Validation of the Cepheid GeneXpert for Detecting Ebola Virus in Semen

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Background. Ebola virus (EBOV) RNA persistence in semen, reported sexual transmission, and sporadic clusters at the end of the 2013–2016 epidemic have prompted recommendations that male survivors refrain from unprotected sex unless their semen is confirmed to be EBOV free. However, there is no fully validated assay for EBOV detection in fluids other than blood.

Methods. The Cepheid Xpert Ebola assay for EBOV RNA detection was validated for whole semen and blood using samples obtained from uninfected donors and spiked with inactivated EBOV. The validation procedure incorporated standards from Clinical and Laboratory Standards Institute and Good Clinical Laboratory Practices guidelines for evaluating molecular devices for use in infectious disease testing.

Results. The assay produced limits of detection of 1000 copies/mL in semen and 275 copies/mL in blood. Limits of detection for both semen and blood increased with longer intervals between collection and testing, with acceptable results obtained up to 72 hours after specimen collection.

Conclusions. The Cepheid Xpert Ebola assay is accurate and precise for detecting EBOV in whole semen. A validated assay for EBOV RNA detection in semen informs the care of male survivors of Ebola, as well as recommendations for public health.

Keywords. Ebola; Semen; Validation; Cepheid Xpert.

During the recent outbreak of Ebola virus (EBOV) in West Africa, assays to detect the virus in blood were readily established, allowing healthcare providers to identify infected individuals, facilitate their admission to Ebola treatment units and mitigate ongoing community transmission through their treatment and isolation. Throughout the epidemic, the US Food and Drug Administration authorized the emergency use of in vitro diagnostics for EBOV detection, but these diagnostics were limited to testing of whole-blood or buccal swab samples [1].

Reports of Ebola viral RNA detected in semen of EBOV disease (EVD) survivors and at least 1 case of documented sexual transmission of the virus has led to interest in detecting EBOV in other body fluids, particularly genital secretions. Findings from a study led by the Centers for Disease Control and Prevention, the World Health Organization (WHO), and the Liberian Ministry of Health suggest that the prevalence of viral persistence and shedding in semen may be as high as 26% among men 7–9 months after recovery from acute EVD [2]. A case of recrudescent EBOV in the cerebrospinal fluid of an EVD survivor and prior reports of detection of the viral RNA in breast milk expand the list of fluids for which detection of the virus is needed [3, 4].

Concerns for the sexual transmission of EBOV has led to recommendations by the WHO that men refrain from unprotected sexual intercourse for at ≥12 months after EVD recovery or until their semen is confirmed to be EBOV free [5]. However, despite this recommendation and the need for accurate testing for EBOV in this and other body fluids, no fully validated assay is available for EBOV detection in fluids other than blood.

We report results of the first clinical validation of a polymerase chain reaction (PCR) assay for EBOV in semen. Given the safety concerns of handling specimens potentially infected with a high-consequence pathogen, the Cepheid GeneXpert PCR platform was selected because RNA isolation, amplification, and detection are contained, and the assay does not require repeat manipulation of the specimen—all features that lead to increased operator safety.

METHODS

Setting

Assay validation was performed at the Phebe Hospital PCR Laboratory in Bong County, Liberia. The laboratory layout follows recommendations from the Centers for Disease Control and Prevention and WHO [6–10]. Briefly, the laboratory is separated into high- and low-risk areas. The high-risk area contains an area to don and doff personal protective equipment, an

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area for sample reception and decontamination, and an area for specimen preparation and virus inactivation within a glove box. The low-risk area contains separate pre- and postamplification rooms.

Assay Platform

The Cepheid Xpert Ebola assay is a rapid, automated test for qualitative detection of the Ebola Zaire virus on the Cepheid GeneXpert Instrument System. The GeneXpert system allows automated extraction and quantitative real-time reverse-transcription (RT) PCR. The Xpert Ebola assay detects EBOV total nucleic acid, amplifying the glycoprotein (GP) and nucleoprotein (NP) genes. Each test cartridge includes a sample adequacy control to ensure that human cells are present in the sample; a Cepheid internal control, an Armored RNA dry bead included in each cartridge to verify adequate lysing of the sample virus and monitor for the presence of inhibitors; and a probe check control to ensure bead rehydration, reaction-tube filling, probe integrity, and dye stability that occurs before RT-PCR [11–14]. This test was granted an emergency use authorization from the US Food and Drug Administration on 23 March 2015.

Samples

The validation procedure was designed to incorporate standards from Clinical and Laboratory Standards Institute and Good Clinical Laboratory Practices guidelines for evaluating molecular devices for use in infectious disease testing [15–21]. For the verification of blood and the validation of semen testing, samples of both body fluids were collected from uninfected individuals and spiked with reconstituted lyophilized EBOV. The lyophilized EBOV stock (donated by Heinzfried Ellerbrok of the Robert Koch Institute, Hamburg, Germany, and originally provided by Stephan Günther from the Bernhard Nocht Institute for Tropical Medicine in Hamburg) originated from a Gueckedou isolate of the Ebola Zaire strain. The virus stock was heat-inactivated and subsequently gamma-irradiated, and the absence of infectivity was confirmed by cultivation experiments. The nucleic acid content of the heat-inactivated and gamma-irradiated virus stock was verified by 3 expert Ebola testing laboratories (personal communication, Heinz Ellerbrok, manuscript in preparation).

