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Ebola virus disease diagnostics, Sierra Leone: analysis of real-time RT-PCR values in clinical blood and oral swab specimens

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Abstract

During the Ebola virus outbreak of 2013–2016, the Viral Special Pathogens Branch field laboratory in Sierra Leone tested approximately 26,000 specimens between August 2014 and October 2015. Analysis of the B2M endogenous control Ct values showed its utility in monitoring specimen quality, comparing results with different specimen types, and interpretation of results. For live patients, blood is the most sensitive specimen type and oral swabs have little diagnostic utility. However, swabs are highly sensitive for diagnostic testing of corpses.

Keywords

Ebola virus; West Africa; qRT-PCR; Housekeeping Gene

Background

The laboratory response to the largest outbreak of Ebola virus (EBOV), species *Zaire ebolavirus*, in West Africa has been an international endeavor, with up to 35 field laboratories from 26 countries having operated at some point during the outbreak. This outbreak proved challenging for even the most seasoned field laboratorians due to the length of staff deployments and the numbers of specimens tested; the Viral Special Pathogens Branch (VSPB), Centers for Disease Control and Prevention (CDC), laboratory response could not have been accomplished without support from various groups within CDC. Of the total 28,652 cases, 15,261 were laboratory-confirmed, illustrating the prodigious total number of specimens tested by EBOV diagnostic laboratories in the affected countries [1].

In August 2014, VSPB set up a molecular diagnostics laboratory at Kenema Government Hospital at the request of the Sierra Leone Ministry of Health and Sanitation and World

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Health Organization. In October 2014, the laboratory moved to the Médecins Sans Frontiéres (MSF) Ebola Treatment Center (ETC) in Bo, Sierra Leone's second largest city. As a result of the new location with improved accessibility and a simple laboratory set-up, approximately 26,000 diagnostic specimens were processed over 14 months [2]. The large number of specimens tested employing the same VSPB EBOV real-time reverse transcription polymerase chain reaction (qRT-PCR) assays allowed for analysis of assay robustness and comparison of blood and oral swab specimen types leading to enhanced result interpretation.

Methods

The requested specimen type for testing live patients for EBOV disease (EVD) was EDTA blood; however, oral swab specimens were submitted from some live patients. The majority of oral swabs were collected from corpses and preserved in viral transport media, but were occasionally submitted dry or in agar tubes. All test results reported here were performed for clinical diagnostic purposes in support of CDC's public health response to the EVD epidemic in Sierra Leone; thus, were not subject to Institutional Review Board requirements.

Processing of suspected and confirmed EBOV specimens in field settings and a complete description of the workflow, personal protective equipment used by laboratorians, and ribonucleic acid (RNA) extraction has been reported [2]. Three separate assays were performed for each specimen: VSPB's qRT-PCR assays targeting EBOV nucleoprotein (NP) or VP40 genes, and a commercial assay detecting human beta-2-microglobulin (B2M) mRNA as an endogenous control (ThermoFisher Scientific; EBOV primer/probe sequences available upon request). The SuperScript™ III Platinum® One-Step qRT-PCR kit (ThermoFisher Scientific) was used with 5 μL RNA per 25 μL reaction. Thermal cycling conditions were 50°C for 15 min; 95°C for 2 min; 40 cycles of 95°C for 15 sec and 55°C for 45 sec. Threshold setting for the qRT-PCR reaction was automatic by the Bio-Rad CFX Manager 2.1 software, and was only manually adjusted if an issue was detected, such as a background spike due to a power interruption. Results were reported as positive, negative, or pending, and the interpretation of results has been previously described [2]. Further interpretation of results categorizing patients into convalescent, acute, and "not a case" as done in previous outbreak responses was initially attempted, but the lack of a universal patient ID number and specimen submission to different field laboratories made this approach impractical. Ct values were analyzed using a non-parametric kernel density estimation to generate the empirical distribution function.

