DEVELOPMENT OF RAPID DIAGNOSTIC TESTS FOR

THE POINT-OF-CARE DETECTION OF

LASSA AND EBOLA VIRAL HEMORRHAGIC FEVERS

AN ABSTRACT

SUBMITTED ON THE 30TH OF SEPTEMBER, 2015

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BY

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ABSTRACT

Lassa virus (LASV; family Arenaviridae) and Ebola virus (EBOV; family Filoviridae) are two of the most lethal pathogens known to man and cause he viral hemorrhagic fevers (VHF) Lassa fever (LF) and Ebola hemorrhagic fever (EHF), respectively. VHFs are sever febrile illnesses which can be fatal in over 50% of cases. LF is endemic to West Africa (Guinea, Liberia, Sierra Leone, and Nigeria) and EHF was thought to be limited to the Congo Basin Region of Central Africa but with the 2014-2015 West African outbreak that range has expanded. The availability of a rapid diagnostic test (RDT) to provide a presumptive diagnostic result for VHF screening is a significant unmet need in the detection and management of VHF outbreaks.

The Viral Hemorrhagic Fever Consortium (VHFC) has developed two RDTs to fill this void with the aid of NIH/NIAID funding. The ReLASV® Antigen Rapid Test is designed as a dipstick-style lateral flow immunoassay and incorporates murine monoclonal antibodies specific for LASV nucleocapsid protein for both capture and detection components. The ReEBOV® Antigen Rapid Test is of similar design and incorporates affinity purified caprine polyclonal antibody specific for EBOV VP40 protein as capture and detection components. Analytical and clinical validation studies were conducted to establish their capacity to detect LF and EHF.

The ReLASV Ag RDT antibody pairing proved to be very specific for LASV clade 4 (Josiah strain) with limited sensitivity to Nigerian strains (clade II & III). A pivotal clinical performance study was conducted in Sierra Leone and revealed test sensitivity is 76.0% (19/25;

95% CI 54.9-90.6%) and specificity is 98.6% (214/217; 95%CI 96.0-99.7%) for IgM sero-negative, acute LF patients resulting in diagnostic likelihood 55.0. The ReEBOV Ag RDT was of similar design and had a LOD of [$3x10^4$ pfu/mL] using EBOV Makona strain. Clinical validation showed sensitivity is 91.1% (195/214; 95%CI 86.5-94.6%) and specificity is 90.2% (175/194; 95%CI: 85.1-94.0%) with a diagnostic likelihood of 9.30. The FDA and WHO granted Emergency Use Authorization for the ReEBOV® Antigen Rapid Test. Both RDTs have demonstrated clinical effectiveness in West African field trials and are now deployed for VHF surveillance.

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FOREWORD

Viral hemorrhagic fever surveillance in the West African countries of Guinea, Liberia and Sierra Leone began as early as 1976 when the US CDC established a laboratory and surveillance program in Sierra Leone where Lassa fever (LF) was particularly severe. The outbreak of civil conflict in Sierra Leone (Blood Diamonds War, 1991 to 2002) and neighboring Liberia interrupted these surveillance efforts. The relief organization Merlin had maintained public health efforts during this period. In 2004, the Mano River Union Lassa Fever Network (MRU LFN) was establish to rebuild and coordinate Lassa fever public health initiatives in Guinea, Liberia, and Sierra Leone. In Sierra Leone this effort was focused on Kenema Government Hospital (KGH) in the eastern district of Kenema which is centrally located in the LF endemic region.

Working in collaboration with Sierra Leone Ministry of Health and Sanitation (MOHS), MRU LFN, WHO, US Army Medical Research Institute of Infectious Disease (USAMRIID), and Tulane University implemented the Lassa Fever Program to build LF testing capacity at the Lassa Fever Lab (LFL) at KGH as well as support the operation of the dedicated 25-bed Lassa Fever Ward. The LFL is a 700 sq. ft. biosafety level 3 (BSL-3) laboratory suite with updated biocontainment equipment and dedication solar power systems. Through the last decade, the labs capacity to perform clinical research and laboratory diagnosis of LF has been significantly improved with the implementation of clinical chemistry, immunoassay and molecular testing (PCR) techniques. Beginning in

2008, development of recombinant Lassa virus protein based immunodiagnostics has enabled the LFL to replace immunoassays derived from viral culture materials. Securing NIH/NIAID funding allowed the development of the ReLASV® diagnostics by the Viral Hemorrhagic Fever Consortium (VHFC) lead by Tulane University researchers. These tests are manufactured under GMP and QSR guidelines at Corgenix Inc. (Broomfield, CO, USA) and have improved standardization of testing in the LFL. The development of the ReLASV® Antigen Rapid Test filled the unmet need for a rapid point-of-care test which allows KGH staff bed-side LF screening capacity and remote testing capability during LF case investigations.

The Lassa Fever Program and the VHFC was engaged at the very beginning of the 2014-2015 West African Ebola Hemorrhagic Fever (EHF) outbreak. Indeed, the KGH staff were in communication with investigators in Guinea to coordinate the potential need for LF laboratory testing at the time Ebola virus was identified as the causative agent. The VHFC responded immediately to add EHF testing capacity to the LFL at KGH. As the EHF outbreak spread across the border to Sierra Leone suspected samples were naturally referred to the LFL for LF screening and newly implemented EHF PCR screening. This vigilance resulted in the identification of the first EHF case in Sierra Leone in May 2015 by Mr. Augustine Goba who is the LFL Director. This discovery was subsequently confirmed by the VHFC and other groups using qPCR methods.

The VHFC also responded to this outbreak by re-initiating its EHF diagnostics program that had successfully developed EHF rapid test prototypes under a previous NIH/NIAID sponsored program. An expedited in-vitro diagnostics development program

quickly identified Ebola virus antigen targets and implemented reagent scale-up and assay design optimization. By Oct. 2015, optimized EHF RDT prototypes were undergoing field trials at KGH and the FDA and WHO were engaged to establish validation protocol approval to support Emergency Use Authorizations.

Having just completed the first field validation testing, the true potential of the ReEBOV Antigen Rapid Test was revealed during the MOHS investigation of a EHF outbreak in the eastern Sierra Leone town of Kono in Dec. 2014. The emergence of EHF in Kono occurred suddenly and had overwhelmed the local medical facility. Suspected EHF samples had been submitted to the LFL at KGH for LF screening but also to the CDC lab in neighboring Bo for EHF confirmation by PCR testing. Samples submitted to the LFL were allowed by MOHS to be screen using the ReEBOV RDT prototype and district medical official soon realized that the RDT was performing with 100% agreement with the CDC confirmatory PCR. The MOHS subsequently took the initiative to deploy LFL staff and the EHF RDT prototype to the hospital in Kono.

Upon arrival, LFL staff began screening suspected VHF cases at the direction of MOHS and local medical staff. This screening program quickly provided presumptive positive results for infected staff and patients which were subsequently confirmed by PCR. Furthermore, there were several cases of presumptive RDT negative cases which would have been transferred to EHF isolation wards without the EHF rapid screening. These negative results were also confirm by PCR. While a promising demonstration of EHF RDT point-of-care testing, RDT screening was discontinued after additional testing capacity had been introduced to the Kono site.

The VHFC continued the ReEBOV RDT validation worked describe herein which resulted in successful Emergency Use Authorizations by the FDA and WHO in Feb. 2015. We dedicate this work to our colleagues and friends who have died during this unprecedented EHF outbreak and during the preceding years working to improved the diagnosis and treatment of LF in Sierra Leone and the Manu River Union.

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Chapter 1. Lassa and Ebola Viral Hemorrhagic Fevers

Lassa and Ebola viruses are two of the most lethal human pathogens know to man. Both viruses cause severe febrile illnesses known as viral hemorrhagic fevers (VHF) which can be fatal in >50% cases. Lassa and Ebola also have wide geographic distribution across sub-Saharan Africa. Lassa virus (LASV; family Arenaviridae) is the etiologic agent of Lassa fever (LF) which is endemic to Nigeria, Guinea, Liberia, and Sierra Leone[1, 2]. Human and animal antibody sero-prevalence has also been detected in the other west African nations of Mali, Burkina Faso, Cote d'Ivoire, Ghana, and Benin[3, 4]. Ebola virus (EBOV; family Filoviridae) has caused periodic and in some instances very large outbreaks of Ebola hemorrhagic fever (EHF) within central Africa including Gabon, Republic of the Congo, Democratic Republic of the Congo (DRC; formerly Zaire), Sudan, Uganda, and Kenya[5-11]. However, a previous Ebola-like isolate in Cote d'Ivoire (Tai Forest virus)[12] and now the ongoing major Zaire Ebolavirus outbreak in Guinea, Liberia, and Sierra Leone have greatly expanded the probable range of EHF.

These VHFs are known to cause significant human to human transmission after exposure to infected animals, most of which are also considered food sources[6, 13]. Combined with apparent overlapping geographic distribution and risk of co-emergence across a wide region, LF and EHF are a continuing threat to sub-Saharan populations[14,

15] and the rest of the world due to well documented exported VHF cases. Without well established and internationally recognized therapeutic drugs or vaccines, both viruses are listed as Select Agents and potential Biowarfare/Bioterrorism pathogens by the United States and other nations. This logically restricts the use of some diagnostic techniques and their use in research and therapeutic development to select high containment labs in the US and internationally.

1.1 VHF Clinical Manifestations

LF and EHF do share similarities in early clinical manifestations which can lead to misdiagnosis of both diseases, often with fatal consequences[16-19]. At time of onset VHF patients will typically present with non-specific signs of febrile illness (fever, malaise, athralgia, myalgia, nausea, anorexia, vomiting, diarrhea, cough, sore throat, or headache) which can lead to misdiagnosis of more common diseases such as malaria or typhoid fever. Increasing morbidity can present as abdominal or chest pain (retrosternal pain), shortness of breath, hiccups, confusion, conjunctival injection, edema (facial or neck), or maculopapular rash may be present. Advanced disease can result in spontaneous abortion, delirium, coma, or hemorrhage (Fig. 1). Coagulation disorders (e.g. disseminated intravascular coagulation, DIC), hypotension, thrombocytopenia, vascular permeability, and hemorrhage contribute to multiple organ failure and shock which can be fatal in >50% of cases[20, 21]. After an incubation period of 1-3wks, VHF can progress from onset to death in 7- 14 days. While some signs and symptoms may be associated with poor prognosis of LF or EHF individually or in small combinations they have poor sensitivity or specificity resulting in weak diagnostic likelihood.

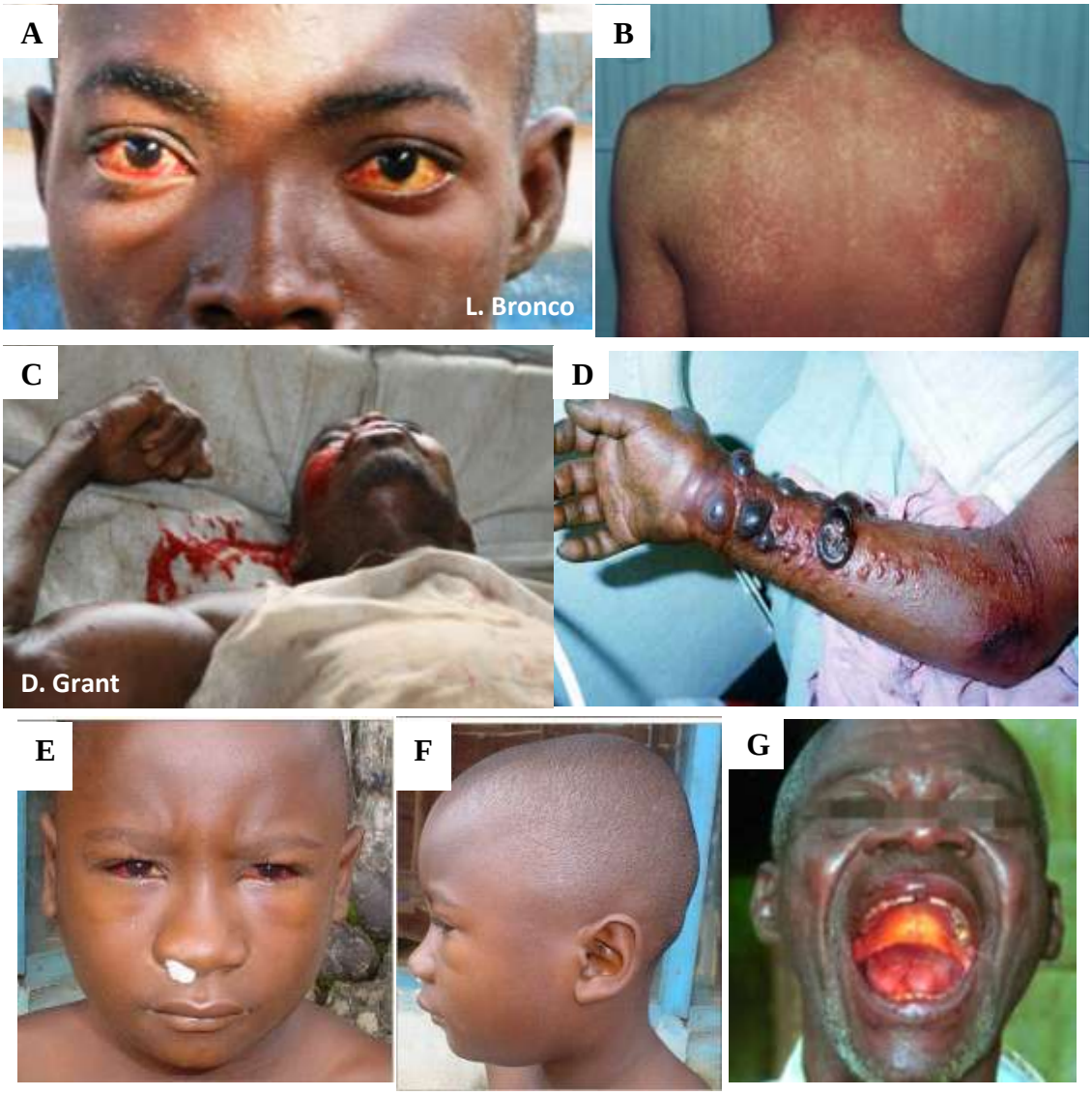


Figure 1. Viral Hemorrhagic Fever Signs

(A) Conjunctival Injection/Conjunctivitis - LF. (B) Maculopapular Rash. (C) Mucosal hemorrhage in advanced LF. (D) Eccyhmosis with bullous hemorrhage. (E) Subconjuctival injection and facial oedema. (F) Facial oedema. (G) Soft and Hard palate erythema. (E and F adapted from Kahn et al. Antiviral Research, 2008;78:103-15)

1.2 Lassa Virus

Arenaviruses, including LASV, are enveloped single-stranded RNA viruses that have a bisegmented genome. The two segments, small (S) and large (L), encoded four genes in an ambisense fashion. The S segment (3.5kb) encodes the viral nucleoprotein (NP) and the glycoprotein precursor (GPC). The L segment (7.2kb) encodes the viral RNA dependent RNA polymerase (L protein) and the Z matrix protein[22]. NP possesses 3' - 5' dsRNA exonuclease activity utilized during viral replication as well as immune evasion activity. Post-translational cleaveage of the GPC generates the stable signal peptide (SSP; 58aa) and the GP1-GP2 precursor. SSP traffics the GP1-GP2 from the endoplasmic reticulum to the Golgi apparatus where it is cleaved into GP1 and GP2 forming mature GPC complexes. The SSP and GPC complex are transported to the cell membrane forming the trimeric glycoprotein spikes of the budded virions. NP stabilizes the viral RNA genome and virion assembly, along with the Z protein and cytoplasmic tails of the GP2, during the viral budding process[23]. The resulting virion is spherical or pleomorphic ranging in diameter from 50 to 300nm. Copies of genome segments and L protein are contained in each virion. On examination by electron microscopy, the virions appear to have large granular particles giving the viruses a sandy appearance from which the virus family derives its name (sandy, Latin "arena") arenaviridae. It has been hypothesized that the granular particles are host cell ribosomes that have been incorporated in to the virions.

Lassa fever was first identified in northern Nigeria in 1969 during the investigation of medical missionaries at Jos Mission Hospital in Jos, Nigeria, that contracted a fatal unidentified febrile illness[24]. One of the nurses, Ms. Lily (Penny) Pinneo was medically evaluated to the US and survived her illness. The first characterized strain, Pinneo, of LASV was isolated from her lab samples[25]. LASV is part of the Old World Arenavirus complex that also includes prototypical arenavirus Lymphocytic Choriomeningitis virus (LCMV), Ippy virus, Mobala virus, and Mopeia virus[17]. LCMV is less pathogenic than Lassa but has a global distribution and can result in miscarriages[26]. The New World arenavirus complex shares common features with Old World arenaviruses including human pathogens Junin, Lujo, and Machupo viruses. Junin virus is known for human to human transmission but is also the one arenaviridae for which a successful vaccine has been developed.

Phylogenic analysis of S segment genes (NP, GP1, GP2) indicate that LCMV has a common ancestor with the Old World Arenaviruses. LASV forms four clades with LASV Pinneo (clade I) forming the earliest divergence but, is not currently in circulation in Nigeria. LASV clade II originated in southern Nigeria and currently has a higher prevalence[27]. LASV clade III originated in north central Nigeria is more closely related to clade IV found in Guinea, Liberia, and Sierra Leone[28]. Molecular dating places the origination of the Nigerian strains (clades I-III) as recent as 1000 years ago followed by spreading and emergence of clade IV in Guinea, Liberia and Sierra Leone (Josiah strain) a few hundred years ago[17].

1.2.1 LASV Pathogenesis

Dendritic cells (DC) and macrophages are early host cell targets for LASV. These cells are professional antigen presenting cells and serve as the first line of defense for viral infection by stimulating both innate and adaptive immune response. The primary cellular receptor for LASV cell entry is α-dystroglycan (α-DG)[29-31]. The viral GP1 attaches to α-DG which initiates clathrin-independent endocytosis. Internalized virions are transported directly to late endosomes bypassing early endosome immune sensors[32]. The transport to the late endosome is Rab5 independent but microtubular-dependent. Acidic shift to pH <5.0 in the late endosomes activates a prefusion GPC complex (a class I fusion protein)[33]. Degradation of α-DG and structural rearrangement of the GP2 leads to membrane fusion and release of viral genome into cytoplasm.

Release of viral proteins into the cytoplasm initiates viral replication processes but also immune suppression and viral pathogenecity properties[34]. During RNA virus replication, double stranded RNA (dsRNA) is produced and constitutes a pathogen-associated molecular pattern (PAMP) that would normally be recognized by the cellular pattern recognition receptors (PRR), retinoic acid-inducible gene I (RIG-I) and melanoma differentiation-associated gene 5 (MDA-5). These receptors initiate signaling pathways that result in translocation of transcription factors including interferon regulatory factor 3 (IRF-3) to the nucleus. Once in the nucleus these transcription factors normally activate the expression of type I interferons (IFN-α,β) initiating the innate immune response to viral infection.

LASV NP contains a functional 3'-5' dsRNA exonuclease activity that inhibits type I IFN expression by degrading viral dsRNA that is produced during viral

replication[35-37]. Over expression of NP can also suppress activation of NFkB or association with RIG-I and IKKε which also leads to the down-regulation of type I IFN expression[38]. LASV Z protein also displays immune suppression activity by interaction with the RIG-I-like receptors which disrupts activation of the mitochondrial antiviral signaling protein (MAVS)[39]. Disruption of this signaling pathway also leads to the inhibition of type-I IFN response.

Severe LASV infections are immunosuppressive in nature due to the failure to induce interferon (IFN) activation, proinflammatory response, and T cell activation[40]. In early infection of the DC, monocytes, and macrophages the associated suppression of IFN induced innate immunity allows the productive replication of LASV. Microphages produce fewer viral particles than DC because type I IFN production is not completely suppressed[41]. DCs are antigen presenting cells distributed throughout the peripheral tissues which function as sentinels for the adaptive immune response. Infected DC migrate to lymphoid tissues from where viral particles disseminate throughout the body infecting cells and tissue including Kupffer cells in the liver, parenchymal cells in the liver and adrenal glands, endothelial cells in a several tissues, and ultimately infection of the epithelium[42, 43].

Disease severity is associated with elevated IL-6, IL-1β, MCP-1, and eotaxin which are released in the dis-coordinated attempt to mount an effective innate immune response. The remaining pro-inflammatory cytokines remain at normal levels and are ineffective in response to LASV viremia[44-46]. The resulting pathology due to increasing viremia is inflammation and cytopathic damage to several tissues and organs including heptocellular necrosis, interstitial pnuemonitis, acute myocarditis, and damage

to reticuloendothelial tissues. Metabolic markers reflect the cellular and tissue damage with significant increases in serum transaminases AST and ALT. If the infection were restricted to the liver these transaminases would be elevated along with alkaline phospahatase (ALP) but bacause the infection and damage is diseminated ALP remains normal or slightly elevated while AST and ALT are increased with AST significantly elevated in proportion to ALT. Hepatocellular damage is reflected in evelvated BUN and reduce renal output. Transient thrombocytopenia is observed without deseminated intravascular coagulation (DIC) which rebound during recovery. Neuropathology in terminal stages is associated with confusion, tremor, seizure, convulsion, and coma.

1.2.2 LF Epidemiology

Human to human transmission of LASV occurs through exposure to bodily fluids. Contact with blood through hemorrhage of mucosal membranes is the most common route. LF patients may also hemorrhagic from injection sites. Transmission during surgical procedures on undiagnosed LF cases has lead to outbreaks among hospital staff. LASV is shed in the urine and antigenemia has been detected in oral fluids. Cases of transmission through sexual contact have been documented and the virus can persist in male semen for several months after LF recovery. LASV has also been detected in breast milk of lactating mothers which may serve as a source of vertical transmission[47].

The incubation time (prodrome phase) after LASV exposure to onset of LF symptoms is typically 1-3weeks. As previously described, early signs are non-specific and delay presentation for clinical diagnosis 3-10days. In cases with rapid onset, the time from onset to fatal outcome can be as short as 5 days so early detection is essential to successful medical intervention. For nonfatal LF, the duration of clinical disease is 2-3

weeks and may include a 10day course of ribavirin therapy. Long term sequalae after LF convalescence has included altered hearing[1, 48, 49].

LASV was first isolated in 1969 from cases of fatal undiagnosed febrile illness in northern Nigeria among missionary healthcare workers[24]. The first nurse fell ill after visiting a clinic in the town of Lassa in northeastern Nigeria. Upon her return to the Missionary Hospital in Jos, Nigeria she experienced an onset of febrile illness and died in one week. Two additional nurse became ill, one succumbed to the illness and the survivor was medically evacuated to the US. Subsequent isolation of this new virus, Lassa virus, was found to be morphologically and genetically related to LCMV and was placed in the Arenaviridae family[25]. Surveillance studies, including a survey of former African missionaries, determined LASV was primarily endemic to Nigeria, Guinea, Liberia, and Sierra Leone. Subsequent studies have revealed seroprevalence across the region of west Africa including Senegal, Gambia, Mali, Burkina Faso, Ivory Coast, Ghana, and Benin[50]. The presumed endemic region corresponds to the Upper Guinean Rainforest of West Africa.

An estimate of 100,000 - 300,000 of cases per year resulting in possibly 5,000 deaths was extrapolated from a 1987 surveillance study of Sierra Leone[51]. This study was conducted in a hyperendemic area where, arguably, the most pathogenic of the three circulating strains (clade IV, Josiah) resides. The authors, who used IFA to detect LASV specific IgM as the laboratory diagnosis[52], believed the 100,000 - 300,000 cases per year to be an overestimate. In actuality, estimating the case rate for such a broad geographic area based on familial clusters in a small but endemic area most likely produces an under estimate. Since this study, health system capacity, improved

surveillance methods, and population-wide education have certainly increased awareness and case reporting in the endemic nations. More recent epidemiologic studies conducted in Guinea and Sierra Leone using improved laboratory diagnostic techniques have enhance our understand of the LF[1, 18, 53-55].

Shaffer et. al. (2014)[18] have reported their findings from the Lassa Fever Program, in Kenema, Sierra Leone which was restarted after the end of the civil conflict - Blood Diamonds War. The Lassa Fever Program which has been supported by Tulane University and the Viral Hemorrhagic Fever Consortium (VHFC.org) has improved LF surveillance and laboratory testing capacity at the Lassa Fever Lab (LFL) at Kenema Government Hospital (KGH) in Kenema, Sierra Leone. Combined with a dedicated Lassa Fever Ward for the treatment of LF, the program has been able to support ongoing clinical research since 2004. A retrospective analysis of clinical and laboratory data collected from 2008 to 2012 which, includes 1740 observations, represents the largest epidemiological survey conduct in this region to date. Enrollment in these studies was based on LF case definition modified from Khan et al. 2008[54].

Laboratory diagnosis of LF was performed on-site in Kenema using ReLASV® ELISAs (Corgenix Inc. Broomfield, CO) for the detection of LASV NP antigen and LASV NP specific IgM and IgG antibodies. These new LF diagnostics are based on LASV-Josiah recombinant viral proteins from which LASV-Josiah specific reagents were developed[56]. Separate clinical studies have validated ELISA performance vs LASV RT-PCR and active cases were confirmed by LASV RT-PCR[48, 49]. Post-acute LF was determined by rising IgM titers or observed IgM to IgG seroconversion by ELISA.

Of the 1740 study patients, 65% (1143/1740) were Ag-/IgM- and therefore considered non-Lassa febrile illness (NLFI). Acute LF cases were defined as Ag+/IgM±, accounted for 11% (190/1740). Remaining suspected LF cases 23% (407/1740) were Ag-/IgM+, this can represent several stages of LF including prior symptomatic or asymptomatic LF due to phenomena of LF IgM persistence[46]. Case fatality rates (CFR) for Acute LF (Ag+/IgM±) was 69% (109/158) for cases with verifiable outcomes. In contrast, CFR for Ag-/IgM+ was 29% (46/161) and for NLFI (Ag-/IgM-) was 36% (65/182). It was noted that NLFI cases were symptomatic for febrile illness and in some cases had significant morbidity. When CFR data was stratified by the presence or absence of LASV specific IgG, CFR for Acute LF and NLFI significantly decrease when IgG+. For Acute LF, only 6 cases presented as Ag+/IgM-/IgG+ and is suggestive of secondary LF infection[51]. For NLFI with IgG+ the significant decrease in CFR may represent recent LF convalescence compared to true NLFI with fatal outcome.

Geographic distribution of cases in 10 of 13 SL districts represents possible expansion of host species in to previously non-endemic areas or possible improvement in LF surveillance. A seasonal peak in LF (Ag+/IgM±) occurs during the Dec-Apr dry season with a reciprocal peak in NLFI in the rainy season. No significant differences in Ag/IgM serostatus was observed between men and women. Peaks in early childhood (0-9yo) and adolescence/early adulthood (15-29yo) occured for admissions and Ag+/IgM± related fatalities, it is unclear to what degree ground breaking healthcare legislation proving free health screening children and lactating mothers affects these distributions or whether they are related to immune status of these previously described risk groups. Lastly this study was not designed as an efficacy trial for ribavirin therapy however,

acute LF (Ag+/IgM-) cases did have a lower CFR 44% with ribavirin vs 92% without ribavirin. NLFI CFR also appeared to benefit from ribavirin but this maybe due to its broad-spectrum antiviral properties.

1.2.3 LF Ecology

The multimammate rodent, *Mastomys natalensis*, has been identified as the primary animal reservoir for LASV[57]. Modeling of LASV in *M. natalensis* has demonstrated the ability to maintain persistent asymptomatic infection. Infectious virus titers could be detected in most organs, tissues, and fluids including blood and urine[58]. In the LASV endemic regions of west Africa, *M. natalensis* cycles between peridomestic and proximal cultivation habitats.

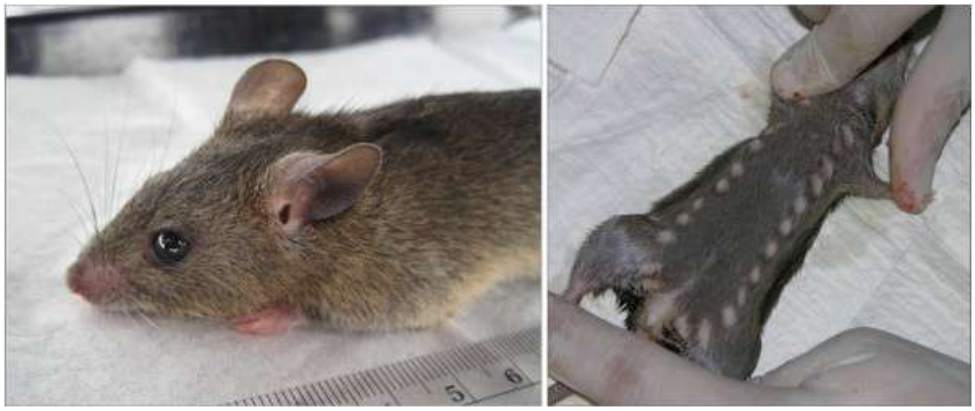


Figure 2. Lassa virus host reservoir *Mastomys natalensis.*
Mastomys sp. range spans sub-Saharan Africa and extends to South Africa. Seroprevalence has been reported as high as 60-80% in *M. natalensis* populations.

A study of abundance of *M. natalensis* and prevalence of LASV within this host species in Guinea has demonstrated that while the LASV prevalence may be higher during the rainy season (proximal cultivation) the transmission likelihood to humans was actually higher in the dry season when the rodents return to peridomestic habitat[59, 60]. This habitat cycling manifests as year-round sporadic transmission with increases during the Nov. - Apr. dry season in Sierra Leone[18].

M. natalensis mate throughout the year so horizontal transmission could not be significantly correlated to rodent age, fertility or mating seasonality[53]. However LASV prevalence in all ages of rodents including very young indicates the likelihood of vertical transmission. *Mastomys* sp. have a large distribution across sub-Saharan Africa[14]. Closely related *Mastomys* sp. as well as genetically related rodents found in endemic areas are also capable of hosting LASV. A recent study in Guinea established horizontal transmission within *Mastomys sp., Lemniscomys striatus, Mus minutoides,* and phylogenically related *Praomys sp.*[61].

The capacity of *M. natalensis* to shed LASV in its excreta combined with scavenging activity in rural west African dwellings enhances host to human transmission potential. Most dwellings in rural west Africa lack rodent-proof construction so direct transmission with contaminated surfaces or bedding is possible[51, 62]. After cultivated crops (rice, cassava, etc.) are harvested they are moved into the dwellings or adjacent structures for storage.

The lack of adequate rodent-proof food and water storage containers provide a route for contamination by rodent excreta[57]. Direct contact transmission through contact with infected rodents, their carcasses, or through consumption of infected rodents

can also occur[13]. Humans can be infected via oral or respiratory mucosa as well as microabrasions of the skin. Outreach teams provide community education to mitigate host - human transmissions through rodent eradication, safe food and water storage, and importance of proper temperature when cooking meat[62]. Training is also provided to understand risk factors associated with human to human transmission includes the role of fomites, contact with bodily fluids, and unsafe sexual contact in the transmission of LF. Identification of clinical manifestations of LF, and instructions for the safe handling and burial of corpses is provided. Prior to the West African EHF outbreak, communities were encouraged to contact the Lassa fever program regarding suspected LF cases or suspected deaths so the cases could be investigated and safe burials provided[47].

Mylne et al. (2015) have recently revised zoonotic niche maps for accessing the risk of VHF transmission to human populations[14]. Their modeling method includes compiling location data for known LF outbreak index cases and confirmed detection of LASV in *Mastomys sp.* in addition to environmental covariants. Secondary transmission such as nosocomial outbreaks are excluded because they do not accurately reflect the source of animal reservoir transmission. Background data representing environment conditions in areas were cases of LF have not been reported was also included in the model.

A total of 374 locations were identified as sites of animal infection or human index cases. The majority of the sites are in countries with active LF surveillance programs. This new model identified vegetation, night-time land surface temperature, environmental suitability for the host reservoir, elevation, and evapotransporation (Fig. 3) as significant covariants. The final probability or risk of transmission captured 95% of all

LF/LASV occurrences and an estimated 37.7 million individuals live in areas considered environmentally suitable for zoonotic transmission of LF. These finding are supported by recent identification of LF seropositive individuals in Mali and the 2014-2015 clustering of LF oubreaks along the Benin - Nigerian border (Kwara and Oyo states).

1.3 Ebola Virus

Filoviruses are enveloped, non-segmented, negative-strand RNA viruses. The viral particles are characteristically filamentous, Ebola having a diameter of 80nm but can reach up to 14,000nm in length. The RNA genome encodes seven proteins including nucleoprotein, viral protein (VP) 24, VP30, VP35, VP40, glycoprotein (GP), and a RNA-dependent RNA polymerase (L; RdRP)[63]. All the genes are monocistronic except for GP that encodes GP1 and GP2 which forms the trimeric surface glycoprotein spikes. VP30 is a viral transcription factor and VP35 is the cofactor for L polymerase. VP40 matrix protein is a peripheral membrane protein and mediates budding and viral particle release. VP24 matrix protein is involved in nucleocapsid capsid formation which is an important role for both viral transcription/replication and virion assembly.

EBOV principal cellular targets are monocytes, DCs and macrophages however the virus can also infect adrenal cortical cells, hepatocytes, fibroblasts, and endothelial cells. The initial targeting of peripheral monocytes, DC, and macrophages followed by release of free virions in the lymph and blood quickly disseminates EBOV to various tissues which, combined with EBOV affinity to several surface receptor targets accounts for its pantropism[64]. Cell surface receptors identified as mediating viral entry include C-type lectins such as asialoglycoprotein on hepatocytes, DC-SIGN and hMGL on DC

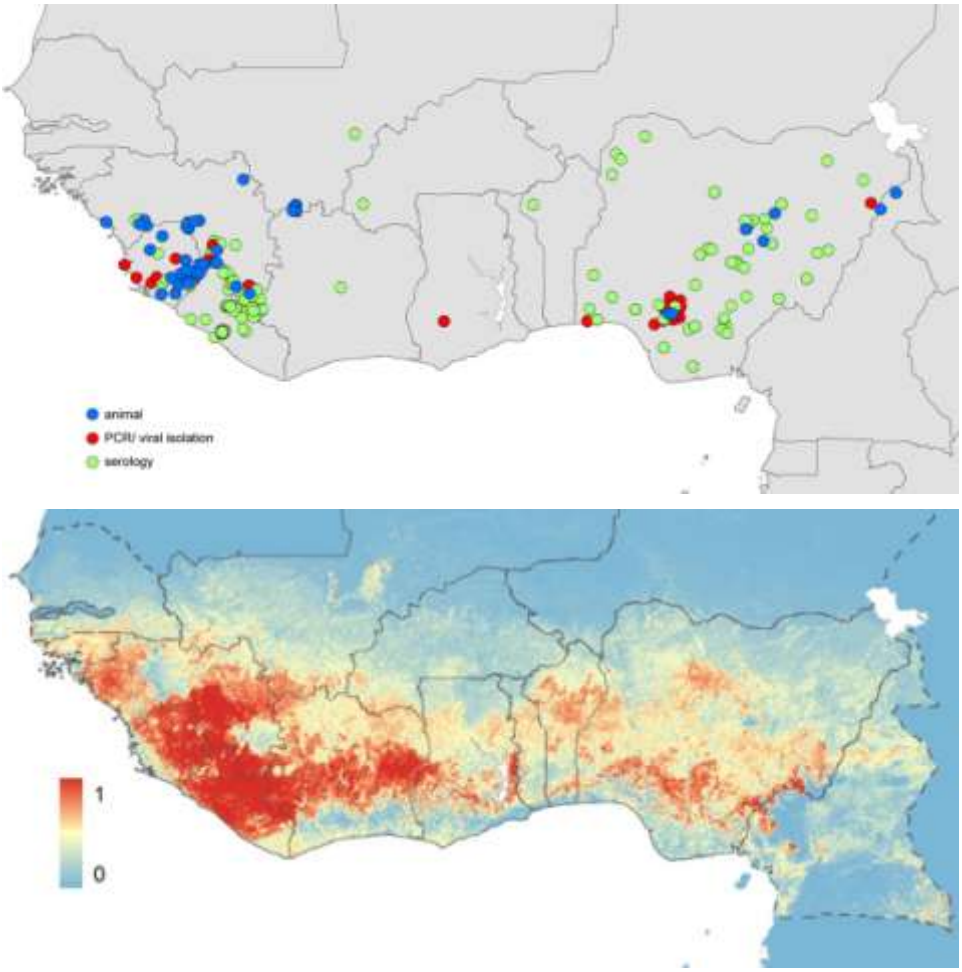


Figure 3. Predicted environmental suitability of zoonotic tranmission of Lassa virus.
(top panel) Reported locations of LASV infection used to build niche maps. (bottom panel) Color from blue (low) to red (high) indicates relative suitability of zoonotic transmission of LASV. Solid borders are countries have reported LF cases. Dashed bordered are countries that have not reported LF cases. (adapted from Mylne et al. Trans R Soc Trop Med Hyg 2015; 109:483-92)

and macrophages, and L-SIGN and LSECtin found on liver and lymph node endothelial cells. Other receptors are the widely expressed β_1-integrins and the TAM

(Tyro3/Axl/Mer) receptor family found on DCs and macrophages. T-cell immunoglobulin and mucin domain 1 (TIM-1) is express in mucosal epithelial cells and may also mediate cell entry[65].

1.3.1 EHF Pathogenesis

EBOV is principally internalized through macropinocytosis forming early endosomes. Caveolin-dependent and clathrin-dependent endocytosis have also been studied in filovirus entry. Once the viral particles are internalized in endosomes, they are trafficked to acidified late endosomes where membrane fusion is initiated by low pH dependent cathepsin B and/or cathepsin L cleavage of GP1. Under these low-pH conditions cleavage of GP1 allows conformational changes and insertion of the GP2 fusion loop into the endosome membrane. This event also triggers extension of the GP2 trimers into the host membranes. Exposure of GP2 heptad repeat (HR1, HR2) domains to the low-pH environment causes the HRs to collapse, bringing the membrane into proximity allowing partial fusion of the membranes and eventual full fusion releasing the viral genome and associated nucleocapsid and matrix proteins into the host cytoplasm.

EBOV VP24 matrix protein has been shown, along with VP40, to act at the transcription/replication level to regulate the virus life cycle between viral protein transcription and initiation of assembly and budding. VP24 also inhibits cellular response to type I and II IFNs by disruption of JAK-STAT pathway[66]. VP24 is capable of binding to karyopherin-α1 and block its binding site for heterogenous nuclear ribonuclear protein complex C1/C2 (hnRNP C1/C2). This prevents karyopherin-α1's functionality as a nuclear transport protein for phorphorylated STAT1 and its associated upregulation of

gene transcription. Redistribution of hnRNP C1/C2 may also play a role in virus replication through its stabilization of viral mRNA.

The VP35 C-terminal region contains an IFN inhibatory domain which funtions to bind and cap the ends of dsRNA generated during viral genome replication. This prevents the activation of PRR RIG-1 pathway and protein kinase R activity. A separate region of VP35 interacts with dsRNA binding protein 76 (BRBP76) mitigating BRBP76's inhibition of viral replication. VP35 also inhibits phosphorylation of several interferon regulatory factors (IFRs) by binding to TBK1/IKKε kinases thus preventing there translocation to the nucleus to upregulate type I IFN α, β expression[66]. Blocking of IKKε kinases activity also interferes in the TLR receptor and RIG-I pathways by accelerating SUMOylation of IFR3/7 which reduces their transcriptional activity.

As stated above Ebola virus GPC is cleaved in to GP1 and GP2 both of which have cytopathic and immune evasion activity. Expression of GP and insertion into the cell membrane leads to the masking and down regulation of adhesion molecules, MHC class I proteins, and EGF receptors. The highly glycosylated mucin-like domain of GP1 induces detachment and down regulation of surface proteins. Release of soluble GP (sGP) through cleavage of GP2 inhibits an effective early adaptive immune response by depletion of circulating GP-specific antibodies shielding infected cells from neutralizing antibody, shutting down cytotoxic cellular immunity[67, 68].

It has also recently been proposed by Gallaher & Garry (2015), that the remaining transmembrane delta peptide from the GP2 cleavage has properties similar to lytic viroporins such as rotavirus NSP4 toxin which could significantly contribute to Ebola cytopathic effect[69]. Within the delta peptide helical structure is a unique 3/4/7

lysine/arginine sequence. It is proposed that late in infection accumulation of these delta peptides in the host cell membrane leads to multimerization of the delta peptides with the 3/4/7 lysine/arginine motifs lining a cationic pore. The accumulation of these ion channels could lead to strong cytopathic effect through efflux of cell contents and disruption of membrane potential. This cytopathic effect may also contribute to excessive fluid loss during acute EHF which requires aggressive fluid replacement therapy.

Combined VP24 and VP35 effectively block the early response to viral infection. VP35 inhibits the detection of of viral dsRNA and initiation or IFN response. The residual activity of IFN α/β that may be produced in infected cells is abrogated by VP24 activity[70]. Thus any recognition of viral infection in the host cell will remain undetected by neighboring cells. In the absence of an early innate immune response unimpeded viral translation and replication lead to exponential release of progeny viron and cytopathic depletion of target host cells such as DC and T cells further retarding effective innate and adaptive responses. The EBOV GPs also contribute to both cytopathic effect and immune evation by the altering the expression of adhesion molecules, display of surface receptors, destabilization of membrane integrity, and release of sGP to neutralize any humoral immune response. All these viral defense mechanism contribute to EBOV pathogenesis and characteristic high morbidity and mortality.

1.3.2 EHF Epidemiology

EBOV can be transmitted to humans through contact with tissue, fluids (saliva, blood) or excreta of infected animals. Large droplet aerosol transmission has also been observed in animal model studies[71]. Transmission can easily occur through

consumption of infected bush meat such as duiker, chimpanzee, gorilla, and bats. Ebola outbreaks since its first discovery have involved secondary human to human transmission and nosocomial transmission incidents which propagate EHF transmission chains making outbreak containment challenging. Human to human transmission occurs through exposure to similar fluids described above as well as semen, sweat and breast milk. In the hospital setting exposure via needlestick or during surgical procedures is a significant concern. Full personnel protective equipment (PPE) and barrier nursing protocols need to be strictly observed and human waste and contaminated bedding correctly disposed. Safe burial practices must be strictly followed to eliminate transmission that would otherwise occur during ritual burial rites that include washing of the corpse.

The incubation period of EHF can be as long as 21 days however cases will typically become symptomatic in 6-12 days. At onset, EHF will present with high fever (40°C), chills, malaise, myalgia, and arthralgia. Morbidity will progress to gastrointestinal signs including anorexia, nausea, vomiting, abdominal pain, and diarrhea. Respiratory signs include chest pain, cough, and shortness of breath. Vascular signs of dizziness, conjunctival injection, and edema may occur. Neurological signs include headache, confusion, delerium, and coma. Hemorrhage typically occurs in fewer than 30% of cases but may include maculopapular rash, petechiae, bleeding at the site of injection, mucosal hemorrhage, haematemesis, hematuria, bloody stool. In acute EHF hepatic and renal involvement are indicated by elevated serum transaminases (AST and ALT), blood urea nitrogen (BUN), cretinine, and proteinuria. Thrombocytopenia will develop along with longer prothrombin times and raised D-dimer as indicators of disseminated intravascular coagulation (DIC).

