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Clinical Validation Trial of a Diagnostic for Ebola Zaire Antigen Detection: Design Rationale and Challenges to Implementation

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Abstract

The current Ebola outbreak in West Africa has affected more people than all previous outbreaks combined. The current diagnostic method of choice, quantitative polymerase chain reaction, requires specialized conditions as well as specially trained technicians. Insufficient testing capacity has extended the time from sample collection to results. These delays have led to further delays in the transfer and treatment to Ebola Treatment Units. A sensitive and specific point-of-care device that could be used reliably in low resource settings by healthcare workers with minimal training would increase the efficiency of triage and appropriate transfer of care. This article describes a study designed to validate the sensitivity and specificity of the ReEBOV™ RDT using venous whole blood and capillary blood obtained via fingerprick. We present the scientific and clinical rationale for the decisions made in the design of a diagnostic validation study to be conducted in an outbreak setting. The multi-site strategy greatly complicated implementation. In addition, a decrease in cases in one geographic area along with a concomitant increase in other areas made site selection challenging. Initiation of clinical trials during rapidly evolving outbreaks requires significant cooperation on a national level between research teams implementing studies and clinical care providers. Coordination and streamlining of approval process is essential if trials are to be implemented in a timely fashion.

Keywords

Ebola; diagnostic; lateral flow; validation trial

Introduction

The largest and most widespread outbreak of Ebola virus disease continues in West Africa, with over 27,514 cases and 11,220 deaths reported worldwide. Through June 2015, the

majority of cases have originated in Guinea, Sierra Leone, and Liberia.¹ The key to interrupting Ebola virus transmission is through identification and isolation of Ebola cases shortly after the onset of symptoms when cases are less likely to transmit the virus.² Ebola has a broad range of signs and symptoms that are similar to many other febrile and gastrointestinal diseases, making identification of Ebola cases by clinical assessment particularly difficult. To better classify Ebola cases, surveillance and clinical case definitions for suspected Ebola cases have been crafted to focus primarily on case sensitivity (Table 1). In some centers, over 50% of suspected cases are ultimately given an alternative diagnosis.³ Laboratory testing is essential to distinguish between Ebola cases and subjects with other illnesses.

Assays to detect Ebola virus nucleic acids have been employed throughout the affected region. The test requires blood collection via venipuncture, safe transport from point of blood draw to laboratory, purification of nucleic acids and testing for Ebola virus nucleic acids. These steps make molecular testing a slow process requiring a high level of laboratory biosafety, staff with high levels of expertise, and use of sophisticated testing equipment that require stable electrical systems. In Sierra Leone, such challenges can significantly increase the reclassification time of suspected to confirmed cases or non-cases.^{4, 5} Ebola suspects are kept in isolation at holding centers or community care centers while awaiting these results, creating additional risks of cross-infection.⁶

Rapid, decentralized testing that does not require specialized laboratories will improve response efforts. Suspected cases from remote areas will not have to travel great distances for testing, which may reduce the delays in testing and treatment seeking that fuel community transmission. Prompt diagnostic feedback will reduce or potentially eliminate the time non-cases spend in isolation wards, which will allow surveillance teams to quickly identify symptomatic contacts, improve border screening, and streamline triage in health care facilities, thereby reducing the burden on non-Ebola services. Prompt feedback will also reduce the complexity of data feedback from testing sites to district Ebola response centers, where poor communication and data systems have hampered response efforts. In this context, Ebola testing using rapid diagnostic tests holds great potential. A rapid diagnostic test with high positive and negative predictive values is ideally suited for screening suspected Ebola cases. A point-of-care test that can reliably detect early Ebola infection can also facilitate rapid case identification in the field. Effective triage of confirmed Ebola cases at community care centers and treatment units could improve survival, open bed-space occupied by non-Ebola cases, and reduce the risk of cross-infection.

For the past decade, Kenema Government Hospital, the regional hospital for the Eastern Province of Sierra Leone, has maintained a clinical research program on Lassa fever, a viral hemorrhagic fever that is endemic in some parts of West Africa.^{7, 8} The first identified cases of Ebola in Sierra Leone were confirmed by Kenema Lassa team members in May 2014.⁹ The existing infrastructure for viral hemorrhagic fever clinical and laboratory research provided opportunities to evaluate novel diagnostic platforms for Ebola virus.

