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SUPPLEMENT ARTICLE

Ebola Virus Disease Diagnostics, Sierra Leone: Analysis of Real-time Reverse Transcription–Polymerase Chain Reaction Values for Clinical Blood and Oral Swab Specimens

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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; endogenous control.

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 owing to the length of staff deployments and the numbers of specimens tested; the laboratory response of the Viral Special Pathogens Branch (VSPB) at the Centers for Disease Control and Prevention (CDC) could not have been accomplished without support from various groups within the CDC. Of the 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, the VSPB set up a molecular diagnostics laboratory at Kenema Government Hospital at the request of the Sierra Leone Ministry of Health and Sanitation and the World Health Organization. In October 2014, the laboratory moved to the Médecins Sans Frontiéres Ebola treatment center in Bo, Sierra Leone's second largest city. As a result of the new location with improved accessibility and a simple laboratory setup, approximately 26 000 diagnostic specimens were processed over 14 months [2]. The large number of specimens that were tested using 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 blood containing ethylenediaminetetraacetic acid (hereafter, "EDTA blood"); however, oral swab specimens from some live patients were submitted. The majority of oral swab specimens were collected from corpses and preserved in

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viral transport medium, but they were occasionally submitted dry or in agar tubes. All tests with results reported here were performed for clinical diagnostic purposes in support of the CDC's public health response to the EVD epidemic in Sierra Leone and, thus, were not subject to institutional review board requirements.

Field processing of specimens in which EBOV was suspected or confirmed and a complete description of the work flow, personal protective equipment used by laboratorians, and RNA extraction method has been reported [2]. Three separate assays were performed for each specimen: VSPB's qRT-PCR assays targeting EBOV nucleoprotein (NP) or viral protein 40 (VP40) genes and a commercial assay detecting human β-2-microglobulin (B2M) messenger RNA (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 of RNA per 25-μL reaction. Thermal cycling conditions were 50°C for 15 minutes, 95°C for 2 minutes, and 40 cycles of 95°C for 15 seconds and 55°C for 45 seconds. 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 identifier and specimen submission to different field laboratories made this approach impractical. Cycle threshold (Ct) values were analyzed using a nonparametric kernel density estimation to generate the empirical distribution function.

RESULTS

A qRT-PCR assay targeting endogenous B2M mRNA was performed 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 swab specimens (with minimal storage time between specimen collection and processing) from patients at Médecins Sans Frontiéres' Bo Ebola treatment center was analyzed (Figure 1*A*) and resulted in a baseline distribution of B2M Ct values (Figure 1*B*). 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 with blood specimens, swab specimens were less consistent and likely had a lower cell count, as demonstrated by higher B2M Ct values (Figure 1*B* 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; >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 owing to poor specimen quality, or problems with RNA extraction or qRT-PCR; 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 analyses. Patient blood specimens were tested when determining whether a symptomatic patient should be admitted to an Ebola treatment center and when recovering patients with EVD could be discharged. To obtain a distribution of initial VP40 Ct values from patients with acute EVD, test results from patients with confirmed EVD who were 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 patients with confirmed EVD could be reliably identified and removed. The distribution of the VP40 Ct values from 1756 acute blood specimens (range, 12–39; mean, 25) indicated 2 populations: the main peak (Ct, approximately 21) and a so-called shoulder appearing at a Ct of approximately 30 (Table 1 and Figure 1*C*), which was not dependent on time after specimen collection (data not shown). In an effort to characterize the shoulder population, a subset of 156 specimens with known outcome (72 from survivors and 84 from individuals who died) was analyzed, and a similar distribution was observed (Figure 1*C*). However, the shoulder was absent from the distribution of the fatal cases (Figure 1*C*), and the peak for the specimens from survivors shifted to the right, indicative of higher Ct values (data not shown).

