Bundibugyo virus (BDBV) (MacNeil et al., 2010; Towner et al., 2008)	unknown for ebolaviruses, frugivorous and/or insectivorous bats suspected; *Rousettus aegyptiacus* frugivorous bats for MARV	unclear; contact with intermediate hosts or reservoir host(s) suspected	high-mortality epizootics amongst non-human primates
		Marburg marburgvirus	Marburg virus (MARV) (Brauburger et al., 2012; Feldmann, 2006; Leroy et al., 2011; Mehedi et al., 2011b) and Ravn virus (RAVV) (Burk et al., 2016; Carroll et al., 2013)			
		Sudan ebolavirus	Sudan virus (SUDV) (Carroll et al., 2013; "Ebola haemorrhagic fever in sudan, 1976. Report of a who/international study team," 1978; McCormick et al., 1983)			
		Tai Forest ebolavirus	Taï Forest virus (TAFV) (Le Guenno et al., 1995)			
		Zaire ebolavirus	Ebola virus (EBOV) (Feldmann & Geisbert, 2011; Groseth et al., 2007; Malvy et al., 2019)			

CURRICULUM VITAE – M. JEREMIAH MATSON

Current positions

NIH/NIAID Graduate Partnership Program Pre-Doctoral IRTA Fellow 2016 – present
Rocky Mountain Laboratories – Hamilton, MT
NIH Clinical Center – Bethesda, MD
MD-PhD Candidate, Marshall University School of Medicine 2014 – present
Marshall University – Huntington, WV

Education

MD-PhD (Candidate) 2014 – 2022 (est.)
Marshall University School of Medicine – Huntington, WV
BTh, Theology, Philosophy, and Ethics
2010 – 2013
Moore College – Sydney, Australia
BS, Biology
2003 – 2007
Cedarville University – Cedarville, OH

Research experience

NIH/NIAID Graduate Partnership Program Pre-Doctoral IRTA Fellow 2016 – present
Laboratory of Virology, Virus Ecology Unit
Rocky Mountain Laboratories – Hamilton, MT and
Emerging Pathogens Section
NIH Clinical Center – Bethesda, MD
Mentors: Dr Vincent Munster, PhD and Dr Daniel Chertow, MD, MPH

- Currently undertaking dissertation research on Ebola virus
- Project focuses on retrospective analysis of over 1,000 patient samples collected in Liberia
- Collaboration with the NIH Clinical Center (Bethesda, MD)

MD-PhD Summer Research Rotations 2014 & 2016
Marshall University School of Medicine – Huntington, WV
Mentors: Dr Hongwei Yu, PhD (2014), Dr Joseph Shapiro, MD (2016)

- Focused on cystic fibrosis pathology involving *Pseudomonas* biofilms (Yu)
- Learned murine techniques (e.g. surgery, BP measurement) and qRT-PCR (Shapiro)
- Mentored one-on-one by the Medical School Dean and active clinician-scientist (Shapiro)

NIH/NIAID Summer IRTA Fellow 2015
Laboratory of Virology, Virus Ecology Unit
Rocky Mountain Laboratories – Hamilton, MT
Mentor: Dr Vincent Munster, PhD

- Spent two months in Dr Munster's lab laying groundwork for GPP
- Sequenced Ebola virus genomes from Liberian patient samples
- Performed phylogenetic and molecular epidemiological analyses on sequences

Visiting Research Fellow
2013
Lens Research Laboratory, Sydney Medical School
The University of Sydney – Sydney, Australia
Mentor: Dr Frank Lovicu, PhD

- Focused on cataract pathogenesis via aberrant TGF-β signaling
- Optimized a NOX4 western blot analysis
- Performed tissue explants of the anterior lens epithelium (rat and mouse)

Research skills

Containment: extensive experience in both BSL-3 and BSL-4 settings
Molecular: including PCR, qPCR, ddPCR, NGS, gel electrophoresis, blotting, ELISA
Microbiological: including cell/tissue culture, general virology, clinical microbiology, and microscopy
Animal work: experience with rats, mice, non-human primates, guinea pigs, and hamsters
Software: including GraphPad Prism, JMP, Geneious, R