Clinical validation of a novel point-of-care diagnostic platform is typically divided into several phases. First, pilot lots of the assay kit are tested on banked samples. After carrying out optimization procedures, investigational use only lots are tested prospectively in the laboratory by experienced technicians using venous whole blood. These results are then compared to a benchmark assay. In the case of Ebola, the benchmark assay is quantitative real-time polymerase chain reaction (qRT-PCR). Finally, the point-of-care device is tested in the field using capillary blood collected by frontline healthcare workers.

Preliminary evaluation of the ReEBOV™ Rapid Diagnostic Test (Corgenix Medical, Inc.) using stored serum specimens was performed at the Viral Hemorrhagic Fever Laboratory at Kenema Government Hospital. Assay kit pilot lots tested against stored specimens posed a range of challenges in relation to the duration and conditions of specimen storage, specimen degradation, and hemolysis. Despite the conditions, the ReEBOV™ RDT demonstrated good agreement with the comparator method (Centers for Disease Control and Prevention PCR as previously described¹⁰). The rapid test gave 91% agreement with PCR positive specimens and 90% agreement with PCR negative specimens (M. Boisen, *in preparation*). Signal range measured through band intensity and uniformity satisfactorily indicated uniform flow of the sample and reagents through the test strip. As the antigen concentration decreases, it is desirable to continue seeing a uniform but progressively weaker signal as the limit of detection is reached. This uniformity aids the operator in the interpretation of low intensity, positive signals.

This article describes a study designed to validate the sensitivity and specificity of the investigational use only lots of ReEBOV™ rapid diagnostic using venous whole blood and capillary blood obtained via fingerprick. The overall goal of this assay is to provide quick bedside identification of Ebola virus positive patients and reduce the risk of transmission. The rapid diagnostic is not intended to replace confirmatory testing by PCR, but rather it is intended to complement the time-consuming PCR technique by providing a rapid preliminary Ebola virus screen to guide appropriate isolation of Ebola virus positive patients. Due to circumstances described in the discussion, our group was unable to initiate subject enrollment. However, we feel that the study design is worthy of detailed description as it offers many lessons in conducting trials during outbreaks.

Study design

We designed a diagnostic accuracy study, with the primary objective of validating the ReEBOV™ Rapid Diagnostic Test. This study was designed to utilize both capillary whole blood from fingerprick and whole blood collected by venipuncture to test the hypothesis that the ReEBOV™ rapid diagnostic will maintain clinical performance of at least 90% sensitivity and at least 95% specificity when compared to qRT-PCR. We chose to simultaneously test the device in centralized laboratories and at decentralized testing centers (Figure 1). This approach was proposed for the following reasons: 1. The need for validation of the rapid diagnostic during the recent outbreak was too urgent to train personnel at all of the testing sites to perform the rapid diagnostic on capillary blood at this stage of testing; 2. Some potential testing sites were not directly connected with a specific Ebola treatment unit. 3. Laboratories were generally not within close proximity to patients and testing centers.

Testing sites currently use venous whole blood collection as part of the standard operating procedures for testing. By maximizing the use of these specimens from these sites, we could decrease the number of required testing sites while improving the likelihood of obtaining a qRT-PCR result for each sample.

Division of performance sites

Centralized venous whole blood testing sites

When the study was designed, there were multiple holding centers throughout Sierra Leone.¹¹ It was impractical to train staff to obtain consent and conduct testing at all holding centers during the outbreak. Blood specimens from suspected Ebola patients were sent from multiple holding centers to centralized laboratories for testing. After qRT-PCR testing was performed, we proposed using the excess blood specimen for evaluating the rapid diagnostic while using the qRT-PCR results and clinical signs and symptoms for official patient diagnosis. Under this scenario, a standard case investigation form would be used to collect data on demographics and clinical signs and symptoms for each suspected case. No direct patient involvement was proposed in this portion of the study and consent would not be obtained for use of these specimens.

Decentralized capillary whole blood testing sites

Samples from suspected Ebola patients for qRT-PCR Ebola testing would be collected by venipuncture at holding centers or community care centers. A drop of blood would also be collected via fingerstick for use on the rapid diagnostic. Confirmatory testing would then be performed at a central facility by qRT-PCR on the whole blood specimen collected by venipuncture. After performing the qRT-PCR testing, the excess blood specimen would also be used for the RDT testing at the central laboratory. Oral consent would be obtained from subjects at these sites. Because the ReEBOV™ RDT kits are only suitable for investigational use, we would not provide the treating physicians or patients with the RDT results.