Of the 1756 acute blood specimens, approximately 77% of initial VP40 qRT-PCR analyses had a Ct of ≤30, and 62% had a Ct of ≤25 (Table 1). The distribution of blood specimen VP40 Ct values was generated on the basis of time after symptom onset, and no pronounced differences were observed; the shoulder population was also seen on each day after symptom onset (Figure 1*D*). For the 442 corpse swab specimens, the VP40 Ct distribution indicated a single population (range, 12–39; mean, 25; Table 1 and Figure 1*C*).

The VP40 qRT-PCR of paired EDTA blood and swab specimens from 52 live patients are shown in Figure 1*A*. All patients but 2 were recovering and undergoing testing for discharge. Thirty-two patients had either EBOV-positive blood and/or swab results. Only 9.4% of blood/swab specimen pairs demonstrated concordant positive EBOV test results, and in 87.5% with positive test results, EBOV RNA was detected in the blood specimen but not in the corresponding swab specimen, one of which was tested for initial diagnosis. One pair (3.1%)

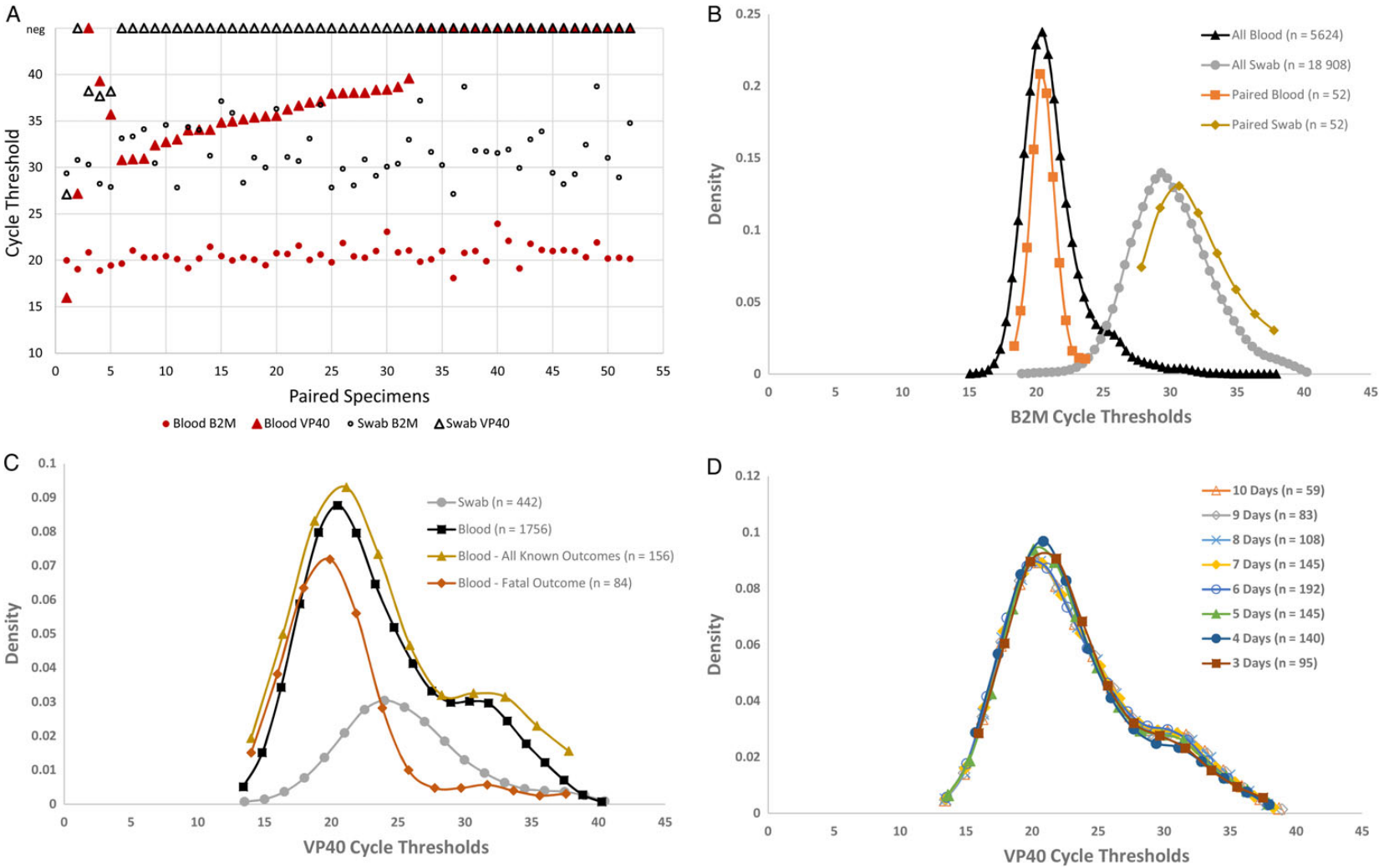

Figure 1. Centers for Disease Control and Prevention Viral Special Pathogens Branch field laboratory blood and oral swab specimen analysis. *A*, Paired blood and oral swab specimen cycle thresholds (Cts) for β-2-microglobulin (B2M) messenger RNA and the gene encoding Ebola virus (EBOV) viral protein 40 (VP40) from 52 live patients. Specimen order was adjusted on the basis of test result. *B*, Nonparametric kernel density estimation (KDE) was used to generate the empirical distribution function for B2M Cts 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 Cts 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 EBOV VP40 Cts for each day after onset of symptoms. Neg, negative B2M or EBOV VP40 result, for which no value was assigned.

Table 1. Quantitative Reverse Transcription–Polymerase Chain Reaction Testing for Endogenous Control β-2-Microglobulin (B2M) Messenger RNA and the Gene Encoding Ebola Virus (EBOV) Viral Protein 40 (VP40) Among Blood and Swab Specimens

Target, Specimen	Specimens, No.	Cycle Threshold			
		Range	Mean	≤25, Specimens, %	≤30, Specimens, %
B2M					
Blood	5624	15–37	21	NA	NA
Swab	18 908	18–40	30	NA	NA
EBOV VP40					
Blood	1756	12–39	25	62	77
Swab	442	12–39	25	55	86

Abbreviation: NA, not applicable.

had an EBOV-positive swab specimen (Ct, 38) and an EBOV-negative blood specimen, 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 to 18.

DISCUSSION