Schieffelin et al. 2014, examined clinical data from the first 106 EHF patients admitted to Kenema Government Hospital, Kenema, Sierra Leone[19]. The majority of cases came from Kailahun District with females comprising 60% of confirmed cases. Mean time from onset to admission was 5.7 ± 0.5 days and the mean time from onset to death was 9.8 ± 0.7 days. Patient age distribution had two peaks with older adolescents and adults 26 to 40 yo. Extrapolating EBOV genome copies from qRT-PCR results, increased CFR correlated with viral load $\geq 10^5$ copies/mL. Increased CFR was also significant for cases >45 yo with a CFR 94% compared to 57% for <21 yo (overall CFR = 74%). They reported no significant difference in from overall CFR and CFR for males and females.

Typical EHF signs described above were observed in the Kenema EHF patients. Fever and headache were the most common for both fatal and non-fatal EHF. Weakness, Dizziness and Diarrhea were significantly ($p < 0.05$) more prevalent in fatal EHF than non-fatal. Hemorrhage was only observed in one patient and other studies have reported less than 5% of EHF case developed hemorrhage[72] Metabolic testing indicated hepatocelluar damage and impaired kidney function. AST and ALT were elevated with a significant increase ($p = 0.009$) in fatal vs non-fatal EHF. Similarly, BUN and creatinine were significantly elevated in fatal vs non-fatal EHF. The higher ratio of AST to ALT indicates damage to tissues apart from the liver is occurring and rhabdomyolysis may be contributing to morbidity and renal manifestations. Excessive fluid loss due to diarrhea will be reflected in the elevated BUN and creatinine.

Hemorrhagic fever caused by a filoviruses was first characterized in 1967 during an outbreak in Marburg and Frankfurt, Germany and Belgrade in the former Yugoslavia

among laboratory workers exposed to blood and tissue of African Green Monkeys (*Cercopithecus aethiops*) imported from Uganda[73]. Marburg virus, Popp strain, was isolated from inoculated guinea pigs and cell culture. During this outbreak there was 31 human cases with 7 fatalities as well as 100% mortality in experimentally infected monkeys. Subsequent Marburg outbreaks in DRC, Angola, Kenya, and Uganda have been intermittent and usually involve small number of human cases[74]. However, in the last two decades much larger Marburg outbreaks have occurred in DRC and Angola. The 1998-2000 Durba, DRC outbreak involved 128 deaths out of 154 cases and was associated with mining activity in northeastern DRC[75]. Genetic analysis identified multiple strains of Marburg virus were being introduced into the mining community is separate spillover events. The 2 year long outbreak allowed investigators to observe two peaks in transmission in the Oct. - Jan. period. The 2004-2005 Ugie, Angola Marburg outbreak was the largest on record (227 deaths/252 cases)[76]. This outbreak involved nearly identical viral isolates indicated a single or few spill-over events. The index case and possible spill-over event were not identified.

Ebola virus emerged in 1976 during large, nearly simultaneous outbreaks in southern Sudan and northern Zaire (now DRC)[11]. The virus isolated from both outbreaks was named after the Ebola river in northern Zaire. The Sudan outbreak had 150 fatalities out of 284 cases for a 53% mortality rate. The second outbreak in Zaire (DRC) resulted in 284 deaths out of 318 cases for a 89% mortality. It was subsequently determined that these outbreaks were caused by two distinct strains, Sudan Ebolavirus and Zaire Ebolavirus. The 1976 outbreak mortality rates remain characteristic for these two strains of Ebola virus.

There was an absence of Ebola cases after 1979 until the 1994 reemergence of EHF in Gabon and the large outbreak in Kikwit, DRC. The size of the Kikwit outbreak (284 deaths out of 318 cases) was increased due to multiple nosocomial transmission events and the occurrence of two superspreaders that were suspected to account for ~20% of the cases[10, 77]. During the second Ebola virus reemergence in the early 2000's, a large outbreak of Sudan Ebolavirus occurred in Uganda. The outbreak emerged from three foci including the town of Gulu, to combine for a total of 425 cases and 173 deaths. One of the last large Ebola outbreaks prior to the 2014 West African outbreak occurred in town of Luebo, DRC between May and Nov. 2007. The significance of this outbreak, which involved 260 cases and 186 deaths, was that the investigation into the index case produced evidence of the direct transmission of EBOV from migrating fruit bats to a human.

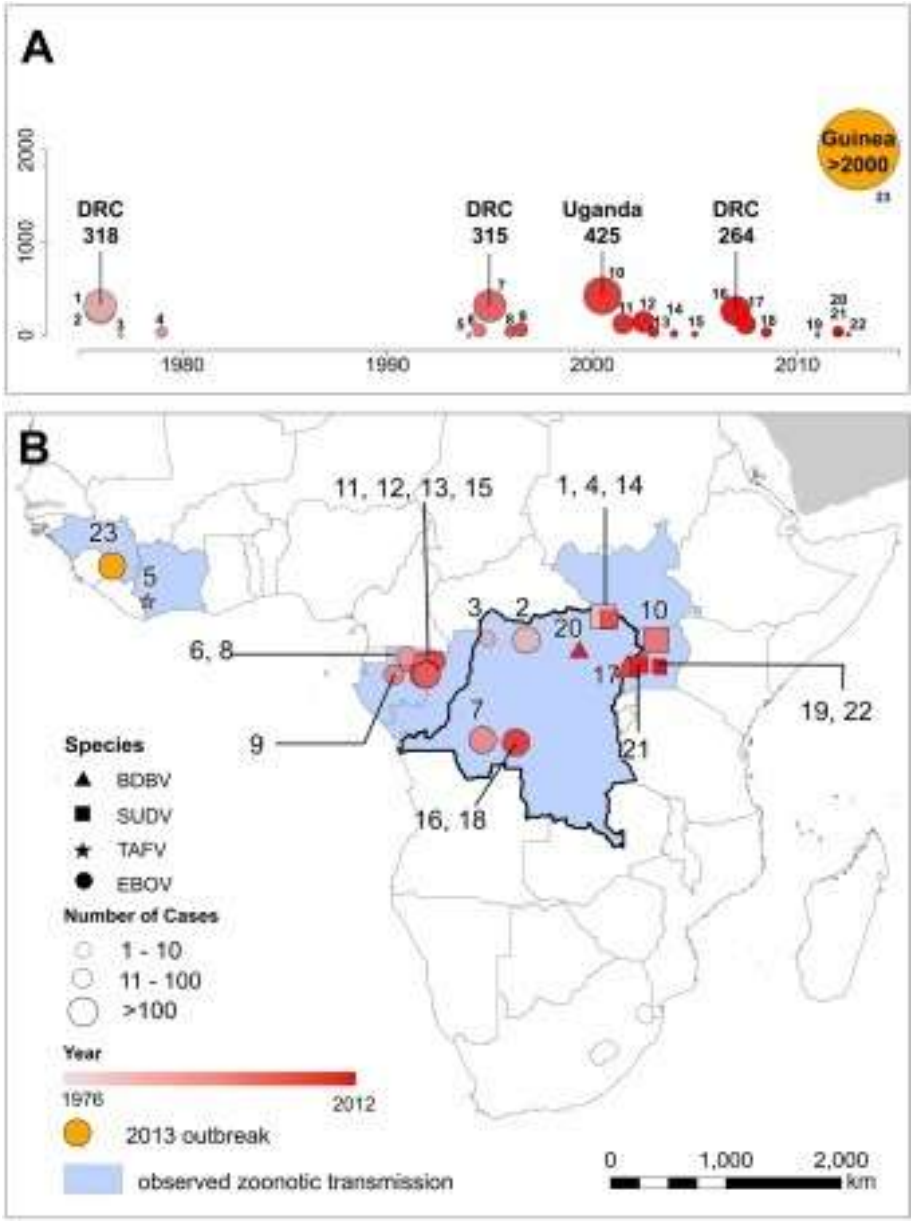


Figure 4. Ebola Hemorrhagic Fever in Humans

(A) 23 reported outbreak of EHF 1976 through 2013. Marker shap denotes Ebola strain, size indicates number ofcases, and color reflects year of index case. Original Zaire(1) and Sudan (2) outbreaks were nearly simultaneous and similar in size. (B) Location of index case for each outbreak. (adapted from Pigott et al. eLife 2014; 3:e04395)

1.3.3 EHF Ecology

Identifying of the natural reservoir for filoviruses had been an elusive effort since the first filovirus, Marburg virus, was discovered. While Marburg virus was the first filovirus discovered, the 100% mortality rate in the imported African green monkeys made them unlikely to be a host species for the virus. During the investigation into the 1976 Sudan Ebolavirus outbreak, insectivorous bats infesting the cotton factory was suspected but no direct evidence was found. The 1994 investigation of the Côte d'Ivoire chimpanzee - human filovirus transmission which lead to discovery of Taï Forest virus was associated with a 25% die-off in the source chimpanzee troop[12, 78]. With the reemergence of EBOV in the mid-1990's several studies were conducted to identify the natural host. EHF spill-over transmission had already identified chimpanzees and gorillas as amplifying intermediate hosts[79]. Swanepoel et al. (1996) attempted experimental infection of several species of plants, mammals, reptiles, and insects[80]. Three varieties of African insectivorous bats (*T. condylura, T pumila*), and fruit bats (*E. wahlbergi*) were able to sustain asymptomatic infection and viral titers reached as high as 10^7 pfu/mL. Virus was recovered from blood, and organs including lungs, spleen as well as bat feces.

Leroy et al. (2005) whose team had investigated the co-incidence of human Ebola outbreaks in Gabon [81] and a large die-off of gorillas in Gabon and DRC[82, 83], conducted a trapping survey of potential host species in the same region of Gabon and DRC[84]. The surveyed animals included 679 bats, 222 birds and 129 small terrestrial animals. Of all the species surveyed three types of fruit bat (*H. monstrosus, E. franqueti,*

and *M. torquata*) had EBOV-specific IgG in their serum and viral RNA was detected in lung and spleen samples (Fig. 5).

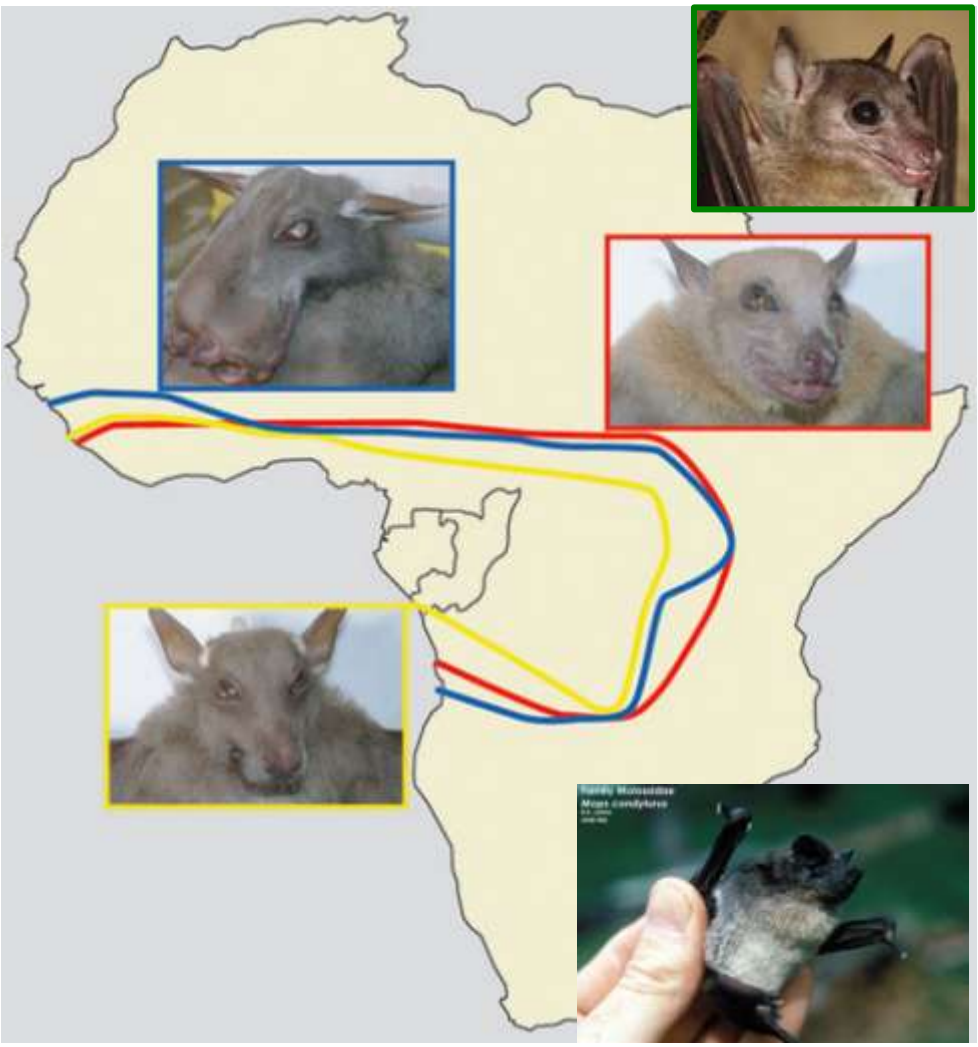


Figure 5. Ebolavirus host reservoir bat species and geographic range

(blue) *H. monstrosus.* (red) *E. franqueti*, and (yellow) *M. torquata.* (green) *R. aegyptiacus.* (lower right) *M. condyluru.* Geographic range of *R. aegyptiacus* and *M. condylurus* includes the upper Guinean rainforest and the Congo Basin. *M. condylurus* is the small insectivorous bat species suspected of being the host reservoir responsible for transmission of the EBOV to the index case for the West African EHF outbreak. (Adapted from Leroy et al. Nature, 2005, 438:575-6)

Following this important study, in 2007 a large Ebola outbreak occurred in Leubo, DRC. Investigation into this outbreak revealed multiple transmission chains spread throughout a dispersed network of villages in remote southern DRC. A large annual migration of fruit bats stops at a pair of river islands to feed in the abandoned palm tree plantation near the villages. The transmission chains led back to the index case and two unreported EHF-like cases. It is a common practice to hunt the fruit bats as a ready source of bush meat during their migratory stop. This is typically conducted with shotguns which exposes the hunters and consumers in the nearby village to potentially infectious blood.

One trader was a man who would frequently carry bats to the villages to sell, it was reported that he developed a mild febrile illness and during his travels possibly infected his small daughter. The 4 yo girl had a rapid onset of Ebola-like febrile illness and died after 4 days. The elderly woman who was the only person to conduct the ritual washing of the child's body also developed Ebola-like febrile illness after a 9 day incubation and subsequently died after transmitting EHF to 11 additional villagers. This outbreak appears to provide the first evidence of direct transmission of EBOV from the natural bat reservoir to humans. The next example of a spill-over event such as this would be the 2014-2015 West African EHF outbreak which has been traced to potential exposure of the index case with a bat colony near the village and reported consumption of the killed bats[85].

Pigott et al. (2014) conducted a zoonotic transmission risk mapping study of EBOV as a companion the Mylne study for LASV (See Ch. 1.2.3)[15]. Using comparable modeling data for EHF index cases (including ape outbreaks), EBOV detection in animal

reservoirs (principally fruit bats), and environmental covariates this study mapped the areas at risk for potential transmission of EBOV from animal reservoir to humans. It should be noted that this model would only include the single index case for the 2014-2015 West African EHF outbreak. The revised risk map predicts environmental suitability for an outbreak in West Africa and identifies that 22.2 million people are predicted to live in areas suitable for zoonotic transmission of EBOV. The highest risk countries continue to be associated with the Congo basin but West African EHF outbreak falls within the risk prediction which should raise concern for the potential of EHF to emerge in Nigeria and Cameroon (Fig 6.).

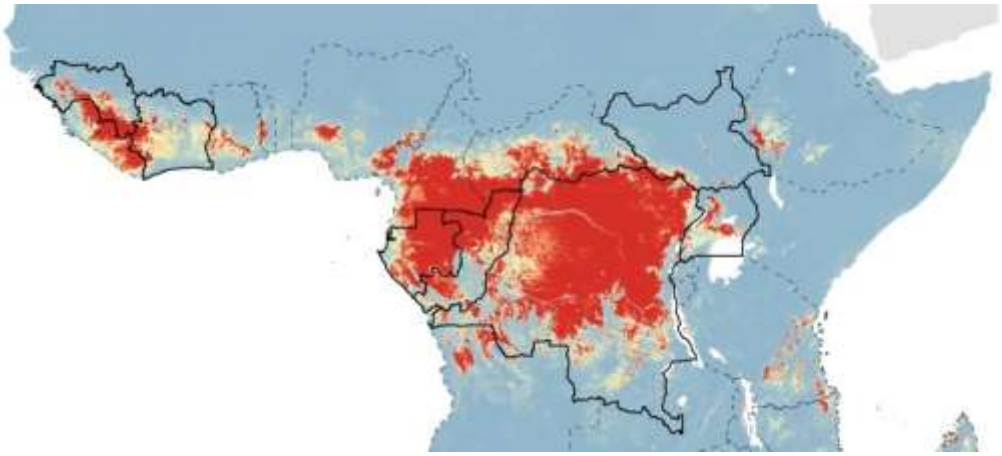


Figure 6. Predicted distribution of zoonotic Ebola virus.

Color from blue (low) to red (high) indicates relative probability of zoonotic transmission of Ebola virus. Solid borders are countries have confirmed human outbreaks. Dashed bordered are countries containing at risk areas for zoonotic transmission. (adapted from Pigott et al. eLife 2014; 3:e04395)

1.4 VHF Treatment, VHF Therapeutics, and Vaccine Development

Currently, there are no regulatory approved therapies for Lassa and Ebola VHF although there are several promising therapeutics and vaccines in clinical trials which have been accelerated in response to the West African EHF outbreak[47, 86]. Since the discovery of these diseases convalescent serum has been used. While studies have shown this to be an ineffective intervention for LF, convalescent serum has continued to be used in the treatment of EHF, particularly in the current outbreak.

Ribavirin has been shown to be effective against LASV infection when administered early in the disease course[87-89]. Ribavirin is a purine (inosine) nucleoside-analogue which has broad antiviral activity for RNA viruses and has been approved for the treatment of HCV and RSV. Its mechanism of action is thought to be inhibition of viral replication by inhibition of inosone monophosphate dehydrogenase activity and 5' cap binding of mRNA resulting in lethal mutagenesis of LASV. During LF, intravenous ribavirin is administered twice a day for 10days in combination with fluid replacement and in some cases blood transfusions.

Ongoing research by the VHFC has lead to the development of several human monoclonal antibodies (huMAbs) that are specific for LASV GPC and NP. To date, eight of these huMAbs have shown to be 100% protective in guinea pig and non-human primates(NHP) in lethal challenge model (Garry et al. unpublished data). This same approach has been used in the development of ZMapp which is a cocktail of chimerized murine mAbs which has shown protective properties against EBOV infection. The ZMapp immunotherapeutic was granted emergency use authorization during the current EHF outbreak and has been used in combination with other antivirals and EBOV

convalescent serum. Due to its production in transgenic tobacco plants, the ZMapp production process is too long to support widespread use. It also has not completed full clinical effectiveness studies to support full FDA approval.

Vaccine development has had limited success, to date attenuated virus and γ-inactivated virus vaccines have not proven effective for either LASV or EBOV. New DNA-based immunizations and DNA vaccines are still in early development stages and clinical effectiveness potential and version have not become available. Recombinant viral vaccines have shown mixed success. These vaccines incorporate LASV or EBOV protein expression in vaccinia virus (rVV) or vesicular stomatitis virus (rVSV)[90]. Currently the rVSV has demonstrated better for both LASV and EBOV are progressing through clinical trials[91].

1.5 VHF Diagnostics

Since the identification LASV and EBOV, classical virology, immunochemistry and molecular biology techniques have been used for laboratory diagnosis of both VHFs. Initially virus isolation, plaque neutralization and Immunoflourescent Assays (IFA) were used and remain gold standards for their use of live virus or specificity to native antigen[52, 92, 93]. Of course these methods take several days of preparation since they are all dependent on virus cultures or material derived from virus cultures. In most cases implementation of these techniques required transfer of clinical samples from west Africa to modern high containment laboratories in Europe or North American. IFA has been successfully used in the field but this has also required laborious preparation of materials and cold chain maintenance during transport of test materials and samples.

Deployment of ELISA to west Africa has been successful but again the early uses of this method were dependent on virus culture derived materials[93-96]. The more recent integration of recombinant viral proteins into these methods has reduced costs and improved biosecurity of these test materials but they still require laboratory capacity and infrastructure which is difficult to maintain in west Africa[97-100]. These same challenges also apply to the implementation of molecular diagnostic techniques such as PCR or quantitative PCR[101-107]. Recently several groups (including the VHFC) have addressed these hurdles to VHF testing in austere laboratory environments by developing rapid immunoassays that incorporate the use of recombinant protein technology[99, 108-112]. However, the final hurdle of method standardization has not been previously address.

The VHFC and Corgenix, Inc. have successfully introduced VHF immunoassay and molecular diagnostics to its clinical research sites in west Africa[46-49, 113, 114]. We have further met the challenge to fulfill the need for low cost, minimal resource dependent, point-of-care rapid tests that satisfy austere and/or remote testing needs in affected West African countries. Herein we present the development and validation of the ReLASV Antigen Rapid Test for the diagnosis of Lassa fever and the ReEBOV Antigen Rapid Test for the diagnosis of Ebola Hemorrhagic Fever. These tests represent the only commercially available VHF RDTs to receive CE/IVD labeling for the diagnosis of LF or Emergency Use Authorization from both the FDA and WHO for presumptive diagnosis of EHF.

PART I. DEVELOPMENT OF ReLASV DIAGNOSTICS

Chapter 2. ReLASV Critical Reagent & Assay Development

2.1 Lassa Virus Critical Reagent Development

LASV recombinant antigens were developed within the VHFC for the production of murine monoclonal antibodies (mAb) and polyclonal antibodies (pAb) for use in in-vitro diagnostics (IVD). LASV recombinant nucleoprotein (rNP) used in final configuration of the ReLASV RDT was developed and optimized for commercial production [56]. LASV recombinant glycoproteins (GPC, GP-1, GP-2) were developed in both bacterial and mammalian expression systems [56, 115]. Recombinant LASV Z matrix protein was donated by Dr. Saphire (Scripps Research Institute, La Jolla, CA). LASV specific polyclonal antiserum was developed in both goats and rabbits using glycoproteins and rNP immunogens (ProSci Inc. Poway, CA; AnaSpec, Inc. Fremont, CA). Antibodies were purified using custom affinity resins prepared with appropriate LASV recombinant proteins. Purified antibody would be absorbed against unrelated tagged protein resins to remove tag-specific (poly-His, MBP) reactivity.

Early antibody detection assays utilized combination microwell coatings of LASV GPC, NP, Z protein. Coating comparisons using banked clinical samples at KGH determined that Lassa patients developed early IgG and IgM titers to all three antigens

however reactivity to rNP only coatings proved equally consistent as the combination coatings. Titers to Z protein could be quite strong but also inconsistent from patient to patient. Z protein coating concentrations were 2 to 4 fold lower than that of GPC or NP which was required to minimize non-specific signal development. Ultimately NP-only coatings were selected as the most cost efficient method while maintaining optimal performance for detection, of IgG and IgM (or IgA) in clinical samples. In order to provide Pan-Lassa specificity an equal mixture of rNP from Clade II, III (Nigerian) and Clade IV (Sierra Leone - Josiah) was used in microwell plate coatings. For LASV NP antigen detection polyclonal antisera was generated with each of these rNPs and the affinity purified IgG blended for use as capture and detector in the ReLASV Pan-Lassa Ag ELISA.

2.2 ReLASV Pan-Lassa IgG/IgM ELISA

The ReLASV® Pan-Lassa IgG/IgM ELISA was developed for screening antibodies during reagent development and clinical samples during field trials. High protein binding 96-well microtiter plates were coated with a mixture of rNP (decribed above) diluted [2µg/mL] in carbonate buffer, pH 9.0. After overnight incubation, coating solution is replaced with Blocking Solution consisting of bovine serum albumin (BSA) diluted in phosphate buffered saline (PBS) containing a blend of sucrose and glycerol. After a second overnight incubation, the Blocking Solution is removed and the coated microwell plates are dried overnight in a humidity controlled room (RH <40%). Coated microplates are sealed in vapor and oxygen barrier mylar bags including dessicant packets to extend shelf-life to +1 year at 2-8°C. Addition of the sucrose/glycerol blend in

Blocking Solution enhances protein coating stability by maintaining protein hydration during long-term storage.

For antibody detection, clinical samples are diluted 1:100 (10µL:990µL) in a high salt Sample Buffer consisting of phosphate buffered BSA plus [1M NaCl] at pH 7.5. The addition of high salt during sample incubation increases the stringency of the coated antigen to antibody binding and thus improves specificity of the assay. Diluted samples and controls are added (100µL/well) directly to the dried, coated microwell plates. Samples are incubated for 30 minutes at ambient temperature (18-32°C). Using a plate washer, samples are aspirated and the microwell plate washed 4 times (300µL/well) using PBS-Tween 20 wash solution. To detect the NP-specific bound antibodies, human IgG, IgM, or IgA-specific secondary HRPO conjugate reagent is added (100µL/well) to the microwell plates and incubated for 30 minutes at ambient temperature. Following secondary antibody incubation, the plates are washed again with PBS-Tween. Tetramethylbenzidine (TMB) substrate is added to the microwell plates (100µL/well) and incubated 15 minutes to allow color development. Stopping Solution [0.36N Sulfuric Acid] is added to quench the color development from the blue TMB radical cation intermediate (λ_{max} = 652nm) to the stable yellow diimine (λ_{max} = 450nm) that is measured (OD450nm) in a plate reading spectrophotmeter.

Controls included in each assay are lyophilized Reference and Negative controls. Both controls are reconstituted with 250µL deionized water prior to testing. Negative control is a bulk control normal (BCN) human serum derived from pooled US donor serum. The Reference is a standardized NP-specific HRPO-antibody bioconjugate reagent. Both controls are diluted as you would a clinical sample (1:100) in Sample

Buffer. The reference is further diluted 3-fold to generate a reference curve. It is also recommended to included a sample from a confirmed LF convalescent patient as an antibody positive clinical reference. A blank well is included in each assay as a No Sample control whose signal is subtracted from the other controls and samples. A normal range signal cut-off for the assay is determined by a panel of individual normal US serums and is equivalent to mean + 3 standard deviations of their resulting signal. Additionally, a bivariate fit of west African control samples is used to determine an indeterminate cut-off signal which represents the limit of antibody sero-negatives within an endemic population.

2.3 ReLASV Pan-Lassa Antigen ELISA

The ReLASV® Pan-Lassa Antigen ELISA has similar design features as the IgG/IgM ELISA. 96-well microtiter plates are coated with a mixture NP-specific affinity purified rabbit IgG that are cross-reactive to LASV clades II, III, IV. Sample Buffer is phosphate buffered BSA plus normal saline [0.15M NaCl], pH 7.5. Sample dilution is decreased to 1:9 (50µL:450µL) of clinical plasma or serum. Sample incubation is increased to 60 minutes in a 35°C oven (if available). The NP antigen-specific HRPO conjugate is the same pAb used for plate coating, labeled with HRPO. The conjugate and TMB substrate incubations are 30 min and 15min (at ambient tempertaure) respectively. Lyophilized Reference and Negative controls are again provided. The reference contains an equal mixture of rNP (clades II, III, and IV) spiked in BCN. The reconstituted Reference and Negative controls are diluted (1:9) in a similar manner as clinical samples. Reference is further 3-fold serially diluted to generate a reference curve. Assay cut-off is

determined using panels of US and West African control serums and is equivalent to mean + 3 standard deviations of their resulting OD450nm.

2.4 ReLASV Antigen Rapid Test

ReLASV® Antigen Rapid Test (ReLASV RDT) was developed to provide a inexpensive, point-of-care testing (POCT) alternative to antigen ELISA described above for use in bed-side triage, austere laboratory conditions, or remote testing situations. Observations during early field trials and case investigations[48, 49] made it apparent that a dipstick-style rapid test would provide the most direct POCT diagnostic result while minimizing the handling of potentially VHF infected clinical specimens. Throughout Sierra Leone and West Africa, clinicians and lab technologists are very familiar with a variety of RDTs particularly for the screening of malaria and sexually transmitted disease. Fingerstick testing is also a common practice and under appropriate use of PPE (gloves, masks, and gowns) the risk of LF transmission is minimal. The added advantage of the dipstick format is once the test strip is inserted into the reaction tube and the tube re-capped, the test strip and sample are completely isolated and can readily be disposed in biohazard waste bin after interpretation.

2.4.1 LASV rNP Specific Monoclonal Antibodies

LASV NP-specific murine monoclonal antibodies were developed by VHFC collaborators (Autoimmune Technologies Inc., New Orleans, LA). Hybridomas were screened using LASV rNP coated ELISA plates and counter screened versus non-relevant antigens and tagged proteins. Candidate mAbs were characterized by Western blot for

linear versus conformational epitopes. Pairing studies for optimal rNP detection were performed in ELISA format by preparing microwell coatings and HRPO-antibody conjugates with each mAb. Similar pairing studies were conducted in LFI format by immobilizing each mAb and preparing gold nanoparticle with each mAb. Two mAbs, 100LN and 1474, emerged as the optimal pairing with specific reactivity and non-overlapping epitopes. Both mAbs are specific for epitopes in the N-terminal RNA-binding domain of LASV NP. MAb 100LN binds to a conformational epitope that is not detectable on denatured or reduced rNP. MAb 1474 binds a linear epitope and proved to be more robust to nanoparticle labeling. Thus, the optimal pairing for development of a NP-specific RDT was mAb 100LN as the capture antibody and mAb1474 as the gold nanoparticle detector antibody.

2.4.2 MAb 1474- Gold Nanoparticle Preparation

The mAb1474 is used to prepare the antibody-nanoparticle conjugate. For this, the classical method of labeling colloidal gold with antibody is used. Size of the nanoparticle used will dictate amount of antibody based on surface area and also color of signal generated during the test. For the ReLASV RDT, a 40nm gold colloid is used which will result in a pink to dark red signal depending on amount of antigen present in the clinical sample. The pH of the gold solution is adjusted using chloride ion free buffer to an optimal pH that will maximize stable absorption of the antibody. Chloride and other negatively charged ions will destabilize gold colloids so, often the labeling antibody will be desalted into low ionic strength buffers. Buffer systems typically used include phosphate, borate, or carbonate depending on the desired labeling pH, in the case of

mAb1474 MOPS free acid buffer is adjusted to pH 7.2 with NaOH, avoiding the introduction of Cl ion.

To prepare the gold conjugate, mAb1474 desalted into 5mM MOPS, pH 7.2 buffer and a [OD_{520nm}=1] gold solution is adjusted from pH 5 to 7.2 by addition of concentrated MOPS, pH 9.0. Antibody is added to the gold solution at an optimized concentration of 5µg/mL under stirring. After incubation of 15mins, the polymer polyvinylpyrrolidone (PVP) is added to permanently stabilized the colloid, protein (BSA) blockers and nonionic surfactants are added resulting in a stabile solution of mono-dispersed antibody-nanoparticles. In preparation for spray deposition the antibody-nanoparticles are concentrated and buffer exchanged by ultra-centrifugation. The bulk solution of antibody-nanoparticle conjugate is aliquoted into centrifuge bottle and centrifuged at 25,000 rcf for 20mins. The supernatant is aspirated off the resulting antibody-nanoparticle pellets. A minimal amount of antibody-nanoparticle spray solution is added to resuspend the antibody-nanoparticle pellets. The pooled concentrated antibody-nanoparticle stock solution is maintained at [$\geq$40 OD_{520nm}] during yield estimate then a final adjustment to [30 OD_{520nm}] with additional spraying solution.

The antibody-nanoparticle spray solution is formulated to optimize the final concentration of buffer, blockers, polymers, or surfactants and to introduce sucrose and/or trehalose in the formulation to stabilize and suspend the antibody-nanoparticle once it is deposited and dried down in the treated polyester ribbons. The formulation of the spray solution is optimized to facilitate "release" of the antibody-nanoparticle from the conjugate pad once it is rehydrated by the flow of the sample. Another important characteristic is that the antibody-nanoparticle must be sufficiently blocked to eliminate

non-specific binding. This will ensure full "clearance" of antibody-nanoparticle through the test strip resulting in minimal background and enhancing the signal generated by specific absorption to the test or control lines.

2.4.3 ReLASV Antigen Rapid Test Design

Dipstick-style rapid test strips are lateral flow immunoassays (LFI) that are typically not enclosed in a plastic module and the sample is added directly to a sample receiving pad. The ReLASV RDT follows a classical design for LFI construction. Upon a polyvinyl backing card coated with biocompatible adhesive, component ribbons are applied in overlapping order to facilitate sample and reagent flow from component to component without interruption. In the order of direction of the chromatographic flow, the ReLASV RDT component ribbons include a sample receiving pad, a colored antibody-nanoparticle conjugate pad, an additional pad for sample and nanoparticle mixing, a nitrocellulose membrane containing immobilized capture and control reagents, and the terminal absorbent pad(Fig. 7).

The sample pad material is a blend of glass fiber and cotton fibers treated with hydrophilic polymers such as vinyl alcohol. The density of the blended fibers is optimized to function as a plasma separator which will release plasma to the test strip but retain red blood cells and other cellular components. The material used for the antibody-nanoparticle and mixing pads is a weave of polyester fibers that receive a hydrophilic treatment of polyvinyl alcohol and surfactant.

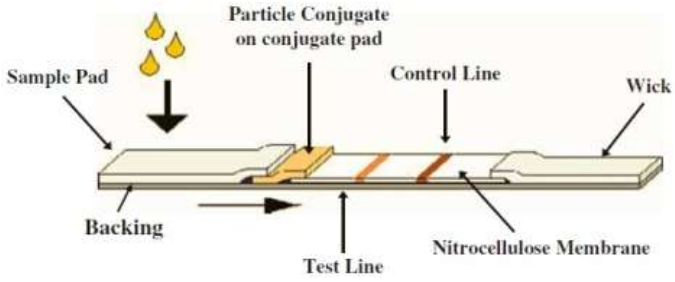


Figure 7. Basic LFI Test Strip Design

Sample and Sample Buffer are added to the Sample Pad which initiates flow through the test strip. As Sample migrates through Conjugate Pad the antibody-nanoparticles are rehydrated and mix with sample. (A third reagent pad used in the ReLASV RDT is not shown) Sample and nanoparticles flow through the stripped nitrocellulose membrane. In the presence of antigen the antibody-nanoparticle is absorbed by the Test Line antibody developing a visual signal. Excess nanoparticle continue to flow through membrane and is captured by anti-murine Control Line antibody. Absorbent Pad (Wick) absorbs excess sample and reagents maintaining test strip flow.

The stock nitrocellulose membrane can be prepared with or without a thin plastic backing for ease of handling. The porosity of the nitrocellulose dictates the flow rate across the membrane. This flow rating is determined by the manufacturer as the time in seconds for water to migrate vertically 4cm. The flow rating can impact assay performance since the rate of flow through the nitrocellulose will dictate the overall rate and interaction of sample-nanoparticle with the immobilized capture antibody and control lines. Nitrocellulose is select for flow rating, and other treatments based on

optimal assay performance. Finally the absorbent pad, or wick pad, is typically a cotton fiber material whose cubic dimensions need to be sufficient to absorb the full amount of sample and buffer used in the test. This will help eliminate reversal of flow during test incubation and prevent development of background signal or false-positive signal on the test line.

The immobilized test and control line antibodies are dispensed onto the nitrocellulose membrane in buffered striping solution in which salts are reduced or eliminated to mitigate denaturing concentrations or crystal formation upon drying. Use of blocking proteins or surfactant is avoided to maximize absorption of test or control antibody to the nitrocelluose. Sucrose and/or trehalose is included to stabilize the dried antibodies and maintain protein hydration. For the ReLASV RDT, the Test Line is the previously mentioned mAb100LN and the control line is pAb goat anti-mouse IgG (Jackson ImmunoResearch Labs, Inc. West Grove, PA) which captures the excess mAb antibody-nanoparticle indicating a valid result. Once the test and control lines are deposited the nitrocellulose, the coated membranes are dried in a forced air oven set.

2.4.4 Sample Buffer Formulation Considerations

The Sample Buffer is the final critical component for proper test performance. For the ReLASV RDT, the Sample Buffer is formulated to facilitate the use of whole blood samples from venous blood draw or fingerstick without hemolyzing the red blood cells. This design goal places restrictions formulation design, it precludes the use of chaotropic agents or strong ion surfactants to nuetralize clinical samples. RDT sample buffers are formulated with increased buffer capacity to neutralize any pH imbalance, hypertonic

NaCl eliminates hemolysis and results in crenated erythrocytes. Addition of chelating compound EDTA sequesters Ca^{2+} preventing coagulation of the released plasma samples as it migrates through the test strip. Use of BSA and the nonionic surfactant Tween reduce non-specific protein interaction and enhance clearance of the unbound antibody-nanoparticles. Polymers or sugars may be added to increase gravity and further mitigate non-specific interactions. Bacterostatic agents (ProClin™; Sigma-Aldrich) prevent microbial growth allowing long-term storage and use. Reducing the use of these additives in a RDT sample buffer is possible but often requires their incorporation into the test strip components (e.g. surfactant blocking of the nitrocellulose membrane) which must be accounted for during critical reagent selection and assay optimization.

2.5 ReLASV Antigen Rapid Test Procedures

The principal of the test is that once a clinical sample is applied to the Sample Pad and the dipstick is insert into a test tube containing Sample Buffer, chromatographic flow is initiated through the test strip. Sample Buffer mixes with the clinical sample and diluted plasma or serum migrates out of the Sample Pad into the conjugate pad. This rehydrates the LASV NP-specific antibody-nanoparticle (mAb 1474) and, if present, NP antigen will be specifically bound to the nanoparticle. The mixing of the sample and nanoparticle continues as the solution migrates through the mixing pad and into the nitrocellulose membrane. When this liquid front reaches the immobilized Test Line any NP antigen bound nanoparticles are specifically captured by the Test Line anti-NP antibody (mAB 100LN). Specific absorption of nanoparticles to the test line generates a visible signal with intensity (pink to red) proportional to the amount of NP antigen in

the sample. Excess, Ag-free nanoparticles continue to migrate through the Test Line and interact with the Control Line. Specific absorption of the murine mAb coated nanoparticles to the immobilized Gt. anti-Mo IgG Control Line generates a second signal indicating proper functioning of the test strip and validating the test line result.

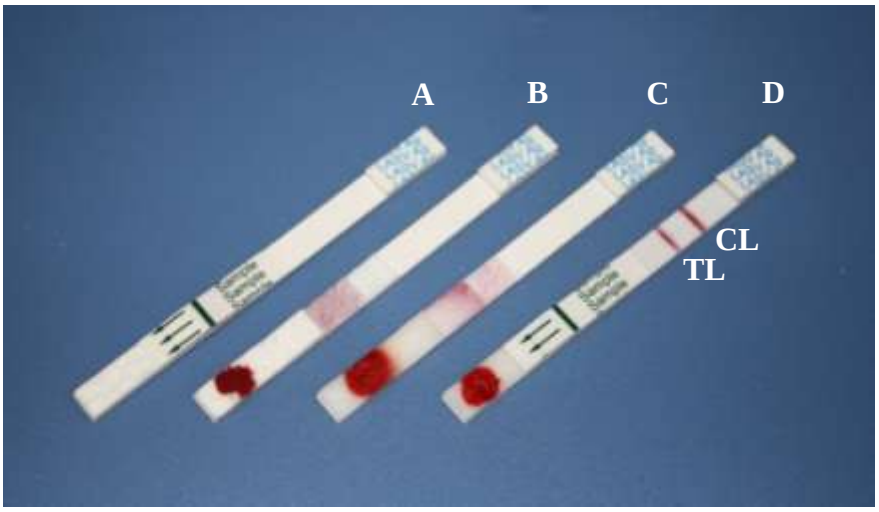


Figure 8. ReLASV RDT

(A) ReLASV RDT test strip. (B) Whole blood sample added to plasma separator Sample Pad (cover tape removed). (C) Initiation of flow through reagent pads, plasma is released from sample pad while retaining red blood cells, sample has rehydrated the antibody-nanoparticles and mixture has flowed onto nitrocellulose membrane. (D) Completed ReLASV RDT test, in the presence of antigen the LASV NP-specific antibody-nanoparticle is absorbed by the Test Line (TL) generating a visual signal and excess nanoparticle is captured by the Control Line (CL).

The assay procedures for the ReLASV® Antigen Rapid Test[116] are as follows:

Assay Procedure
Remove appropriate amount of dipsticks for testing one Negative Control, on Positive Control, and the required patient samples.

For Control Samples

1. Prepare separate tubes for Negative and Positive Controls.
2. Add 4 drops of Sample Buffer to an appropriate size culture tube (10x75mm).
3. Add on drop of control onto the center of the Sample Pad (below arrows).
4. Insert the Rapid Test dipstick (arrows down) into the culture tube containing Sample Buffer. Replace tube cap.
5. Allow Rapid Test dipstick to develop for 15-25 minutes before visual interpretation.

For Patient Samples

1. Add 4 drops of Sample Buffer to an appropriate size culture tube (10x75mm).
2. Transfer 30µL of serum, plasma, or whole blood onto center of the Sample Pad (below arrows).
3. Insert the Rapid Test dipstick (arrows down) into culture tube containing Sample Buffer. Replace tube cap.
4. Allow the Rapid Test dipstick to develop for 15-25 minutes before visual interpretation.

Interpretation of Results

1. The ReLASV® Antigen Rapid Test results should be compared to the Negative Control and included Visual Aid to assist in interpretation.
2. For a positive patient result on the ReLASV® Antigen Rapid Test, a line ranging from faint pink to dark red should form across the Test Zone.
3. For a negative patient result no line should be detected across the Test Zone. Compare patient results to the Negative Control.
4. If available a permanent record should be made by digital photography.