Awards and recognitions

2020

- Fellows Award for Research Excellence (FARE) – NIH ($1,500)
- American Society for Virology – Travel Award for ASV 2020 ($500)
- 32nd Annual Marshall University Research Day – Best Basic Science Oral Presentation (student category)

2019

- Infectious Diseases Society of America – Grants for Emerging Researchers Mentorship award ($4,000)
- Infectious Diseases Society of America/NIAID – selected & funded to attend ID Research Careers Meeting
- Infectious Diseases Society of America – Travel Award ($500)
- Infectious Diseases Society of America – selected for IDWeek Mentorship Program

2018

- Global Virus Network – selected for 5th annual short course (University of Maryland/Johns Hopkins/NIH)
- Infectious Diseases Society of America – Medical Scholar Award ($2,000)
- American Society for Microbiology – Microbe Travel Award ($1,500)
- American Society for Microbiology – Young Ambassador (2018-2020)
- Smithsonian Institution/American Society for Microbiology – Community Outreach Award ($1,000)

2017

- American Society of Tropical Medicine and Hygiene – Benjamin H. Kean Fellowship ($5,000)
- NIH OITE Graduate Partnership Program Symposium – Travel Award ($1,750)

Professional associations

American Society for Microbiology
American Society of Tropical Medicine and Hygiene
American Society of Virology
Infectious Diseases Society of America

Presentations

1. **Matson, M.J.**, Anzick, S.L., Feldmann, F., Martens, C.A., Sprecher, A., Giuliani, R., Feldmann, H., Massaquoi, M., Chertow, D.S., Munster, V.J. Vero E6 cells are more sensitive and provide greater sequence fidelity for the isolation of Ebola virus from clinical samples compared to Huh7 cells. Oral presentation ASV Filovirus Workshop (remote due to COVID-19). June 18, 2020.
2. **Matson, M.J.**, Feldmann, F., Ricotta, E., Massaquoi, M., Munster, V.J., Chertow, D.S. Ebola virus disease and bacterial co-infections. Oral presentation at 32nd Annual Marshall University Research Day, Huntington, WV USA. March 6, 2020.
3. **Matson, M.J.**, Massaquoi, M., Sprecher, A., Giuliani, R., Edwards, J.K., Dekker, J.P., Ricotta, E., Drake, S.K., Feldmann, F., Feldmann, H., Munster, V.J., Chertow, D.S. Bacteremia is not commonly detected in Ebola virus disease. Poster presentation at IDSA/NIAID ID Research Careers Meeting: Bethesda, MD USA, June 6-8, 2019 and at IDWeek 2019: Washington, DC USA. October 2-6, 2019. Conference abstract available at doi.org/10.1093/ofid/ofz360.1991
4. **Matson, M.J.**, Munster V.J. Analysis of patient samples from the ELWA-3 Ebola treatment unit in Monrovia, Liberia. Oral presentation at the Global Virus Network 5th Annual Short Course: University of Maryland, Baltimore, MD USA. August 5-11, 2018.
5. **Matson M.J.**, Stock F., Shupert W.L., Bushmaker T.J., Feldmann F., Bishop W.B., Frank KM, Dekker JP, Chertow DS, Munster VJ. Inactivation of maximum containment viruses and identification of bacterial co-infections. Poster presentation at ASM Microbe 2018, Atlanta, GA USA. June 7-11, 2018.
6. **Matson M.J.**, Chertow, D.S., Munster, V.J. Ebola virus disease: an introduction to the 2013-2016 epidemic. Oral presentation at Marshall University Biomedical Research Symposium. October 2017; Huntington, WV.
7. **Matson, M.J.**, Chertow, D.S., Munster, V.J. Ebola virus disease and sepsis. Poster presented at: NIH GPP Research Symposium, NIH Main Campus, Bethesda, MD USA. February 23, 2017.