The testing of approximately 26 000 specimens by 1 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 performed 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 1*A* and 1*B*) [3]. Specimen quality in general was a concern because of 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 an EBOV-negative result and a B2M value of >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 that EBOV-negative specimens with B2M results in the higher Ct range not be relied on and to proceed with safe burial practices.

During the 2013–2016 EVD outbreak, considerable interest was expressed for testing oral swab specimens 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 1*A*) demonstrate consistently higher Ct values (by up to 14) for the EBOV target genes in swab specimens as compared to blood specimens, indicating that swab specimens are considerably less sensitive. This is consistent with findings from previous filovirus outbreaks in the Republic of Congo and Angola and in several animal studies [5–8], which indicated that swab specimens are suitable 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 swab specimens; however using swab specimens for diagnosis of EBOV in live patients will lead to false-negative results.

Analysis of 1756 initial VP40 Ct values for patients with acute EBOV infection identified 2 populations: the main population, with peak Ct values at approximately 21, and a second shoulder population around Ct values of approximately 30 (Figure 1*C* and 1*D*). Based on previously observed differences between Ct values for acute specimens from Ebola survivors versus those for individuals who died of Ebola [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 1*C*). However, the shoulder was absent in the analysis of fatal cases (Figure 1*C*), suggesting that survivors with lower viral loads contribute to the shoulder. The same distribution of VP40 Ct values was seen on each day after symptom onset (Figure 1*D*), 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 into 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 were developed during the course of the outbreak [11]. Considering that approximately 77% of patients with acute infection presented with an initial Ct of ≤30, it is critical that rapid diagnostic test specificity and sensitivity calculations include specimens with a clinically relevant range of Ct values (ie, specimens with Ct values of >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 swab specimens from these individuals should be discouraged.

Notes

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