After initial testing with the assay under evaluation and the benchmark assay, patients admitted to Ebola Treatment Centers would be repeatedly tested by qRT-PCR during the course of their hospitalization to monitor Ebola antigenemia. Repeat testing with the ReEBOV™ RDT would be performed whenever qRT-PCR testing is performed with the precise testing schedule as determined by the treating physicians. Current practice standards dictate that Ebola patients should be tested for Ebola virus clearance by qRT-PCR when they are asymptomatic for at least 48 hours. A capillary blood sample would be collected at the time of whole blood collection. If the Ebola patient continued to have evidence of Ebola virus circulation (i.e., continued positive qRT-PCR), they would be retested following the Ebola treatment center's standard practice (generally after another 48 hours). The qRT-PCR test is the official diagnostic test and the benchmark assay.

Clinical and demographic data—Clinical and demographic data would be collected on all suspected Ebola cases at study entry using the standard case investigation forms for public health surveillance and disease outbreak response. This data would be obtained from

the Sierra Leone Ministry of Health and Sanitation at the end of the study, rather than by study personnel.

Participants

Patients would be eligible for enrollment in the decentralized capillary blood testing portion of the study if they meet the World Health Organization case definition for Ebola (Table1).¹² Patients who are unable to cooperate with the fingerstick or venous blood draw or unable to provide consent would be ineligible. All samples received at participating centralized laboratory testing centers during the study period would be eligible for study use.

Testing and interpretation procedures

The ReEBOV™ Antigen Rapid Tests would be evaluated according to the manufacturer's instructions. Briefly, after informed consent is obtained at the holding centers, four drops of sample buffer would be added to 5ml capped polypropylene round-bottom tube (Falcon, Corning). A safety lancet (CoaguChek® Lancets, Roche) would be used to perform a finger stick. One to two drops of blood (approximately 30ul) would then be placed on the sample pad of the dipstick. All study personnel would be instructed to oversaturate the sample pad. The dipstick would then be placed into the snap cap tube containing the buffer. After replacing the cap and placing the tube upright into a rack, the dipstick would be allowed to develop for 15 to 25 minutes. The procedure would be standardized across centralized lab facilities where 30ul of whole blood would be added to the sample pad via pipette. The package insert includes step-by-step visual instructions. In addition, the manufacturer produced an interpretation card that assists with test interpretation.

At all testing sites, the tests would be read by two independent observers for a dichotomous response of test positivity or negativity. In cases of disagreement between the two readers, a third independent reader would serve as a tie-breaker. A visual aid supplied by the manufacturer would be made available to assist readers with interpretation.¹³ A solid control line would be required on the dipstick to yield a valid result. A pink to red line across the test area would be considered a positive result. Although the visual aid provided in the kit allows for a semi-quantitative interpretation on a scale of 0 to 5, we would ask readers to interpret the test as positive or negative only. All results would be recorded on standardized forms. Due to infection control concerns, a picture of the form would then be taken via tablet and then sent wirelessly to a password-protected computer outside of the testing area or laboratory.

The positive and negative controls included in the test kit are lyophilized. They are reconstituted by adding 0.250 ml of laboratory-grade water. Controls would be performed each time a new kit is opened and then on a weekly basis. The controls should be stored at 4°C between tests. However, refrigeration is not always available at all holding centers. At such sites, the controls would be replaced on a weekly basis. The qRT-PCR diagnostic serves as the benchmark assay. Although a commercially available kit exists (RealStar® Ebolavirus RT-PCR Kit 1.0, Altona Diagnostics, Hamburg, Germany) and is the assay preferred by the WHO Ebola Diagnostic Assessment Committee, it is not universally used in

testing centers. For all test samples, the qualitative interpretation (positive or negative) would be recorded with the quantitative. These results would be sent wirelessly to the research staff by participating laboratories.

Clinical data

Clinical data, including age, gender, temperature, pulse, respiration rate, and clinical signs and symptoms, would be collected from using case investigation or laboratory forms for all consented subjects at the isolation center.