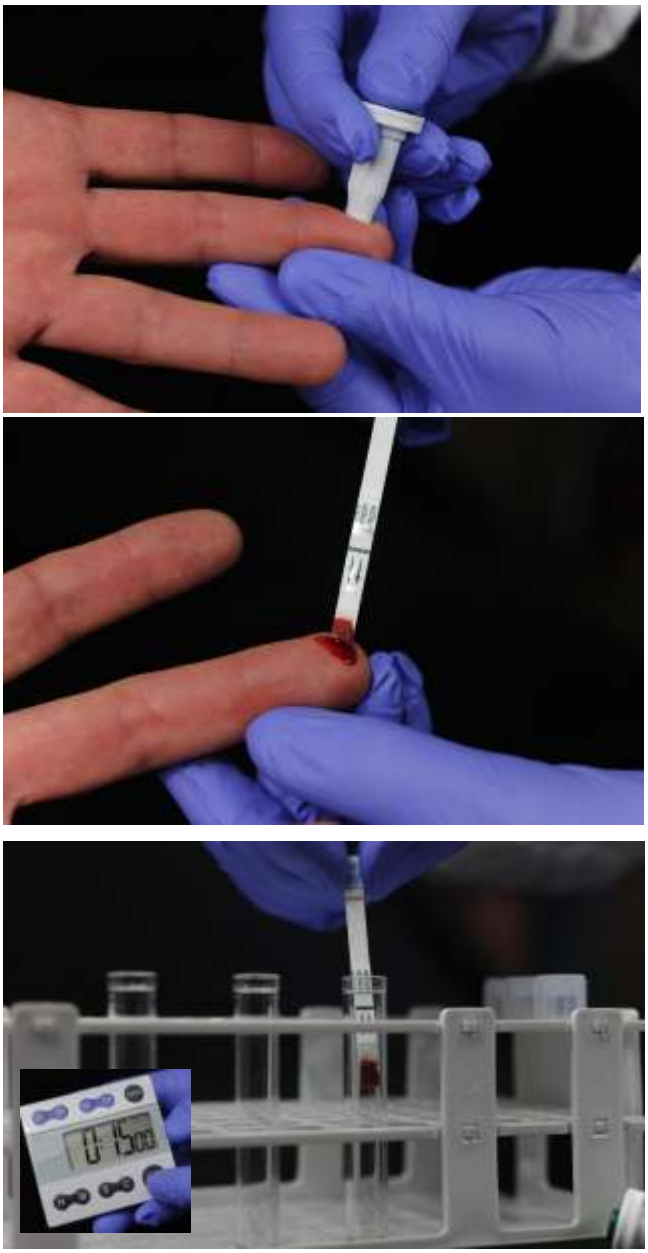


Figure 9. Performing Fingerstick Test

(top panel) Finger is cleaned with alcohol swab then fingerstick performed using retractable safety lancet. (middle panel) Full blood drop (~30µL) is allowed to develop. Test strip Sample Pad is touched directly to the blood drop which is absorbed into Sample Pad material. (lower panel) ReLASV RDT test strip is inserted into test tube containing four drops (~200µL) of Sample Buffer to initiate flow through test strip. RDT incubation is 15 - 25 minutes at ambient temperature.

ReLASV Ag Rapid Test Kit materials include 120 ReLASV dipsticks in four sealed mylar (vapor barrier) pouches, two dropper bottles of Positive Control (rNP spiked Hu. serum), two dropper bottles of Negative Control (Hu. serum), and four dropper bottles of Sample Buffer. The convex tips of the control bottle deliver ~30µL drops and the large concave tips of the Sample Buffer bottles deliver ~50µL drops. For field testing, 10x75mm snap-cap test tubes are included with general laboratory supplies.

Figure 10. ReLASV® Antigen Rapid Test Kit

The test kit includes 4 x 30 test strips and Sample Buffer, Positive and Negative Controls, and Instruction for Use. Visual Aids are included in Instruction for Use to assist in interpretation of test results. Reagents and test strips require 2-8°C storage but are stable at elevated room temperatures during testing.

Chapter 3 ReLASV Antigen Rapid Test Analytical Validation

3.1 RDT Signal Interpretation and Measurements

The ReLASV RDT is designed to be read visually which results in a subjective interpretation by the operator. The resulting data is categorical (or nominal) when recorded as a positive or negative result. However, the Package Insert's visual aid provides a 5 level scale to guide scoring of the signal intensity which produces ordinal data. During performance evaluations the ReLASV RDT has also been read using the Quant-Gold Lateral Flow Reader (LFR; Qiagen GmbH, Germany). The LFR measures the reflectance (mV) as it scans the test strip. Absorbance (A_{520nm}) at the test line and control line result in reduction of total reflectance. Peak integration software provides a absolute reflectance (total mV - measured mV) for both test and control line. A ratio of reflectance (mV test ÷ mV control) is calculated to normalize data for fluctuations in background (absolute reflectance) from strip to strip.

The uncontrolled variable in the RDT measurements is development of non-uniform signal intensity within the test line or control line itself. At low analyte levels, variable flow rates of the nanoparticles through the membrane (e.g. channeling, edge effect, differential porosity) can result in non-uniform signal development within the Test Line. The LFR reader will detect the total reflectance of the non-uniform signal however, the operator will subjectively score the most intense portion of the Test Line signal.

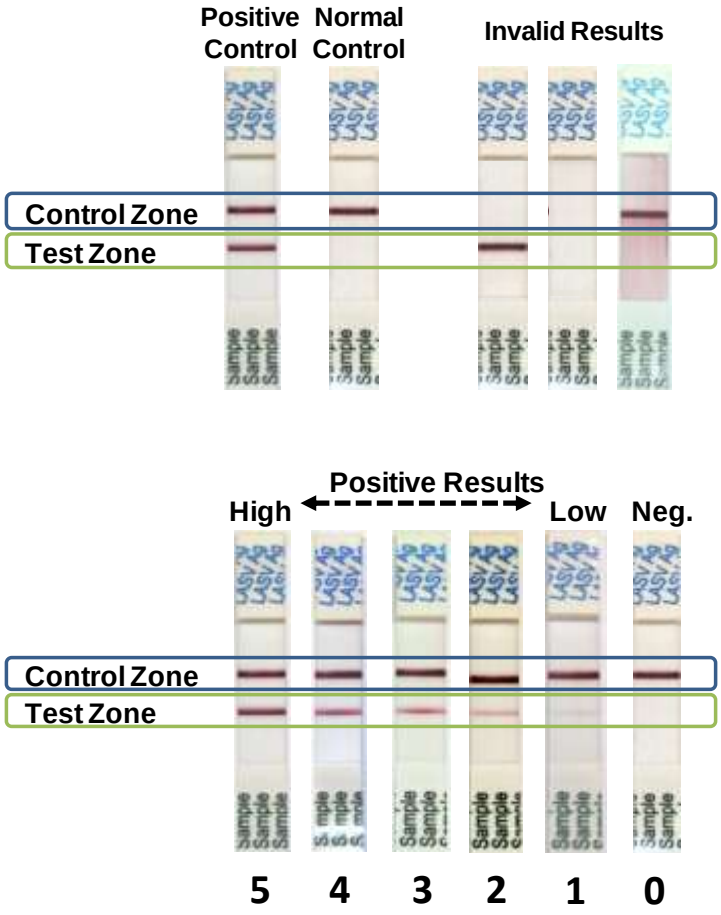


Figure 11. ReLASV Visual Aid and Scoring Scale

Test Zone and Control Zone help orient operator to correct position of Test and Control Lines. (top) Example of Positive Control and Negative Control results provided. Invalid results demonstrate, from right to left, high background, absent Control Line or Test Line, and absent Control Line. All are invalid results requiring retesting. (bottom) Example of Test Line signal gradient, operators required to score 0 - 5 based in intensity of Test Line signal. Results evaluated as ordinal data.

3.2 Validation Methods

Validation of RDT performance follows the Clinical & Laboratory Standards Institute (CLSI) guidelines User Protocol for Evaluation of Qualitative Test Performance[117], Protocols for Determination of Limits of Detection and Limits of Quantitation[118], Interference Testing in Clinical Chemistry[119], Method Comparison and Bias Estimation Using Patient Samples[120].

3.2.1 RDT Signal Kinetics

Optimal incubation time of ReLASV RDT determined using kinetic scan function of Quant-Gold LFR and development software. rNP was serially diluted in bulk normal control serum (BCN) to concentrations to generate both low, moderate, and high visual scores. Quant-Gold LFR programmed to conduct reflectance (mV) scan in 90sec intervals over 25 minutes. Test strip was place in LFR tray and 30µL of sample (rNP diluted in BCN) added to the sample pad. Two drops of Sample Buffer were added directly to the Sample Pad to initiate flow within the test strip. Tray is closed and LFR kinetics run started. After 3 and 5 minutes a drop of Sample Buffer is added to the Sample Pad to maintain flow during the remaining program. Peak integration software calculates the absolute Test and Control Line reflectance then calculates a ratio of the two to normalize any background.

RDT Kinetics Results

Plotting incubation time (sec) vs. mean reflectance (mV) reveals that at a shortened 5 minute "Quick Read" the high and moderate antigen level reached ~80% of

their full signal. After minimum of 15 minute incubation the RDT was at full (100%) of its visual signal as measured by the reflectance Test/Control ratio. For lower antigen concentrations the full 15 minute incubation is necessary to allow reagent clearance of the membane and full visual signal (Fig. 12).

Conclusion

During RDT validation testing the sample incubation will be a minimum of 15 minutes before visual signal interpretation and signal will not be read after 25 minutes.

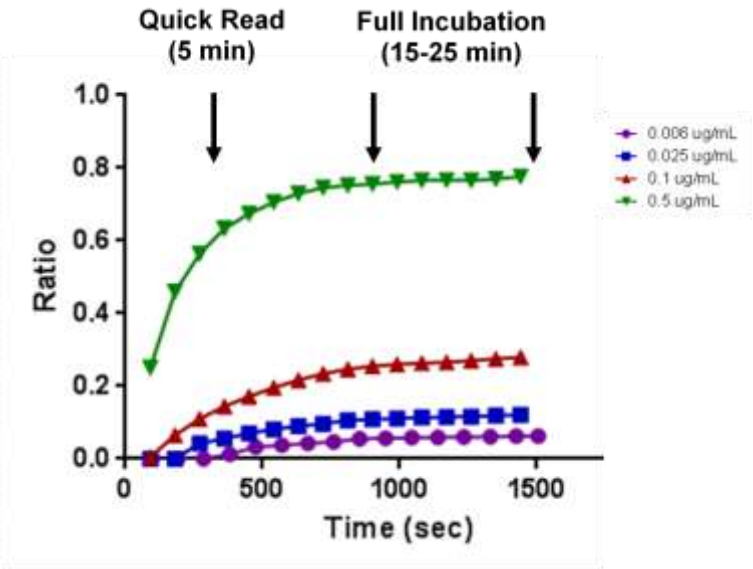


Figure 12. ReLASV RDT Kinetics Scans.

Reflectance measurements (mV) at intervals of 90sec. during 25 min. incubation reveals moderate to high RDT Test Line signals reach ~80% full strength at 5mins. 100% full strength signal for all analyte levels reached between 15 - 25 mins.

3.2.3 Determination of Limit of Blank, Cut-off, Limit of Detection

For qualitative tests such as visually read RDTs, the Cut-off is the analyte level at which 50% of observed results are positive[117]. For comparison, the Limit of Detection is the analyte level at which 95% of the observed results are positive [118]. Limit of Blank was determined using four normal human serums (PSG, Inc., Huntingdon Valley, PA) tested in duplicate by two operators. Testing was performed over three consecutive days using two lots of ReLASV RDT to generate n=96 observations. Serial dilutions (2-fold) of LASV rNP were prepared using the same four normal human serums. rNP concentration for each dilution spanned the anticipated qualitative Cut-off level [0.0015 - 0.025µg/mL]. Sufficient volume of each dilution is prepared to allow same replicate testing as the Limit of Blank determination. Results are read visually after 15min incubation and signal intensity scored on 0-5 scale using the Package Insert visual aid (Fig. 13).

3.2.4 Interfering Substances

Interfering substances screening was performed to verify that drugs in common use for the treatment of febrile illness and parasitic disease in west Africa will not interfere with ReLASV RDT signal development[119]. BCN serum was spiked with rNP antigen at the Limit of Detection [0.006 µg/mL]. Interfering substances (Table 1) are diluted to 20X the recommended testing concentration in appropriate solvent. Prior to testing a 1:20 dilution of each interfering substance (20X stock) is prepared in the rNP LOD sample. Solventcontrols are also prepared by adding 1:20 volume into rNP LOD

sample as well. Five replicates of all samples are tested. A substance is considered a non-interferent if the median score vs control solvent is not altered (Tables 1-3).

3.2.5 Cross-Reactant Screening

Specificity of the ReLASV RDT was challenged with a panel of inactived viruses (Tulane U. New Orleans, LA). Virus stock were estimated to be $\geq 10^5$pfu/mL. As in the interfering substance testing, a stock of rNP diluted in BCN at the LOD [0.006 µg/mL] was prepared. Each cross-reactant (Table. 4) is diluted 1:20 in both rNP LOD sample and BCN with spiked rNP. All samples were tested in triplicate. Viruses were considered cross-reactive if it generated false-negative on rNP LOD sample or false-positive on the BCN sample.

3.2.6 Serum vs plasma bias

The serum vs plasma bias estimate was determined in order to validate serum, plasma, and whole blood as acceptable sample types for the ReLASV RDT[120]. Plasma was compared to serum based on availability of banked specimens at KGH. Plasma was considered concomitant with whole blood due to use of a plasma separation sample pad in test strip and EDTA having been determined to be a non-interferent. Sample matrix bias was evaluated with 24 matched pairs of clinical samples (n=9; KGH) and donated normal samples (n=3; Corgenix) with serial dilutions of spiked rNP. Clinical samples and rNP dilution levels [0.03 - 0.5 µg/mL] were selected to generate signals within the range of the ReLASV RDT. All samples were tested in triplicate and the median score determined for statistical analysis.

3.2.7 Prozone Effect

ReLASV RDT was challenged with high levels of rNP spiked in serum to determine if the test strip would generate false-negative when saturated with analyte producing a Prozone effect. Undiluted rNP was serially diluted (2-fold) to near the LOD [0.01 - 1200.00 µg/mL] using BCN. Duplicate samples were tested at each level. Test strips were incubated 15 mins before visual scoring. Duplicates at all levels except [300µg/mL] were scored the same.

3.3 Statistical analysis

Data were analyzed using JMP v8-9 (SAS Institute, Inc., Cary, NC). RDT assay Cut-off and Limit of Detection by contingency analysis with Chi-square test and distribution of ordinal data for blank and diluted rNP levels. Median scores for replicates during Interfering Substance and Cross-reactant testing were calculated. To facilitate a bias estimate of the sample type, median score ordinal data was converted to continuous for a Matched Pairs analysis. Difference probability (Prob>|S|) was determined by Wilcoxon signed-rank test.

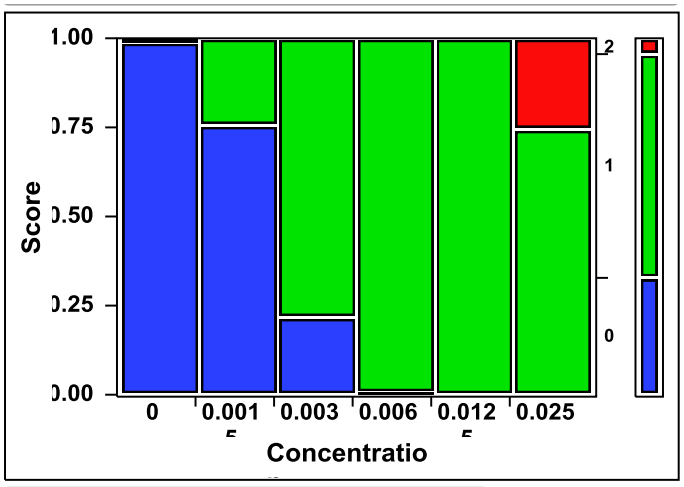
3.4 Validation Results

Determination of RDT Cut-off shows that at rNP concentration of [≥0.003µg/mL] that RDT is positive for >50% quantile (78.1%; 75/96) of results which meets Cut-off determination requirements. At [0.006µg/mL] rNP the RDT is positive at the >97.5 quantile (99.0%; 95/96) which meets Limit of Quantitation specifications. Results were reproducible for two lots of ReLASV RDT (Fig.13).

Interfering substances testing of included drugs in common use for the treatment of febrile illness including ribavirin, malaria therapies, antibiotics, anti-helmintics. and analgesics. None of these substances interfered with ReLASV RDT signal development for spike rNP near the analytical Cut-off (Tables 1,2,3).

Cross-reactant screening of Arenaviridae nearest-neighbors and febrile illness pathogens did not identify any agents capable of generating false-negative when spiked into rNP positive serum or false-positives when spiked into pooled normal human serum (Table 4).

Matrix bias testing of match LF positive clinical serum and plasma samples and rNP spiked contrived samples using individual donor serum and plasma tested in three replicates which were visually scored(Table 5). In order to perform matched pairs analysis median score was converted to continuous data. Mean difference of matched pairs was 0.0833, producing a correlation r = 0.957. No significant bias (p = 0.6250) was observed by Wilcoxon signed rank analysis of the paired samples(Fig. 14).



Contingency Table

Concentration	Score			
Count / Row %	0	1	2	
0	95 98.96	1 1.04	0 0.00	96
0.0015	73 76.04	23 23.96	0 0.00	96
0.003	21 21.88	75 78.13	0 0.00	96
0.006	1 1.04	95 98.96	0 0.00	96
0.0125	0 0.00	96 100.00	0 0.00	96
0.025	0 0.00	71 74.74	24 25.26	95
	190	361	24	575

Tests

N	DF	-LogLike	RSquare (U)
575	10	286.56861	0.6303

Test	ChiSquare	Prob>ChiSq
Likelihood Ratio	573.137	<.0001 *
Pearson	525.983	<.0001 *

Warning: 20% of cells have expected count less than 5, ChiSquare suspect.

Figure 13. RDT Cut-off and Limits of Detection

At rNP concentration of [0.003µg/mL] RDT is positive 78.1% (75/96) which satisfies Cut-off determination. At rNP concentration of [0.006µg/mL] RDT is positive 99.0% (95/96) which satisfies Limit of Detection determination.

Table 1. Interfering Substance I for ReLASV Validation

Substance	Conc. Tested	Solvent	Median Score	Replicates	Result
Ribavirin (Loading Dose 75kg Adult)	4.5g/dL (30mg/kg/5L)	npH_2O	1	1,1,1,1,1	No Effect
Amodiaquin (Loading Dose 2g Adult)	3.93g/dL	npH_2O	1	1,1,1,1,1	No Effect
Arthemeter (Loading Dose 75kg Adult)	0.48g/dL (3.2mg/kg/5L)	npH_2O	1	1,1,1,1,1	No Effect
Artesunate (Loading Dose 152.4mg Adult)	0.314g/dL	DMSO	1	1,1,1,1,1	No Effect
Chloroquine (Loading Dose 1g Adult)	2.06g/dL	npH_2O	1	1,1,1,1,1	No Effect
Mefloquine (Lodaing Dose 1250mg Adult)	2.58g/dL	DMSO	1	1,1,1,1,1	No Effect
Quinidine Gluconate	15µmol/L	npH_2O	1	1,1,1,1,1	No Effect
Quinidine Gluconate	6.2µmol/L	npH_2O	1	1,1,1,1,1	No Effect
Quinine	148 µmol/L	npH_2O	1	1,1,1,1,1	No Effect
Proguanil	4mg/dL	Acetonitrile	1	1,1,1,1,1	No Effect
Atovaquone	15mg/dL	DMSO	1	1,1,1,1,1	No Effect
Biguanide	20mg/dL	npH_2O	1	1,1,1,1,1	No Effect
Doxycycline	67.5 µmol/L	npH_2O	1	1,1,1,1,1	No Effect
Amoxicillin	206µml/L	npH_2O	1	1,1,1,1,1	No Effect
Ampicillin	152 µmol/L	npH_2O	1	1,1,1,1,1	No Effect
Ciprofloxacin	30.2µmol/L	0.1N HCL	1	1,1,1,1,1	No Effect
Ceftriaxone	1224µmol/L	npH_2O	1	1,1,1,1,1	No Effect
Clarithromycin	26.7µmol/L	Acetone	1	1,1,1,1,1	No Effect

Table 2. Interfering Substances II for ReLASV Validation

Substance	Conc. Tested	Solvent	Median Score	Replicates	Result
Clindamycin	89.1µmol/L	npH_2O	1	1,1,1,1,1	No Effect
Metronidazole	701µmol/L	npH_2O	1	1,1,1,1,1	No Effect
Tetracycline	33.8µmol/L	npH_2O	1	1,1,1,1,1	No Effect
Praziquantel (Loading Dose 1270mg Aduilt)	2.62g/dL (20mg/kg/4.85L)	DMSO	1	1,1,1,1,1	No Effect
Acetaminophen (Paracetamol)	199µmol/L	npH_2O	1	1,1,1,1,1	No Effect
Acetaminophen (Paracetamol)	33µmol/L	npH_2O	1	1,1,1,1,1	No Effect
Ibuprofen	2425µmol/L	0.1N NaOH	1	1,1,1,1,1	No Effect
Promethazine	4.22 µmol/L	npH_2O	1	1,1,1,1,1	No Effect
ALT	2000units/L	npH_2O	1	1,1,1,1,1	No Effect
AST	2000units/L	npH_2O	1	1,1,1,1,1	No Effect
Alkaline Phosphatase (ALP)	140units/L (High)	npH_2O	1	1,1,1,1,1	No Effect
Alkaline Phosphatase (ALP)	20units/L (Low)	npH_2O	1	1,1,1,1,1	No Effect
Bilirubin	15mg/dL (High)	0.1N NaOH	1	1,1,1,1,1	No Effect
Bilirubin	1mg/dL (Low)	0.1N NaOH	1	1,1,1,1,1	No Effect
Conj. Bilirubin	5mg/dL (High)	npH_2O	1	1,1,1,1,1	No Effect
Conj. Bilirubin	0.2mg/dL (Low)	npH_2O	1	1,1,1,1,1	No Effect
EDTA (short draw)	25mM	npH_2O	1	1,1,1,1,1	No Effect

Table 3. Solvent Controls for Interfering Substances Stock Solutions

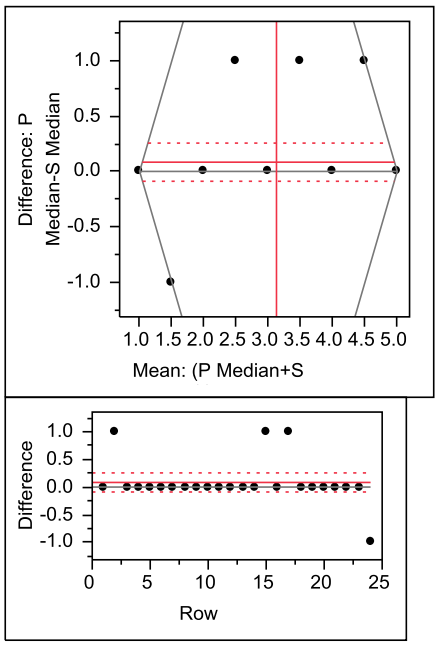
Solvent Controls	Conc. Tested	Solvent	Median Score	Replicates	Result
npH_2O Control	npH_2O	-	1	1,1,1,1,1	No Effect
0.1N NaOH Control	0.1N NaOH	-	1	1,1,1,1,1	No Effect
0.1N HCl Control	0.1N HCl	-	1	1,1,1,1,1	No Effect
Acetone Control	1.5% Acetone	-	1	1,1,1,1,1	No Effect
DMSO Control	50% DMSO	-	1	1,1,1,1,1	No Effect
Acetonitrile Control	60:40 (1mg/mL)	-	1	1,1,1,1,1	No Effect

Table 4. Cross-reactant and Nearest Neighbor Pathogens Screening

Virus	Type-Strain	Positive Median; Scores	Negative Median; Scores	Cross-reactivity
PBS Control	-	1; 1,1,1	0; 0,0,0	-
Arenaviridae (NW)	Tamiami	1; 1,1,1	0; 0,0,0	None
Arenaviridae (NW)	Tacaribe	1; 1,1,1	0; 0,0,0	None
Arenaviridae (NW)	Junin	1; 1,1,1	0; 0,0,0	None
Arenaviridae (NW)	Pichinde	1; 1,1,1	0; 0,0,0	None
Arenaviridae	LCMV	1; 1,1,1	0; 0,0,0	None
Flaviviridae	DENV-1	1; 1,1,1	0; 0,0,0	None
Flaviviridae	DENV-2	1; 1,1,1	0; 0,0,0	None
Flaviviridae	DENV-3	1; 1,1,1	0; 0,0,0	None
Flaviviridae	DENV-4	1; 1,1,1	0; 0,0,0	None
Herpesviridae	CMV-Townes	1; 1,1,1	0; 0,0,0	None
Influenza	A-Memphis	1; 1,1,1	0; 0,0,0	None
Influenza	A-New Caledonia	1; 1,1,1	0; 0,0,0	None
Paramyxoviridae	Parainfluenza 1	1; 1,1,1	0; 0,0,0	None
Paramyxoviridae	Parainfluenza 3	1; 1,1,1	0; 0,0,0	None
Paramyxoviridae	Measles-Edmonston	1; 1,1,1	0; 0,0,0	None
Paramyxoviridae	Mumps	1; 1,1,1	0; 0,0,0	None
Paramyxoviridae	RSV-Long Strain	1; 1,1,1	0; 0,0,0	None
Paramyxoviridae	RSV-A2 Strain	1; 1,1,1	0; 0,0,0	None
Poxviridae	Rabbit pox	1; 1,1,1	0; 0,0,0	None
Poxviridae	Vaccinia	1; 1,1,1	0; 0,0,0	None
Rhabodvirus	VSV	1; 1,1,1	0; 0,0,0	None

Table 5. Serum - Plasma Matrix Bias Scores

Patient	PANEL	Serum A	Serum B	Serum C	Plasma A	Plasma B	Plasma C	Serum Median	Plasma Median
G2795-1	LF Pos	2	2	2	2	2	1	2	2
G2770-1	LF Pos	3	3	3	3	4	4	3	4
G2615-1	LF Pos	3	3	2	3	3	2	3	3
G2585-1	LF Pos	3	3	4	3	3	3	3	3
G2554-1	LF Pos	2	3	2	2	3	2	2	2
G2511-1	LF Pos	2	2	3	2	3	2	2	2
G2384-1	LF Pos	5	5	5	5	5	5	5	5
G2349-1	LF Pos	5	5	5	5	5	5	5	5
G2723-1	LF Pos	5	5	5	5	5	5	5	5
DS 0.50	Spiked	5	4	5	5	4	5	5	5
DS 0.25	Spiked	5	4	3	4	4	4	4	4
DS 0.125	Spiked	4	3	3	4	3	3	3	3
DS 0.0625	Spiked	2	2	2	2	2	2	2	2
DS 0.03125	Spiked	2	1	1	2	1	1	1	1
DTS 0.5	Spiked	5	3	4	5	5	5	4	5
DTS 0.25	Spiked	4	4	4	5	4	3	4	4
DTS 0.125	Spiked	4	2	2	4	2	3	2	3
DTS 0.0625	Spiked	3	2	1	3	2	1	2	2
DTS 0.03125	Spiked	2	1	1	2	1	1	1	1
KS 0.50	Spiked	5	5	5	5	4	5	5	5
KS 0.25	Spiked	4	4	4	5	4	4	4	4
KS 0.125	Spiked	4	3	3	4	3	3	3	3
KS 0.0625	Spiked	2	2	2	2	2	2	2	2



P Median	3.16667	t-Ratio	1
S Median	3.08333	DF	23
Mean Difference	0.08333	Prob > |t|	0.3277
Std Error	0.08333	Prob > t	0.1639
Upper 95%	0.25572	Prob < t	0.8361
Lower 95%	-0.0891		
N	24		
Correlation	0.95699		

Wilcoxon Signed Rank

	P Median-S Median
Test Statistic S	2.500
Prob>|S|	0.6250
Prob>S	0.3125
Prob<S	0.6875

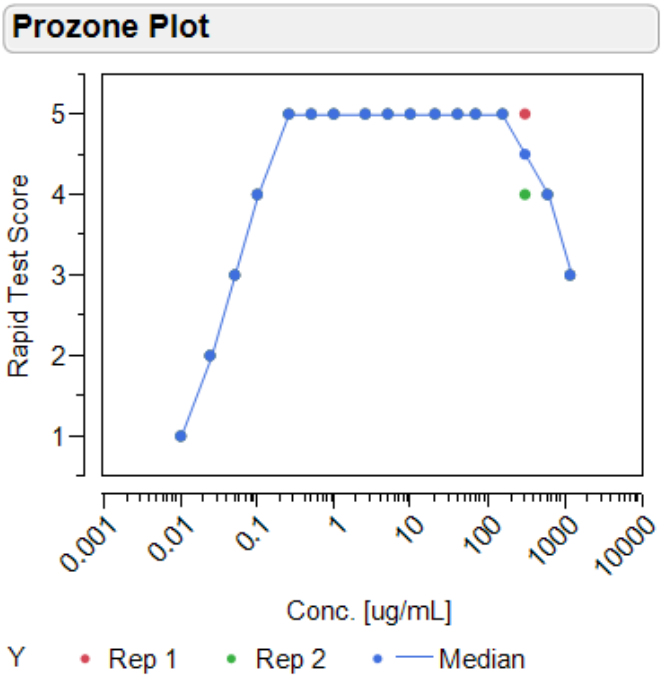
Figure 14. Match Pairs Analysis of Serum-Plasma Bias Testing

Matched pair median RDT ordinal visual scores (n=24) converted to continuous data to analyzed matrix bias. Mean difference equaled 0.083 and correlation was r =0.957. Signed rank test (p=0.6250) indicates no significant bias for serum or plasma clinical and contrived samples.

Prozone Effect evaluation demonstrated a decrease at highest rNP concentrations from a maxium signal level (score 5) to moderate signal level (score 3) without abolishing positive reactivity (Table 6). Incomplete prozone effect occurred 6-logs above RDT cut-off concentration and 3-log above upper limit of RDT signal scale.

Table 6. ReLASV Prozone Effect Screening and Plot

Conc [µg/mL]	Rep 1	Rep 2	Mean
1200	3	3	3
600	4	4	4
300	5	4	4.5
150	5	5	5
70	5	5	5
40	5	5	5
20	5	5	5
10	5	5	5
5	5	5	5
2.5	5	5	5
1	5	5	5
0.5	5	5	5
0.25	5	5	5
0.1	4	4	4
0.05	3	3	3
0.025	2	2	2
0.01	1	1	1



3.5 Validation Conclusions

ReLASV Ag Rapid Test demonstrates rNP antigen sensitivity down to a qualitative cut-off of [0.003µg/mL] which is equivalent to [90 pg/test]. Interfering substances screening reveal no RDT signal interference by ribavirin which is commonly used as antiviral therapy for acute LF. We also observed no interference by anti-malarial, anthelmintic, antibacterial, and common analgesic medications which are often prescribed to patients prior to their referral for LF screening. Serum transaminases (AST and ALT) are often elevated in acture LF and showed no interference with RDT signal. EDTA (which simulates a short venous whole blood draw), bilirubin and conjugated bilirubin were non-interferants. Hemogloblin (simulating hemolyzed blood samples), lipemic serum, and rheumatoid factor were not tested as they are already designated as test limitations.

Nearest Neighbor and Cross-reactant pathogen screening did not identify any cross-reactivity or interference by New World Arenaviruses or Old World LCMV. ReLASV RDT also demonstrated no cross-reactivity or interference by Filoviridae (Dengue virus), CMV, Influenza, Paramyxoviridae, or representatives of Poxviridae and Rhabodoviridae. This indicates the RDT should not generate false results with viruses that are known to cause febrile illness. Malaria cultures were not available for analytical screening.

Serum - Plasma Bias testing included both LF clinical samples and rNP contrived samples. Plasma was considered concominate with whole blood testing due to the incorporation of plasma separation in the test strip. Visually scored replicate tests representing the full range of RDT signal demonstrated good reproducibility. Matched

pairs analysis revealed no significant difference (p= 0.6250) between serum and plasma RDT signal generation. This will enable inclusion of both serum and plasma clinical samples during clinical validation studies. Further, the use of high titer clinical samples in Matrix Bias testing combined with no observed prozone effect indicates that acute LF cases are most likely to not generate false-negatives due to severe LASV antigenemia.

The ReLASV Antigen Rapid Test demonstrated high sensitivity to rNP antigen with a 6 log concentration range in analyte detection. The RDT demonstrated an absence of matrix bias or interference by non-LASV febrile illness pathogens or febrile illness medication. Combined these attributes indicate excellent analytical performance that would predict potentially significant clinical effectivness for the detection of LF NP antigenemia.

Chapter 4: ReLASV Antigen Rapid Test Clinical Validation

4.1 Research Aim and Study Design

The pivotal clinical study "Preclinical Development of Recombinant Antigen Diagnostics for Lassa fever - Pivotal Diagnostics Clinical Protocol" was conducted to support the safety and effectiveness of the ReLASV® Antigen Rapid Test Kit (Corgenix, Inc., Broomfield, CO). Donald S. Grant, M.D. was the Principal Investigator at KGH, Kenema, Sierra Leone which served as the primary site. Secondary sites for referral of suspected LF cases were Panguma Mission Hospital, Panguma, Sierra Leone (Dr. Augustine Mama, Medical Superintendent) and Dixon Memorial Hospital, Segbwema, Sierra Leone (Dr. Missa, Physician). The study protocol was approved by Tulane University (IRB Study No. 09-00332) and the Sierra Leone Review and Ethics Committee. The aims of the study were to validate sensitivity and specificity of the ReLASV RDT using whole blood from venipuncture (venous) or fingerstick (capillary), plasma, or serum.

The clinical hypothesis was the ReLASV RDT device will maintain a clinical Sensitivity $\geq$ 70% and Specificity $\geq$ 95% when compared to LASV RT-PCR. A secondary objective was the field validation of ReLASV Ag and IgG/IgM ELISAs. The null hypothesis would be that the RDT is not capable of significantly detecting LF compared to triage by the clinical signs (case definition) of LF alone.

The ReLASV RDT pivotal clinical study design was compliant with appropriate sections of current Good Clinical Practices (cGCP) and followed current regulations:

- Protection of Human Subjects (21 CFR Part 50)
- Financial Disclosure by Clinical Investigators (21 CFR Part 54)
- Institution Review Board (21 CFR Part 56)
- Investigational Device Exemptions (21 CFR Part 812)

While the study was entirely conducted outside United States jurisdiction, sample and data management was held compliant with Health Insurance Portability and Accountability Act (HIPAA; 45 CFR Part 46). All personally identifiable record and data were maintained at the primary study site in Kenema. Tulane personnel (Site Manager, Database Manager) were responsible for de-identification of data transferred or transmitted from the study site. Data collection process included assignment of "G-####" series identification number and sequentially logged in log book by KGH personnel. The G-number was included on Informed Consent forms, Pre-Admission Eval and Admission forms. Laboratory samples (Blood, urine, breast milk) were submitted de-identified with only the G-number. Laboratory sample and results logs were maintain with only de-identified G-numbers. Scanned or electronic clinical and laboratory records had identifiable data removed by software. Data and records were only transmitted via secure file sharing applications.

4.2 Subject Population

4.2.1 Enrollment Number and Sample type

The clinical study was designed to integrate multiple testing centers to collect venous and or fingerstick whole blood sample data. Proposed sites included previously mentioned Kenema Government Hospital, Panguma Mission Hospital and Dixon Memorial Hospital. Gondoma Referral Center in Bo, Sierra Leone is also a regrular source of referred suspected LF cases.

All suspected LF cases would be enrolled based on inclusion criteria (see below). Close contacts, community contacts, nosocomial contacts and laboratory contacts are also eligible for enrollment.

4.2.2 Inclusion and Exclusion Criteria - LF Case Definition

Inclusion criteria was all suspected LF cases admitted to Lassa ward at KGH and referral cases from secondary referral sites and close contact of confirmed LF cases. Exclusion criteria was hemodynamic instability as determined by attending physician. Close contact, community contacts and nosocomial contacts were eligible for enrollment. There was no age limitation on enrollment; children were defined as under 18 years old (yo).

LF Case Definition[1]

- Known exposure to confirmed LF case
- Reported or documented temperature ≥ 38°C for less than 3 weeks (with absence of local inflammation) and a combination of at least 2 major LF signs and/or 3 minor LF signs.

Major Signs:

- Bleeding
- Edema (face or neck)
- Conjunctival injection
- Spontaneous Abortion
- Petechial or hemorrhagic rash
- Tinnitis or altered hearing
- Absence of response to anti-malarial or antibiotic therapy

Minor Signs:

- Headache
- Sore throat
- Cough
- Nausea
- Vomiting
- Diarrhea
- Anorexia
- Dizziness
- Weakness
- Abdominal pain
- Retrosternal Pain
- Myalgia or arthralgia

[1]Case definition adapted from Khan et al., Antiviral Research 78(2008) 103-15[54]

4.2.3 Informed Consent and Patient Data Management

Suspected cases that satisfy the inclusion criteria competed the Tulane IRB approved Informed Consent form. For Children (<18 yo), a parent or guardian were required to provide informed consent. Children between ages 7 and 17 yo, were required to complete an Assent form. If a study subject was too ill, a relative could provide written consent for them. For suspected cases that died prior to securing consent, samples could

be enrolled based on the determination that human subject requirements no longer apply. Case Contact forms included investigation data and consenting sections.

4.2.4 Sample Size

Based on previous field study enrollment we expected a ratio of 10: 1 LF negative to LF positive cases. This would require enrollment of approximately 1000 suspected LF cases in order to obtain 100 confirmed acute LF cases. The minimum sample size estimate was based on a margin of error for a 95% CI using two-sided type I error at 0.05 (Table 7)[121].

Table 7. Number of required suspected cases for level of sensitivity (or specificity) and margin of error[1]

	Margin of Error[2] for 95% CI				
Sensitivity or Specificity	0.02	0.05	0.10	0.15	0.20
0.70	401	103	72	46	26
0.75	340	95	68	45	26
0.80	306	88	64	43	25
0.85	265	80	59	40	24
0.90	217	70	53	37	25
0.95	162	60	55	40	25

[1] Derived using the CI interval formula for a single proportion by Weiss et. al. (2003)

[2] Defined as half of the confidence interval width

Based on the sample size estimate, a minimum enrollment of 102 confirmed LF positives is needed to show the true sensitivity was no less than 70.0% (72/102; 95% CI 58.2 - 81.0%). A minimum of 162 LF negatives is need to show the true specificity was no less than 95.0% (154/162; 95% CI 90.5 - 97.8%). The enrollment targets of 100 case and 900 controls far exceeded the numbers required to meet the performance estimates. These calculations were performed with two-sided type I error at 0.05.

4.2.5 Data Analysis

Studies to evaluate the performance will follow CLSI guidance EP12-A2, User protocol for Evaluation of Qualitative Test Performance[117]. The reference method will be LASV RT-PCR. The following outcomes will be measured for the ReLASV RDT:

- Sensitivity
- Specificity
- Positive Predictive Value (PPV)
- Negative Predictive Value (NPV)
- Diagnostic Likelihood Ratio
- Accuracy

4.3 Study Procedures

4.3.1 Sample Collection

Samples will be tested by LASV RT-PCR to aid in the diagnosis of LF in conjunction with clinical signs and symptoms. ReLASV RDT testing will be performed at presentation and results scored as presumptive results while awaiting RT-PCR confirmatory test results. During the course of hospitalization ReLASV ELISAs and RDT will be used to monitor LASV antigenemia and anti-LASV sero-reactivity. Long-term follow-up testing will be conducted to screen for convalescent IgM and IgG titers. Patient will be tested at least 3 times but no more than 5 times during their hospitalization.

Samples from enrolled subjects or contacts were collected by fingerstick (capillary whole blood) or intravenous blood draw (venous whole blood, plasma, or serum). Pre-Admission Exam, Admission, Clinical Forms, and Case Investigation forms were used to compile clinical data, patient history, physical exam, and vital signs. Blood draws were planned periodically from day 1 through day 10 which corresponds to the duration of ribavirin therapy. One plasma tube (edta, purple top) and one serum tube (red top) were collected for a daily total no more than 10mL (adults) or 5mL (children). All blood draws were at the discretion of attending physician.

Small blood volumes (typically 5 mL) for VHF screening were collected from study subjects in vacutainer tubes by experienced phlebotomists. For serum. the blood was allowed to coagulate for 20 min at room temperature, and then the serum was separated from the coagulated blood by centrifugation (200 g for 20 min at room temperature). For plasma, whole blood-EDTA tubes were by centrifugation (200 g for 20 min at room temperature) and plasma supernatant removed by pipette. Aliquots of the

plasma and serum were used immediately for testing, and the remaining serum was stored in cryovials at −20°C for future use.

Human Subjects

The clinical research, including all human subjects tested at the KGH, was approved by the Sierra Leone Ethics and Scientific Review Committee. Suspected LF cases and close contacts of confirmed cases were eligible for enrollment in this study as outlined by the study protocol approved by Tulane University's Institutional Review Board (IRB) and following the National Institutes of Health/National Institutes of Allergy and Infectious Diseases (NIH/NIAID) guidelines governing the use of human subjects for research. Human immunodeficiency virus and hepatitis virus testing was not performed due to IRB and ethics committee considerations.

Only KGH staff were involved in the administration of healthcare to patients at the KGH Lassa Ward. All medical decisions, were at the sole discretion of the attending KGH Lassa Ward physician.

4.3.2 Methods

Day 1 laboratory testing included:

Finger Stick

- ReLASV Antigen Rapid Test

Whole Blood (EDTA, purple top)

- ReLASV Antigen Rapid Test*
- Centrifuge for plasma → ReLASV Antigen Rapid Test*
- Bank plasma

Serum (red top)

- ReLASV Antigen Rapid Test*
- ReLASV Ag, IgG, and IgM ELISA
- viral RNA extraction for RT-PCR
- Piccalo Metabolic Panel

*Serum, plasma, or whole blood run as needed for sample matrix comparisons and other performance evaluations based on sample availability.

For the purposes of a point-of-care test validation the Day 1 test results satisfy completion of study. However, for clinical research purposes additional samples were collected on days 2, 3, 4, 7, 10. ReLASV tests, RT-PCR, and metabolic testing were conducted using these samples to monitor laboratory results through course of therapy. Discharged patients were retested during a 180 day follow-up program conducted by Outreach Team. Follow-up clinical data and laboratory results were maintained in the same de-identified database for analysis.

ReLASV® Antigen Rapid Test

As previously described (See Ch. 2.5.1), whole blood, plasma, or serum samples were tested on the ReLASV RDT. Four drops (~200µL) of Sample Buffer was added to labeled 10x75mm test tubes. For testing, 30µL of sample was added to a RDT dipstick using vapor barrier pipet tips. Dipsticks were then inserted into test tube incubated for 15 min before reading the test line signal visually. After scoring the RDT signal, the dipstick was removed from the test tube for measuring reflectance (mV) of the test signal using a ESEQuant Lateral Flow Reader (LFR; Qiagen Lake Constance, GmbH). Each test, visual score and reflectance were recorded on ReLASV RDT worksheets and digital photograph taken. RDT measurements were also transferred to secured lab database and manually entered into lab results log book. RDT results were also recorded on the Lab Results Worksheet compiled for each patient sample.

LASV RT-PCR

LF was confirmed by RT-PCR of a highly conserved region of the LASV S segment genome[101]. RNA was extracted from serum samples of suspect LF patients using the QiAmp Viral RNA Mini Kit (Qiagen N.V., Netherlands). Purifed viral RNA is amplified by RT-PCR using Superscript III RT (Thermo Fisher Scientific, Carlsbad, CA) and primers 36E2 and LVS339-rev. The amplified product is a 318 nucleotide fragment corresponding to positions 4 to 322 of LASV S RNA genome that encodes LASV GPC. The amplicons are resolves on 2% E-gels (Thermo Fisher Scientific) and UV images (tiff format) are captured for visual analysis.