Results

A qRT-PCR assay targeting endogenous B2M mRNA was run for each specimen to monitor specimen quality and serve as an extraction and qRT-PCR control. A subset of 52 blood specimens and paired oral swabs (with minimal storage time between specimen collection and processing) from MSF's Bo ETC patients was analyzed (Figure 1A) and resulted in a baseline distribution of B2M Ct values (Figure 1B). The distribution and mean B2M Ct values of the remaining 24,428 blood and swab specimens were similar to those of the small subset. Compared to blood, swab specimens were less consistent and likely had lower cell

count as demonstrated by higher B2M Ct values (Figure 1B and Table 1). The comparison of B2M values of EBOV-positive and EBOV-negative specimens demonstrated that the EBOV result did not impact the B2M Ct distribution (data not shown). To determine how storage time affected the B2M Ct value, the average Ct values based on time from collection to testing were analyzed; over 92% of specimens were tested within 4 days of collection, 99% were tested within 1 day of receipt by the laboratory, and no difference was observed for the average B2M Ct values for any time point (data not shown). In approximately 1% of all specimens tested, B2M was not detected possibly due to poor specimen quality, RNA extraction, or qRT-PCR problems; 85% of the B2M negative specimens were attributed to swab specimens.

The distributions of Ct values for both EBOV targets were very similar, with the NP assay being slightly less sensitive; therefore, only the VP40 data were used for the remaining Ct value analyses. Patient blood was tested when determining whether a symptomatic patient should be admitted to an ETC and when recovering EVD patients could be discharged. In order to obtain a distribution of initial VP40 Ct values from patients with acute EVD, test results from confirmed patients being tested for discharge were omitted from analysis. However, given difficulties in identifying and following patient movement between facilities, it is likely that not all subsequent specimens for EVD confirmed patients could be reliably identified and removed. The distribution of the VP40 Ct values from 1,756 acute blood specimens (range 12-39, mean 25) indicated 2 populations: the main peak (Ct ~21) and a “shoulder” appearing at Ct ~30 (Table 1, Figure 1C), which was not dependent on time post specimen collection (data not shown). In an effort to characterize the “shoulder” population, a subset of 156 specimens with known outcome (72 survivors, 84 deaths) was analyzed and a similar distribution was observed (Figure 1C). However, the “shoulder” was absent from the distribution of the fatal cases (Figure 1C), and the peak for the survivor specimens shifted to the right indicative of higher Ct values (data not shown).

Of the 1,756 acute blood specimens, approximately 77% of initial VP40 qRT-PCR results of acute patients had a Ct value ≤30, and 62% were ≤25 (Table 1). The distribution of blood specimen VP40 Ct values was generated based on time post symptom onset, and no pronounced differences were observed; the “shoulder” population was also seen on each day after symptom onset (Figure 1D). For the 442 corpse swab specimens, the VP40 Ct distribution indicated a single population (range 12-39, mean 25) (Table 1, Figure 1C).

The VP40 qRT-PCR of paired EDTA blood and swab specimens from 52 live patients are shown in Figure 1A. All patients, except for 2, were recovering and undergoing testing for discharge. Thirty-two of the patients had either positive blood and/or swab results. Only 9.4% of blood/swab pairs demonstrated concordant positive EBOV test results and in 87.5% with positive test results, EBOV RNA was detected in blood but not the corresponding swab specimen, one of them being tested for initial diagnosis. One pair (3.1%) had a positive swab (Ct 38) and negative blood result, although a curve below the threshold was observed for the blood specimen. Overall, the difference between the paired blood and swab specimen B2M Ct values ranged from 7–18.

Discussion

The testing of approximately 26,000 specimens by one field laboratory provided an opportunity to evaluate the results of VP40 and B2M assays and describe the characteristics and suitability of blood and oral swab specimens. The turnover of laboratory workers was high, with teams rotating in and out every 4 weeks. However, the data show the robustness of the assays despite the changes in personnel and the lack of temperature and humidity controls in the laboratory space and during specimen transport.