Sample size assessment

At the time of study design, the sample size assessment was based on the ability to estimate 95% confidence interval half widths for the rapid diagnostic test's sensitivity, specificity, and positive predictive value. Based on positivity rates early in the outbreak, we anticipated a 2:1 ratio of patients with negative to positive diagnostics, and we aimed to enroll 600 subjects to obtain 400 antigen- negative controls and 200 antigen-positive cases. Pilot studies estimated the rapid diagnostic test's sensitivity, specificity, and positive predictive value as 90%, 95%, and 90%, respectively. Conditional on these estimates, the 200 cases would be sufficient for estimating the rapid diagnostic's sensitivity to within 4.2 percentage points of their true values. The 400 controls would be sufficient for estimating the rapid diagnostic's specificity to within 2.1% of its true value. We also anticipated a 2:1 ratio of patients with negative to positive test results. Conditional on this, the 200 positive-test individuals would be sufficient for estimating the rapid diagnostic's positive-predictive value to within 4.2 percentage points of their true values.

Ethical issues and approval

Strict infection control measures are in place at all Ebola treatment and holding centers because of the severity and high mortality rate of Ebola and concerns for transmission. These measures require that all items coming in contact with suspected Ebola patients be incinerated. Due to these restrictive measures coupled and low levels of literacy, we would be unable obtain written consent. For those cases where excess blood samples would be collected at centralized testing centers, study personnel would not have access to patient information and waivers of informed consent would be requested. Witnessed verbal informed consent would be obtained at sites collecting fingerprick samples for capillary whole blood. A script describing the study purpose and procedures would be read to each subject. An informed consent log would document study subjects who verbally consent to enroll in the study.

Approval for this study was obtained from the Sierra Leone Ethics and Scientific Review Committee and the Tulane University Human Research Protection Program and the protocol was reviewed by the WHO Ebola Diagnostic Assessment Committee. All aspects of the study, including specimen collection, Ebola testing, data entry, and data analysis would be performed independently of the of ReBOVTM rapid diagnostic's manufacturer (Corgenix, Inc).

Discussion

This study was designed to rapidly and efficiently determine the sensitivity and specificity of the ReEBOV™ rapid diagnostic during the largest Ebola outbreak in history. The immediate need for such a test required that the typical testing strategy focusing on initial use in controlled settings be replaced with validation testing in more “intended use” environments. The design described above has several strengths. First, the use of two testing venues would simultaneously allow us to evaluate the rapid diagnostic test’s performance on fresh samples both under controlled conditions and in the field. Second, by having samples tested at both holding centers and central laboratories, we could evaluate discrepancies between sample types.

Unfortunately, significant limitations exist in the study design and the outbreak environment. First, the design utilizing holding centers, centralized laboratories and Ebola treatment center required significant coordination with multiple organizations, many of which are operated by nongovernmental organizations not familiar with research. Although most of those approached expressed great interest in evaluating a rapid diagnostic test, they faced significant hurdles. At the time, many nongovernmental organizations lacked the organizational infrastructure to approve research study participation. Most centralized laboratories and holding centers were experiencing severe personnel limitations and frequent staff rotation. Coupled with stringent biosafety measures, the limited number of personnel prohibited staff from participating in study procedures. Second, the immediate need for the test during this outbreak also prevented full development of the test. The test strip design does allow for semi-quantitative interpretation, but we chose to limit interpretation to a qualitative read-only that expressed the results as either positive or negative. The semi-quantitative interpretation would require the development of a proficiency panel as well as more extensive training of laboratory personnel prior to trial initiation. Given the urgent need to start the trial and the numerous study sites required, such work was not feasible.

The performance of the qRT-PCR was also a significant concern. There was a strong desire by regulatory agencies to use a commercially available, validated test as the benchmark assay. At the time of study design, the RealStar® Ebolavirus RT-PCR Kit 1.0 (Altona Diagnostics, Hamburg, Germany) was the only commercially available assay. This posed several significant problems. First, the test was difficult to procure and not widely used in Sierra Leone at the time of the study design. Second, it was validated on a limited number of qPCR machines that were not available in many centralized laboratories. Third, most Ebola testing centers in Sierra Leone were using this kit to validate their mobile laboratories and then reverted to a “home brew” kit. Fourth, there is some evidence that the RealStar® Ebolavirus RT-PCR Kit 1.0 has inferior sensitivity at lower viral loads.¹⁴ However, this may be due to the fact that the kit was being used in settings that were not consistent with the manufacturer’s recommendations. Finally, it is unclear if qRT-PCR results are equivalent amongst laboratories. Throughout this outbreak, qRT-PCR results have been reported as the number of cycles to positivity (cycle time values) rather than as viral loads. Reporting viral loads would require the use of a standard curve and would significantly increase the workload of the already overburdened testing centers. These reporting practices resulted in

varying “cut-offs” from laboratory to laboratory. In other words, the benchmark assay did not have defined, standardized thresholds for test positivity and negativity.