Peer-reviewed publications

1. **Matson MJ**, et al. Bacteremia is not commonly detected in Ebola virus disease patients by diagnostic microbiology or targeted deep sequencing. *In preparation*
2. **Matson MJ***, Ricotta ER*, et al. Reevaluation of triage Ebola virus load in Liberia by droplet digital PCR: epidemiological findings and methodological advantages. *In preparation*
3. **Matson MJ**, et al. Ebola virus isolates from clinical samples maintain consensus sequence but lose quasispecies diversity. *In preparation*
4. Yinda, CK*, Morris DH*, […], **Matson MJ**, […], Munster VJ. The effect of temperature and humidity on the stability of SARS-CoV-2. *In preparation*
5. Avanzato VA*, **Matson MJ***, et al. Shedding of infectious SARS-CoV-2 from a chronically infected patient for over three months. *In preparation*
6. Fischer RJ, […], **Matson MJ**, […], Munster VJ. Stability of SARS-CoV-2 in wastewater. *In preparation*
7. **Matson MJ** and Munster, VJ. Ebola virus: uniquely challenging amongst the hemorrhagic fever viruses. *In preparation*

8. Fisher RJ, Purushotham J, van Doremalen N, Sebastian S, Meade-White K, Cordova K, **Matson MJ**, Feldmann F, Haddock E, LaCasse R, Saturday G, Lambe T, Gilbert S, Munster VJ. ChAdOx1-vectored Lassa fever vaccine elicits a robust cellular and humoral immune response and protects guinea pigs against lethal Lassa virus challenge. *In revision*
9. Figueroa D, […] **Matson MJ**, et al. Development and validation of portable, field-deployable Ebola virus point-of-encounter diagnostic assay for wildlife surveillance. *In revision*
10. **Matson MJ**, Anzick SL, Feldmann F, Martens CA, Drake SK, Feldmann H, et al. Bacillus paranthracis Isolate from Blood of Fatal Ebola Virus Disease Case. Pathogens. 2020;9(6):475.
11. Fischer RJ, Morris DH, van Doremalen N, Sarchette S, **Matson MJ**, Bushmaker T, et al. Effectiveness of N95 Respirator Decontamination and Reuse against SARS-CoV-2 Virus. Emerg Infect Dis. 2020 Jun 3;26(9).
12. **Matson MJ**, Yinda CK, Seifert SN, Bushmaker T, Fischer RJ, van Doremalen N, et al. Effect of Environmental Conditions on SARS-CoV-2 Stability in Human Nasal Mucus and Sputum. Emerg Infect Dis. 2020 Jun 8;26(9).
13. **Matson MJ**, Chertow DS, Munster VJ. Delayed recognition of Ebola virus disease is associated with longer and larger outbreaks. Emerg Microbes Infect. 2020;9(1):291-301.
14. Schulz JE, Seifert SN, Thompson JT, Avanzato V, Sterling SL, Yan L, Letko MC, **Matson MJ**, et al. Serological Evidence for Henipa-like and Filo-like Viruses in Trinidad Bats. J Infect Dis. 2020 May 11;221(Supplement_4):S375-S82.
15. Seifert SN, Schulz JE, **Matson MJ**, Bushmaker T, Marzi A, Munster VJ. Long-Range Polymerase Chain Reaction Method for Sequencing the Ebola Virus Genome From Ecological and Clinical Samples. J Infect Dis. 2018 Nov 22;218(suppl_5):S301-S4.
16. **Matson MJ**, Stock F, Shupert WL, Bushmaker T, Feldmann F, Bishop WB, et al. Compatibility of Maximum-Containment Virus-Inactivation Protocols With Identification of Bacterial Coinfections by Matrix-Assisted Laser Desorption/Ionization Time-of-Flight Mass Spectrometry. J Infect Dis. 2018 Nov 22;218(suppl_5):S297-S300.

*denotes shared first-authorship

Teaching experience

Biomedical Research After School Scholars (volunteer) 2016 – present

NIH community outreach program – Hamilton, MT

- Led a team of four volunteer researchers
- Wrote course materials and designed hands-on experiments for 7th and 8th grade students
- Ran after school science education programs at four local middle schools throughout the year

High School Biology and Chemistry Teacher (0.5 FTE) 2010 – 2014

Matrix HSC Education (www.matrix.edu.au) – Sydney, Australia

- Provided accelerated year 11 and 12 lecture and laboratory instruction at a government-accredited site
- Developed course content, including lecture slides, handout materials, experiments, and examinations
- Delivered timely assessment and feedback to students