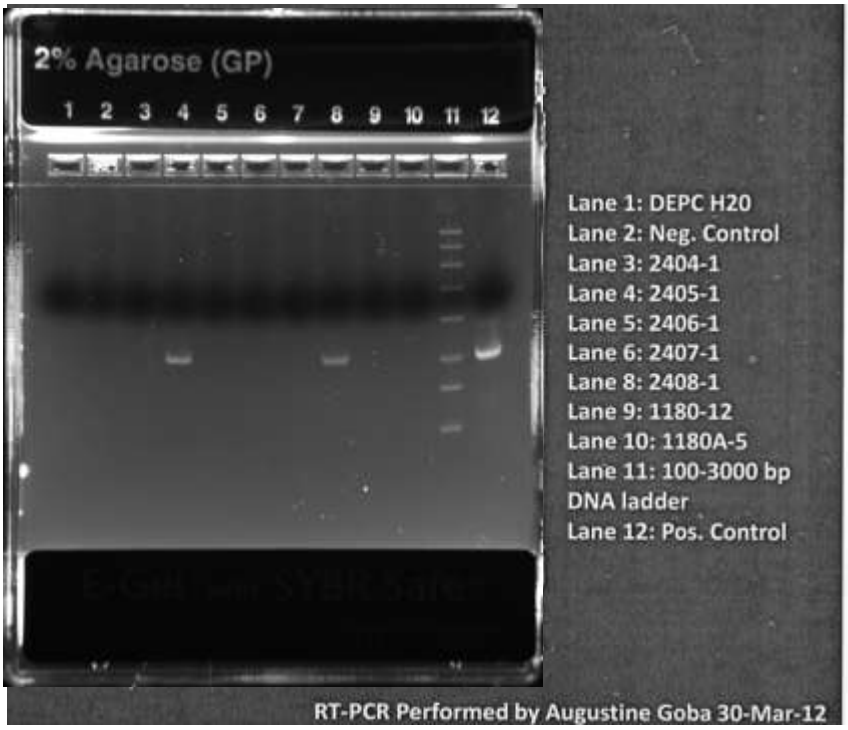


Figure 15. Example of LASV RT-PCR Image Analysis

Each RT-PCR gel included DEPC, Negative Control, base-pair DNA ladder, and a Positive extraction control using a previously confirmed clinical sample. Once gel run was complete two operators confirmed the presence of 318bp LASV amplicon. (Image A. Goba)

ReLASV® Antigen ELISA

ReLASV Ag ELISA sample dilution and addition to the 96-well microwell plate is performed in the biohood using vapor barrier pipet tips. Each plasma or serum sample is diluted 1:9 (50µL:450µL) in Sample Diluent using 2mL deep well sample blocks (ThermoFisher, USA) and mixed by pipet. Reference and Negative Controls are reconstituted with 250µL diH_2O (Direct-Q, Millipore, USA). Both Reference and Negative Control were diluted 1:9 (50µL:450µL) in Sample Diluent and mixed by pipet.

The Reference was further serially diluted 3-fold by five successive transfer of 125µL into 250µL Sample Diluent. A confirmed LF antigen positive clinical sample (from LFL sample bank) was also prepared as a second internal reference. Samples are tested in duplicate wells (100µL/well) for a maximum of 40 per plate including clinical sample reference (strip-well columns 3-12). Reference dilution curve, negative control, and reagent blank are added to strip-well column 1-2 (see plate map Table 8). Positive cut-off of OD_{450nm} = 0.085 was previously established[48, 49].

ReLASV® IgM/IgG ELISA

ReLASV IgG/IgM ELISA sample dilution and addition to the 96-well microwell plate is performed in the biohood using vapor barrier pipet tips (Denville Scientific, USA). Each plasma or serum sample is diluted 1:99 (10µL:990µL) in Sample Diluent using 2mL deep well sample blocks (ThermoFisher, USA) and mixed by pipet. Reference and Negative Controls are reconstituted with 250µL diH_2O (Direct-Q, Millipore, USA). Both Reference and Negative Control were diluted 1:99 (10µL:990µL) in Sample Diluent and mixed by pipet. The Reference was further serially diluted 3-fold by five successive transfer of 250µL into 500µL Sample Diluent. An LF convalescent IgG/IgM positive clinical sample was also prepared as a second internal reference. Samples are tested in duplicate wells (100µL/well) for a maximum of 40 per plate including clinical sample reference (strip-well columns 3-12). Reference dilution curve, negative control, and reagent blank are added to strip-well column 1-2 (see plate map Table 8). Positive cut-offs of OD_{450nm} = 0.170 for IgG and OD_{450nm} = 0.226 for IgM was previously established[48, 49]

Table 8. Example Plate Map for ReLASV ELISA Tests

	1	2	3	4	5	6	7	8	9	10	11	12
A	Kit Ref		1- Ref*		9-Sample		17-Sample		25-Sample		33-Sample	
B	Diln 1		2-Sample		10-Sample		18-Sample		26-Sample		34-Sample	
C	Diln 2		3-Sample		11-Sample		19-Sample		27-Sample		35-Sample	
D	Diln 3		4-Sample		12-Sample		20-Sample		28-Sample		36-Sample	
E	Diln 4		5-Sample		13-Sample		21-Sample		29-Sample		37-Sample	
F	Diln 5		6-Sample		14-Sample		22-Sample		30-Sample		38-Sample	
G	Neg Ctrl		7-Sample		15-Sample		23-Sample		31-Sample		39-Sample	
H	Blank		8-Sample		16-Sample		24-Sample		32-Sample		40-Sample	

*Clinical Reference consisting of previously confirmed sample.

4.3.3 Statistical analysis

Data were analyzed using v11.0 (SAS Institute, Inc.) and GraphPad Prism v. 6.04 (GraphPad Software, Inc.) Hypotheses involving dichotomous response variables were tested using Student's t test, Fisher's exact test or logistic regression with receiver operating characteristics (ROC). Binomial proportion comparisons use to establish significance across contingency estimates. Analyses were two-tailed, with a significance threshold set at $p<0.05$. Confidence intervals (95%): Agresti and Coull (1998)

4.4 Clinical Study Results

4.4.1 Study Enrollment

The initial study enrollment was 246 Suspected LF cases and 88 Contacts, including household and community contacts. Gender distribution within suspected LF cases was significantly different with 147 females and 98 males (p<.0001) however the age distribution (mean ± SD) was equivalent (p=0.7997) with females 25.4 ± 15.3yo and males 25.9 ± 13.8yo (Fig. 16).

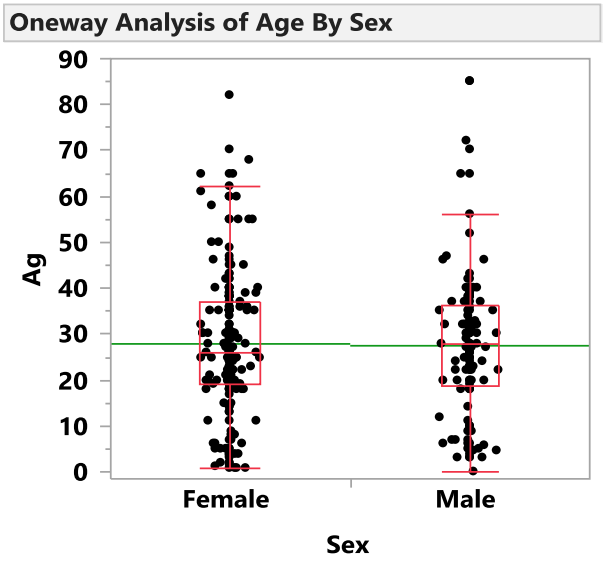


Figure 16. Age and Sex Matching in Suspect LF Cases

Sex distribution of 147 female vs. 98 male (p<.0001). Age means comparison was 25.4yo famale vs 25.9yo male (p=0.7997)

Age distribution of case vs. contact is similar with inter-quartile ranges of 18-35yo for cases and 22-42yo for contacts (Figure 17). However, the distribution means are significantly different (p<.0001) with mean case age 25.7yo (SD=14.7yo) vs mean contact age 33.7yo (SD=17.5yo). The difference in age distribution is due in part to a higher proportion of cases (22.0%) under 15yo versus contacts (10.2%) and a higher number of contacts (22.7%) over 45yo versus cases (7.3%)

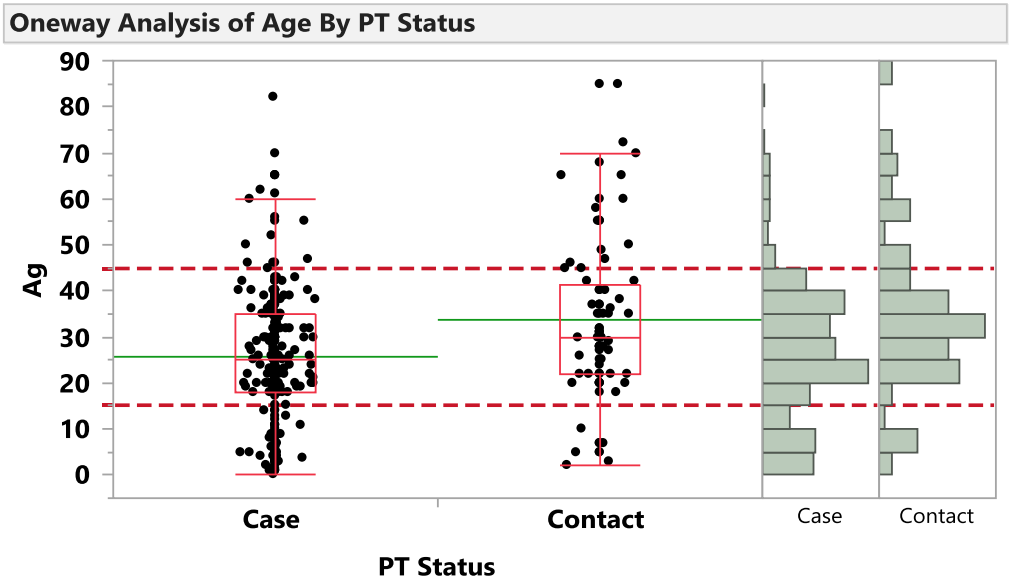


Figure 17. Age Bias in Case vs Contact Enrollment

A higher number of 0-15yo were enrolled as suspected LF Cases 22.0% (54/246) compared to contacts 10.2% (8/88) and is proportionally significant (p=0.0015). Conversely, a high number of >45yo were enrolled as contacts 22.7% (20/88) vs. cases 7.3% (18/246) which also proportionately significant (p<.0001). There was no significant difference in the proportion of 15-45yo (p=0.518).

4.4.2 Geographic patient distribution

Cases in this analysis adequately reflect LF case distribution reported in larger epidemiological studies of LF in Sierra Leone[18]. Confirmed or suspected cases were enrolled from 9 of 13 districts primarily located in the central, southern, and eastern regions of Sierra Leone (Table 9). Isolated cases are also referred for testing from the northern districts of Bombali and Koinadugu but are not part of this dataset. The highest frequency of confirmed and suspected cases come from Kemema district which is home to the primary study site at KGH and one of the main referral sites, Panguma Mission Hospital. The 2nd and 3rd highest case enrollments come from Bo and Kailahun districts, respectively (Fig. 18). These districts contain the other two primary referral sites: Gondama Referral Center (GRC) in Bo and Dixon Memorial Hospital in Segbwema. Many of the enrolled cases from Bonthe, Moyamba, Pujehun, and Tonkolili districts are first seen at GRC then referred to KGH.

Table 9. LF Confirmed Case, Suspected Case, and Case-Contact by SL District

District	Confirmed LF Cases	Suspected LF Cases	Case Contacts
Bo	10	10	20
Bombali	-	1	1
Bonthe	-	1	8
Kailahun	7	6	13
Kenema	31	163	16
Kono	1	-	-
Moyamba	1	1	6
Pujehun	5	5	17
Tonkolili	1	-	5
Total	56	187	86

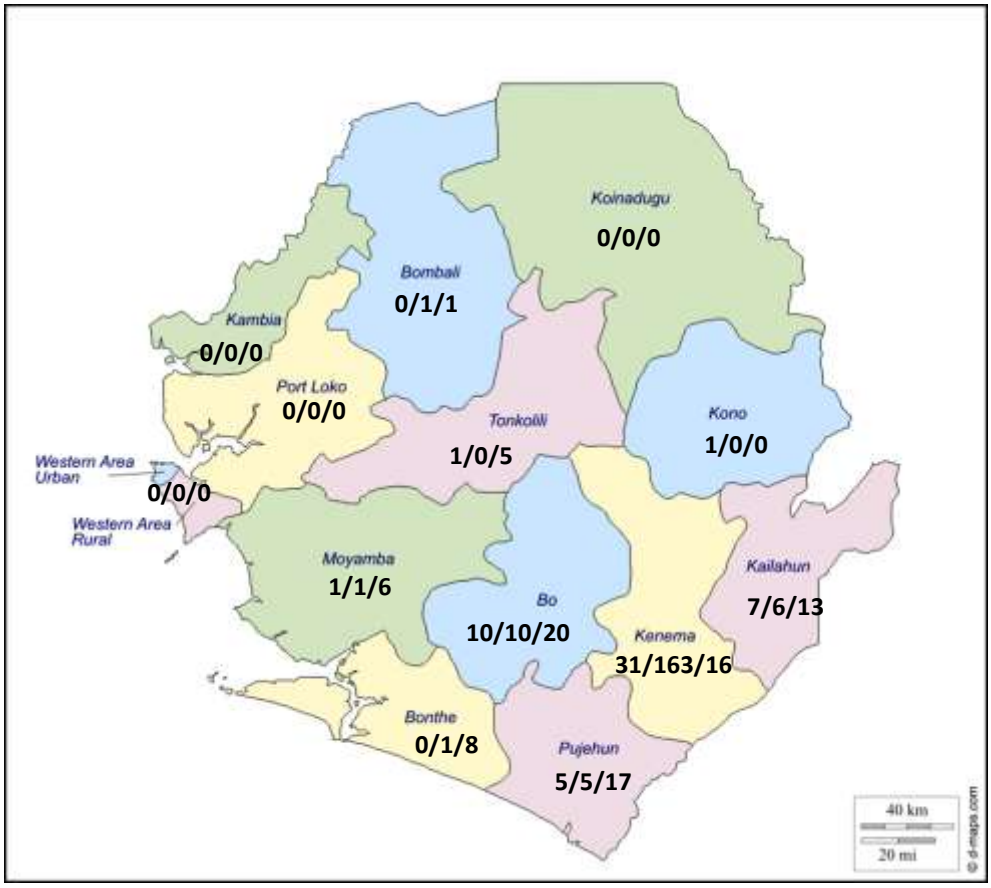


Figure 18. Distribution of LF Confirmed, Suspected, and Contacts in Sierra Leone. Confirmed LF case, suspected LF case, and Case-contacts is concentrated in the Kenema, Bo, Kailahun, and Pujehun Districts. LF cases from the south and west of Kenema will often be referred by MSF Hospital in Bo District. LF cases from endemic areas in northern Kenema District and Kailahun District will most often directly present to Kenema Government Hospital. (In order: **Confirmed/Suspected/Contacts**)

4.4.3 Signs and Symptoms at Presentation

The LF case definition used for enrollment satisfied the study design. Confirmed LF cases reported a median of 3 major signs (range 0-5) excluding fever. Confirmed LF cases also reported a median of 7 minor signs (range 0-11). This tally includes lack of response to anti-malarials and/or antibiotics as a single major sign. Any types of bleeding or pain were also scored singularly.

For Major LF Signs (Fig 19) reported or documented fever was recorded for 53% (95^{th}% CI 43-56) of suspected cases. Only 3 LF patient contacts presented with febrile illness. Lack of response to Anti-malarial or antibiotic therapy was the most frequent major sign at presentation (66% and 68% respectively), and these two classes of drugs were frequently (60% of cases) used in combination. The frequent self-prescribed or administered use of these drugs without laboratory determination undermines their diagnostic value as a major LF sign. However, lack of response to anti-malarials and antibiotics is a key clinical indicator of viral infection. Edema and conjunctival injection (or conjunctivitis), despite low frequency (21% and 14% respectively), provided the highest diagnostic value of any major LF sign. Bleeding as a major sign also had low frequency (40%) and poor overall diagnostic value. Diagnostic value of specific types of bleeding, such as bleeding at injection sites, vaginal bleeding, and bloody or blackened stools, was significant. Spontaneous abortion (or stillbirth) was not recorded in significant frequency, but in confirmed LF cases 9 of 10 pregnant women had failed pregnancies. Altered or complete loss of hearing and hemorrhagic rash did not occur with significant frequency.

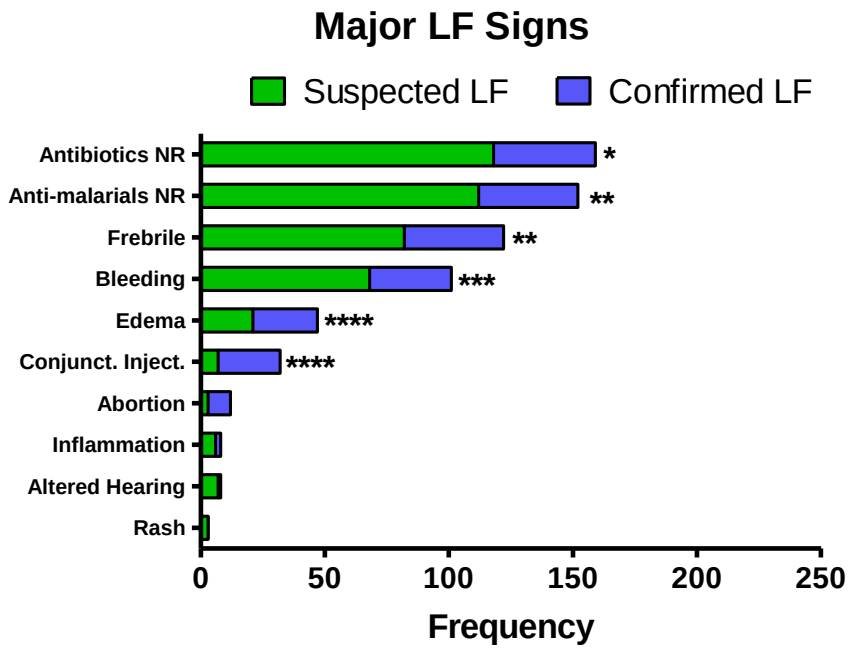


Figure 19. Frequency of LF Case Definition Major Signs.

Major clinical signs of LF providing significant diagnostic likelihood (Chi square $p < 0.05$) do not occur with sufficient frequency to provide adequate Sensitivity or Positive Predictive Value (PPV) for LF case triage. Bleeding PPV = 35.9%; Edema PPV = 55.3%; Conj. Inj. PPV = 78.1% (Chi Square Test; *p<0.05, **p<0.010, ***p<0.001, ****p,0.0001)

Minor LF signs (Fig 20) occurred with more frequency than major signs, but most have minimal diagnostic value independently. Most of the signs are non-specific in nature and can occur during the course of general febrile illnesses. Headache, pain, vomiting, cough, and sore throat were among the most frequent signs recorded, often in combination. These signs are also frequently reported at onset of symptoms during

investigation of confirmed LF cases, or in referral notes. Several low frequency minor signs (retrosternal pain, convulsions, diarrhea, sore throat, and crepitus) provide significant diagnostic likelihood in confirmed LF cases. Anorexia was frequently reported in confirmed LF cases but was not included in the original enrollment evaluation.

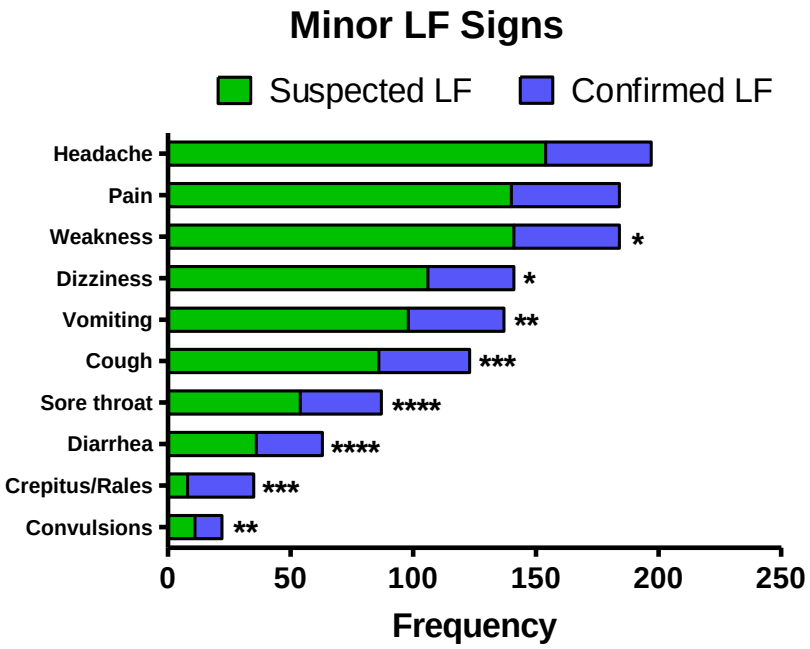


Figure 20. Frequency of LF Case Definition Minor Signs

Minor clinical signs of LF are non-specific and present in higher frequency which generates poor diagnostic likelihood despite significant association with LF ($p<0.05$). Less frequent signs retrosternal pain, Crepitus, and Convultions have increase diagnostic likelihood due to association with higher morbidity levels. (Chi Square Test; *$p<0.05$, **$p<0.010$, ***$p<0.001$, ****p,0.0001)

4.4.4 ReLASV RDT Clinical Performance

For RDT performance analysis, the database included 46 Confirmed LF (PCR+), 193 Suspected LF (PCR-), and 86 Case-Contacts (asymptomatic, PCR-). Initial performance estimate for the ReLASV RDT revealed assay Sensitivity 58.7% (27/46; 95% CI 43.2 - 73.0%) and Specificity 98.6% (275/279; 95% CI 96.4 - 99.6%). The high specificity contributed to a high negative predictive value (PPV = 93.5%) and diagnostic likelihood of 40.9 (Table 10). Failure to achieve confirmed LF target enrollment was a significant contributing factor to error in sensitivity estimate. However, the sensitivity was below the clinical hypothesis of 70% and warranted investigation. Analysis eliminated the Case-Controls as a source of discordant results since they are 100% PCR negative.

Table 10. Clinical Performance of ReLASV Ag RDT

Clinical Performance	ReLASV RDT Positive	ReLASV RDT Negative	Total
LASV RT-PCR Positive	27	4	31
LASV RT-PCR Negative	19	275	294
Total	46	279	325
Sensitivity	58.7% (27/46)	95% CI 43.2 - 73.0%	
Specificity	98.6% (275/279)	95% CI 96.4 - 99.6%	
PPV	87.1% (27/31)	95% CI 70.2 - 96.4%	
NPV	93.5% (275/294)	95% CI 90.1 - 96.1%	
Accuracy	92.9% (302/325)	95% CI 89.6 - 95.3%	
Likelihood	40.9 (p<.0001)		

ReLASV Ag ELISA performance improved vs RT-PCR generating sensitivity 80.0% (28/35; 95% CI 63.1- 91.6%) and Specificity 99.0% (207/209; 95% CI 96.6 - 99.9%). We cannot rule out a sampling bias in the improved performance because Ag ELISA results were unavailable for 50 suspected LF cases and 31 case-contacts. Agreement between ReLASV Ag ELISA & RDT was high at 95.9% (233/243; 95% CI 92.5 - 97.9%). The possibility of interference by developing IgM or IgG titers in post-acute LF cases was recognized. Previous performance estimates found strong inverse correlation of antigenemia and IgM/IgG sero-reactivity by ELISA. Previous clinical research had also revealed persistent LASV-specific IgM titers in LF survivors[46].

A Non-reference Standard Analysis was performed to elucidate any LASV-specific antibody interference, LF Case-Contacts were excluded from this analysis (Table 11)[122]. ReLASV IgM ELISA positives accounted for 68% of RDT false-negatives (13/19) these samples also had a significantly higher IgG seropositive rate of 41.0% (25/61) compared to the overall IgG seropositive rate of 24.3% (58/239). Based on the natural history of LF the apparent IgM interference could be associated with post-acute LF case that are in the process of seroconversion to IgM/IgG sero-positive.

Table 11. Three-way Discrepant Analysis Incorporating Non-reference Standard (IgM ELISA) and Reference Standard (RT-PCR)

ReLASV RDT	ReLASV IgM ELISA	Total Subjects	RT-PCR Reference Standard		ReLASV IgG Seropositive
			Pos	Neg	
Pos	Pos	9	8	1	11.1%[#]
Pos	Neg	22	19	3	13.6%[#]
Neg	Pos	**61**	**13**	**48**	**41.0%****
Neg	Neg	147	6	141	19.7%
	Total	239	46	193	24.3%

[#] prevalence p values are not valid due to small sample size.

We determined that reassessment of ReLASV RDT performance was justified for IgM and/or IgG sero-negative samples to determine antibody interference bias. Contingency analysis excluding IgM or IgG ELISA positives increases RDT Sensitivity for the IgM sero-negatives analysis however, the performance estimate looses statistical power due to reduce sample size. Specificity of the RDT is maintained at a level statistically comparable to original performance evaluation (Table 12).

Table 12. ReLASV RDT Performance with Antibody Sero-Negative Samples

	Sensitivity	Specificity
Overall	58.7% (27/46)	98.6% (275/279)
IgM Sero-Neg	76.0% (19/25); p = 0.145	98.6% (214/217); p = 0.962
IgG Sero-Neg	66.7% (26/39); p = 0.450	99.5% (195/196); p = 0.332
G/M Sero-Neg	78.3% (18/23); p = 0.108	99.4% (164/165); p = 0.424

Final ReLASV RDT performance evaluation was conducted using IgM ELISA sero-negative samples and case-contact samples. Performance reflects high Specificity and negative predictive value (NPV). Sensitivity is increased to 76% and PPV to 86.4% however due to the reduced sample size the one-sided margin of error is greater than 0.10. As a "Rule-out" triage test the high Specificity generates a diagnostic likelihood of 55.0. Accuracy with RT-PCR remains high with an incremental increase to 92.9% (302/325; 95% CI 89.6 - 95.3%).

Table 13. ReLASV RDT Performance with IgM Sero-negative Samples.

IgM Sero-negative Performance	ReLASV RDT Positive	ReLASV RDT Negative	Total
LASV RT-PCR Positive	19	3	22
LASV RT-PCR Negative	6	214	220
Total	25	217	242
Sensitivity	76.0% (19/25)	95% CI 54.9 - 90.6%	
Specificity	98.6% (214/217)	95% CI 96.0 - 99.7%	
PPV	86.4% (19/22)	95% CI 65.1 - 97.1%	
NPV	97.3% (214/220)	95% CI 94.2 - 99.0%	
Accuracy	96.3% (233/242)	95% CI 93.0 - 98.1%	
Likelihood	55.0 (p<.0001)		

4.5 Natural history of Lassa fever

Our on-going clinical studies in addition to previous LF studies have shown the Lassa virus infection in most cases follows the concomitant model of both cellular and humoral immune responses to viremia. However, several key features of LASV infection include soluble GP-1 release, high mortality rates, and sustained IgM titers during convalescence. Our studies indicate that the humoral response to the LASV NP antigen produces the dominant antibody titer for the detection and diagnosis of LF. This strong immune response to LASV NP antigen results in almost complete inverse correlation of antigenemia and IgG seropositvity in LF patients(Fig. 21).

Time from onset of symptoms to pre-admission testing was a mean of 8.6 days (CI 7.3-9.9; SD 4.5 days, N=46). Time from onset for Ag+ confirmed cases was a mean of 8.6 days (CI 6.7-10.4; SD 4.6, N=25), and for IgM+ confirmed cases the mean was 8.7 days (CI 6.6-10.7; SD 4.5, N=21). Fourteen cases presented as IgM+ and seroconverted to IgG+. Time from onset for these IgM+ cases was mean of 8.5 days (CI 6.5-10.4; SD 3.4, N=14), and seroconversion to IgG+ was a mean of 14.3 days (CI 11.7- 16.8; SD 4.4, N=14).

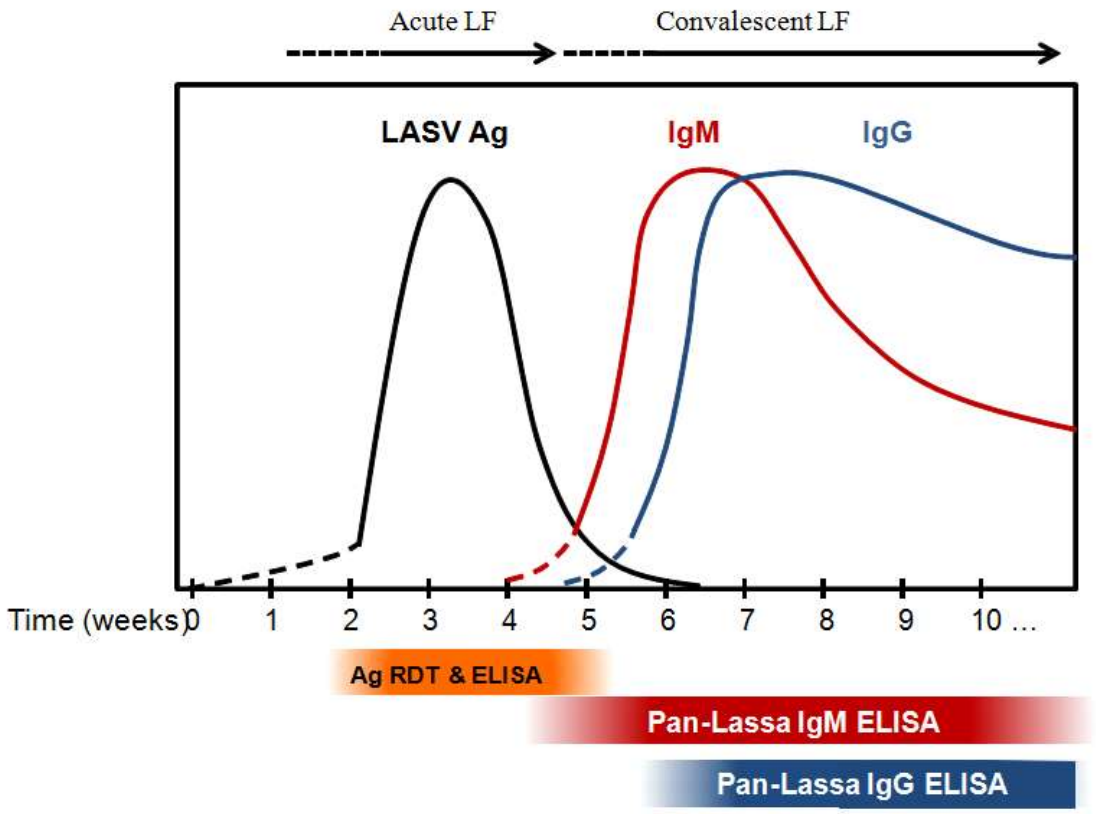


Figure 21. The Natural History of Lassa fever

The incubation period for Lassa fever is typically 2-3 week. LASV titers and circulating antigenmeia increase during the acute phase of LF and may be detected by ReLASV Antigen RDT and ELISA (CFR 50-70%). If a successful humoral immune response is initiated, developing LASV specific IgM followed by IgG emerge and replace antigenemia in the post-acute or early convalescent phase which can be detected by ReLASV IgG/IgM ELISA. During early convalescence CFR significantly decreases from ~28% to ~15%.

Outcome analysis demonstrates the sensitivity if the ReLASV RDT to acute LF. The case fatality rate for all febrile suspected LF cases was 14.4% (35/243). The CFR remained unchanged for ReLASV IgG ± IgM positives at 14.9% (15/101). Factoring in the ReLASV Ag ELISA or RDT (± IgM), the CFR rises to 27.8% (27/97). Finally the use of the ReLASV RDT to screen for acute LF leads to a peak CFR of 70.0% (21/30). Confirmation of results by LASV RT-PCR reduced the acute LF CFR slightly to 56.8% (25/44) by detection of additional PCR+/IgM+ post-acute LF cases (Fig. 21).

Analysis of LF antibody response compared the IgM/IgG ELISA results to previously described natural history of Lassa fever. The ReLASV IgM/IgG negatives represent LF antibody sero-negative cohort in a LF endemic area. For the acute LF, antibody sero-negative group of suspected LF case 16% (23/140) are actually confirmed LF (LASV PCR+) confirming that this imunologically naive group is susceptable to LASV infection (Table 14). In the case-control group as expected from previous analysis there is no active LF (0/47), by PCR in this group (Table 15). The LF seronegative rate is approximately 55% for both suspected LF cases and case-contact groups.

Evaluation of IgG+ for both groups indicates a potential range of 22% to 38% IgG LF convalescences. After removing 5 PCR+ which represent potential LF reinfections, the apparent "true" LF IgG convalescence is 26.3% (88/334). Finally, evaluation of IgM positive (± IgG) after removal of PCR+, reveals the potential level of LASV-specific IgM persistence at 15% to 21% (case-contacts vs non-LF).

Table 14. ReLASV IgG/IgM ELISA Results for Suspected LF

LF Cases	IgM +	IgM -	Total
IgG +	26	33	59
IgG -	47	140	187
Total	73	173	246

Table 15. ReLASV IgG/IgM ELISA Results for Case-Controls

Case-Control	IgM +	IgM -	Total
IgG +	6	28	34
IgG -	7	47	54
Total	13	75	88

4.6 Clinical Study Conclusions

The ReLASV Ag Rapid Test did not entirely meet the performance goals as stated in the clinical hypothesis. Enrollment of suspected case and collection of necessary laboratory data was approximately half of the study's minimal sample size target. A large proportion of the enrolled patients appear to be post-acute or LF convalescent cases. The increased frequency of LASV-specific IgG/IgM interfered with antigen-specific RDT performance. Re-evaluation of the ReLASV RDT with IgM sero-negative data improved sensitivity to 76% which meets the clinical hypothesis but reduced sample size can only provide a minimal sensitivity of 54.9% based on the 95% confidence interval. However the ReLASV RDT demonstrated excellent specificity (>95%) throughout the performance analysis. These results combine to generate a Diagnostic Likelihood >40 and accuracy with LASV RT-PCR >92%.

With a Diagnostic Likelihood >40, the ReLASV RDT rejects the null hypothesis that the RDT will be no more effective at detection of LF than the clinical case definition based in part on non-specific clinical signs.

The ability of this test to detect acute (antibody sero-negative) LF is reflected in the elevated CFR = 70% for RDT positive compared to the low ~15% CFR generated by apparent early LF convalescent IgG/IgM positive cases. The pairing of high Specificity and Positive Predictive Value (>85%) validates the ReLASV Ag Rapid Test to be a valuable POCT rapid diagnostic for the triage of suspected LF cases.

PART II. DEVELOPMENT OF ReEBOV DIAGNOSTICS

Chapter 5. ReEBOV Diagnostics Design and Analytical Validation

5.1 Ebola Critical Reagent Development

The emergence in 2014 of the West African EHF outbreak, required more tactical approach to IVD design to expedite optimization and deployment. In order to fast track RDT development and secure an Emergency Use Authorization (EUA) from the Federal Drug Administration (FDA) only a limited number of field trails would be possible compared to the more extensive field testing during the ReLASV RDT development.

During the NIH SBIR R43 Phase I grant (2010-2013), recombinant filovirus glycoproteins (rGP), VP40 (rVP40), and NP (rNP) were prepared for antibody production and assay development [123-126]. For ReEBOV Ag RDT, N-terminal truncated VP40EΔ40 (rVP40) was used for improved stability and yield during production [127]. Fidelity of the EBOV rGP to native protein was previously establish by reactivity of the human mAb KZ52 donated by Drs. Saphire and Burton at TSRI[123, 127]. In June 2014, early field testing of rVP40 and rNP ELISA coatings demonstrated reactivity of suspected EHF serums to these recombinant proteins and further strengthened confidence in antibody reagents that had previously been developed and purified against them[128]. This included polyclonal caprine serum that was prepared and affinity purified antibody

held in reserve from the R43 Phase I program. Ultimately the decision was made to focus RDT development toward detection of EBOV VP40 antigen due reagent availability and promising feasibility studies.

5.2 Statistical Analysis

Data were analyzed using v11.0 (SAS Institute, Inc.) and GraphPad Prism v. 6.04 (GraphPad Software, Inc.) Hypotheses involving dichotomous response variables were tested using Student's t test, Fisher's exact test or logistic regression with receiver operating characteristics. Binomial proportion comparisons use to establish significance across contingency estimates. Analyses were two-tailed, with a significance threshold set at $p<0.05$. Confidence intervals (95%): Agresti and Coull (1998)

5.3 ReEBOV ELISA Design

ReEBOV® Ag and IgG/IgM ELISAs would include both rVP40 and rNP-specific multiplexed reagents. ReEBOV ELISA development followed closely that of the previously developed ReLASV ELISA tests. For ReEBOV IgG/IgM ELISA, 96-well plate coatings included a mixture of both EBOV rVP40 and rNP. Preparation of the remaining kit components was not altered from that of ReLASV® Pan-Lassa IgG/IgM ELISA, other than the anti-EBOV bioconjugates used in the lyophilized ReEBOV IgG/IgM Reference (See Ch. 2.2). For ReEBOV Ag ELISA an equal mixture of affinity purified Gt. anti-rVP40 and Gt. anti- rNP were used on microwell plate coating. These same pAb's were conjugated to HRPO and mixed together in the detector reagent. EBOV rVP40 and rNP were also diluted in serum and lyophilized as the ReEBOV Ag

Reference. The remaining components of this assay were not altered from that of the ReLASV® Pan-Lassa Ag ELISA (See Ch. 2.3). Aside from reconstituting the lyophilized References and Negative Control with deionized water, all ReEBOV assay procedures were identical to the ReLASV ELISA testing procedures.

5.4 Validation of ReEBOV IgG/IgM ELISAs

ReEBOV® IgG/IgM ELISA coatings were validated for specificity towards EBOV antibodies. Non-human primate antisera collected from control primates in EBOV therapeutic studies was donated by collaborator Dr. T. W. Geisbert at Galveston National Lab, University of Texas Medical Branch, Galveston TX. Briefly, NHP convalescent EBOV antisera was serially diluted in IgG/IgM Sample Diluent and incubated on EBOV rNP or rVP40 plate coatings. BCN was also serially diluted and tested as non-specific controls. After incubation (30min at ambient temperature) and washing with PBS-Tween, the ELISA plates were incubated with anti-Hu IgG peroxidase conjugates which are cross-reactive with both human and non-human primate IgG. Following the reagents incubation and plate washing, TMB substrate was added and microwell plate incubated 15min for signal development. Positive ELISA signals by the NHP antisera indicated the recombinant antigen coatings were capable of detecting live EBOV induced antisera from experimentally infected NHPs (Fig. 22).

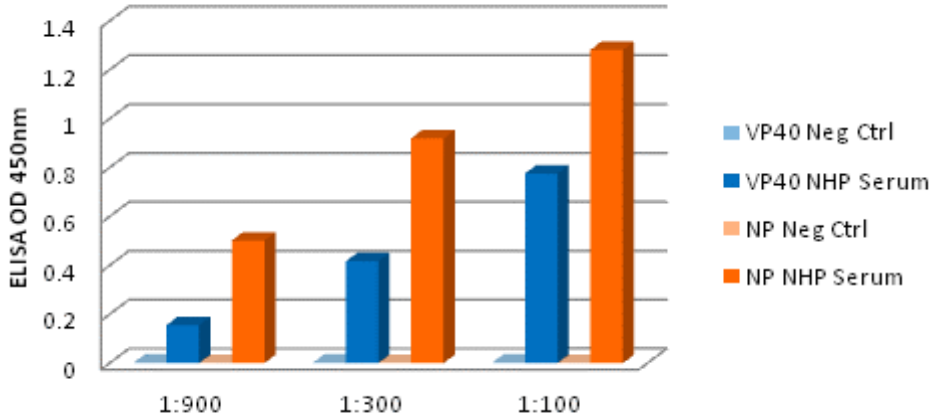


Figure 22. Non-Human Primate EBOV Antiserum in ReEBOV IgG ELISA
Titerable reactivity to coated rNP and rVP40 by NHP EBOV antisera demonstrates immunoreactive fidelity of the recombinant antigens to antisera induced by live virus.

Field validation of the ReEBOV IgG/IgM ELISA at Kenema Government Hospital was conducted using serum and plasma samples from qRT-PCR confirmed EHF positives (n=83), suspected EHF cases (n=110), and US normal serum (N=40). Samples were diluted 1:99 (10µL:990µL) in IgG/IgM Sample Diluent and added to ELISA microwells coated with a 1:1 mixture of EBOV rNP:rVP40. Sample and reagent additions are 100µL/well and all samples are tested in duplicate. Microwell plates were incubated at ambient temperature (25-32°C) for 30mins. Following plate washing with PBS-Tween, diluted Gt. anti-Hu. IgG or IgM conjugated to HRPO was added to the microwells. Incubation followed by plate washing was repeated. TMB substrate was added to the microwells and incubated 15min at ambient temperature to allow color development (Fig. 23 A & B).

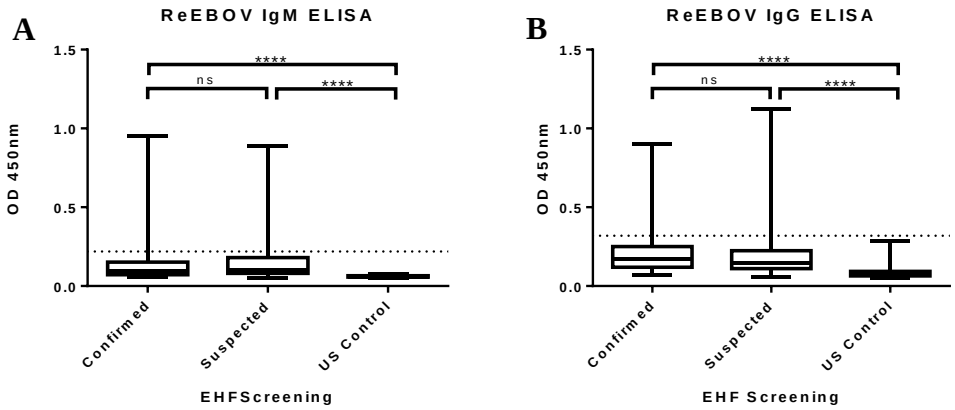


Figure 23A & B. ReEBOV IgG/IgM ELISA Field Validation Testing
Mean testing showed significant difference in Confirmed and Suspected patient samples vs US Controls for both IgM and IgG ELISA. (A) ReEBOV IgM ELISA Cut-off was set at OD = 0.200 (dotted line). (B) ReEBOV IgG ELISA Cut-off was set at OD = 0.320 (Fisher's exact, **** p<.0001)

5.5 Validation of ReEBOV Antigen ELISA

The ReEBOV® Antigen ELISA Test is designed in similar fashion as the ReLASV Pan-Lassa Ag ELISA (See Ch. 2.3) using previously developed EBOV specific antibodies and recombinant antigens[128]. The microwell plate coating is 1:1 mixture of affinity purified EBOV rNP and rVP40 specific caprine IgG. Once the microwell plates are coated and blocked, they are sealed in oxygen and vapor barrier mylar bags. The same mixture of EBOV rNP and rVP40 specific antibody used for plate coating is also labeled with HRPO for use as detection reagent. rNP and rVP40 antigens are diluted in BCN and lyophilized for use as an antigen Reference, BCN is also lyophilized for use as a Negative Control.