The future utility of this test also presents a potential limitation in that it will not allow for predicting the specific strain for a subsequent outbreak. Therefore, it is unknown how well the ReEBOV™ rapid diagnostic will perform in the future. However, it has been shown *in vitro* to perform similarly in the detection of both the Makona and Mayinga strains of Ebola as well as Sudan and Bundigbugyo EBOV (M Boisen, in preparation). The ReEBOV™ rapid diagnostic does not detect Reston Ebola or Marburg virus, and Tai Forest EBOV has not been tested.

Several significant lessons were learned between inception of the study design and attempts to initiate subject enrollment and sample testing. In an outbreak setting with rapidly changing hotspots and fluid implementing partners, identifying performance sites with sufficient patient numbers poses a great challenge. When the study was conceived, a significant number of suspected Ebola patients were still presenting to hospitals and outreach units in Kenema and its surrounding districts. The initial study design assumed that a sufficient number of potential subjects would be present in the catchment area of our long-standing research team based at Kenema Government Hospital. However, by the time the protocol was fully approved, the number of patients in our base area was minimal and hospitalizations were peaking in other districts. Ultimately, we were unable to initiate this study, and a modified version of this study design was performed by another group.¹⁴

Based on our experience, several things could improve study design and implementation during outbreaks. Most importantly, a centralized organization coordinating all investigational activities would greatly reduce the time from study approval to subject enrollment. Although there was a desire to coordinate amongst teams, each team operating in the region was struggling to complete their individual role in the response or move forward with their own research goals for much of the first year of the outbreak. Personnel were stretched very thin in all laboratories. With little flexibility in human resources, coordination and collaboration was particularly challenging. In addition, for those seeking to initiate studies, a significant amount of effort and time was spent identifying appropriate collaborators. A central, coordinating body might remedy this issue. Also, incorporating agreements for research use of clinical excess samples into memorandums of understanding and material transfer agreements between ministries of health and partners involved in the response would also help streamline the study.

The need for coordination among research groups is exemplified by the simultaneous planning and initiation of rapid diagnostic test development and vaccine efficacy studies. Both the rVSV and Ad5 vaccines currently under investigation are live vaccines that express the Ebola glycoprotein.^{15, 16} A protein detection assay that targets Ebola glycoprotein may detect this protein for some period after vaccination, leading to lower test specificity and unnecessary admissions. Any admission to an Ebola treatment unit due to a false positive test result will result in exposure to confirmed patients and increased risk of transmission. Because the ReEBOV™ rapid diagnostic detects the Ebola matrix VP40 protein, there is no expectation of decreased specificity among those receiving the vaccination. However,

coordination among teams studying novel vaccines and diagnostic platforms to test for such reactivity is advisable.

For validation studies of novel diagnostic platforms, liberalization of the benchmark assay is also essential in a way that does not compromise study validity. This is especially true in the case of Ebola testing where no gold standard test is available. Similarly, for competing emerging infections, identification of the most appropriate comparator test may be impossible. For the validation studies for the ReEBOV™ rapid diagnostic, the WHO Ebola Diagnostic Assessment Committee agreed to allow the rapid diagnostic to be compared to any qRT-PCR protocol being used in WHO-recognized laboratory facilities. Further quality assessment was performed in a more controlled setting using banked samples and the sole commercially available test.

Ultimately, there is a delicate balance between response to current epidemics and development of tools to assist in future epidemics, with the highest priority always placed on response to current threats. There is no other time but during an outbreak to conduct research on Ebola. Building mechanisms for the development of tools in an outbreak context will ensure that these activities complement, rather than impede, response efforts.

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Figure 1.
Study design

Table 1**Ebola Virus Disease Suspected WHO Case Definition¹²**

Any person suffering or having suffered from a sudden onset of high fever and having had contact with:	
-	a suspected, probable or confirmed Ebola case
-	a dead or sick animal
OR: any person with sudden onset of high fever and at least three of the following symptoms	
-	headache
-	anorexia/loss of appetite
-	lethargy
-	aching muscles or joints
-	breathing difficulties
-	vomiting
-	diarrhea
-	stomach pain
-	difficulty swallowing
-	hiccups
OR: any person with inexplicable bleeding	