An endogenous control B2M assay was routinely run on each specimen to control for specimen quality, RNA extraction/integrity, and amplification. The analyses of B2M values for blood specimens resulted in a narrow distribution curve as expected for a defined specimen type. The wide range of Ct values observed for swabs reflects the heterogeneity of this specimen type due to collection technique differences resulting in variable number of cells in the specimen, dilution factor based on the device used, and possibly degradation from host enzymes and bacteria in the oral cavity (Figure 1A and 1B)[3]. Specimen quality in general was a concern given the sometimes long distances between collection site and the laboratory with no reliable cold chain in place. However, the B2M data indicate that up to 4 days from the time of collection to testing had no negative impact on test results, which is in agreement with published human and experimental animal studies [4–6]. In regard to result interpretation, ideally blood and swab specimens with a negative Ebola result and a B2M value above 30 and 35, respectively, should be disregarded and a new specimen collected and tested. As this is impractical in most field situations, it is recommended for negative Ebola specimens with B2M results in the higher Ct range not be relied on and to proceed with a safe burial.

During the 2013–2016 EVD outbreak, considerable interest was expressed for testing oral swabs from live patients, given the acceptance of this collection method by both patients and clinicians. However, the data set from paired blood and oral swab specimens (Figure 1A) demonstrate consistently higher Ct values for the Ebola target genes (by up to 14 Cts) in swabs compared to blood specimens, indicating that swabs are considerably less sensitive. This is consistent with findings from previous filovirus outbreaks in Republic of Congo and Angola and several animal studies [5–8], which indicated that swabs are suitable specimens only for severely ill patients and corpses. Especially for deceased patients, the anticipated high virus load [5–8] and safety concerns with performing cardiac puncture in field settings also support the continued use of swabs; however using swabs for the diagnosis of live patients will lead to false negative results.

Analysis of 1,756 initial VP40 Ct values of acute patients identified 2 populations; the main population at Ct ~21 and a second population at Ct ~30 (Figures 1C and 1D). Based on previously observed differences between Ct values of acute specimens from Ebola survivors versus fatal outcome [9–10], the question arose as to whether the higher Ct “shoulder” may predominantly represent cases destined to survive. When 156 specimens with known outcomes were analyzed, a similar distribution was observed (Figure 1C). However, the “shoulder” was absent in the analysis of fatal cases (Figure 1C) suggesting that survivors with lower viral loads contribute to the “shoulder”. The same distribution of VP40 Ct values

was seen on each day post symptom onset (Figure 1D), in contrast to the expectation that in the first few days, viral loads would be lower and increase during the course of illness either until death or until they begin to decrease for recovering patients. This result strongly suggests that the dates provided for symptom onset were inaccurate and cannot be relied upon for clinical or diagnostic purposes.

The data and analysis of the VSPB EBOV test results also provide insights in how to integrate new diagnostic tools in the outbreak response. While qRT-PCR is still used as the gold standard for EVD diagnosis, several rapid diagnostic tests (RDTs) were developed during the course of the outbreak [11]. Considering that approximately 77% of acute patients presented with an initial Ct value ≤30, it is critical that RDT specificity and sensitivity calculations include specimens with a clinically relevant range of Ct values (i.e., specimens with Ct values >30).

In summary, the B2M data demonstrated that RNA extraction and qRT-PCR assays established in the field were robust and that delays from collection to testing and a lack of cold chain did not have a measurable impact on test results. For live patients, blood continues to be the most sensitive specimen type and collection of oral swabs from these individuals should be discouraged.