Field validation of the ReEBOV Ag ELISA was conducted using serum and plasma samples from qRT-PCR confirmed EHF positives (n=83), suspected EHF cases (n=110), and US normal serum (N=40). Samples were diluted 1:9 (50µL:450µL) in Ag Sample Diluent and added to coated microwells. Sample and reagent additions are 100µL/well and all samples are tested in duplicate. Sealing tape was applied and microwell plates were incubated in 35°C oven for 1hr. Following plate washing with PBS-Tween, diluted Gt. anti-EBOV-HRPO detection reagent was added to the microwells. Incubation was for 30min at ambient temperature, followed by plate washing. TMB substrate was added to the microwells and incubated 15min at ambient temperature to allow color development (Fig. 24).

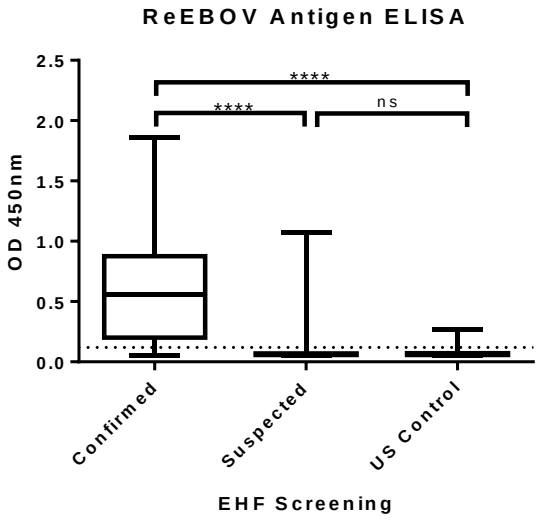


Figure 24. ReEBOV Ag ELISA Field Validation Testing

Mean testing showed significant difference in Confirmed vs Suspected and US Controls. No significant difference is seen between Suspect and US Controls. ReEBOV Ag ELISA Cut-off was set at OD = 0.120 (Fisher's exact, **** p<.0001)

Clinical performance analysis of the ReEBOV Ag ELISA was conducted as part of the ReEBOV Ag RDT EUA Clinical Validation. Means testing of Ag ELISA OD for qPCR positive and negative was significant (p<.0001) and verified the OD 0.120 assay cut-off (Fig. 25). A contingency analysis was run using EBOV qRT-PCR as the reference method (Table 16), results show ReEBOV Ag ELISA Sensitivity = 81.9% and Specificity = 97.4% resulting in Diagnostic Likelihood = 31.1.

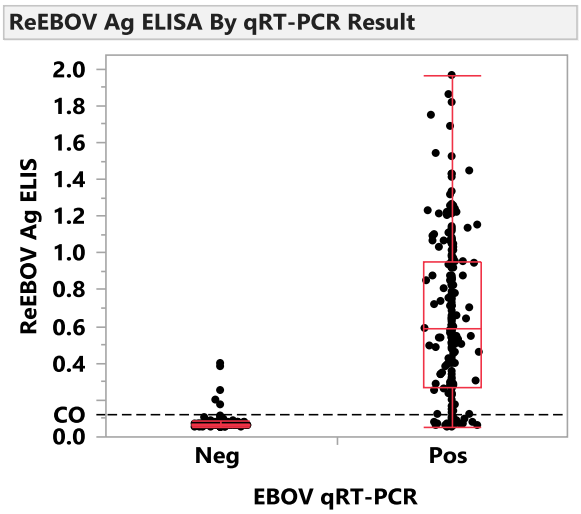


Figure 25. ReEBOV Ag ELISA by qPCR

Mean comparison of ReEBOV Ag ELISA OD450nm by EBVOV qPCR is significant (p<.0001) and confirmed Ag ELISA OD cut-off = 0.120.

Table 16. Clinical Performance of ReEBOV Ag ELISA

	qPCR Pos	qPCR Neg	Total
ReEBOV Ag+	172	5	177
ReEBOV Ag-	38	185	223
Total	210	190	400
Sensitivity (%)	81.9 (172/210)	95% CI: 76.0% - 86.9%	
Specificity (%)	97.4 (185/190)	95% CI: 94.0% - 99.1%	
PPV (%)	97.2 (172/177)	95% CI: 93.5% - 99.1%	
NPV (%)	83.0 (185/223)	95% CI: 77.4% - 87.7%	
Accuracy (%)	89.3 (357/400)	95% CI: 85.8% - 91.9%	
Likelihood	31.1	AUC 0.932	

5.6 ReEBOV Antigen Rapid Test Design

ReEBOV® Antigen Rapid Test (ReEBOV RDT) required several formulation changes partly due to the use of affinity purified pAb (caprine) instead of mAb (murine) used in the ReLASV RDT. This also presented the opportunity to introduced several RDT process improvements and manufacturing efficiencies. The most notable modification was switching to a borate buffer system (pH 9) for the preparation of pAb conjugated nanoparticles which was dictated by the stability of the pAb itself. Also notable was a change in nitrocellulose membrane for manufacturing considerations. The RDT sample buffer formulation also required additional fortification with increased concentrations of blocking agents and surfactants to mitigate non-specific binding of the immobilized pAb in test line and pAb antibody-nanoparticles. The use of caprine pAb naturally required change of the control line to anti-caprine specificity. Again, except for reconstituting the lyophilized Reference and Negative Controls, the ReEBOV RDT procedures were identical. Additional visual aids are included for procedural steps and test interpretation.

The assay procedures for the ReLASV® Antigen Rapid Test[116] are as follows:

Assay Procedure
Remove appropriate amount of dipsticks for testing one Negative Control, on Positive Control, and the required patient samples.

For Control Samples

1. Prepare separate tubes for Negative and Positive Controls.
2. Add 4 drops of Sample Buffer to an appropriate size culture tube (10x75mm).
3. Add on drop of control onto the center of the Sample Pad (below arrows).
4. Insert the Rapid Test dipstick (arrows down) into the culture tube containing Sample Buffer. Replace tube cap.
5. Allow Rapid Test dipstick to develop for 15-25 minutes before visual interpretation.

For Patient Samples

1. Add 4 drops of Sample Buffer to an appropriate size culture tube (10x75mm).
2. Transfer 30µL of serum, plasma, or whole blood onto center of the Sample Pad (below arrows).
3. Insert the Rapid Test dipstick (arrows down) into culture tube containing Sample Buffer. Replace tube cap.
4. Allow the Rapid Test dipstick to develop for 15-25 minutes before visual interpretation.

Interpretation of Results

1. The ReLASV® Antigen Rapid Test results should be compared to the Negative Control and included Visual Aid to assist in interpretation.
2. For a positive patient result on the ReLASV® Antigen Rapid Test, a line ranging from faint pink to dark red should form across the Test Zone.
3. For a negative patient result no line should be detected across the Test Zone. Compare patient results to the Negative Control.
4. If available a permanent record should be made by digital photography.

In response to the West African EHF outbreak, Corgenix, Inc. approached the FDA for guidance on design validation specifications that would be required for an Emergency Use Authorization application. During the same period the WHO issued a separate guidance for POC immunoassays (RDTs)[129]. The original ReEBOV RDT design features satisfied many of the design specification requested by both FDA and WHO. Through a collaborative effort with the FDA the Draft Interactive EUA Review Template For Serological Assays-Antigen was issued in Oct. 2014. Included within this document were analytical performance testing guidelines including Limit of Detection, Specificity, Cross Reactivity, Interfering Substance, and Mock Clinical Evaluation using contrived samples.

5.7 ReEBOV RDT Analytical Validation

Validation study sites included UTMB-Galveston National Lab, Galveston, TX where all live EBOV, VHF virus nearest neighbor testing, and non-human primate sample testing was performed. Infectious disease agent cross-reactive testing was performed at Tulane University, New Orleans, LA and CDC Fort Collins, CO. Analytical performance using recombinant VP40 antigen was conducted at Corgenix, Inc., Broomfield, CO. Whenever possible a record of results were made by digital photography before test strips had dried.

5.7.1 ReEBOV RDT Range and Limits of detection

The RDT assay range and limit of detection (LOD) were determined for both rVP40 antigen and live Zaire EBOV. Range of the RDT was determined in preliminary

LOD testing. For rVP40 antigen, testing was conducted by serial dilution of rVP40 into BCN serum in a concentration range of [40.0 - 10,000 ng/mL]. For live Zaire EBOV, testing was conducted by serial dilution of Zaire EBOV (Makona) serially diluted in donor whole blood in a concentration range of [$1.00E^4$ - $1.00E^7$ pfu/mL]. Each dilution level was tested with 5 replicates. Test strips were incubated a minimum of 15mins before visually scoring. The preliminary LOD was considered the lowest dilution in which all five test strips were positive by visual interpretation. Verification of the preliminary LOD was performed by testing twenty replicates of diluted rVP40 or Zaire EBOV at and near the preliminary LOD dilution levels.

LOD Results:

Preliminary LOD for rVP40 spiked into BCN was equal to 160.0ng/mL = 4.7ng/test (Table 17). Verification of the spiked rVP40 LOD was conducted at 2.35ng/mL and 4.7 ng/mL. At 4.7ng/mL, 100% of the 20 replicates was positive and represents the LOD (Table 18). LOD verification was repeated with rVP40 spiked into donor whole blood. LOD concentrate was 2-fold higher at 9.4 ng/test.

Preliminary LOD for Zaire EBOV in whole blood $1x10^6$ pfu/mL (Table 19). Verification of Ebola virus LOD in whole blood was confirmed by 100% positive of the replicates (Table 20).

Table 17. Preliminary LOD – rVP40 Antigen in Serum

	ng/mL	ng/test	Replicates (5)					Result
VP40 Ag in Serum	10000	300	+	+	+	+	+	Positive
	5000	150	+	+	+	+	+	Positive
	2500	75.0	+	+	+	+	+	Positive
	1250	37.5	+	+	+	+	+	Positive
	625	18.8	+	+	+	+	+	Positive
	312	9.4	+	+	+	+	+	Positive
	160	4.7	+	+	+	+	+	LOD
	80	2.3	+	-	-	+	+	Negative
	40	1.2	-	-	-	-	-	Negative
	Control		-	-	-	-	-	Negative

Table 18. Verification of LOD - rVP40 Antigen in Serum and Whole Blood

	Serum LOD		Whole Blood LOD	
Measure #	**2.35ng/test**	**4.7ng/test**	**4.7ng/test**	**9.4ng/test**
1	+	+	-	+
2	+	+	+	+
3	+	+	+	+
4	+	+	+	-
5	+	+	+	+
6	+	+	+	-
7	+	+	+	+
8	+	+	+	+
9	+	+	+	+
10	+	+	-	+
11	-	+	-	+
12	+	+	+	+
13	+	+	+	+
14	+	+	+	+
15	-	+	+	+
16	+	+	-	+
17	+	+	-	+
18	+	+	+	+
19	+	+	-	+
20	+	+	-	+
%Pos	90.0	100.0	65.0	90.0

Table 19. Preliminary LOD of Ebola Virus in Whole Blood

	pfu/mL	pfu/test	Replicates (5)					Result
Zaire Ebola virus in Serum	1×10^7	3×10^5	+	+	+	+	+	Positive
	2×10^6	6×10^4	+	+	+	+	+	Positive
	1×10^6	3×10^4	+	+	+	+	+	LOD
	4×10^5	1.2×10^4	+	-	-	+	+	Negative
	1×10^5	3×10^3	-	-	-	-	-	Negative
	8×10^4	2.4×10^3	-	-	-	-	-	Negative
	1×10^4	300	-	-	-	-	-	Negative
	Pos Control		+	+				Positive
	NegControl		-	-				Negative

Table 20. LOD Verification of Ebola Virus in Whole Blood

	2×10^6 pfu/ml	1×10^6 pfu/ml
1	**1**	**1**
2	**2**	**1**
3	**2**	**2**
4	**2**	**1**
5	**1**	**1**
6	**1**	**2**
7	**2**	**1**
8	**2**	**1**
9	**1**	**1**
10	**2**	**1**
11	**2**	**1**
12	**2**	**1**
13	**2**	**1**
14	**2**	**1**
15	**2**	**1**
16	**2**	**1**
17	**2**	**1**
18	**2**	**1**
19	**1**	**1**
20	**2**	**1**
% Pos	**100%**	**100%**

5.7.2 Interfering Substances

Interfering substances screening was conducted in accordance with CLSI approved guideline EP7-A2[119]. The rVP40 antigen was diluted in BCN at a concentration near the LOD [0.2µg/mL ≈ 6.0ng/test]. Substance and concentrations to be tested were found in EP7-A2, Appendix C or D. for substances not listed the equivalent of a 20X stock for the normal adult dosage diluted in 5L (blood volume) was prepared. Prior to testing, the 20X substance stock is diluted in an aliquot of the dilute rVP40 sample and BCN without antigen. Each substance and solvent control is tested in 5 replicates. Additional levels of select substances were later request by the FDA. A substance was considered non-interferant if the antigen spiked median score did not differ from the solvent control or generate false-positive signal in BCN.

Interfering Substances Results

For the biologic substances (Table 21), hemoglobin failed due to the interference with positive signal development and interpretation due to heavy background staining of the nitrocellulose membrane. Rheumatoid factor also failed due to false-positive signal above 39IU/mL spiking concentration. Bilirubin also stained the nitrocellulose membrane yellow but this did not interfere with negative or positive signal development.

None of the Drug In Common Use (Table 22) interfered with positive or negative signal development or interpretation including serum aminotransferase (AST, ALT).

Table 21. Interfering Substances - Biologics

Substance	Conc. Tested	Solvent	Result
Hemoglobin	100mg/mL	npH2O	Fail
Hemoglobin	20 g/dL	npH$_2$O	Fail
Serum Protein	50mg/mL	npH2O	Pass
Serum Protein	35mg/mL	npH2O	Pass
HAMA	800 ng/mL	Serum	Pass
HAMA	182.1units/mL	Serum	Pass
HAMA	2.65units/mL	Serum	Pass
Rheumatoid Factor	60units/mL	npH2O	Pass
Rheumatoid Factor	15units/mL	npH2O	Pass
Rheumatoid Factor (RF)	2000 IU/mL	Serum	Fail
RF Serial Diln	1050 IU/mL	-	Fail
RF Serial Diln	350 IU/mL	-	Fail
RF Serial Diln	117 IU/mL	-	Fail
RF Serial Diln	39 IU/mL	-	Pass
Bilirubin	25 mg/dL	0.1N NaOH	Pass
Bilirubin	15mg/dL (High)	0.1N NaOH	Pass
Bilirubin	1mg/dL (Low)	0.1N NaOH	Pass
Conj. Bilirubin	5mg/dL (High)	npH2O	Pass
Conj. Bilirubin	0.2mg/dL (Low)	npH2O	Pass

Table 22. Interfering Substances - Drugs In Common Use

Substance	Conc. Tested	Solvent	Result
EDTA (short draw)	25mM	npH2O	Pass
Ribavirin (Loading Dose 75kg Adult)	4.5g/dL (30mg/kg/5L)	npH2O	Pass
Arthemeter (Loading Dose 75kg Adult)	0.48g/dL (3.2mg/kg/5L)	npH2O	Pass
Artesunate (Loading Dose 152.4mg Adult)	0.314g/dL	DMSO	Pass
Quinidine Gluconate	15µmol/L	npH2O	Pass
Quinidine Gluconate	6.2µmol/L	npH2O	Pass
Quinine	148 µmol/L	npH2O	Pass
Doxycycline	67.5 µmol/L	npH2O	Pass
Ampicillin	152 µmol/L	npH2O	Pass
Ciprofloxacin	30.2µmol/L	0.1N HCL	Pass
Acetaminophen (Paracetamol)	199µmol/L	npH2O	Pass
Acetaminophen (Paracetamol)	33µmol/L	npH2O	Pass
Ibuprofen	2425µmol/L	0.1N NaOH	Pass
Aspirin (ASA)	21.6mg/mL	Ethanol	Pass
Promethazine	4.22 µmol/L	npH2O	Pass
ALT	2000units/L	npH2O	Pass
AST	2000units/L	npH2O	Pass

5.7.3 Cross-reactants and Nearest Neighbor Testing

Nearest Neighbor and Cross-reactant screening was conducted at multiple sites depending on the required Bio-Safety Level (BSL) for the agents (virus, bacteria, or parasite) tested. Testing was performed in a similar manner as Interfering Substance previously described. BCN serum with or without rVP40 antigen diluted to [0.2µg/mL ≈ 6.0ng/test] was distributed or prepared on-site. BSL-4 viruswere tested at UTMB, BSL-3 vaccine virus strains were tested at CDC, Fort Collins, and remaining BSL-2, -3 pathogens were tested at Tulane Health Science Center since they were not inactivated. Concentration tested of pathogen fluctuated due to stock available. Every attempt was made to maintain a dilution factor of ≤ 5% of the antigen spiked BCN or no antigen BCN to retain serum matrix integrity. All pathogens were tested in triplicate. Pathogens were considered non cross-reactive if they did not produce false-positive or false-negative signals.

Cross-Reactant Testing Results

None of the Cross-reactants tested interfered with positive or negative signal development or interpretation (Tables 23 & 24). Lassa viruses and Junin virus were not screened for false-negative generation but clinical Lassa virus cross-reactivity was not observed during field trails in Sierra Leone and LASV rNP has been tested and shown not to cross-react in EBOV RDT (data not presented). Paramyxoviridae was positive on 1 of 3 negative serums but recorded as non-reactive due to 5/6 correct RDT results.

Table 23. Cross-reactant Pathogens - Set 1

Virus/Microbe Parasite	Type-Strain (Isolate)	Concentration Tested	Positive Scores	Negative Scores	Reactivity
Lassa Virus	Josiah (CDC#057562)	$8.8x10^4$ pfu/mL	nd	---	None
Lassa Virus	Nigeria-Clade II (Saurwald)	$1.5x10^4$ pfu/mL	nd	---	None
Junin Virus	Espindola (P3790)	$2.25x10^4$ pfu/mL	nd	---	None
CCHF Virus	IBAR 10200	$5.0x10^4$ pfu/mL	+++	---	None
Yellow Fever Virus	CDC vaccine strain	not available	+++	---	None
Rift Valley Fever Virus	CDC vaccine strain	not available	+++	---	None
Chikungunya Virus	CDC strain	not available	+++	---	None
Flaviviridae	DENV-1	$>10^5$ pfu/mL	+++	---	None
Flaviviridae	DENV-2	$>10^5$ pfu/mL	+-+	---	None
Flaviviridae	DENV-3	$>10^5$ pfu/mL	+++	---	None
Flaviviridae	DENV-4	$>10^5$ pfu/mL	+++	---	None
HIV-1	JC/pMT4R5	1.0×10^3 pfu/mL	+++	---	None
HIV-1	Ba-1/MT4R5	1.0×10^3 pfu/mL	+++	---	None
HIV-1	JFM/pMt4R5	1.0×10^3 pfu/mL	+++	---	None
Herpesviridae	CMV-Townes	8.47×10^4 pfu/mL	+++	---	None
SIV	17E/MH161	1.0×10^3 pfu/mL	+++	---	None
EBV	B95-8	1.0×10^3 pfu/mL	+++	---	None
HSV1	-	5.5×10^4 pfu/mL	+++	---	None

Table 24. Cross-reactant Pathogens - Set 2

Virus/Microbe Parasite	Type-Strain (Isolate)	Concentration Tested	Positive Scores	Negative Scores	Reactivity
Influenza	A/P8/8/34 H1N1	9×10^9 pfu/mL	+++	---	None
Paramyxoviridae	Measles-Edmonston	4.67×10^3 pfu/mL	+++	-+-	None
Rhabodvirus	VSV	Unknown	+++	---	None
Arenaviridae (NW)	Pichindae	2.81×10^4 pfu/mL	+++	---	None
Trypanosoma	cruzi	1.0×10^8 parasite/mL	+++	---	None
Plasmodium	falciparum	1.0×10^7 parasite/mL	+++	---	None
Toxoplasma	gondii	Unknown	+++	---	None
Pseudomonas	aeruginosa	1.0×10^5 cell/mL	+++	---	None
Shigella	Serogroup A	Unknown	+++	---	None
Streptococcus	pnuemoniea	1.0×10^8 cell/mL	+++	---	None
Salmonella	Typhi	1.0×10^6 cell/mL	+++	---	None
Yersinia	Pseudo-tuberculosis	1.0×10^6 cell/mL	+++	---	None

Nearest-neighbor filovirus screening was conducted at UTMB-Galveston Nat'l Lab BSL-4 facilities. Only normal human serum (BCN) was spiked with filoviruses. The ReEBOV RDT was reactive to both Makona and Mayinga Zaire EBOV. The RDT was also cross-reactive to Sudan and Bundibugyo EBOV at 10^3 - 10^4 pfu/mL (Table 25). Reston virus and Marburg Virus were non-reactive.

Table 25. Nearest-Neighbor Filoviridae Screening

Virus/Microbe Parasite	Type-Strain (Isolate)	Concentration Tested Pfu/mL	Negative Scores	Reactivity
Ebola Virus	Zaire (Mayinga)	$3.3x10^6$	+++	Yes
Ebola Virus	Zaire (Makona)	$1.3x10^5$	+++++	Yes
Ebola Virus	Sudan-Gulu (200011676)	$3.75x10^5$	+++	Yes
Ebola Virus	Sudan-Gulu (200011676)	$3.75x10^4$	+++	Yes
Ebola Virus	Bundibugyo (200706291)	$1.38x10^4$	+++	Yes
Ebola Virus	Bundibugyo (200706291)	$1.38x10^3$	+++	Yes
Ebola Virus	Reston (AZ-1435)	$6.75x10^3$	---	None
Marburg Virus	Musoke	$2.2x10^5$	---	None

5.7.4 High-dose Prozone Effect

The ReEBOV RDT was challenged with high-dose rVP40 antigen levels to determine if acute viremia and associate antigemia were capable of generating prozone effect resulting in false-negative results. Undiluted stock of rVP40 antigen [2.6mg/mL] was 2-fold serially diluted in BCN down to [0.2µg/mL] resulting in a range of [6ng to 39µg/test]. Five replicates of each level was tested.

Result

Absence of prozone effect at high rNP concentrations satisfies assay design goals.

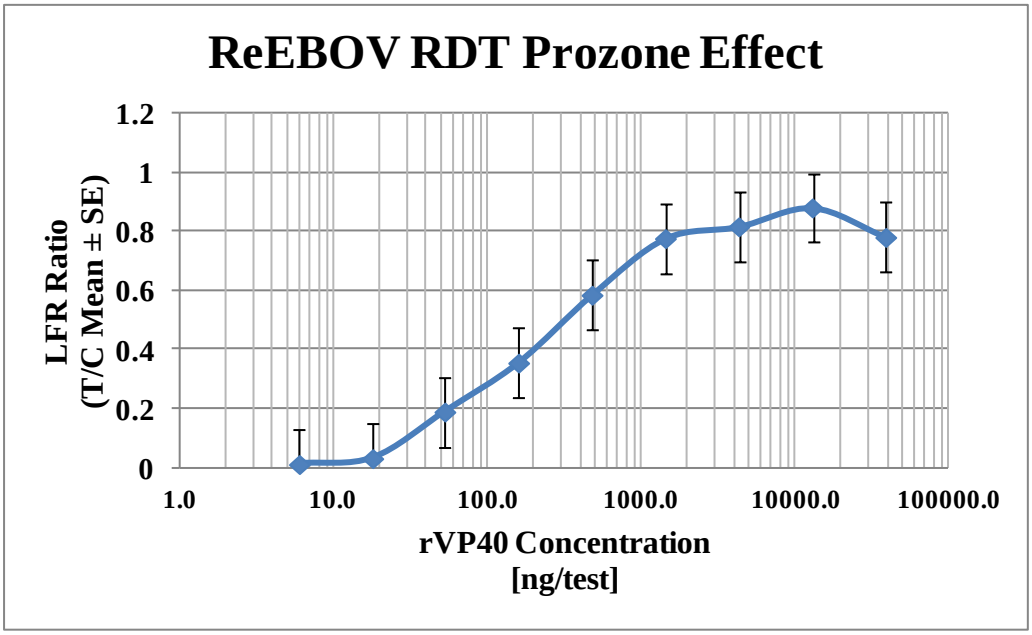


Figure 26. Prozone Effect Screening

No significant Prozone Effect was observed up to [39µg/test].

5.7.5 RDT Repeatability

Thirteen volunteers donated venous whole blood collected in EDTA-vacutainers (BD Medicine). Randomized whole blood samples were spiked with rVP40 antigen at the LOD [9.4ng/test] and 2-fold series upward to [600ng/test]. Extra samples were spiked with rVP40 at LOD and 2-fold above. A total of 75 replicates of unspiked whole blood was tested for assay specificity. All samples were blinded and tested according to Package Insert. After 15min incubation, each dipstick was read by three technicians. Scores were tabulated based on agreement of three technicians.

Results

The three RDT readers agreed on un-spiked sample scoring 92% (69/75) and 100% (75/75) for 2 out of 3 RDT readers. For rNP spiked samples, 2 of 3 readers agreed 58% (21/36) for [9.4 ng/mL] which would correspond with the Qualitative Assay Cut-off. At [18.8ng/mL] all three reader agreed 100% (36/36) which would correspond with LOD.

Table 26. Inter-operator Repeatability and Specificity

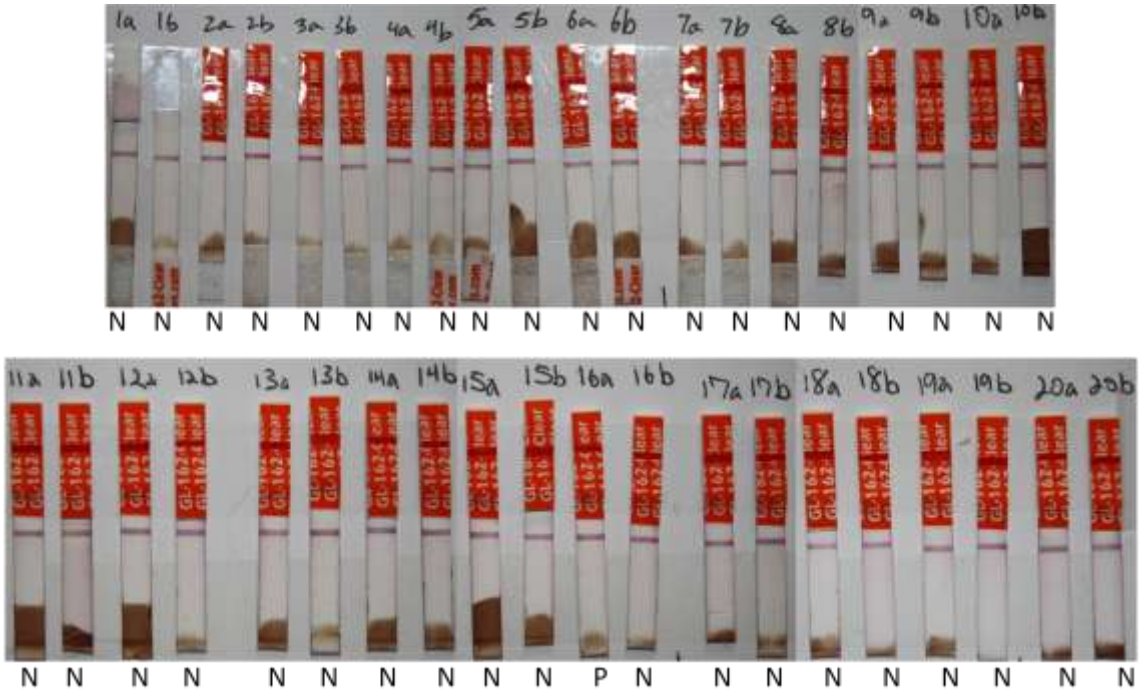
	rVP40 Spike level [ng/test]	No. of Samples	Observations by 3 individual readers			
			# of specimens and % of total specimens at each spike level			
			3/3 Pos	2/3 Pos	1/3 Pos	0/3 Pos
Un-spiked	0	75	0% (0/75)	0% (0/75)	8% (6/75)	92% (69/75)
Spiked	9.4	36	47.2% (17/36)	11.1% (4/36)	22.2% (8/36)	19.4% (7/36)
Spiked	18.8	30	96.7% (29/30)	0% (0/30)	3.3% (1/30)	0% (0/30)
Spiked	37.5	5	100% (5/5)	0% (0/5)	0% (0/5)	0% (0/5)
Spiked	75.0	7	85.7% (6/7)	0% (0/7)	0% (0/7)	14.3% (1/7)
Spiked	150	6	100% (6/6)	0% (0/6)	0% (0/6)	0% (0/6)
Spiked	300	4	75% (3/4)	0% (0/4)	0% (0/4)	25% (1/4)
Spiked	600	6	100% (7/7)	0% (0/7)	0% (0/7)	0% (0/7)

5.7.6 Mock Specificity & Sensitivity

Specificity of Fingersticks

During donor blood collection, 20 healthy US volunteers agreed to fingerstick screening. Each volunteer provided duplicate fingersticks. One full drop (~30µL) was allowed to developed then transferred directly to the plasma separation Sample Pad by touching the dipstick directly to the drop. This was repeated to collect a second sample from each volunteer. Dipstick were immediately inserted into test tube containing Sample Buffer to initiate development of RDT Result. After 15min incubation, results were scored blinded by a second technician.

Table 27. Fingerstick Specificity Panel

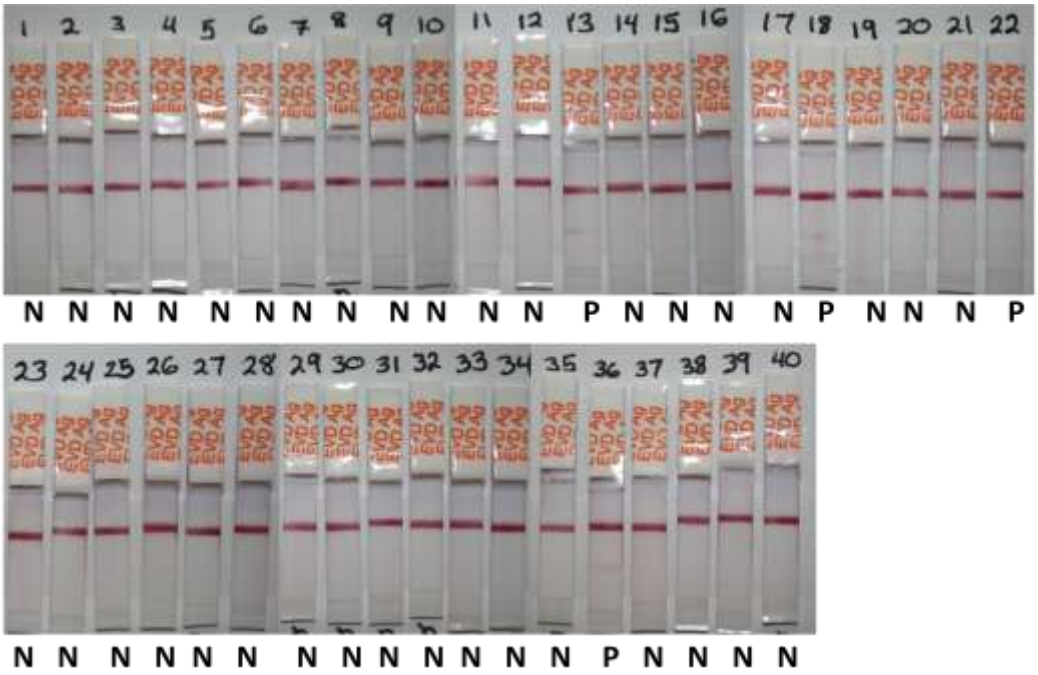


Specificity Evaluation using US Normal Serum Panels

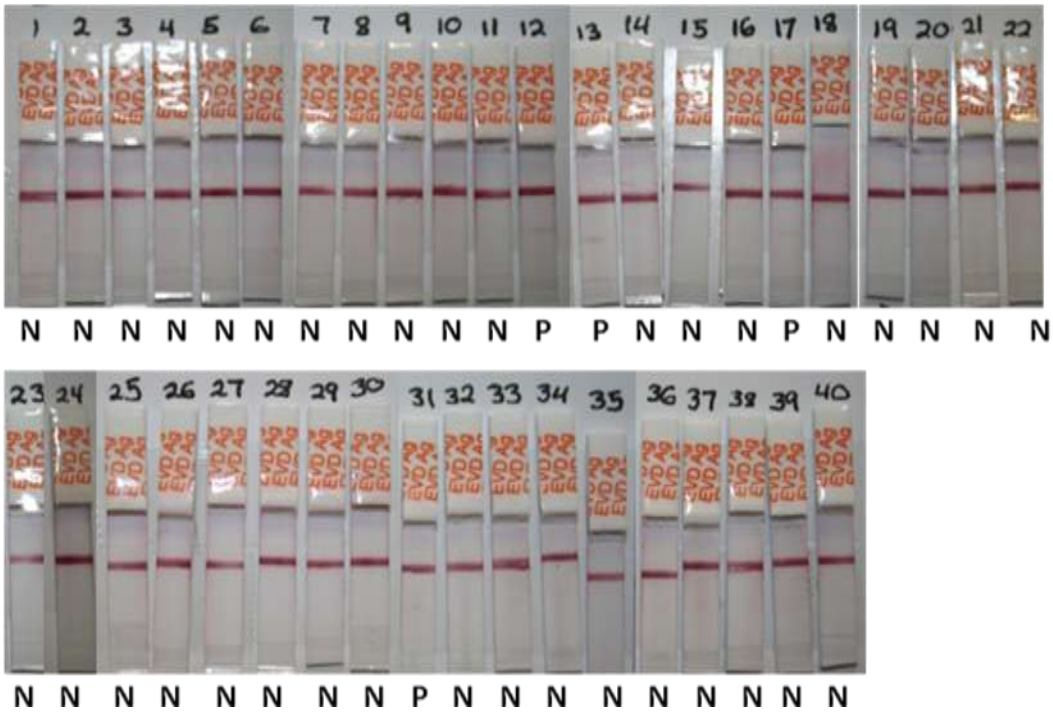
Specificity of serum samples was evaluated using US normal serum panels developed from commercial donor serum. A total of four panels representing N=138 individual serum donors were tested. Each sample was tested individually. A second technician scored the results independently.

Table 28. US Normal Serum Panels

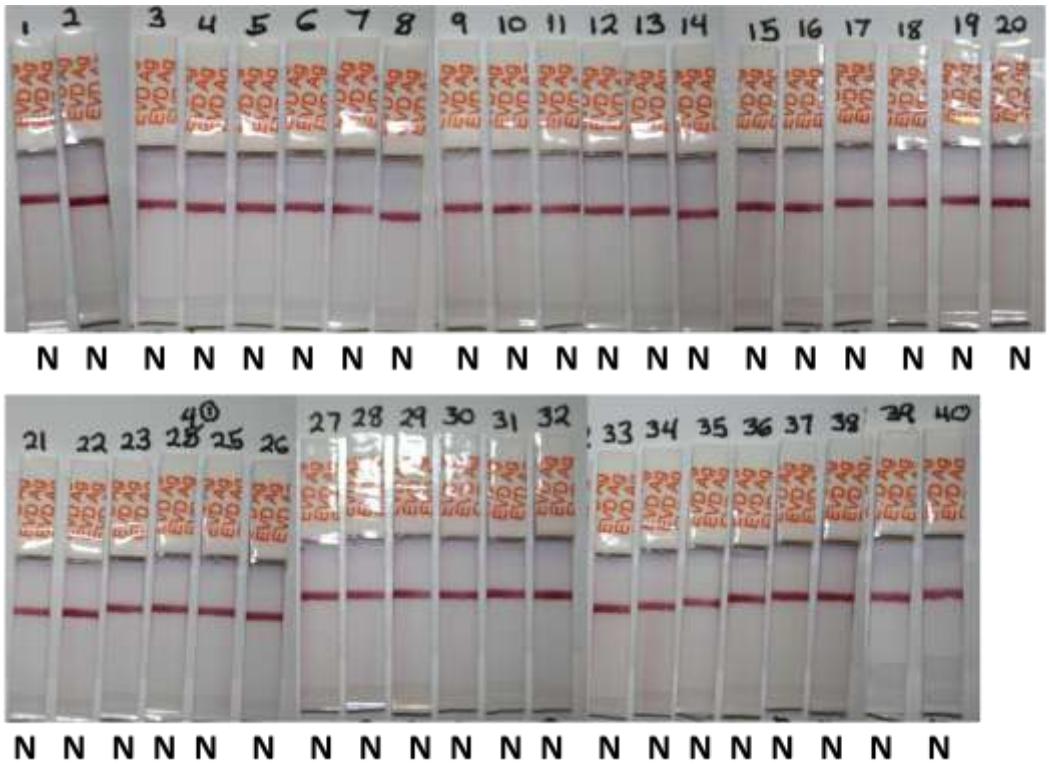
Panel B5



Panel B6



Panel B7



PSG Panel

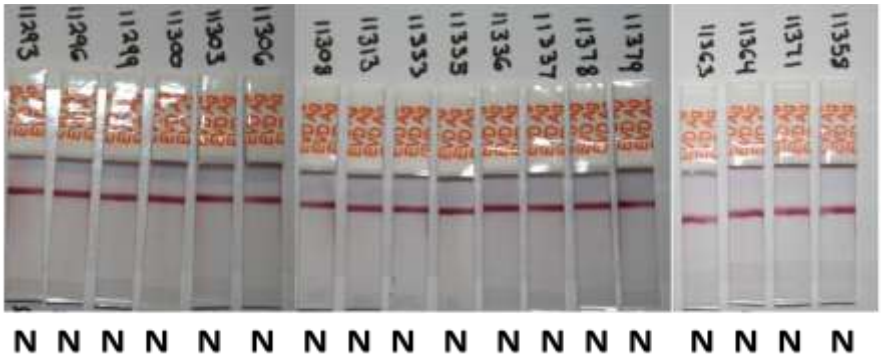


Table 29. Specificity of ReEBOV Ag RDT with US Normal Serum Panels

	RDT Negative	RDT Positive	% Agreement	95th % CI
No Antigen	131	7	94.9 (131/138)	89.8 – 97.9%

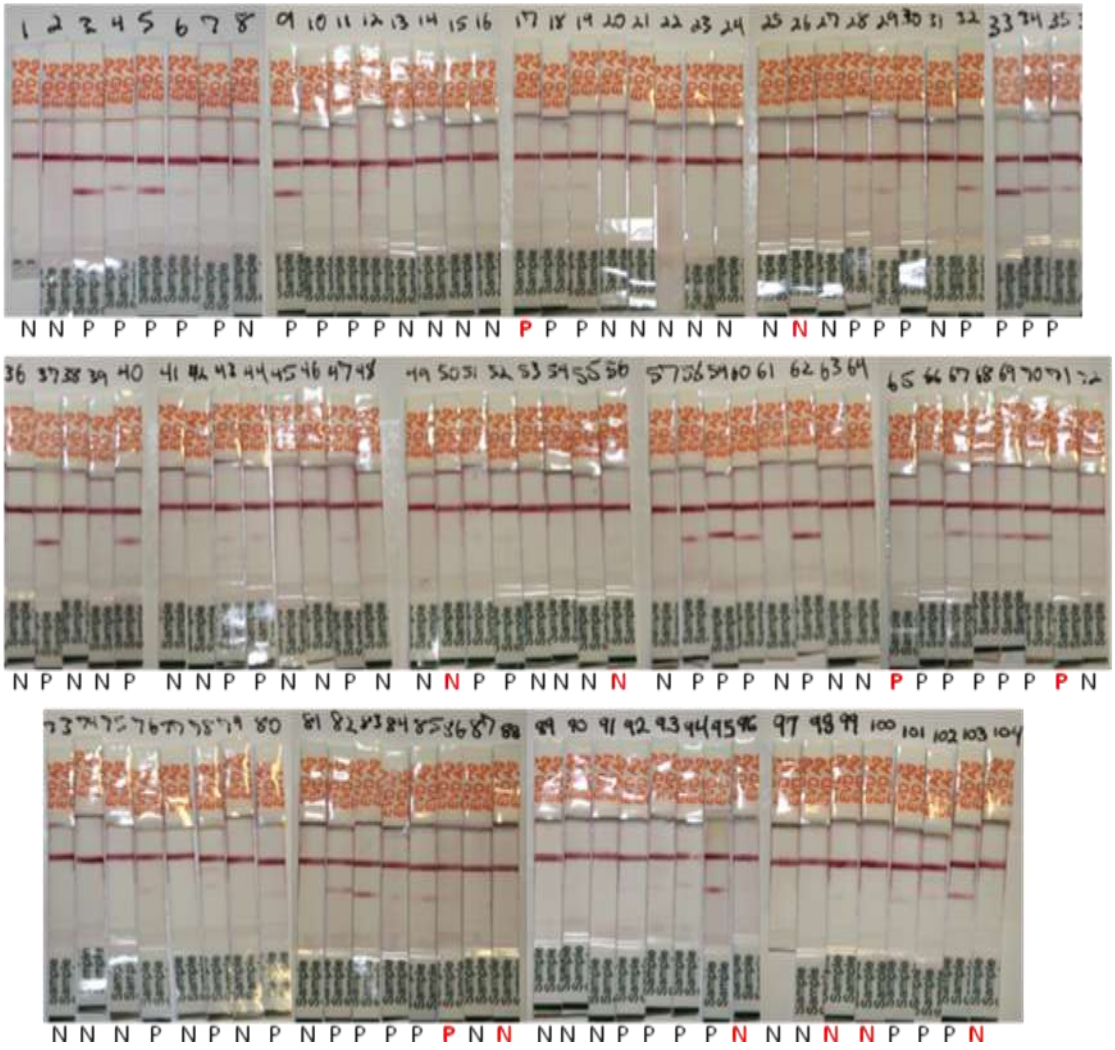
Mock Clinical Study using serum samples

Blinded, randomized design was augmented to verify reproducibility of LOD determined with pooled Bulk Normal Control.

Table 30. Distribution of Mock Clinical Study Samples by Donor Serum

Count Row %	Spiked rVP40 Serum Level (ng/test)								Total % Total
	0.0	**4.7**	**9.4**	**18.8**	**37.5**	**75.0**	**150**	**300**	
430	12 50.0	6 25.0	3 12.5	0	0	3 12.5	0	0	24 4.20
431	18 46.2	9 23.1	9 23.1	0	0	3 7.7	0	0	39 6.82
432	18 42.9	9 21.4	9 21.4	3 7.1	0	3 7.1	0	0	42 7.34
434	18 42.9	9 21.4	9 21.4	0	3 7.1	3 7.1	0	0	42 7.34
435	18 48.7	7 18.9	6 16.2	3 8.1	0	0	3 8.1	0	37 6.47
436	18 45.0	8 20.0	6 15.0	2 5.0	3 7.5	0	3 7.5	0	4 6.99
437	18 47.4	9 23.7	6 15.8	3 7.9	0	0	2 5.3	0	38 6.64
438	18 43.9	9 22.0	6 14.6	3 7.3	0	3 7.3	2 4.9	0	41 7.17
440	11 50.0	6 27.3	0	0	3 13.6	0	0	2 9.1	22 3.85
441	18 42.9	9 21.4	6 14.3	0	3 7.1	0	0	6 14.3	42 7.34
442	17 41.5	9 22.0	6 14.6	0	3 7.3	0	3 7.3	3 7.3	41 7.17
443	18 42.9	9 21.4	6 14.3	0	3 7.1	0	0	6 14.3	42 7.34
444	11 47.8	6 26.1	0	0	3 13.0	0	0	3 13.0	23 4.02
445	12 36.4	6 18.2	9 27.3	6 18.2	0	0	0	0	33 5.77
446	12 36.4	6 18.2	9 27.3	3 9.1	0	0	3 9.1	0	33 5.77
447	12 43.5	6 18.2	9 27.3	3 9.1	0	3 9.1	0	0	33 5.77
Total	249 43.5	123 21.5	99 17.3	26 4.6	21 3.7	18 3.2	16 2.8	20 3.5	572

Table 31. Mock Clinical Performance Using Serum - Set 1



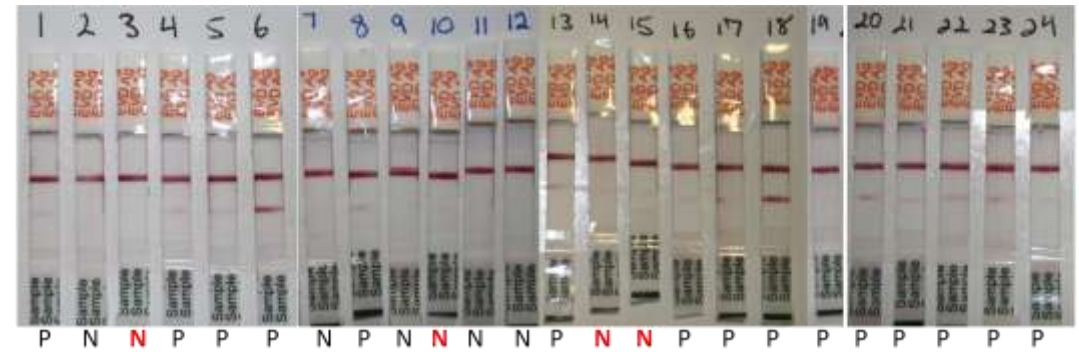
Key:

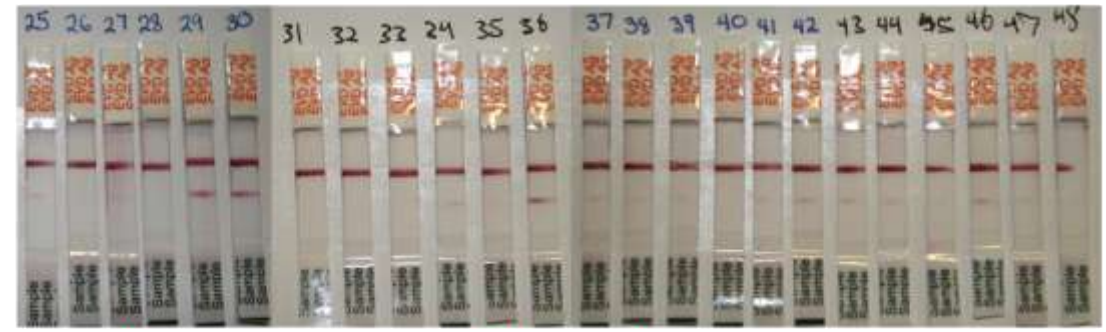
P = At least 2 of 3 observations scored as Positive

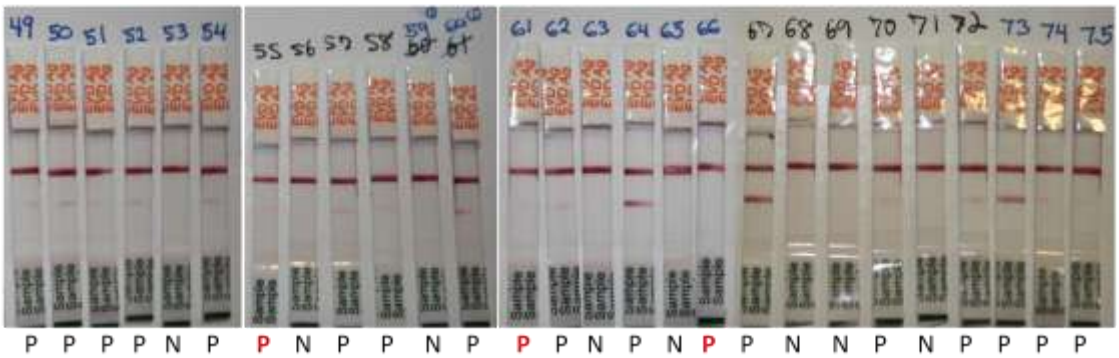
N = At least 2 of 3 observations scored as Negative

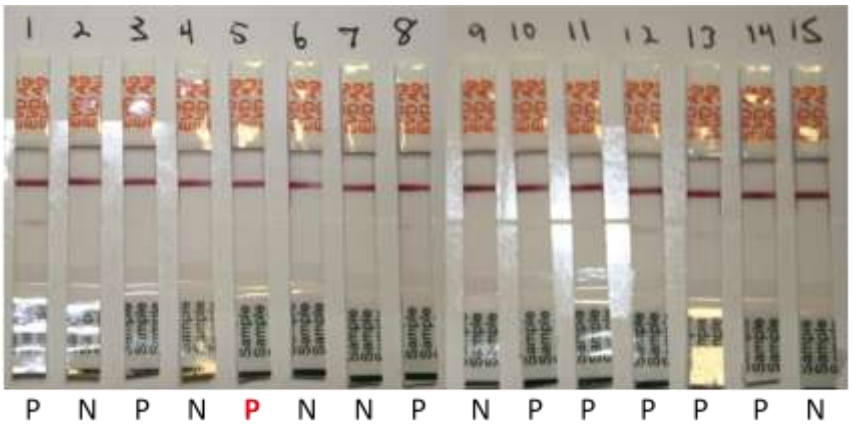
Red= discordant with unspiked or spiked VP40 antigen

Table 32. Mock Clinical Performance Using Serum - Set 2









When read in triplicate, serum not spiked with VP40 antigen totaled 249 observations or 43.5% of design. Serum spiked with VP40 antigen spanning LOD to upper limit of test [4.7 to 300 ng/test] totaled 323 observations. Individual donors did not contribute more the 8% of total observations. Three additional donors (445 – 447) were added to the design in Sets 2 & 3 to make up for observation lost first round donors (430, 440, 444) with depleted samples. Serum samples stored at 2-8°C and VP40 Antigen was spiked on day of test.

Table 33. Percent Agreement - Contrived Serum Samples

Serum	RDT Negative	RDT Positive	% Agreement	95th % CI
No Antigen	200	49	80.3 (200/249)	74.8 – 85.1%
rVP40 Antigen ≥ 4.7 ng/test	23	300	92.9 (300/323)	89.5 – 95.4%
rVP40 Antigen ≥ 9.4 ng/test	7	193	96.5 (193/200)	92.9 – 98.6%

Evaluation of the % Agreement of the two lower levels indicates they are near or above the target specification of ≥95% agreement with LOD. At ≥4.7 ng/test, Agreement is at 92.9% (300/323) and is not significantly different from target of 95% (307/323; p=0.248) which is captured in the confidence interval of 89.5 to 95.4%. The next level ≥9.4 ng/test exceeds the %Agreement specification but is also not significantly different than the target (p=0.431)

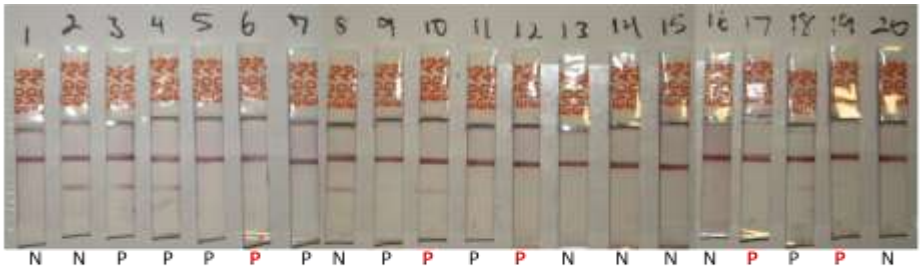
Mock Clinical Study using venous whole blood samples

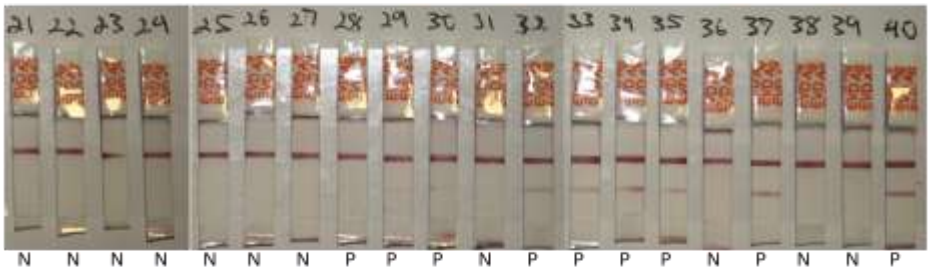
A blinded, randomized design was augmented to evaluate two levels near the LOD to verify reproducibility of LOD determined using a single randomly selected whole blood sample.

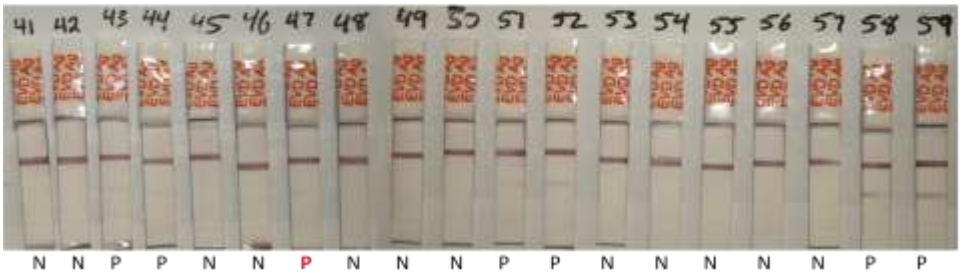
Table 34. Distribution of Mock Clinical Study Samples by Donor Whole Blood

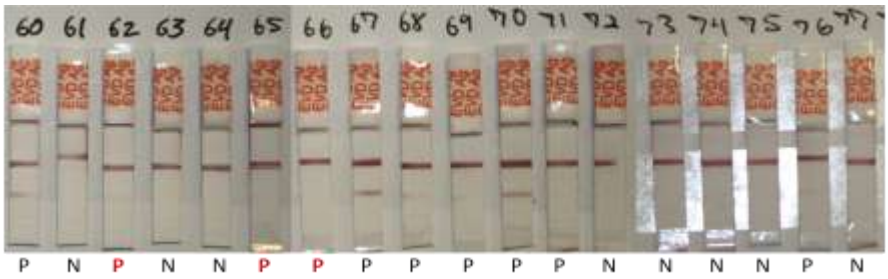
Count Row %	Spiked rVP40 Whole Blood Level (ng/test)								Total % Total
	0.0	9.4	18.8	37.5	75.0	150	300	600	
430	18 42.9	9 21.4	9 21.4	3 7.1	0	3 7.1	0	0	42 7.82
431	18 46.1	9 23.1	9 23.1	0	0	3 7.7	0	0	39 7.26
432	18 42.9	9 21.4	9 21.4	3 7.1	0	3 7.1	0	0	42 7.82
434	18 42.9	9 21.4	9 21.4	0	3 7.14	3 7.14	0	0	42 7.82
435	18 46.1	9 23.1	6 15.4	3 7.7	0	0	3 7.7	0	39 7.26
436	18 42.9	9 21.4	6 14.3	3 7.1	3 7.1	0	3 7.1	0	42 7.82
437	18 46.1	9 23.1	6 15.4	3 7.7	0	0	3 7.7	0	39 7.26
438	18 42.9	9 21.4	6 14.3	3 7.1	0	3 7.1	3 7.1	0	42 7.82
440	18 42.9	9 21.4	6 14.3	0	3 7.1	3 7.1	0	3 7.1	42 7.82
441	18 42.9	9 21.4	6 14.3	0	3 7.1	0	0	6 14.3	42 7.82
442	18 42.9	9 21.4	6 14.3	0	3 7.1	0	3 7.1	3 7.1	42 7.82
443	18 42.9	9 21.4	6 14.3	0	3 7.1	0	0	6 14.3	42 7.82
444	18 42.9	9 21.4	6 14.3	0	3 7.1	0	3 7.1	3 7.1	42 7.82
Total	234 43.6	117 21.9	90 16.8	18 3.4	21 3.9	18 3.4	18 3.4	21 3.9	537

Table 35. Mock Clinical Performance Using Whole Blood - Set 1









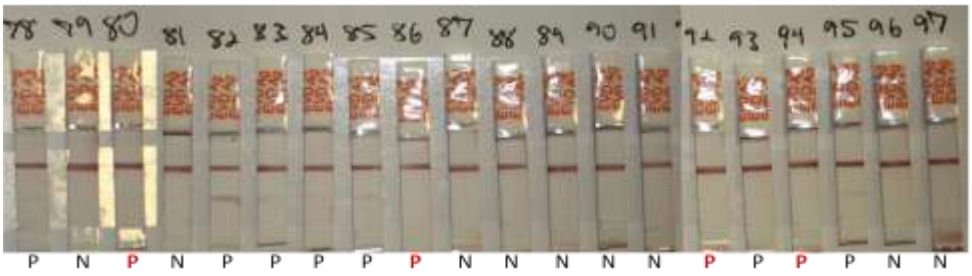
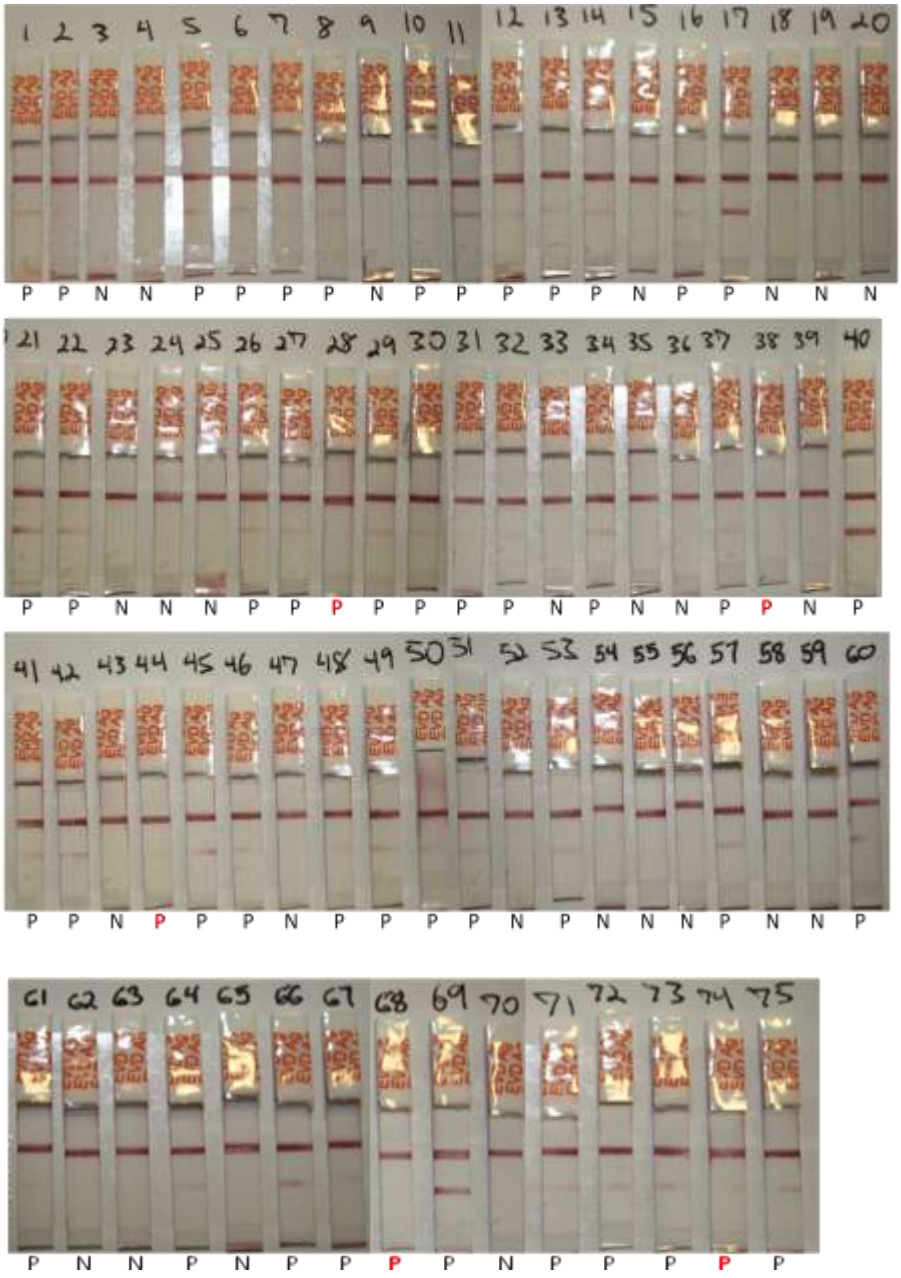


Table 36. Mock Clinical Performance Using Whole Blood - Set 2



Key:

P = At least 2 of 3 observations scored as Positive

N = At least 2 of 3 observations scored as Negative

Red= discordant with unspiked or spiked VP40 antigen

When read in triplicate, whole blood not spiked with VP40 antigen totaled 234 observations or 43.6% of design. Whole blood spiked with VP40 antigen spanning LOD to upper limit of test (9.4 to 600 ng/test) totaled 303 observations. Individual donors did not contribute more the 8% of total observations

Table 37. Mock Clinical Study Performance, Agreement with Specifications

Whole Blood	RDT Negative	RDT Positive	%	95th % CI
No Antigen	226	8	96.6 (226/234)	93.4 – 98.5%
rVP40 Antigen = 9.4 ng/test	46	71	60.7 (71/117)	51.2 – 69.6%
rVP40 Antigen = 18.8 ng/test	2	90	97.8 (90/92)	92.2 – 99.7%

Analysis of false-positives at the first and second antigen levels indicates that the effective LOD when antigen is spiked into a panel of fresh whole blood is 18.8 ng/test. The first level of [9.4 ng/test] was only detected 60.7% (71/117; CI 51.2 – 69.6%) which would satisfy a qualitative cut-off. The second level of [18.8 ng/test] was detected 97.8% (88/90; CI 92.2 – 99.7%) and would satisfy LOD criteria.

5.8 Validation Conclusions

The Analytical Validation of the ReEBOV Ag RDT demonstrated the sensitivity and specificity of the RDT to detect Zaire EBOV including the Makona strain which is responsible for the current West African EHF outbreak. The limit of detection for the RDT was determined to be [4.7 ng/test] for serum and [18.8ng/test] for whole blood. Range of the RDT extended up to [38.0µg/test] without significant Prozone Effect. Limit of detection for live EBOV (Makona strain) was equivalent to [$3x10^4$ pfu/test] when spiked in whole blood.

The RDT successfully demonstrated that drugs in common use including ribavirin, antibiotics, anti-malarials, anti-helmetics, and anti-febrile medication (e.g. paracetamol). Interference by hemoglobin and rheumatoid factor was observed in contrived samples and will be listed as limitations of the test however, the fact that intended sample type is whole blood should mitigate hemoglobin interference. Preliminary Rf screening at Kenema Hospital indicates Rf positive clinical samples are low prevalence and do not significantly bias RDT results.

Specificity of the RDT was demonstrated by the lack of cross-reactivity with non-filovirus febrile illness pathogens including Lassa virus, Dengue virus, Influenza and *P. falciparum*. Nearest-neighbor testing revealed the RDT to be sensitive to the three types of EBOV (Zaire, Sudan, and Bundibugyo) known to cause human EHF including the current Zaire EBOV - Makona strain responsible for the current West African EHF outbreak.

During mock clinical performance validation study inter-operator agreement when reading RDT test results was >95% for negative samples and contrived samples

above the LOD. Positive percent agreement (sensitivity) with expected results was >90% for contrived serum and whole blood samples. Negative percent agreement (specificity) was also >90% for fingersticks, venous whole blood, and US normal serum panels.

Mock sensitivity for rNP spiked serum was determined testing replicates at the LOD (n=99) and at levels above LOD (n=101). Sensitivity for rNP spiked whole blood was determined by testing replicates at the LOD (n=90) and levels above the LOD (n=96). ReEBOV RDT mock sensitivity is summarized in Table 38. ReEBOV RDT mock specificity is summarized in Table 39.

Table 38. Mock Sensitivity Summary

Sample	% Positive Agreement (Sensitivity)	95% CI
Serum ≥9.4 ng/test	96.5 (193/200)	92.9 - 98.6%
Whole Blood ≥18.8 ng/test	93.0 (173/186)	88.3 - 96.2%

Table 39. Mock Specificity Summary

Sample	% Negative Agreement (Specificity)	95% CI
US Normal Panel	94.9 (131/138)	89.8 - 97.9%
Donor Serum Replicates	80.3 (200/249)	74.8 – 85.1%
Donor Whole Blood Replicates	95.0 (399/420)	92.5 - 96.9%
Fingersticks	97.5 (39/40)	86.8 - 99.9%

Chapter 6 ReEBOV Antigen Rapid Test Clinical Validation

The 2014-2015 EHF outbreak proved to be very challenging environment to quickly establish sufficient laboratory capacity to the affected countries. In Sierra Leone, the disease emerged in Kailahun and Kenema Districts of the Eastern Province. At the time, the only hospital lab with the capacity to routinely run both molecular diagnostics (RT-PCR) and immunoassays (ELISA, Western & RDT) for VHF was the Lassa Fever Lab (LFL) at KGH. As the EHF outbreak spread, international health organizations (i.e. US CDC, MSF, WHO) set up additional temporary labs or added testing capacity to existing labs throughout the country. Based on WHO guidance, these new testing sites all used qRT-PCR as their EHF confirmatory test method. The centralized nature of qRT-PCR test methods created difficult logistical problems transporting samples from the surveillance teams and ETU's. Biosecurity protocols and data management issues created further delays in the reporting of laboratory results. When the outbreak had reached national emergency status, the volume of samples exceeded lab capacity resulting in delays up to 5 days or more for reported results. Against this backdrop, Corgenix and its VHFC partners worked with the FDA and WHO to expedite the validation of the ReEBOV RDT to provide a POCT method for EHF screening. The simplicity of the RDT format also presented the opportunity to decentralized EHF screening and empower

surveillance teams to conduct remote RDT testing. The study described herein offered the first proof of the clinical capacity of an EHF RDT.

Authors Note:

The dynamic course the 2014-2015 Ebola outbreak compromised the full implementation of clinical study design as described below. By November 2014, the Ebola outbreak in Kenema District had subsided. Due to the severity of the case load at KGH during June - Sept. 2014, the hospital was no longer treating EHF cases. A holding center was still accepting suspected EHF cases for transport to the IFRC ETU located between the cities of Kenema and Bo. The South African/WHO testing center in Freetown had disengaged from the VHFC study and PHE Kerry Town ETU was working with Partner's In Health (PIH) group to develop an independent study protocol. Tulane's Lassa Fever Program at KGH continued to maintain the EHF sample bank and continued to receive EHF samples from IFRC and other ETU's for LF and EHF diagnostic screening. We proceeded with clinical validation studies of the ReEBOV RDT with the support of both the FDA and WHO using the available banked serum and plasma EHF samples and additional fresh whole blood samples that continued to be received at the Lassa Fever Lab.

6.1 Research Aim and Study Design

Field trials were conducted at KGH to assess the clinical effectiveness of the ReEBOV® Ag Rapid Test (Corgenix, Inc., Broomfield, CO) in support of Emergency Use Authorization applications to both the US FDA and the WHO. The Principal Investigator was Dr. John S. Schieffelin (Tulane University School of Medicine). The co-

investigators were Robert F. Garry, PhD and Matt L. Boisen (Tulane University School of Medicine) and Dr. Donald Grant and Augustine Goba (Viral Hemorrhagic Fever Program, Kenema, Sierra Leone). The principal study site would be Kenema Government Hospital, Kenema, Sierra Leone. The surplus banked samples had been submitted to the Lassa Lab for Lassa fever screening before and during the 2014-2015 EHF outbreak in the eastern province of Sierra Leone. Enrollment and initial screening of these surplus, de-identified samples was performed under several Tulane IRB approved study protocols including "Roles of Protective or Pathogenic B Cell Epitopes in Human Lassa Fever and Ebola Virus Disease" (Tulane IRB 09-00419), "Epidemiologic and Clinical Characterization of an Ebola Outbreak, Sierra Leone, 2014" (Tulane IRB 14-631715), and "Development of a Recombinant Antigen Diagnostic for Ebola Zaire Antigen Detection" (Tulane IRB 14-682862). These studies also received approval from the Sierra Leone Ethics and Scientific Review Committee. Since the ReEBOV development grant (R44 AI088843) only supported the pre-clinical development of Filovirus diagnostics, ReEBOV RDT clinical validation and commercialization was funded separately by awards from Bill & Melinda Gates Foundation (OOP1123820) and the Paul Allen National Philanthropic Trust.

The ReEBOV RDT study design was compliant with appropriate sections of current Good Clinical Practices (cGCP) and followed current regulations:

- Protection of Human Subjects (21 CFR Part 50)
- Financial Disclosure by Clinical Investigators (21 CFR Part 54)
- Institution Review Board (21 CFR Part 56)
- Investigational Device Exemptions (21 CFR Part 812)

The study protocol for the ReEBOV RDT clinical validation, "Development of a Recombinant Antigen Diagnostic for Ebola Zaire Antigen Detection", had the primary objective of validating the diagnostic performance of the ReEBOV RDT compared to the EBOV qRT-PCR reference method [130]. The aims of the study was to validate sensitivity and specificity of the ReEBOV RDT using whole blood from venipuncture (venous) or fingerstick (capillary). Exploratory aims were also approved for assay validation using saliva, breast milk, and urine. Ultimately due to limitations imposed on the study design, banked plasma and serum would be used to establish the RDT performance.

The stated clinical hypothesis was that the ReEBOV RDT diagnostic would maintain clinical performance of no less than 90% sensitivity and 95% specificity when compared to qRT-PCR. This represents an alternative hypothesis that the RDT will be substantially equivalent to the reference method, qRT-PCR. The null hypothesis would be that the RDT is capable of significantly detecting EHF compared to the standard of care for triage which in this case would be the clinical signs (case definition) of EHF.

While the study was entirely conducted outside United State jurisdiction, sample and data management was held compliant with Health Insurance Portability and Accountability Act (HIPAA; 45 CFR Part 46). All personally identifiable record and data were maintained at the primary study site in Kenema. Tulane personnel (Site Manager, Database Manager) were responsible for de-identification of data transferred or transmitted from the study site. Data collection process included assignment of "G-####" series identification number and sequentially logged in log book by KGH personnel. The G-number was included on Informed Consent forms, Pre-Admission Eval and

Admission forms. Laboratory samples (Blood, urine, breast milk) were submitted de-identified with only the G-number. Laboratory sample and results logs were maintain with only de-identified G-numbers. Scanned or electronic clinical and laboratory records had identifiable data removed by software. Data and records were only transmitted via secure file sharing applications.

6.2 Subject Population

6.2.1 Numbers and Sample Types

The clinical study was designed to integrate multiple testing centers to collect venous and or fingerstick whole blood sample data. Proposed sites included KGH and IFRC ETU in Kenema District, Public Health England Kerry Town ETU, and WHO sponsored South African Testing Center in Freetown. Enrollment goals for KGH and Kerry Town ETU were 250 each and the South Africa/WHO testing center enrollment would be 1,000. Total enrollment for the three sites would be 1,500 suspected cases.

All suspected EHF cases would be enrolled based on inclusion criteria (see below). Close contacts, community contacts, nosocomial contacts and laboratory contacts are also eligible for enrollment[131].

6.2.2 Inclusion and Exclusion Criteria - EHF Case Definition

Inclusion criteria was all suspected EHF referred or admitted to the participating sites in Sierra Leone. Exclusion criteria was hemodynamic instability as determined by treating physician, patient unable to cooperate with fingerstick and/or blood draw, and

patient unable to give verbal consent. There was no age limitation on enrollment; children were defined as under 18 years old (yo).

Case Definition for Ebola[1]

- Any person, alive or dead, suffering or having suffered from a sudden onset of high fever (≥ 39°C) and having had contact with:
  - a suspected, probable, or confirmed Ebola case
  - a dead or sick animal
- Any person with sudden onset of high fever and at least three of the following symptoms:
  - headaches
  - anorexia
  - stomach pain
  - vomiting
  - diarrhea
  - lethargy
  - myalgia/arthralgia
  - difficulty swallowing
  - difficulty breathing
  - hiccups
- any person with inexplicable bleeding
- any sudden, inexplicable death

[1]Case definition recommendations for Ebola or Marburg virus diseases. WHO Interim Guideline, 9 Aug 2014[131]

6.2.3 Informed Consent and Patient Data Management

Due to EHF biosafety considerations for the patient and medical staff for the collection of written documentation within treatment or holding centers, the study protocols were granted a waiver of written informed consent for enrollment and waiver of written informed consent for use of excess, de-identified blood samples in accordance

with 45 CFR 46.116(d)(1-4). Verbal informed consent would be obtained from all enrolled suspected EVD cases at each site prior to collection of whole blood, saliva, breast milk or urine. The enrolled subjects will receive a de-indentifying (G-####) study number used to label their laboratory samples and clinical data. Centralized testing centers using excess whole blood samples for this study would not obtain consent. A WHO approved case investigation form would be used to collect clinical data, symptoms and contact information for each suspected case. The study would observe all the privacy and security requirements outlined by HIPAA. All personally identifiable data for this study would be maintained and stored only at the Kenema site. Tulane personnel would be responsible for ensuring the de-identification of the core data needed for this study.

6.2.4 Sample Size

Sample size could not be based on prevalence since Ebola outbreaks are sporadic, the region affected was new to this disease, and at the time of study submission the Ebola outbreak was the largest ever recorded. The sample size estimate was based on the WHO target performance specifications for sensitivity and specificity. The minimum sample size estimate was based on a margin of error for a 95% CI using two-sided type I error at 0.05 (Table 40)[121].

The estimated EHF case rate was 3:1 negative to positive confirmed cases and the hypothesized clinical sensitivity was 90% and specificity was 95%. Based on the required sample size a minimum enrollment of 240 confirmed EHF positives is needed to show the true sensitivity was no less than 90% (216/240; 95% CI 85.5 - 93.2). The minimum of 170 confirmed EHF negative is needed to show the true specificity was no

less than 95% (162/170; 95% CI 90.8 - 97.7). The enrollment targets of 500 case and 1500 controls far exceeded the numbers required to meet the performance estimates.

Table 40. Number of required suspected cases for level of sensitivity (or specificity) and margin of error[1]

	Margin of Error[2] for 95% CI				
Sensitivity or Specificity	0.02	0.05	0.10	0.15	0.20
0.75	1941	340	95	45	26
0.80	1697	306	88	43	25
0.85	1405	265	80	40	24
0.90	1066	216	70	37	25
0.95	683	162	60	40	25

[1] Derived using the CI interval formula for a single proportion by Weiss et. al. (2003)

[2] Defined as half of the confidence interval width

6.2.5 Data Analysis

Studies to evaluate the performance will follow CLSI guidance EP12-A2, User protocol for Evaluation of Qualitative Test Performance[117]. The reference method will be EBOV qRT-PCR. The following outcomes will be measured for the ReEBOV RDT:

- Sensitivity (Positive Percent Agreement)
- Specificity (Negative Percent Agreement)
- Positive Predictive Value (PPV)
- Negative Predictive Value (NPV)
- Diagnostic Likelihood Ratio
- Accuracy

6.3 Study Procedures

6.3.1 Sample Collection

As noted previously, enrollment of active EHF cases at KGH site was not possible but several whole samples were transferred from the nearby IFRC ETU. The majority of samples included in this study were from the Lassa Fever Lab sample bank. Clinical data collected for these samples (including patient outcome data) has been sequestered by the SL Ministry of Health and is not available for analysis of morbidity and mortality correlation with laboratory results.

During the initial stages of the Sierra Leone EHF outbreak serum was primarily collected for Lassa fever screening then later Ebola screening. As the EHF outbreak progressed and testing guidelines developed, sample collection switched over to whole blood collection required by EBOV qRT-PCR methods. This creates a temporal transition in the sample bank with older EHF samples predominately banked serum and more recent samples being predominately banked plasma.

Description of approved sample types and collection methods

Samples will be tested by EBOV qRT-PCR to aid in the diagnosis of EVD in conjunction with clinical signs and symptoms. Fingerstick testing will be performed at presentation and results scored as presumptive results while awaiting qRT-PCR confirmatory test results. During the course of hospitalization ReEBOV ELISAs and RDT will be used to monitor EBOV antigenemia and anti-EBOV sero-reactivity. Long-term follow-up testing will be conducted to screen for convalescent IgM and IgG titers.

Patient will be tested at least 3 times but no more than 5 times during their hospitalization.

Capillary blood will be collected by fingerstick. A trained technician will clean finger with alcohol swab an perform fingerstick with safety lancet. Initial drop is typically wiped away and a second drop allowed to develop. Once a full drop (~30µL) forms, it is directly transferred to the Sample Pad of the ReEBOV RDT by touching droplet to the dipstick. The ReEBOV RDT is immediately inserted into test tube containing Sample Buffer and proceed with incubation and interpretation of the test.

Whole blood will also be collected by venipuncture using EDTA whole blood collection tubes and serum tubes. Trained phlebotomists who performed both the fingerstick and venepuncture followed strict biosafety precautions according to WHO blood collection guidelines[132]. Collected samples were transported to laboratory for diagnostic EHF screening including RDT and qRT-PCR. After testing, whole blood may be centrifuged and surplus plasma banked for later testing. This study protocol also allows for the collection of saliva, breast milk, and urine. Modifications to RDT procedure may be required for testing these clinical matrices.

Human Subjects

The clinical research, including all human subjects tested at the KGH, was approved by the Sierra Leone Ethics and Scientific Review Committee. Suspected EHF cases and close contacts of confirmed cases were eligible for enrollment in this study as outlined by the study protocol approved by Tulane University's Institutional Review Board (IRB) and following the National Institutes of Health/National Institutes of Allergy

and Infectious Diseases (NIH/NIAID) guidelines governing the use of human subjects for research. Human immunodeficiency virus and hepatitis virus testing was not performed due to IRB and ethics committee considerations.

Only KGH staff were involved in the administration of healthcare to patients at the KGH Lassa Ward. All medical decisions, were at the sole discretion of the attending KGH Lassa Ward physician. Small blood volumes (typically 5 mL) for VHF screening were collected from study subjects in vacutainer tubes by experienced phlebotomists. For serum. the blood was allowed to coagulate for 20 min at room temperature, and then the serum was separated from the coagulated blood by centrifugation (200 *g* for 20 min at room temperature). For plasma, whole blood-EDTA tubes were separated by centrifugation (200 *g* for 20 min at room temperature) and plasma supernatant removed by pipette. Aliquots of the plasma and serum were used immediately for testing, and the remaining serum was stored in cryovials at −20°C for future use.

6.4.2 Methods

Whole blood samples from suspected VHF cases were delivered to the Lassa Fever Lab and entered into Sample Log after assignment of de-identifying G-series number. To maintain biosecurity, all manipulations of the clinical samples were performed in laminar flow hoods within the Lassa Fever Lab by staff wearing full PPE including disposable Tyvek coveralls, rubber boots, N91 masks and full length face shields. An aliquot of each sample is taken for whole blood viral RNA extraction and whole blood RDT screening. Remaining sample is centrifuged to separate plasma which was aliquoted for sample banking and additional screening of plasma in RDT and ELISA.

Previously banked samples were thawed in the biohood, remixed, and blinded for RDT screening. US normal serum panels or SL asymptomatic controls were included in sample screening for internal negative reference.

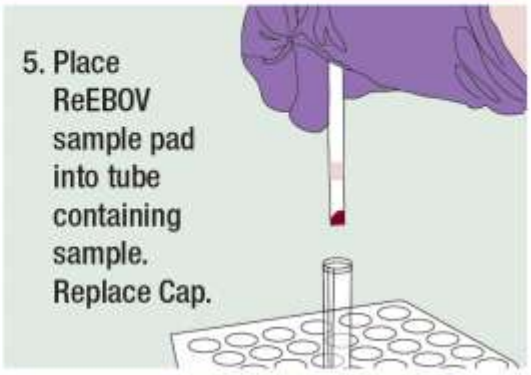
ReEBOV Antigen Rapid Test

As previously described (See 5.3), whole blood, plasma, or serum samples were tested on the ReEBOV RDT. Four drops (~200µL) of Sample Buffer was added to labeled 10x75mm test tubes. For testing, 30µL of sample was added to a RDT dipstick using vapor barrier pipet tips. Dipsticks were then inserted into test tube incubated for 15 min before reading the test line signal visually. After scoring the RDT signal, the dipstick was removed from the test tube for measuring reflectance (mV) of the test signal using a ESEQuant Lateral Flow Reader (LFR; Qiagen Lake Constance, GmbH). Each test, visual score and reflectance were recorded on ReEBOV RDT worksheets and digital photograph taken. RDT measurements were also transferred to secured lab database and manually entered into lab results log book. RDT results were also recorded on the Lab Results Worksheet compiled for each patient sample.

ReEBOV™ Antigen Rapid Test Instructions

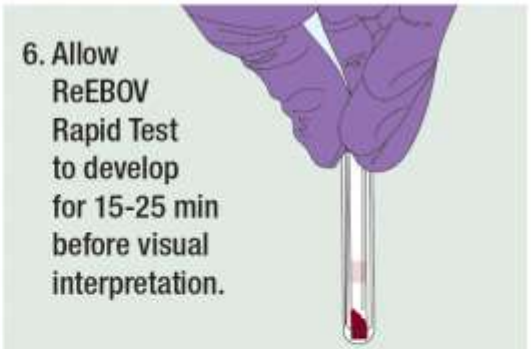
For Detection of Ebolavirus VP40 Antigen

1. Add 4 drops Sample Buffer to plastic tube.



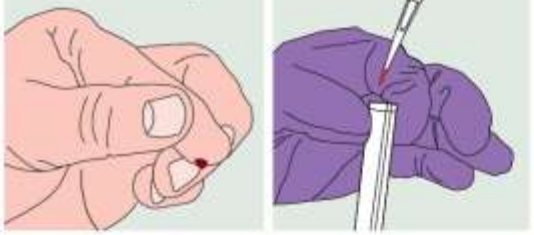
5. Place ReEBOV sample pad into tube containing sample. Replace Cap.

2A. Use safety lancet to perform finger stick. *or* 2B. Use a venipuncture.

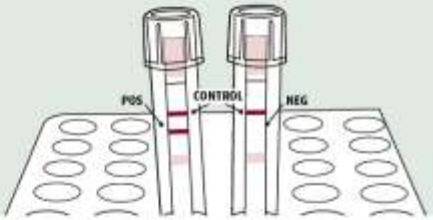


6. Allow ReEBOV Rapid Test to develop for 15-25 min before visual interpretation.

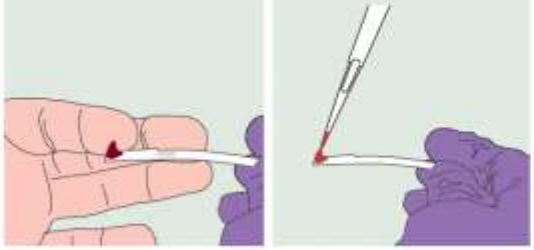
3A. Allow large drop of blood to develop. *or* 3B. Remove blood with pipette



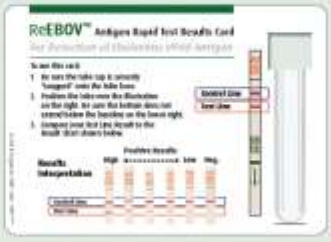
7. Visual Interpretation
   - Top line is control stripe
   - Bottom line is a positive test line



4. Transfer drop of blood on to ReEBOV Rapid Test sample pad.



8. Use the Visual Aid card provided to assist in result interpretation.



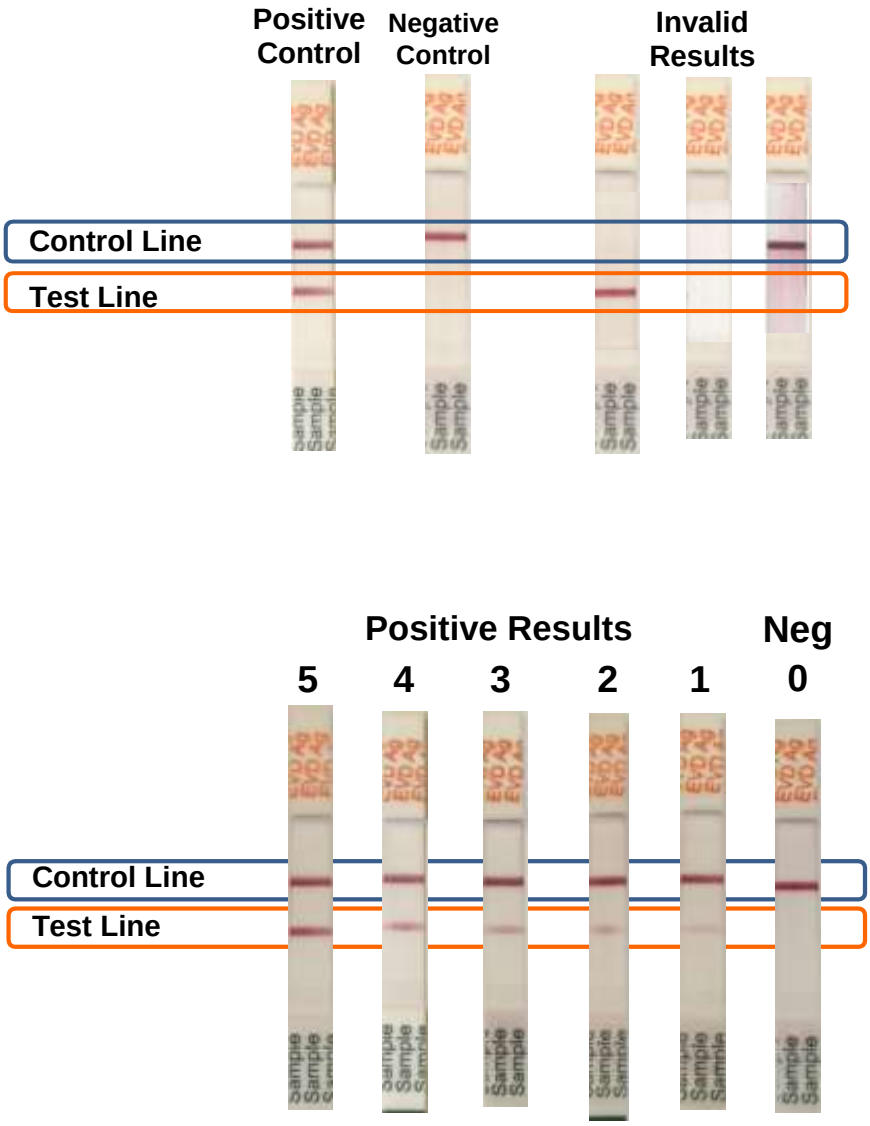


Figure 27. ReEBOV Antigen Rapid Test Visual Aid

The package insert visual aid provides the operator with a reproduction of valid Positive and Negative Control results and a panel of invalid test results to aid in correct interpretation of the rapid test. A scale of Test Line signal intensity is also provided to help guide results interpretation.