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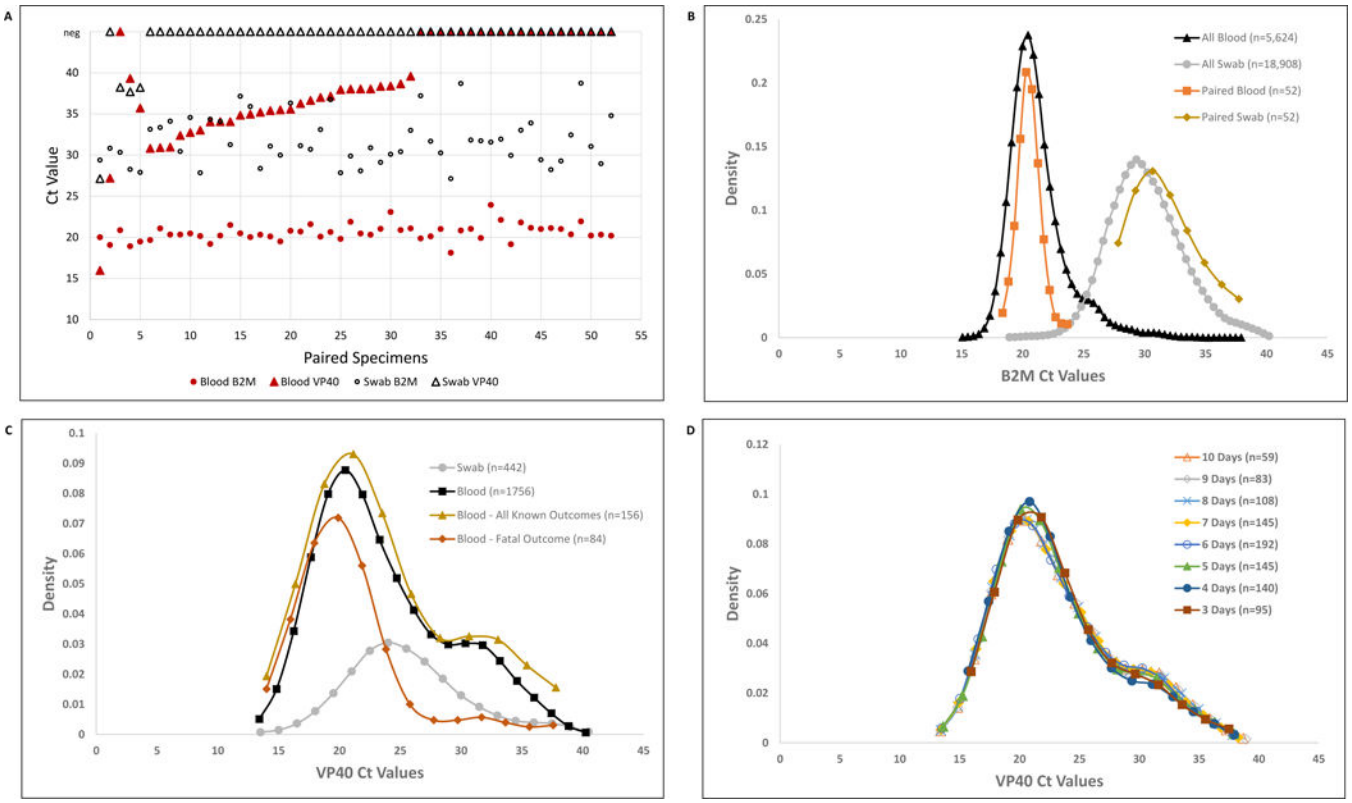

Figure 1.

Centers for Disease Control and Prevention (CDC) Viral Special Pathogens Branch (VSPB) field laboratory blood and oral swab specimen analysis. A) Paired blood and oral swab specimen Ct values for B2M and EBOV VP40 from 52 live patients. Specimen order was adjusted based on result. Neg refers to a negative B2M or EBOV VP40 result and no value is assigned. B) Non-parametric kernel density estimation (KDE) was used to generate the empirical distribution function for B2M Ct values for a subset of paired blood and oral swab specimens and for the total blood and oral swab populations. C) Distribution by KDE of EBOV VP40 Ct values for blood and oral swab specimens, and distribution of a subset of blood specimens with a known outcome and a fatal outcome. D) Distribution by KDE of Ebola VP40 Ct values for each day post onset of symptoms.

Table 1

Quantitative Reverse Transcription-Polymerase Chain Reaction Testing for Endogenous β-2-Microglobulin (B2M) Messenger RNA and the Gene Encoding Ebola Virus (EBOV) Viral Protein 40 (VP40) Among Blood and Swab Specimens

Measurement	Category	Count	Ct Range	Ct Mean	% Ct ≤25	% Ct ≤30
B2M	Blood	5624	15-37	21	NA	NA
	Swab	18908	18-40	30	NA	NA
Ebola VP40	Blood - All	2263	12-40	26	54	66
	Blood - Acute	1756	12-39	25	62	77
	Swab	442	12-39	25	55	86

NA = Not Applicable