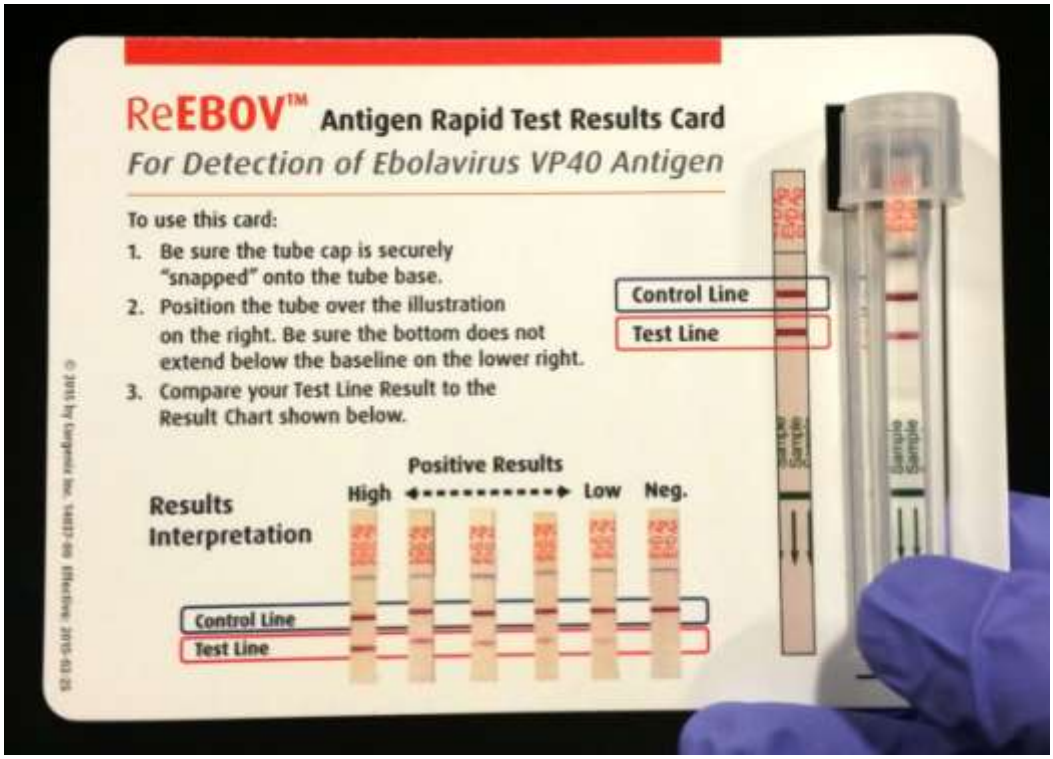


Figure 28. ReEBOV Antigen Rapid Test Kit Results Card

The Results Card visual aid was developed with the FDA and WHO collaboration to aid ReEBOV RDT operators in test results interpretation. The card provides a full scale image of a developed rapid test and hole to align the capped tube. Correct alignment allows inexperienced operators to correctly identify the Control and Test Line signals. A scale of Test Line signal aids in test interpretation.

ReEBOV IgM/IgG ELISA

ReEBOV IgG/IgM ELISA sample dilution and addition to the 96-well microwell plate is performed in the biohood using vapor barrier pipet tips. Each plasma or serum sample is diluted 1:99 (10µL:990µL) in Sample Diluent using 2mL deep well sample blocks and mixed by pipet. Reference and Negative Controls are reconstituted with

250µL diH_2O (Direct-Q, Millipore, USA). Both Reference and Negative Control were diluted 1:99 (10µL:990µL) in Sample Diluent and mixed by pipet. The Reference was further serially diluted 3-fold by five successive transfer of 250µL into 500µL Sample Diluent. An EHF convalescent IgG/IgM positive clinical sample was also prepared as a second internal reference. Samples are tested in duplicate wells (100µL/well) for a maximum of 40 per plate including clinical sample reference (strip-well columns 3-12). Reference dilution curve, negative control, and reagent blank are added to strip-well column 1-2 (see plate map Fig. 41). Positive cut-offs of OD_{450nm} = 0.320 for IgG and OD_{450nm} = 0.220 for IgM was previously established (See Ch. 5.3).

ReEBOV Antigen ELISA

ReEBOV Ag ELISA sample dilution and addition to the 96-well microwell plate is performed in the biohood using vapor barrier pipet tips. Each plasma or serum sample is diluted 1:9 (50µL:450µL) in Sample Diluent using 2mL deep well sample blocks and mixed by pipet. Reference and Negative Controls are reconstituted with 250µL diH_2O (Direct-Q, Millipore, USA). Both Reference and Negative Control were diluted 1:9 (50µL:450µL) in Sample Diluent and mixed by pipet. The Reference was further serially diluted 3-fold by five successive transfer of 125µL into 250µL Sample Diluent. A confirmed EHF antigen positive clinical sample (from LFL sample bank) was also prepared as a second internal reference. Samples are tested in duplicate wells (100µL/well) for a maximum of 40 per plate including clinical sample reference (strip-well columns 3-12). Reference dilution curve, negative control, and reagent blank are

added to strip-well column 1-2 (see plate map Fig. 41). Positive cut-off of OD_{450nm} = 0.120 was previously established (see Ch. 5.4).

Table 41. Example Plate Map for ReEBOV ELISA Tests

	1	2	3	4	5	6	7	8	9	10	11	12
A	Kit Ref		1- Ref*		9-Sample		17-Sample		25-Sample		33-Sample	
B	Diln 1		2-Sample		10-Sample		18-Sample		26-Sample		34-Sample	
C	Diln 2		3-Sample		11-Sample		19-Sample		27-Sample		35-Sample	
D	Diln 3		4-Sample		12-Sample		20-Sample		28-Sample		36-Sample	
E	Diln 4		5-Sample		13-Sample		21-Sample		29-Sample		37-Sample	
F	Diln 5		6-Sample		14-Sample		22-Sample		30-Sample		38-Sample	
G	Neg Ctrl		7-Sample		15-Sample		23-Sample		31-Sample		39-Sample	
H	Blank		8-Sample		16-Sample		24-Sample		32-Sample		40-Sample	

*Clinical Reference consisting of previously confirmed sample.

EBOV qRT-PCR

Viral RNA was extracted from 140µL of clinical samples (fresh whole blood or banked serum and plasma) using the QIAmp Viral RNA Mini Kit (Qiagen GmbH, Germany) as per the manufacturer's recommended protocol. Final elution of viral RNA was 80µL of AVE buffer and stored at -20°C until needed. qRT-PCR reactions were set-up as described in Trombley et. al., 2010[130]. qRT-PCR was performed using the SuperScript™ III One-Step RT-PCR with Platinum *Taq* DNA Polymerase (Life Technologies). Master Mix was scaled to the number of PCR samples and controls. Forward and reverse primers were used for both Zaire EBOV GP (F2000, R2079) and NP (F565, R640) as well as FAM-probe for both.

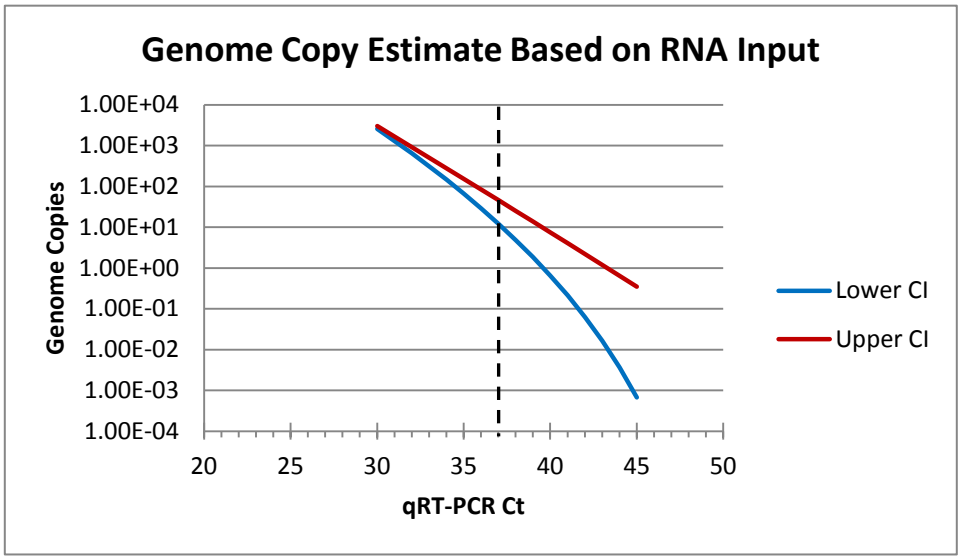
qPCR Program "EBOV.RT"

View:		Lid: 105^{o}C
LASV.RT		Vol: 26uL
1 = 50.0^{o}C	For 15:00	RT of RNA into cDNA
2 = 95.0^{o}C	For 5:00	Denaturation of RT
3 = 95.0^{o}C	For 0:01	Denature cDNA
4 = 60.0^{o}C	For 0:20	Annealing of Primers, extension, annealing of probes, release of fluorescent dye*
5 = GOTO	3, 44 times	# of PCR cycles
6 = 40.0^{o}C	For 0:30	Final extension

*A single fluorescence read is measured at the end of each 60^{o}C step.

qRT-PCR samples were analyzed on an Applied Biosystems StepOnePlus™ RT-PCR System. Total program run time ~ 1 hour.

EBOV qRT-PCR cut-off of ≥ 37cycles was established by non-linear regression analysis of an Ebola virus RNA standard (Primer Design, UK). The standard curves generated were used to estimate a Ct value near the lower limit of detection. At Ct = 37 was the lowest Ct with the mean-SD >10 genome equivalents.



RNA copies	Ct	Ct SD	lower 95%CI	upper 95%CI
1000000	19.95	0.062	19.89	20.01
100000	24.32	0.042	24.28	24.36
10000	27.90	0.076	27.83	27.98
1000	31.44	0.145	31.29	31.58
100	35.22	0.653	34.57	35.87
10	37.22	0.772	36.45	37.99

Ct	# genomes	
	lower 95%CI	upper 95% CI
45	6.69E-04	0.347
43	0.016	1.201
41	0.213	4.106
39	1.856	13.892
37	12.315	46.553
35	66.930	154.733
33	313.048	510.742

Table 42. Regression analysis of Primer Design quantified Ebola virus RNA Standard

Confidence Intervals for RNA copy Ct non-linear regression established Ct=37 as the highest number of cycles to detect ≥ 10 genome equivalents.

6.4.3 Statistical analysis

Data were analyzed using v11.0 (SAS Institute, Inc.) and GraphPad Prism v. 6.04 (GraphPad Software, Inc.) Hypotheses involving dichotomous response variables were tested using Students t test, Fisher's exact test or logistic regression with receiver operating characteristics. Binomial proportion comparisons use to establish significance across contingency estimates. Analyses were two-tailed, with a significance threshold set at $p<0.05$. Confidence intervals (95%): Agresti and Coull (1998)

6.5 Study Results

Total enrollment in the clinical study was 408 samples including 196 plasma and 212 serum samples with paired RDT and qRT-PCR data. Age and sex distribution data was not available. The confirmed EHF (qRT-PCR+) prevalence rate was 52.45% (214/408) overall. EHF prevalence for plasma samples was. 61.32% (130/212) vs. 42.86% (84/196) for serum which was significant ($p = 0.0002$). A preliminary Logistic Fit analysis comparing serum and plasma Response Operator Characteristics (ROC) revealed similar performance (Fig.29). The plasma testing area under curve (AUC) was 0.970 and generated an optimal sensitivity of 96.0% and specificity of 93.4% at a qPCR Ct = 35.3. For serum the AUC was 0.937 and generated sensitivity of 89.2% and specificity of 90.4% at a qPCR Ct =37.38.

Comparison of the ReEBOV RDT clinical performance by sample type revealed an apparent significant difference (Table 43). Plasma generated greater specificity (97.3%; 109/112) than sensitivity (85.7%; 72/84). Serum produced the opposite performance with sensitivity (94.6%;123/130) than specificity (80.5%; 66/82). However

the distribution of data was similar between sample types (Fig. 30) so a means comparison was performed.

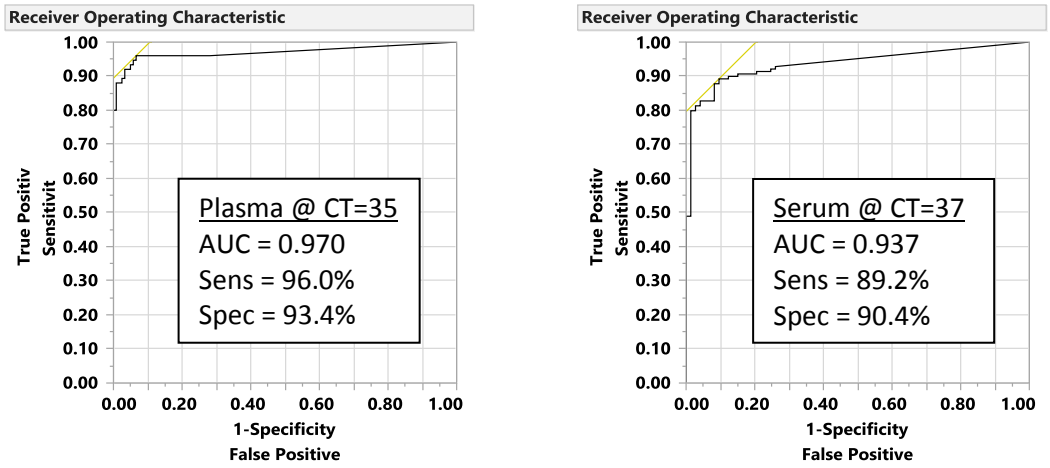


Figure 29. Logistical Fit - ROC of RDT vs qRT-PCR for Serum vs Plasma Bias

Plasma had slightly higher overall performance sensitivity at 96.0% and specificity at 93.4% with a qPCR Cut-off of Ct ≥ 35. Serum generated sensitivity of 89.2% and specificity of 90.4% using qPCR cut-off Ct ≥37.

Table 43. ReEBOV RDT Performance Bias by Sample Type

Using previously described qPCR Ct ≥ 37 cut-off, evaluation of RDT performance shows significant advantages for sensitivity or specificity. However, neither sample type has overall superiority.

Sample Type	RDT % Sensitivity		RDT % Specificity	
Serum	94.6 (123/130)	CI 89.2 - 97.8	80.5 (66/82)	CI 70.3 - 88.4
Plasma	85.7 (72/84)	CI 76.4 - 92.4	97.3 (109/112)	CI 92.4 - 99.4
P value	0.0254		<.0001	

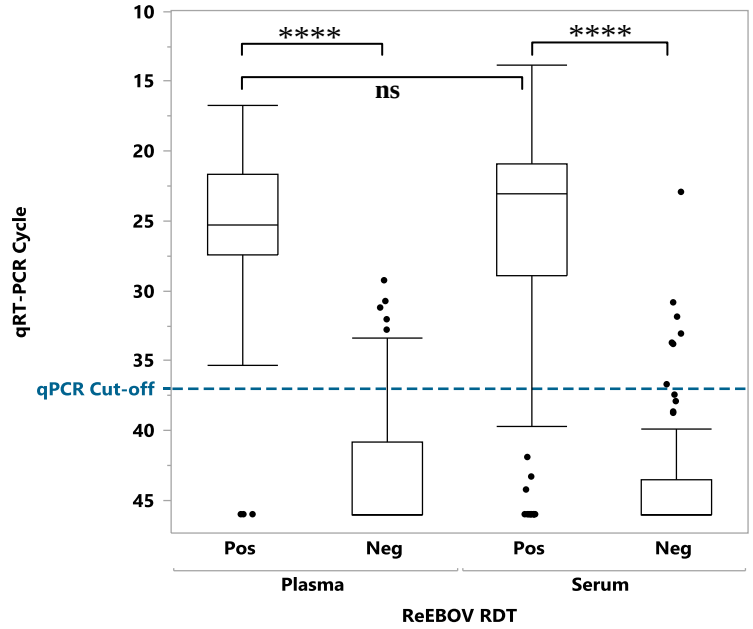


Figure 30. Means Testing of qRT-PCR by Sample Type and RDT Result. Testing the qPCR Ct Mean ± SD variance of the RDT positives and negative revealed no significance difference. This would indicate that except for outliers plasma and serum have similar Ct distributions.

Table 44. qRT-PCR Ct Means Testing Data For RDT Plasma - Serum Bias Test.

	Rapid Test Positive		Rapid Test Negative	
Sample Type	Plasma	Serum	Plasma	Serum
Ct Mean	25.54	26.07	43.57	43.78
Ct SD	6.01	8.01	4.27	4.61
SEM	0.6940	0.6794	0.3882	0.5396
N	75	139	121	73
p value	p = 0.6164		p = 0.7478	

Means testing (Table. 44) confirmed no significant difference in the reference method qRT-PCR Ct mean ± SD between plasma and serum for RDT positives (p=0.6164) and RDT Negatives (p=0.7478). Based on this finding the combined dataset would be used for the clinical performance estimate. Another contributing factor is that by keeping plasma and serum separate, neither satisfies the minimum sample size estimate for 0.95 sensitivity (or specificity) within a 0.05 margin of error for 95% confidence interval.

The visual scoring of the ReEBOV RDT also revealed a significant correlation of qRT-PCR and RDT signal however, the qRT-PCR cycles maintain an inter-quartile range of CT 20.8 - 26.5 for RDTs scored 2 or higher (Fig. 31). Mean comparison for qRT-PCR CT between the higher RDT scores (e.g. score 2 vs 4, Mean CT p = 0.0002) was significant however, given the subjective nature of the RDT scoring attempting to assign a linear correlation between qPCR (viral RNA) and RDT signal (viral antigenemia) is not appropriate.

The relationship of ReEBOV RDT scoring to EHF qPCR demonstrates high positive predictive value (PPV) when visual signal is scored 2 or higher (Table 45). However, the number of samples at each signal levels is not sufficient to establish PPV with less than a 0.05 margin of error (95th% CI). The combined PPV for the moderate (2-3) and high (4-5) visual scoring is 97.0% (161/166; 95% CI 93.1 - 99.0%) with a margin of error 0.03.

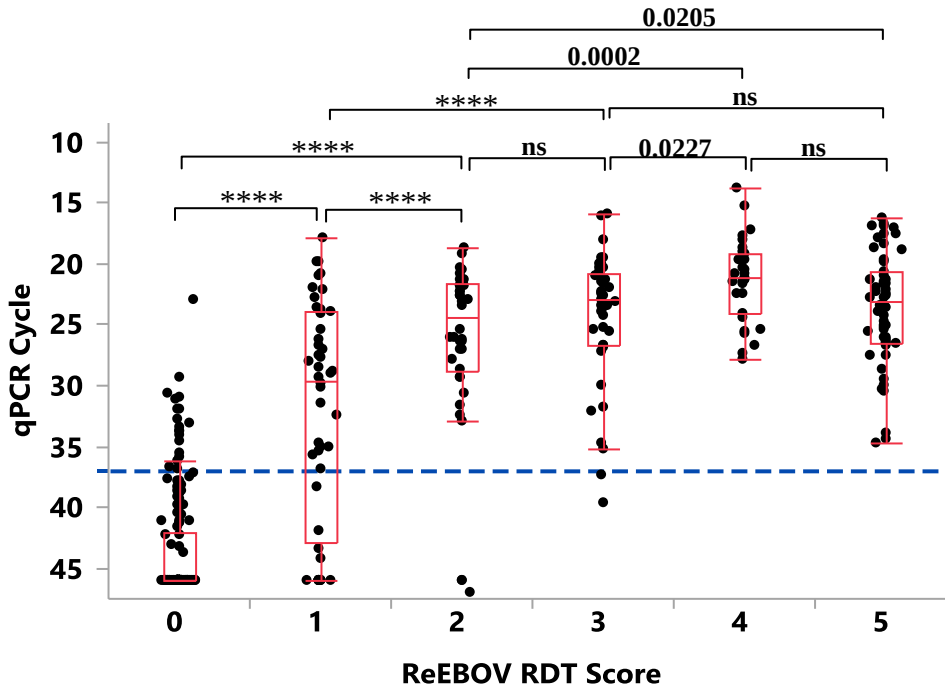


Figure 31. Means comparison of qRT-PCR Ct by visual RDT Score. There is a significant relationship between negative (0) signal and the development of low to moderate (1-3) signal. However, at a score ≥2 the PCR Ct's remain positive with an inter quartile range of 20.8 - 26.5.

Table 45. Positive Agreement of Stratified ReEBOV RDT Visual Scores

RDT Visual Score	qRT-PCR Pos.	qRT-PCR Neg.	PPV	95th% CI	Performance Signal ≥ 2
5	56	0	100	96.3 - 100%	PPV 97.0% (161/166) 95th% CI 93.1 - 99.0%
4	31	0	100	88.8 - 100%	
3	39	2	95.1	83.5 - 99.4%	
2	35	3	92.1	78.6 - 98.3%	
1	34	14	70.8	56.7 - 81.9%	

Preliminary RDT performance estimate by qRT-PCR Ct mean testing and Logistic Regression indicate significant diagnostic capability in detecting EHF. Using the previously described qRT-PCR cut-off of Ct ≥ 37, a mean difference of 17.7 Ct's resulted in p <.0001. The interquantile range of Ct's was 21.3 - 28.2. The upper 90% quantile of RDT negatives was equal to the Ct 37 cut-off (Fig. 32A). The lower 90% quantile for RDT positives was at Ct = 35.5. The ROC analysis actually recommends Ct 35.4 as the optimal qPCR cut-off but assessing the assay performance at Ct 36.9 estimates sensitivity at 91.1% (195/214; 95% CI 86.5 - 94.6%) and specificity 90.2% (175/194; 95% CI 85.1 - 94.0%) with AUC = 0.949(See Fig 32B.)

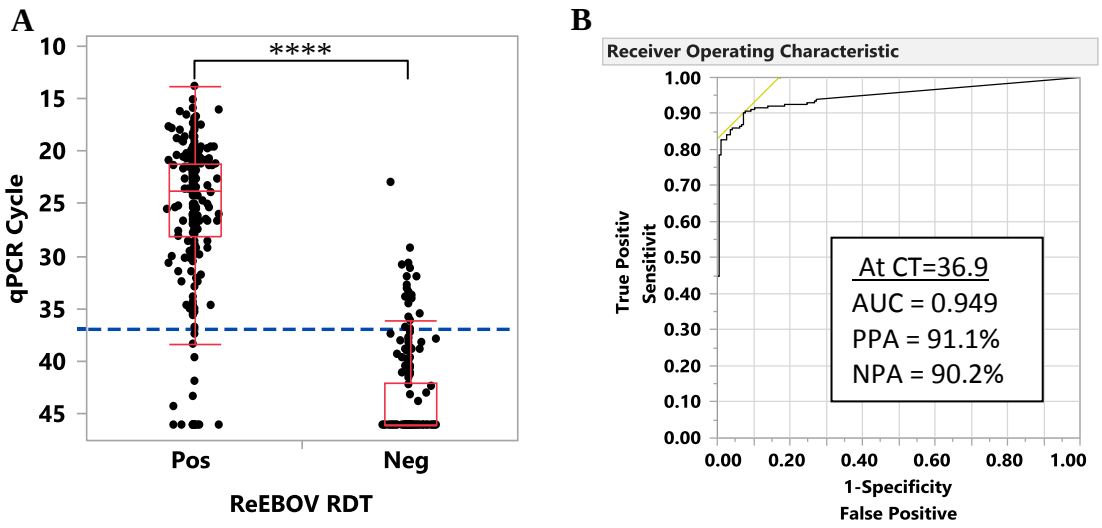


Figure 32. Means Testing and Logistic Fit Analysis of ReBOV RDT vs qPCR.

(A) Mean comparison of RDT results and qPCR Ct generate significant (p<.0001) difference in Mean Ct with the current qPCR cut-off at the 90th% quantile of RDT negatives. (B) Logistic Fit and ROC analysis confirm the Ct=37 qPCR cut-off and estimates clinical performance of ReEBOV RDT at 91.1% Positive Agreement and 90.2% Negative Agreemant with EBOV qRT-PCR. (Fishers exact p<.0001****)

The ReEBOV RDT clinical performance failed to rejects the clinical hypothesis that RDT sensitivity will meet or exceed 90% and that the specificity will meet or exceed 95% (Table 46). Performance testing generated a sensitivity of 91.1% (195/214; 95% CI 86.5 - 94.6%) which comparison of binomial proportions (Ho $_{p}1 = {}_{p}2$) with the minimum required sample size (216/240) shows there is no significant difference (p=0.684) and the margin of error was only 0.041 despite not reaching minimum target enrollment for confirmed EHF positives.

Table 46. Clinical Performance of ReEBOV Antigen Rapid Test

Clinical Performance	ReEBOV RDT Positive	ReEBOV RDT Negative	Total
qRT-PCR Positive	195	19	214
qRT-PCR Negative	19	175	194
Total	214	194	408
Sensitivity	91.1% (195/214)	95% CI 86.5 - 94.6%	
Specificity	90.2% (175/194)	95% CI 85.1 - 94.0%	
PPV	91.1% (195/214)	95% CI 86.5 - 94.6%	
NPV	90.2% (175/194)	95% CI 85.1 - 94.0%	
Accuracy	90.7% (370/408)	95% CI 87.5 - 97.2%	
Likelihood	9.30 (p<.0001)		

The specificity of 90.2% (175/194; CI 85.1- 94.0) failed to exceed 95% however the binomial proportion of an equivalent (n) with 95% specificity (184/194) reveals a marginally significant difference (p = 0.0823). Accuracy of ReEBOV RDT to qRT-PCR is 90.7% (370/408; 87.5 - 97.2%) which compared to the minimum sampling of (162/170) for 95% estimate again revels no significant difference in the two estimates (p = 0.662). Diagnostic likelihood for a positive ReEBOV RDT is 9.30 and is as much as 4-fold higher than clinical diagnostic likelihood based solely on case definition without laboratory testing[19, 133, 134].

Discrepant analysis using the ReEBOV Ag ELISA as a non-reference standard indicates that the 14 false-positive may be due to interference by IgG/IgM sero-positive samples reflected in the 67.6% sero-positive rate for RDT+/ELISA- samples[122]. The 18 false-negatives are associate with both higher qPCR CT range which spans the qPCR Ct cut-off of ≥37 cycles and elevated antibody sero-positive rate as well (55.5% vs 38.6% overall, p=0.0167). The 38.6% antibody sero-positive is a potential source of bias due to interference in an antigenemia test by apparently post-acute EHF patient enrollment.

Table 47. Three-way discrepant analysis incorporating Non-reference Standard (Ag ELISA) and Reference Standard (qRT-PCR)

ReEBOV RDT	ReEBOV Ag ELISA	Total Subjects*	qRT-PCR Reference Standard		IgG/IgM Seropositive
			Pos	Neg	
Pos	Pos	175	171[1]	4	25.9%
Pos	Neg	34	20[2]	14	67.6%
Neg	Pos	2	1	1	0%
Neg	Neg	189	18[3]	171	55.5%
	Total	400	211	190	38.6%

*8 samples ReEBOV Ag ELISA not done

[1]qPCR CT Mean 23.7 ± SD 5.0

[2]qPCR CT Mean 29.8 ± SD 4.8

[3]qPCR CT Mean 33.5 ± SD 2.2

Logistical analysis of the RDT performance ± sero-positives determined that EBOV specific IgG does reduce RDT performance. ROC analysis demonstrates IgG sero-positives reduced sensitivity to 83.3% (40/48; 95% CI 70.2 - 91.6%) with a slight increase in specificity to 91.8% (56/61; 95% CI 81.8 - 96.8%) when using a Ct = 37.4 cut-off (AUC 0.897). Conversely, absence of IgG sero-positives enhanced RDT performance sensitivity or specificity depending on the Ct cut-off used. The ROC analysis revised the cut-off for optimal assay performance down from 37 to 30.7 Ct's. Without a change to the Ct 37 cut-off, sensitivity is enhanced to 94.4% (151/166; 95% CI 89.5 - 97.2%) with a reciprocal decrease in specificity to 88.5% (115/130; 95% CI 81.7 -

93.0%). By reducing the qPCR cut-off to the optimal Ct = 30.7 there is a reversal to an enhanced specificity of 98.5% (128/130; 95% CI 94.8 - 99.9%) and lowered sensitivity 88.8% (142/160; 95% CI 82.8 - 92.9%). The overall affect IgG sero-positivity has on the ReEBOV RDT (compared to the overall diagnostic likelihood of 9.6) is that by screening out IgG sero-positives patients who are possibly EHF post-acute or EHF convalescent, you significantly increase the diagnostic likelihood ratio 8.2 → 57.7 (when Ct 37 → 30.7) for identifying acute EHF patients who potentially pose a higher transmission risk. It should be noted that there was actually a slight increase in diagnostic likelihood to 10.2 for the IgG sero-positive samples. Small sample size can be an additional source of error in this likelihood estimate.

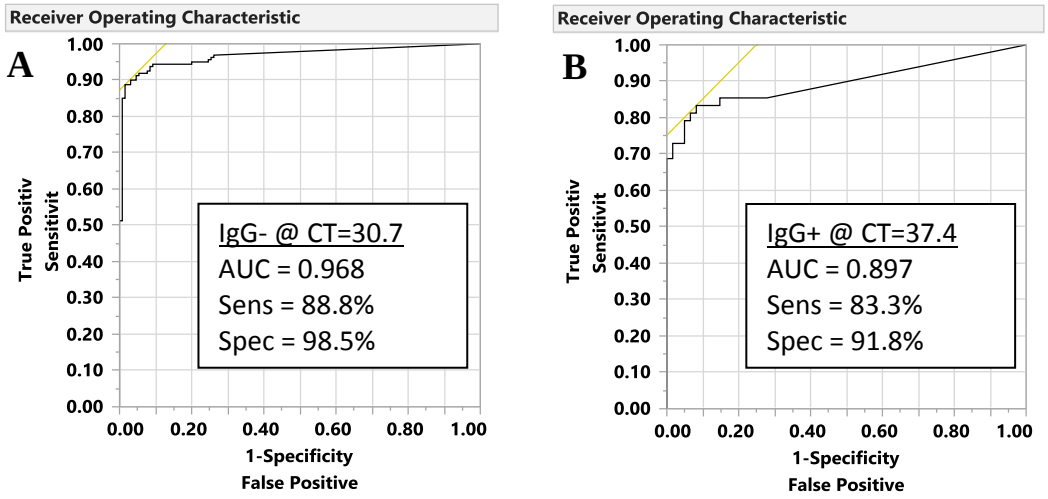


Figure 33. Logistic Regression of RDT Performance by IgG Sero-positivity.
(A) Removing IgG sero-positives increased NPV resulting in enhance RDT diagnostic likelihood of 57.7 when combined with re-optimized Ct = 30.7 cut-off. (B) IgG positive samples are still detected by RDT with sufficient performance to generate a diagnostic likelihood of 10.2.

Correlation of qRT-PCR and ELISA data reveals the relationship of apparent EBOV viral load as represented by qPCR Ct and EHF immune response. The correlation of EBOV antigenemia and viremia (qPCR) is observed in Fig. 34A and results in R^2 = 0.628. This also reflects the discordance between immunoassay detectable antigenemia and the variability in assigned qPCR cut-offs between 30 - 40 Ct. The accurate assignment of qPCR Ct cut-off has a large influence on performance estimates of EHF immunodiagnostics including the ReEBOV assay presented here. In Fig. 34B, the variate in immunodiagnostic performance is represented by the emergence of early IgG convalescent titers in cases where circulating viral genetic material is detected by qPCR. Again the qPCR Ct cut-off can impact perceived immunoassay performance in the absence of clinical observations. Fig. 34B & C reflect the sharp transition from positive antigenemia to IgM/IgG seroconversion and its influence on EHF survivability. As seen in LF, the ability of EBOV viremia to outpace an effective immune response and the pathogenic manifestations of the resulting EHF predict dire prognosis for EHF cases as witnessed in the current West African outbreak.

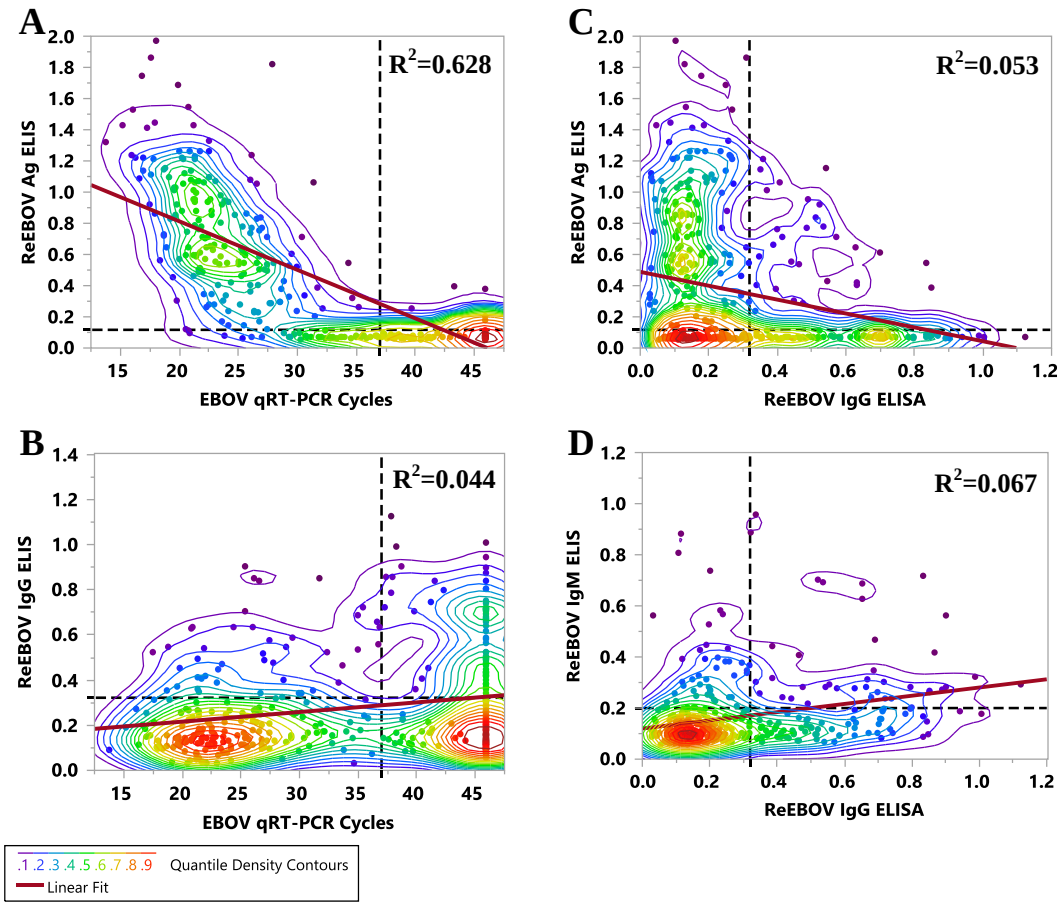


Figure 34. Correlation of EHF Viral Load, Antigenemia, and IgG Sero-positivity

Correlation charts displaying quantile density heatmaps of data distribution and linear regression. A) A significant correlation between antigenemia and qRT-PCR (R^2 = 0.628). B) Lack of correlation between IgG sero-positive and qRT-PCR (R^2 = 0.044) density heatmap revealing possible IgG+/PCR- EHF survivors. C) Strong inverse correlation (R^2 = 0.054) demonstrates seroconversion from positive antigenemia to IgG sero-positive. D) Poor correlation (R^2 = 0.067) between IgM and IgG sero-positive reflects IgM→IgG class-switching during early convalescence. (Dashed lines are qPCR CT and ELISA OD_{450nm} cut-offs. Observations n=396.)

6.6 Clinical Study Conclusion

The clinical validation study of the ReEBOV Antigen Rapid Test established the diagnostic performance as sensitivity 91.1% (195/214; 95% CI: 86.5 - 94.6%) and specificity 90.2% (175/194; 95% CI: 85.1 - 94.0%) vs. EBOV qRT-PCR reference method. Accuracy with qRT-PCR was 90.7% (370/408; 95% CI: 87.5 - 97.2%) and the resulting diagnostic likelihood was 9.3. This performance estimate failed to reject the clinical hypothesis that the RDT would be substantially equivalent to qRT-PCR or exceed it. However, the RDT did reject the null hypothesis that the diagnostic likelihood would be no better than the ability of the EHF case-definition alone as capable of accurately diagnosing EHF.

Indeed the capacity of the RDT diagnostic likelihood to increase to 57.7 in the absence of apparently early IgG convalescent patients and a more accurate qRT-PCR cut-off reveals the ReEBOV RDT to potentially be the most effective laboratory confirmation of acute EHF especially since it is designed as a point-of-care test and offers the ability to produce a 15min time to result during the EHF triage process or at the ETU bedside.

This potential for the ReEBOV Antigen Rapid Test to provide rapid presumptive detection of EHF was recognized by both the US FDA and WHO and was granted Emergency Use Authorization by both organizations in Feb. 2015

PART III. IMPACT OF VHF POINT-OF-CARE TESTING

Chapter 7: VHF Surveillance and Fevers Of Unknown Origin

As has been previously described both LF and EHF present with non-specific signs of febrile illness early after onset. Even as morbidity increases the signs and symptoms of VHF can still be misdiagnosed as other endemic diseases such as drug resistant malaria or in the case of the current EHF outbreak, LF was rightfully suspected until EHF was confirmed. Prior to the current EHF outbreak in Sierra Leone, the Lassa Fever Program at KGH was supporting disease surveillance efforts in that region by assisting in case investigations both within SL and cross-border collaborations with Guinean and Liberian case investigations. Arising from this clinical research was the need to understand what non-Lassa pathogens were circulating within the LF endemic region and what impact those diseases had on LF laboratory diagnosis. The authors had also initiated development of EVD immunoassays supported by a NIAID SBIR R43 (Phase I) grant, guided by their experience with LF immunoassays. Prior to the emergence of EHF in Guinea, Boisen et al. (2015) conducted a series Fevers of Unknown Origin surveys of the LFL sample bank to elucidate sources of non-Lassa febrile illness, including the possible involvement of filoviruses. Prior to the outbreak of EVD in West Africa

7.1 FUO Screening Methods

Samples included in this retrospective study had previously been enrolled and consented under the Tulane sponsored protocols "Pre-clinical Development of Recombinant Antigen Diagnostics for Lassa Fever - Pivotal Diagnostics Clinical Protocol" (Tulane IRB 09-00332) and "Roles of Protective or Pathogenic B Cell Epitopes in Human Lassa Fever" (Tulane IRB 09-00419) which were also approved by the Sierra Leone Ethics and Scientific Review Committee.

Among patients meeting the case definition of LF, who were evaluated at the KGH Lassa Ward from 2008 to 2012, only 11% (190/1,740) were LASV antigenemic. Over the same time span, 65% (1,143/1,740) of suspected LF patients tested negative for either LASV antigen or anti-LASV IgM, and therefore were considered to have a non-Lassa febrile illness (NLFI). The NLFI patients had a similar range of morbidity as acute LF cases which accounts for their high case fatality rate (CFR) of 36%.

FUO Case Classifications:

- **LF** (Lassa fever) defined as PCR+ (or Ag+when PCR data were unavailable).
- **LPE** (LASV prior-exposure) defined as PCR–IgM+ (or Ag–IgM+when PCR data were unavailable).
- **Non-Lassa febrile illness (NLFI)** defined as PCR–IgM– (or Ag–IgM– when PCR data were unavailable).

Statistical analysis

Data were analyzed using SAS v9.3 (SAS Institute, Inc.). Hypotheses involving dichotomous response variables were tested using Fisher's exact test or logistic regression. For logistic regression models with multiple independent predictors, all pairwise interaction terms were included in the model. Count outcomes were modeled using Poisson regression, and distributional comparisons were carried out using the Kolmogorov–Smirnov approach. Gaussian kernel smoothing was used to smooth the categorical serostatus age distributions. Analyses were two-tailed, with a significance threshold set at $p<0.05$.

7.2 FUO ELISA Survey

A series of NLFI patient sera (fever of unknown origin; FUO) were selected for testing based on apparent morbidity. Symptomatic patients were identified from the Lassa Program database based on their cumulative symptoms. Patients with moderate to high symptom frequencies were selected in part based on the volume of the available sample to perform the numerous assays (Table 48).

A total of 77 sera were analyzed in non-LF tropical disease ELISA kits, according to the manufacturer's instructions. FUO testing included DENV Detect™ NS-1 antigen and IgM/IgG, West Nile Virus Detect™ IgM/IgG (InBios Int'l Inc., Seattle WA); Chikungunya IgM/IgG, Typhus IgM/IgG (Genway Biotech Inc. San Diego CA), and Leptospira IgM (Core Diagnostics, Birmingham UK). Assay performance was monitored

with the use of internal controls, and cutoffs were determined according to the manufacturer's instructions for individual kits.

Table 48. Febrile Illness Signs and Symptoms on Referral for Suspected LF

Characteristic	LF (n=29)	LPE (n=24)	NLFI (n=24)	Total (n=77)	p-Value[a]
Fatal outcome	13/29 (45)	4/24 (17)	4/24 (17)	21/77 (27)	0.04[*]
Fever	24/26 (92)	21/23 (91)	23/24 (96)	68/73 (93)	0.86
Headache	15/25 (60)	13/23 (57)	14/24 (58)	42/72 (58)	1.00
Weakness	12/25 (48)	18/23 (78)	16/24 (67)	46/72 (64)	0.10
Dizziness	8/25 (32)	6/23 (26)	14/24 (58)	28/72 (39)	0.06
Muscle pain	6/25 (24)	3/23 (13)	7/24 (29)	16/72 (22)	0.43
Retrosternal pain	11/25 (44)	8/23 (35)	11/24 (46)	30/72 (42)	0.73
Coughing	13/25 (52)	13/23 (57)	12/24 (50)	38/72 (53)	0.91
Vomiting	12/25 (48)	12/23 (52)	16/24 (67)	40/72 (56)	0.42
Diarrhea	7/25 (28)	5/23 (22)	8/24 (33)	20/72 (28)	0.72
Bleeding	8/25 (32)	8/23 (35)	6/24 (25)	22/72 (31)	0.81
Sore throat	6/25 (24)	9/23 (39)	9/24 (38)	24/72 (33)	0.47
Abdominal pain	7/25 (28)	8/23 (35)	12/24 (50)	27/72 (38)	0.29
Runny nose	0/25 (0)	0/23 (0)	1/24 (4)	1/72 (1)	0.65
Conjunctivitis	4/25 (16)	2/23 (9)	2/24 (8)	8/72 (11)	0.72
Jaundice	0/25 (0)	0/23 (0)	1/24 (4)	1/72 (1)	0.65
Rash	0/25 (0)	0/23 (0)	0/24 (0)	0/72 (0)	—
Deafness	0/25 (0)	0/23 (0)	1/24 (4)	1/72 (1)	0.65
Confusion	3/25 (12)	1/23 (4)	1/24 (4)	5/72 (7)	0.61
Shortness of breath	1/25 (4)	0/23 (0)	1/24 (4)	2/72 (3)	1.00
Neck swelling	3/25 (12)	3/23 (13)	1/24 (4)	7/72 (10)	0.61

Values are expressed as mean (sample size).
[a]*p*-Value for Fisher's exact test for association among the three comparison groups.
—, insufficient data for calculating Fisher's exact test statistic.[*]$p<0.05$.
LASV, Lassa virus, PCR, polymerase chain reaction.

After the emergence of the 2014-2015 EHF outbreak additional FUO testing was performed to assess potential cross reactive between the ReLASV and ReEBOV ELISA reagents. Included in that study was an additional survey of Old World Hantavirus IgG sero-reactivity (IBL Int'l Corp, Toronto, Canada) in response to growing evidence of novel Hantavirus strains circulating in West Africa including Sangassou virus and reports of Hantavirus-like pathogenicity in neighboring Guinea[135, 136].

Results

The IgG seroprevalence for Flaviviruses Dengue and West Nile (45% and 54% respectively) was actually higher than Lassa IgG (39%) followed by Chikungunya virus (27%) and Typhus (18%), a Leptosproidum IgG test was not available. For IgM seroprevalence Leptosporidium (38%) was highest followed closely by Chikungunya (35%) and Typhus (29%) (Table 49). The screening for OW Hantavirus IgG reactivity indicated 6% seroprevalence rate which comparable to reported rates in other West African Hantavirus surveys which used similar Hantavirus antigen specificities (Fig.38).

Multivariate analysis of the FUO ELISA data was performed to determine the level of cross-reactivity (Fig. 36). Correlation (R^2) of each bivariate fit reveals the only significant cross-reactivity between Dengue virus IgG and West Nile virus IgG. Both of these are Flavivriuses so cross-reactivity is not entirely unexpected. However, background information of prior vaccination for Yellow fever, another Flavivirus, is not available an could prove to be a source of non-specific signal in Dengue and West Nile

Table 49. Fevers of Unknown Origin Seropositivity Rates for Suspected LF

Seroreactivity	LF (n=29)	LPE (n=24)	NLFI (n=24)	Total (n=77)	p-Value[a]
LASV IgG	7/29 (24)	12/24 (50)	11/24 (46)	30/77 (39)	0.11
DENV IgG	10/29 (34)	12/24 (50)	13/24 (54)	35/77 (45)	0.35
DENV IgM	5/29 (17)	7/24 (29)	8/24 (33)	20/77 (26)	0.40
CHIK IgG	7/29 (24)	7/24 (29)	7/24 (29)	21/77 (27)	0.90
CHIK IgM	11/29 (38)	7/24 (29)	9/24 (38)	27/77 (35)	0.83
Lepto IgM	9/29 (31)	12/24 (50)	8/24 (33)	29/77 (38)	0.39
WNV IgG	10/25 (40)	13/21 (62)	13/21 (62)	36/67 (54)	0.26
WNV IgM	8/25 (32)	5/21 (24)	3/21 (14)	16/67 (24)	0.38
Typhus IgG	4/25 (16)	5/20 (25)	3/21 (14)	12/66 (18)	0.66
Typhus IgM	9/25 (36)	7/20 (35)	3/21 (14)	19/66 (29)	0.22

Values are expressed as mean (sample size).

[a]*p*-Value for Fisher's exact test for testing for association among the three comparison groups.

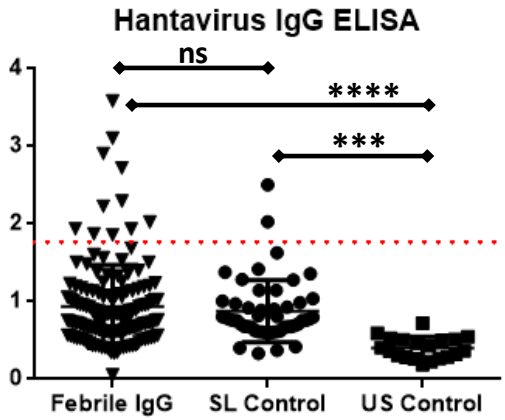


Figure 35. Old World Hantavirus IgG Survey

Lassa Fever Program sample bank reveals IgG sero-reactivity to Old World Hantavirus (possibly Sangassou virus) representing a 6% seroprevalence which is comparable to prior West African surveys. (Fisher exact test p < 0.001***, p<.0001****)

virus immunoassays. Dengue and West Nile virus are not reported in high frequency in Sierra Leone but West Nile virus was detected by PCR (see Ch 7.3).

Clustering if clearly positive Chikungunya virus IgG/IgM samples are observed and Chikungunya IgM sero-prevalence has been reported in nearby Bo city as well as previous reports in Guinea and Senegal [137, 138]. O'nyong-nyong virus is another Alphavirus from the Semliki Forest Complex and has been reported in nearby Cote d'Ivoire so cross-reactivity with Chikungunya virus in the absence of clear PCR evidence cannot be ruled out [139]. Leptosporidium IgM sero-reactivity was not included in the multivariate analysis but diplayed not significant cross-reactivity with the FUO assay panel. Importantly, the sero-reactivity of Dengue, WNV, Chikungunya, and Typhus do not appear to significantly cross-react with ReLASV IgG/IgM ELISA which is also the same reagent system as the ReEBOV IgG/IgM ELISA.

Bivariate analysis of ReLASV and ReEBOV ELISA (Fig. 37) reveals no significant cross-reactivity between these assays for IgG (R^2 = 0.13) however there is clear evidence of patients with seroconversion for both VHFs. This analysis also reveals the absence of LF (Fig. 37B &C) during the western Sierra Leone EHF outbreak. This probable under-reporting of LF in a previously endemic area may be due to the fear and social stigma associated with EHF suspicion causing LF cases to not seek clinical referral.

As expected, the Bivariate fit of ReLASV IgG and ReEBOV Ag ELISA shows prior LF IgG convalescence is not protective for EHF (Fig. 38). The strong clustering alone the ReLASV IgG axis and the ReEBOV Ag axis establish that these tests are not cross-reactive and reveal the obviously acute EHF cases with prior exposure to LASV.

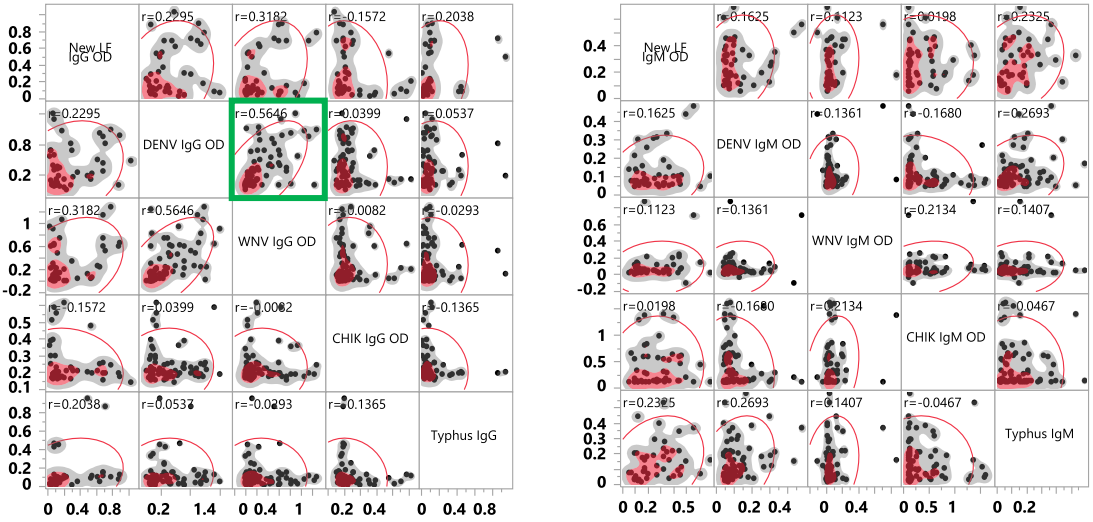


Figure 36. Multivariate Analysis of FUO Assay Cross Reactivity

Multivariate scatter plots were prepared to analyze potential cross-reactivity within the FUO ELISA screening. Quantile density shading (pink to gray) is applied and correlation of individual bivariate fits included. (Left) The only significant IgG cross-reactivity (r=0.5646) is seen the Flavivirus tests: Dengue vs West Nile (green square). Probable cases of dual or multiple convalescent titers is observed such as Lassa virus and Flavivirus convalescence. Cross-reactive titers induced by Yellow fever virus immunization cannot be ruled out. (Right) No significant correlations are observed in IgM screening. Relative OD450nm signal are lower than IgG ODs and generally reflect an indeterminate range of these immunoassays.

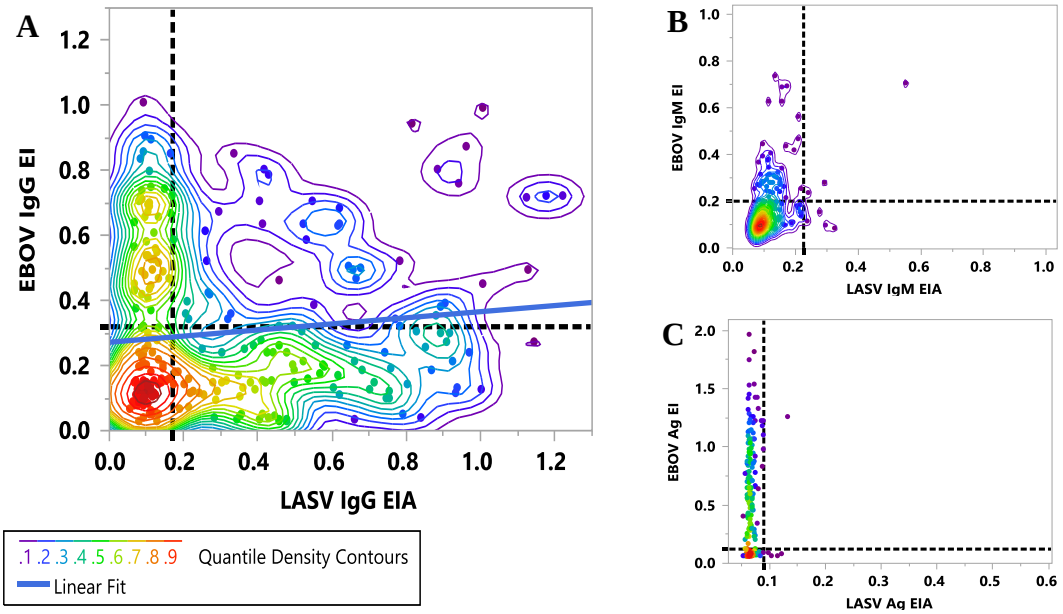


Figure 37. ReLASV and ReEBOV IgG ELISA Correlations

(A) IgG ELISA bivariate fit shows poor correlation of Lassa and Ebola assays (R^2 = 0.13, n=262) . Similar lack of correlation exists for (B) IgM ELISAs (R^2 = 0.182, n=266) and (C) Antigen ELISAs (R^2 = 0.010, n=266). These results reflect the absence of reported Lassa fever cases during the SL EHF outbreak.

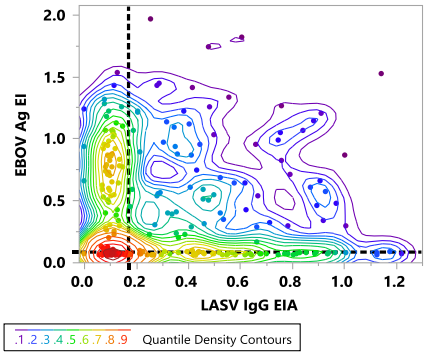


Figure 38. ReEBOV Ag and ReLASV IgG ELISA Non-correlation

Bivariate fit of ReEBOV Ag ELISA and ReLASV IgG ELISA confirms lack of protection by LF Convalescence in this EHF naive population.

7.3 FUO PCR survey of potential human pathogens

A panel of fever-causing agents with historical seroprevalence, sporadic emergence, or endemic status in Western Africa was chosen for a screen of viral and microbial agents present in subjects with acute LF, previous exposure to LASV, or NLFI. PCR analysis was performed on 41 of the original panel of 77 sera, due to lack of sample volume from the remaining 36 sera. The agents analyzed in the PCR survey were LASV, Zaire ebolavirus (EBOV), Marburg virus (MBGV), Crimean-Congo hemorrhagic fever virus (CCHFV), Rift Valley fever virus (RVFV), dengue virus (DENV), yellow fever virus (YFV), West Nile virus (WNV), chikungunya virus (CHIKV), *Leptospira interrogans*, *Rickettsia prowasekii*, *Salmonella typhi*, *Bacillus recurrentis*, *Plasmodium falciparum*, and *Plasmodium vivax*. Infectious agent or family-specific oligonucleotides and corresponding minigene amplicons were identified in the published literature. Inclusion criteria for PCR methodologies used in this study were previous testing on live agents, obtained from *in vitro* or *in vivo* sources, validation on nearest neighbors, and testing in controlled studies. Nucleic acids were isolated from 100 µL of patient serum using either a Qiagen QIAmp Viral RNA Mini kit or a Genomic DNA Isolation kit (Qiagen). Oligonucleotides, buffers, and amplification conditions were as previously described [140].

Results:

Forty-one sera were screened by RT-PCR and conventional PCR using a panel of oligonucleotides designed for specific detection of amplicons from 13 febrile agents

(Table 50). Nine sera tested positive for LASV (9/41, 22%), six were WNV positive (6/41, 15%), and 32 were *P. falciparum* positive (32/41, 78%). A single patient sample was positive for a 419 nt amplicon corresponding to a conserved filoviral L gene segment (1/41, 2.0%). Five samples tested positive for multiple infectious agents (5/41, 12%), with three double positives for *P. falciparum*and WNV (3/41, 7%), one double positive for *P. falciparum* and LASV (1/41, 2%), and one triple positive for LASV, WNV, and *P. falciparum* (1/49, 2%) (Fig. 39).

The study did not identify *P. vivax* positive malaria (0/41, 0%), although at least one patient with this parasite has been identified over the time period studied (unpublished results) and malaria cases diagnosed by thin smear microscopy are generally not referred for VHF screening unless they do not responsed to anti-malarial therapy. This study did not identify genetic material in the serum of FUO patients corresponding to YFV, DENV, CHIKV, CCHF, RFV, *S. typhi* STY1599, *Leptospira* int & RFA, *R. prowasekii*, and *B. recurrentis* amplicons.

7.4 Potential Exposure of Subjects with NLFIs to a Filovirus.

Phylogenic analysis of the EBOV isolates that have emerged in the West African countries of Guinea, Sierra Leone, and Liberia suggest the EBOV has been in the Guinean rain forest for several decades. To determine the possibility that human

Table 50. Positivity Rates for FUO Assessed by PCR

Competing illnesses	LF (n=10)	LPE (n=18)	NLFI (n=13)	Total (n=41)	p-Value[a]
P. falc.	9/10 (90)	13/18 (72)	10/13 (77)	32/41 (78)	0.312
P. vivax	0/10 (0)	0/18 (0)	0/13 (0)	0/41 (0)	—
Filovirus	0/10 (0)	1/18 (6)	0/13 (0)	1/41 (2)	0.641
WNV	2/10 (20)	2/18 (11)	2/13 (15)	6/41 (15)	0.803
YFV	0/10 (0)	0/18 (0)	0/13 (0)	0/41 (0)	—
DENV	0/10 (0)	0/18 (0)	0/13 (0)	0/41 (0)	—
CHIKV	0/10 (0)	0/18 (0)	0/13 (0)	0/41 (0)	—
RVF	0/10 (0)	0/18 (0)	0/13 (0)	0/41 (0)	—
CCHF	0/10 (0)	0/18 (0)	0/13 (0)	0/41 (0)	—
S. typhi	0/10 (0)	0/18 (0)	0/13 (0)	0/41 (0)	—
Lepto.	0/10 (0)	0/18 (0)	0/13 (0)	0/41 (0)	—
R. prowasekii	0/10 (0)	0/18 (0)	0/13 (0)	0/41 (0)	—
B. recurrentis	0/10 (0)	0/18 (0)	0/13 (0)	0/41 (0)	—

Values are expressed as mean (sample size).

[a]*p*-Value for Fisher's exact test for testing for association among the three comparison groups.

—, insufficient data for calculating Fisher's exact test statistic.

WNV, West Nile virus; YFV, yellow fever virus; DENV, dengue virus; CHIKV, chikungunya virus; RVF, Rift Valley fever virus; CCHF, Crimean-Congo hemorrhagic fever virus.

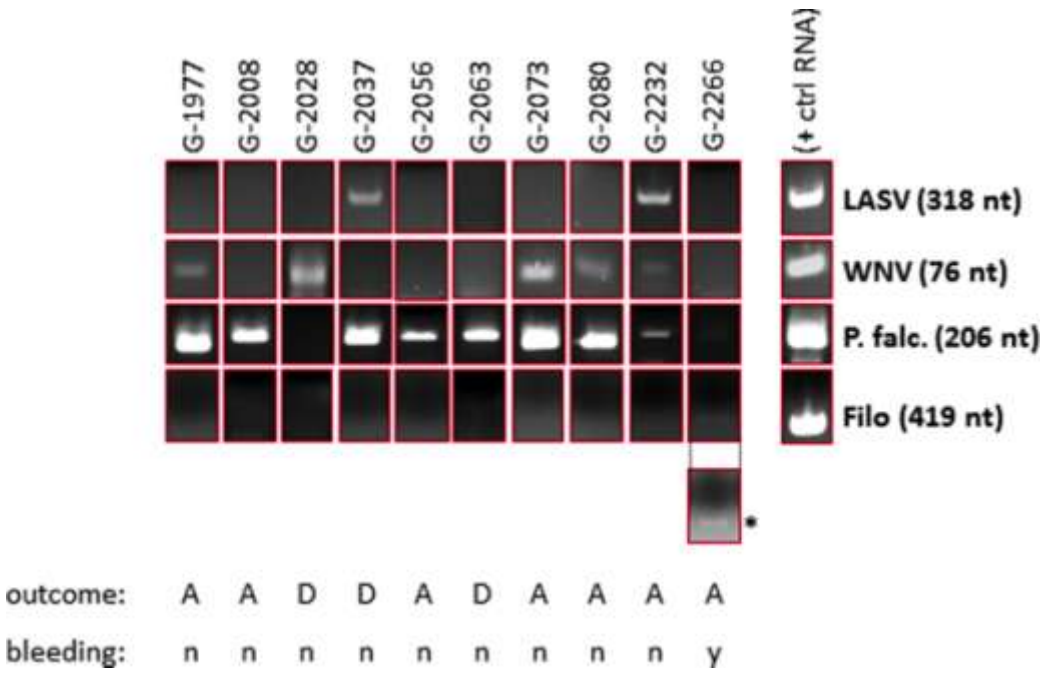


Figure 39 Representative FUO PCR panel showing single, dual, and triple infections identified by PCR screening in a subset of FUO patients.

A subset of patients in the study tested positive by PCR for non-Lassa febrile illnesses (NLFIs), such as *Plasmodium falciparum* (G-2008, G-2056, G-2063), *P. falciparum* and West Nile virus (WNV; G-1977, G-2073, G-2080), or WNV alone (G-2028). One patient tested positive for Lassa virus (LASV) and *P. falciparum* (G-2037), and another was triple positive for LASV, WNV, and *P. falciparum* (G-2232). One patient in the study had a very low level of *P. falciparum* antigen and tested positive in a filoviral reverse transcription (RT)-PCR screen (G-2266). Positive control RNAs were generated by runoff *in vitro* transcription reactions from a T7 promoter immediately upstream of the corresponding amplicon. The outcome (A=alive; D=deceased) and bleeding (n=no bleeding; y=bleeding) status is shown *below* each set of panels

exposures to filoviruses may have occurred prior to the current outbreak, a serosurvey was performed on samples collected between June 2011 and March 2014 (*n*=242). Significant IgG seroreactivity was noted in samples collected in the years 2012–2014. A conservative cutoff OD_{450}=0.360, established on the 80th percentile obtained with a panel of normal sera from SL donors (*n*=14), which intersected with three times the mean O.D. generated by a U.S. normal sera panel (*n*=13;Fig 40A) resulted in a seropositivity rate of 22% (53/242; Fig 40B).

Only three sera from the data set represented in Fig. 39 (patient IDs 2008, 2126, 2281) were also analyzed in the retrospective EBOV IgG study. Serum from the sample that tested positive in the filovirus-specific PCR in January 2012 (G-2266) was not available for testing in the EBOV IgG/IgM ELISA, preventing further characterization of this potential early case of EBOV infection without acute manifestation of EVD. The remaining serum volume was also not sufficient to permit reamplification of filoviral sequences, for cloning of selected amplicons, or for deep sequencing analysis.

Patient G-2266 was a 26-year-old Sierra Leonean female who presented to the KGH LFW from another KGH ward as a suspected LF case. She presented with fever, weakness, dizziness, retrosternal pain, and bleeding at the time of presentation to the KGH LFW on January 17, 2012. Following a diagnosis of a NLFI, the patient was not admitted or followed as a suspected VHF case. The patient was LASV antigen-negative by LFI, ELISA, and PCR, and negative in *P. falciparum* and WNV PCR screens. She registered positive titers for LASV IgM, DENV IgG, CHIKV IgM, WNV IgM and IgG, and typhus IgM. Her serum cytokine profile was unremarkable, registering low levels of IL-10 and IL-8, at 20 pg/mL and 126 pg/mL respectively.

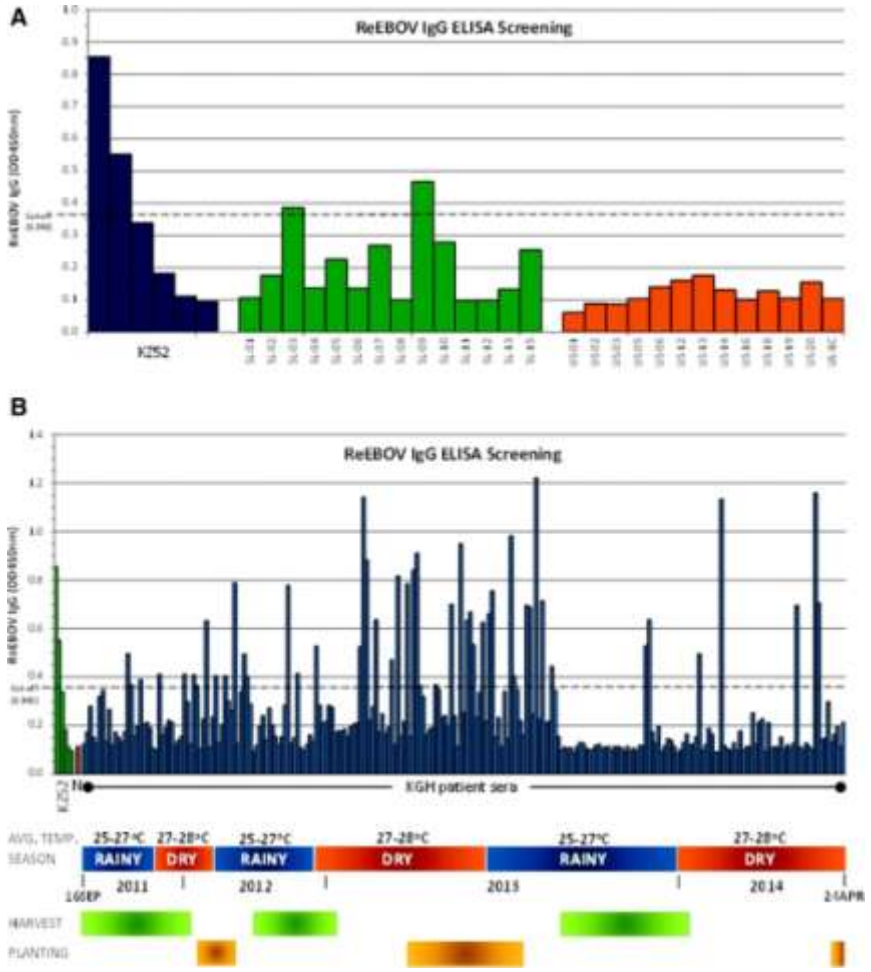


Figure 40. FUO Survey of EBOV IgG Reactivity Using ReEBOV IgG ELISA

(A) OD 450nm Cut-off determination for ReEBOV IgG ELISA (GP, VP40 combo coating) using the EBOV GP-specific KZ52 Hu. mAb over a titration range (*blue bars*), a panel of 14 SL normals (*green bars*), and 13 US normals (*brown bars*). Conservative cutoff OD 450 nm=0.360, established at the 80th percentile of SL and US Normals. (B) ReEBOV IgG ELISA screening of KGH Sample Bank collected between 2011 and March 2014 (*n*=242). Reference mAb KZ52 (green bars), a negative control serum (*orange bar*), and patient serums (*blue bars*). A temporal distribution of sera analyzed, from mid-September 2011 to late April 2014, and corresponding dry and rainy seasons, and average temperatures (in °C) are graphically represented. These studies did not identify a correlation between seasons and the emergence of EBOV sero-reactivity.

7.5 FUO Survey Conclusion

In our experience conducting VHF clinical research in eastern Sierra Leone, cases are often referred to our lab for VHF screening without overt signs of hemorrhage or other vascular manifestations (edema, conjunctival injection). Many of these febrile cases have significant morbidity which has cause fatalities. The fevers of unknown origin survey are conducted to elucidate the causative source of these cases and identify emergence of new pathogens. This study identified significant seroprevalence for Flaviviruses, Alphaviruses, Hantaviruses, Typhoid fever, Leptospirosis, malaria, and evidence of Ebola virus circulation prior to the 2014-2015 EHF outbreak. Patients in this survey often have multiple convalescent titers and there is evidence of co-infection.

After the subsidence of the current EHF outbreak it will be difficult to assess the extent of EBOV circulation prior to the emergence in neighboring Guinea. This is due to the well documented occurrence of asymptomatic EHF, as well as asymptomatic LF[141, 142]. Zoonotic surveys will have to be conducted to assess how extensively EBOV is circulating host reservoir species as well as serological evidence of EBOV exposure in secondary reservoirs such as the Sierra Leone chimpanzee population

The FUO testing also provides valuable evidence that further validates the specificity of the VHF diagnostic testing we have established at KGH. In the presence of both LF and EHF in Sierra Leone, accurate diagnosis is critical for proper isolation of these patient and exclusion of non-VHF cases to minimize secondary nosocomial transmission of either VHF.

Chapter 8. Discussion - VHF RDT Clinical Utility

The development of the VHF RDTs fulfills one of the VHFC's core goals to provide diagnostic solutions for the management of VHF disease. Our diagnostic development efforts began in 2008 with NIH/NIAID funding for the development LF critical reagents and diagnostic tests. In 2010, we received NIH/NIAID funding to pursue similar reagent and test development for Filoviruses. Assembling a team of collaborator's from industry and academia in the US and West Africa, the VHFC has successfully launch the commercial diagnostics described here and also continues to pursue small molecule and huMAb therapeutics.

Assay specificity plays a critical role in the RDT clinical effectiveness. Both ReLASV and ReEBOV RDTs target viral proteins that are high in copy number per virion. The Lassa test is specific for the nucleocapsid (NP) protein. The murine mAbs generated against LASV NP creates a very specific pairing including a conformational epitope. Nearest-neighbor and cross-reactant testing revealed the test to be specific for LASV Josiah (clade IV) and field trials in Nigeria demonstrated limited cross-reactivity with the Nigerian strains (clade II & IIII). The VHFC is continuing the development of Nigeria strain specific and pan-reactive Lassa critical reagents. Cross-reactant testing conducted outside the scope of this paper (Boisen et al. unpublished data (2015)) has demonstrated no cross-reactivity with malaria positive clinical samples. Of all the endemic pathogens in West Africa, malaria is of most concern because acute LF cases in Sierra Leone are overwhelmingly misdiagnosed for malaria unless they are directly

referred as suspected LF to KGH. There is also the potential for chronically infected malaria cases to develop a co-infection the LASV.

In the clinical validation of the ReLASV RDT there was age and sex bias which may be due to cultural and health policy influence. In 2010, Sierra Leone instituted a new policy offering free healthcare for pregnant women, lactating mothers, and children in an effort to reduce childhood, pregnancy related mortality. This important policy change made healthcare more accessible to these high risk groups. The sex bias of +50% female could be due to the combination of easier access by women to medical care and cultural tradition of women being the primary caregiver to children. This causes bias of LF cases to children and young mothers.

The distribution of LF cases during the clinical study reflects the traditional LF endemic region. The three districts with the highest case load also possess the three principle referral centers (Panguma, Segbwema, and Bo City). LF referrals from Central and Southern districts frequently present for febrile illness referral in Bo City then are sent to KGH for LF screening. Case investigation and contact tracing is more difficult in the north and south due to distance and logistics.

As stated in the clinical performance claims for ReLASV RDT (Ch. 4.4) the generation of Diagnostic Likelihood >40.9 clearly rejects the null hypothesis that the RDT will perform no better than reported clinical signs of LF. In this study two trends are observed that affects the diagnostic likelihood of signs included in the case definition. The first trend involves the higher frequency signs including fever, headache, pain, weakness, vomiting which due to their frequency (>50%) of cases results in reduce specificity. This combination reduces the Positive Predictive Value (PPV; <50%) and the

Diagnostic Likelihood fails to rise significantly above 2.0. The second trend involves lower frequency LF signs which can represent increased morbidity. Signs such as convulsions, retrosternal pain, edema, conjunctival injections, and bleeding from injection site all have significant associate with LF by Chi square testing ($p<0.05$) and can have high specificity for LF but their low frequency means they fail to detect the majority of LF cases either individually or in combination. Since specificity drives both the PPV (>80%) and Diagnostic Likelihood (>3) calculations both of the performance parameters are elevated creating the performance characteristics of a "Rule-In" test by these more specific signs.

However, in the wake on the West African EHF outbreak it must be recognized that all of these potential "Rule-In" LF signs could lead to misdiagnosis of EHF or vice versa. Obviously all of these febrile illness signs can indicate significant morbidity however potential co-circulation of LF and EHF as well as FUO pathogens make accurate laboratory and point-of-care based diagnosis essential for proper VHF case management. Both of the VHF RDTs presented here fulfills the previously unmet need for VHF point-of-care screening.

The ReLASV RDT clinical performance validation revealed the test to be very specific (98.6%) resulting in high PPV (87.1%) and Diagnostic Likelihood (40.9) despite sensitivity (58.7%) below the target specification (70%). Discrepant analysis indicated that 13 (28%) of the RDT false-negative were IgG+ LF convalescent raising the possibility of RDT interference by IgG/IgM sero-positive. Subsequent performance analysis after removal of IgM+ &/or IgG+ confirmed the antibody interference which suggests that 20% or more of the enrolled suspected LF were LF post-acute or LF

convalescent. Further interrogation of the IgG/IgM ELISA results showed that IgG+ convalescent sample had a minimal affect and that Post-Acute LF (IgM+, IgG±) interfering with RDT sensitivity The revised clinical performance using IgM seronegative did increase sensitivity to 76% (19/25) however the smaller sample size reduces the statistical power of this estimate. Remarkably, the other performance parameters did not significantly change with specificity 98.6%, PPV 86.4% and resulting Diagnostic Likelihood increased to 55.0. This performance profile justifies the use of the ReLASV RDT as a Rule-in Test for the detection of acute LF. Availability of mortality data support RDT screening utility with a CFR 77% when RDT+/IgM-; cases that were Ag+, IgM± dropped to a CFR 25% and LF IgG convalescent CFR 14.9% was not significantly different than CFR 14.4% of the general febrile cases.

Since the clinical validation and awarding of CE/IVD marking by the EU, the ReLASV Ag RDT has been used as the daily LF screening method. During morning rotations the medical staff will refer cases of undiagnosed febrile illness to the VHF Lab for Lassa screening. Typically a whole blood tube and serum tube is drawn by trained phlebotomists and transferred to the VHF Lab for de-identification and logging into the VHF Lab database. Rapid test results will be confirmed by newly validated LASV qRT-PCR and ReLASV ELISA to assess full immune profile of each case. The ReLASV RDT is also used in outbreak investigations by the Lassa Program Outreach Team. In these cases a technician from the VHF Lab will accompany the Outreach Team to referral hospitals or clinics to screen suspected cases or case-contacts.

The analytical validation of the ReEBOV RDT demonstrated strong predictive performance based on its sensitivity to both recombinant VP40 antigen and live EBOV

when spiked in donor whole blood. Further the RDT cross-reacted with all three EBOV viruses (Zaire, Sudan, and Bundibugyo) known to cause EHF in humans without displaying cross-reactivity to non-EBOV VHFs and other febrile illness pathogens and agents representative of diseases endemic to the EHF outbreak region. Interference testing identified only two substances, Hemoglobin and Rhuematoid factor, that caused false signals in contrived serum samples. Neither was observed to be a source of discordant results during field testing because grossly hemolyzed samples do not occur with high frequency and those sample are typically rejected on arrival as unsuitable for RDT testing and a second sample requested or alternate method used for screening. A brief study using Rhuematoid Factor ELISA and latex aggulutination tests found no significant frequency of high titer Rf samples. Normal serum and whole blood specificity testing indicated that we should expect a range in specificity between 75 - 100%.

The Clinical Validation study in fact generated Specificity of 90.2% (175/194; 95% CI: 85.1 - 94.0) and Sensitivity of 91.1% (195/214; 95% CI: 86.5 - 94.6%). The resulting PPV 90.2% and the Diagnostic Likelihood was 9.30. These performance estimates fell just below the WHO guideline targets however binomial proportion comparison between actual and target was not significant given sample size. For example the RDT sensitivity was 91.1% (195/214) to achieve 95% sensitivity the proportion would need to be (203/214). Binomial proportion comparison (Ho p1=p2) was (195/214) = (203/214) did not generate a significant difference ($p = 0.130$).

Despite the clinical performance being slightly under target. The diagnostic likelihood of 9.3, PPV = 91.1%, and accuracy vs qPCR = 90.7% (370/408; 95% CI 87.5 - 97.2%), the ReEBOV RDT still makes an impressive case as a rule-in test. This

argument for clinical utility would be easier if morbidity and mortality data was available but is not unfortunately. Based on our experience with ReLASV RDT, a change to the ReEBOV RDT clinical performance vs acute EHF amy be found in IgG sero-negative patients. Removing the IgG+ samples and performing a Logistic Regression with ROC analysis results in the RDT being optimized to a lower Ct cut-off = 31. This lower Ct Cut-off produces a sensitivity = 88.8% and specificity = 98.5% with the resulting diagnostic likelihood = 59.2. So much like the performance targeting acute LF for the ReLASV RDT the same holds true for the ReEBOV RDT. This gains importance when you consider the lack of patient data exchange between ETUs and Labs. In the case of KGH VHF Lab we have no way to verify at what point in the course of EHF disease each sample has been drawn from. Also this performance estimate was based entirely on banked plasma and serum instead of the whole blood samples the RDT was designed for.

The WHO also conducted an independent clinical study of the ReEBOV RDT. This study was performed in the Freetown area of Sierra Leone at two sites. The European Mobile Laboratory at Hastings tested fresh venous whole blood only. The African Union/European Mobile Laboratory at Prince of Wales tested fresh venous whole blood and frozen plasma samples. The reference method was the RealStar® Filovirus Screen RT-PCR Kit 1.0 (Altona Diagnostics, GmbH). The frozen plasmas tested were collected between Dec. 2014 and Feb. 2015. A total of 147 fresh venous whole blood and 146 frozen plasmas were tested. The combined WHO clinical performance for the ReEBOV RDT was sensitivity 91.8% (89/97; 95% CI: 84.4 - 96.4%) and specificity 84.6% (165/195; 95% CI: 78.8 - 89.4%) with a diagnostic likelihood = 5.96

There was concern that there was a performance bias between whole blood and plasma but that is not a clear comparison because the number of PCR+ whole blood samples was too low (n=23) and the PCR- plasma (n=66) was also below an appropriate a sample size. Another confounding observation was that the apparent qPCR Ct cut-off = 30 for which there was no qualifying data provided. Because these issues could not be resolved, the WHO left the analysis intact. In comparison with the Corgenix study (banked plasma & serum) there was no statistically significant difference from the WHO sensitivity and specificity estimates for fresh whole blood and banked plasma.

Sensitivity: Corgenix 91.1% vs. WHO 91.8%; p=0.855

Specificity: Corgenix 90.2% vs. WHO 84.2%; p=0.0966

The WHO was very helpful in providing this independent clinical performance assessment specificity of 90.7% for fresh whole blood which helps confirm the whole blood specificity of 95.0% from the analytical validation. Based on their findings, including an independent analytical assessment, the WHO accepted the ReEBOV® Antigen Rapid Test in Feb. 2015 and placed the test in on their approved products list for the presumptive diagnosis of EHF. Shortly thereafter the FDA also granted the RDT and Emergency Use Authorization.

The FDA however, had decided to modify our dataset by only allowing the banked plasma data and excluded the use of any qPCR cut-off. This resulted in sensitivity dropping from 91.1% to 62.1% and specificity increasing from 90.2% to 96.7%. The increase in specificity caused the diagnostic likelihood to actually increase to 18.8. Adjusting the sensitivity estimate down so dramatically would make launching the test

into West Africa difficult in order to help with the still active EHF outbreak. Further complicating the situation was the lack of a WHO clinical use guidance to provide recommendations on how to implement the ReEBOV RDT use within the ETUs and EHF labs.

A second independent study had been started in Feb. 2015 and was conducted by jointly by Partners In Heath (Boston, MA, USA) and Public Health England (Porton Down, UK)[143]. For this study the primary sites would be the Public Health England field reference laboratory in Port Loko, Sierra Leone. Samples enrolled in the study would be referrals from several sites and clinics in western Sierra Leone. The reference method would again be the RealStar Filovirus Screen RT-PCR kit 1.0 (Altona Diagnostics, GmbH) . Importantly, this would be the first study to fully model the Point-of-Care Testing concept by conducting fingersticks as well as fresh venous whole blood screening.

The total enrollment was 105 fingersticks and 277 venous whole blood. The clinical performance for both fingerstick and whole blood was nearly identical. Fingerstick testing produce sensitivity 100% (28/28; 95% CI: 87.7 - 100%) and specificity 92.2% (71/77; 95% CI: 83.8 - 97.1%). For whole blood the sensitivity was 100% (45/45; 95% CI: 92.1 - 100%) and specificity 92.2% (214/232; 95%CI: 88.0 - 95.3%). Agreement between fingerstick and whole blood was 93.0% (80/86; 95% CI:85.4 - 97.4%) with all discordant results being weak (score 1) false-positives. The diagnostic likelihood's were 12.8 for fingerstick and 14.3 for fresh whole blood. The success if the ReEBOV RDT represents to most effective use of a RDT in an EHF triage scenario.

The concept of performing rapid diagnostic detection of VHFs in the field has been pursed since Lassa virus and Ebola Virus were first identified. While it is true the original viruses were isolated in western labs when they were first discovered. It is also true that as soon as the viral cultures could be optimized IFA materials were prepared for field testing in the subsequent LF and EHF outbreaks. As new diagnostic technologies emerged they too would be applied towards field testing and disease surveillance. VHF immunoassay techniques have adapted over the years from ELISA tests which used inactived viral cultures and murine ascites for plate coatings and detection reagents, to the used of purified reagents, and more recently expanding to other technologies such as biosensors and LFI while replacing the culture based materials with recombinant viral proteins. The question has never been are these new technologies capable of accurate diagnosis of VHF. The real question is what is the most practical solution for providing this testing capacity to healthcare services in the affected areas.

The endemic regions for LF and EHF can be very hostile to modern laboratory equipment. Several countries have establish Virology Institutes to bring modern high containment lab capabilities to Western and Central Africa. These facilities are located in larger cities that have the capacity to provide the necessary infrastructure to maintain these labs. The international response to previous outbreak, in particular EHF, has evolved over the past decades from small investigative teams and limited equipment to more coordinated and rapid responses capable of setting up field hospitals, labs, and surveillance teams. When there are isolated cases this approach can be very efficient containing an emerging outbreak. However even in more recent outbreaks, prior to even the west African EHF outbreak, the locations where these outbreaks emerged are remote

enough that they grow to involve dozens to hundreds of cases and contacts before outbreak management teams can arrive in force and in some cases laboratory diagnosis is still conducted elsewhere[7, 76, 144].

What has been missing from the outbreak surveillance is the capacity to deploy simple rapid tests in these remote communities in order to conduct more proactive VHF surveillance. Even in small country like Sierra Leone, where LF is endemic and a regional lab at KGH is available, the time from onset to medical interventions can be 5-10 days and often include misdiagnosis and multiple referrals. For acute LF, delays this long can have fatal consequences. This situation has fueled the desire to distribute rapid diagnostic tests to the community health officers to shorten the delay in medical intervention. This same remote testing model could be repeated in larger Nigeria and across the Congo Basin.

Rapid diagnostic tests such as the ReLASV RDT for LF and the ReEBOV RDT for EHF possess the right design features to fill the void in remote testing. The key feature they provide is that very little infrastructure is needed to run them. As a visually read test there is not mandatory equipment or power requirements. They can easily be operated with basic disposable lab supplies. RDTs are also very stable and typically don't require refrigeration for storage. Furthermore they can be produced at much lower costs than more complex tests such as RT-PCR. Training requirements are also very basic. The VHFC will continue to development rapid, robust testing solutions for VHF surveillance and case management. The encroachment of man into VHF host reservoirs will continue to present opportunities for disease transmission and VHF outbreaks. VHF RDT's represent the best approach to fill the void in active disease surveillance methods.

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Biography

Matthew L Boisen is a native of California where he attended Monta Vista High School in Cupertino. Matt completed his undergraduate studies at San Jose State University and received a Bachelors of Science degree in Medical Microbiology & Immunology in December 1990. While at SJSU, Matt conducted characterization studies of *Borellia burgdoferi* (Lyme Disease) murine monoclonal antibodies specific for heat shock proteins that he developed for his advisor Dr. John Boothby.

Over the following 25 years, Matt has held several position in the private biotech industry specializing product development of rapid point-of-care diagnostics for infectious diseases including enteric pathogens, sexually transmitted diseases, and biowarfare agents. At his current position as Program Director at Corgenix Inc. (Broomfield CO), Matt has served as chief designer of viral hemorrhagic fever *in-vitro* diagnostics in collaboration with Dr. Robert F. Garry of Tulane University and the Viral Hemorrhagic Fever Consortium. Matt has conducted extensive field trials in West Africa in support of his research and product development. Matt was accepted into the Tulane University School of Medicine Graduate Studies in Biomedical Sciences in 2010. Matt currently lives in Frederick, Colorado with his wife Shelly and daughters Morgan and Michaela.