Virus stock was diluted by genomic equivalents in the sending laboratory. For our work, the lyophilized EBOV samples were reconstituted with 100 µL of Molecular Grade RNase-, DNase- and protease-free water (Corning), and serial dilutions were prepared using the donor specimens. Aliquots of spiked samples were prepared and tested over 3 days, then a new vial of lyophilized virus was reconstituted and samples were prepared for use over the next 3 days. This procedure was repeated until all testing was completed. Testing of all validation samples occurred within 72 hours after collection to evaluate stability of the gene targets (GP and NP) and sample integrity for each sample type. Statistical and logistical factors were considered when determining acceptable stability limits; limits were defined as acceptable if the probit analysis of analytical limits of detection on day 3 shows results within ±0.25 $\log_{10}$ copies/mL of those achieved on day 1.

Blood

Because EBOV RNA detection in blood has previously been validated using the Cepheid GeneXpert PCR platform, sensitivity and repeatability were verified by testing 40 EBOV-spiked positive and 20 negative donor samples on each instrument in local conditions [22–26]. Blood was collected in two 4-mL EDTA tubes from 4 donors, was spiked immediately after collection with the reconstituted virus stock to achieve a dilution series of samples containing 10^2–10^6 copies/mL, and was aliquoted into multiple sets of complete dilution series that consisted of 5 concentrations run in singlet over 4 days (n = 20) to show repeatability and 2 concentrations run in duplicate (n = 16) to verify sensitivity and repeatability experiments over the same 4 days. The negative (undiluted) donor samples were run 5 times each day (total n = 20). The first set of the dilution series samples was tested the same day as collection and spiking; the remaining sets were stored at 2°C–8°C until testing.

Semen

The validation of EBOV RNA detection in semen was then performed using 150 spiked samples and 25 negative samples tested on each instrument (n = 300 and n = 50, respectively). Semen was collected in 3-oz 88.72 mL sterile collection cups from 6 donors. Panels of logarithmic dilutions were prepared to test in triplicate for 5 days to show repeatability (n = 30 for each concentration), and 3 concentrations were tested in duplicate for 15 days to establish sensitivity and further confirm repeatability (n = 70 for each concentration). Semen was allowed to liquefy for 30–45 minutes before spiking with the EBOV stock in the same manner used for blood, the first set of the dilution series was tested on the day that spiking was completed, and the remaining sets were stored at 2°C–8°C until testing.

Assay

All tests were performed using a single Xpert Ebola Test lot across all days of testing. Samples were tested on 2 GeneXpert Instruments, and testing was rotated between 2 technicians each day. Concordance between the 2 GeneXpert instruments was verified by testing a WHO Proficiency Panel II (provided by the Robert Koch Institute) consisting of 11 blinded samples. The lyophilized proficiency panel samples were reconstituted with 100 µL of Corning Molecular Grade RNase-, DNase- and Protease-free Water, and the entire reconstituted volume was processed and tested according the manufacturer's package insert [11]. Briefly, 100 µL of sample was added to the kit lysis

buffer. After a 10-minute incubation period at ambient temperature (15°C–28°C), 1000 µL of sample/lysis buffer was added to the Xpert Ebola Cartridge and loaded onto the GeneXpert module for testing.

Blood samples were processed and tested per the manufacturer's package insert, and semen samples (100 µL) were also processed and tested in the same manner as blood, with 1 additional step. After the initial incubation period, each semen sample was treated with 100 µL of the reducing agent, dithiothreitol (1 mol/L; Sigma-Aldrich) for an additional incubation of 10 minutes at ambient temperature (15°C–28°C) to reduce viscosity. Test results were interpreted by the GeneXpert Dx System from measured fluorescence signals based on algorithms embedded within the assay software [11]. Samples are considered positive if either target gene (GP or NP) was detected; the instrument identifies which targets are detected.

Controls

A set of commercial controls, the SeraCare Ebola Control Bundle, was obtained from Cepheid [27]. One positive control (rEbolaGP-NP, containing both gene targets) and 1 negative control (human serum) from the bundle was tested each day that testing occurred and cycle threshold (Ct) values of the controls were plotted as part of the quality control and assurance for stability of the instrument and test. The semen extraction controls consisted of negative donor semen (undiluted), tested in triplicate over 5 days and in singlet over an additional 10 days on each instrument (n = 50); the EBOV-spiked samples with nominal concentrations of 10^6 copies/mL were used as positive controls to validate the inactivation procedure, tested in singlet over the 15 days on each instrument (n = 30).

Specimen Biosafety Procedure

The specimen biosafety protocol for Ebola samples consists of a thorough decontamination of the primary sample container (cooler box), secondary container (specimen bag), specimen and paperwork by spraying with 0.5% sodium hypochlorite solution (10% bleach) prepared fresh weekly. Contact time was ≥10 minutes for each step. Once the sample was decontaminated, entered into the laboratory specimen log, and given a laboratory-specific identification number, it was placed into the glove box and prepared for PCR testing. For the GeneXpert Ebola test, 100 µL of semen was added to the lysis buffer and incubated for 10 minutes. The inactivated sample was then wiped with an alcohol wipe (70% ethanol) and passed through the exit chamber where it underwent another 10% bleach cleansing step (10 minutes of contact time). This decontamination procedure was performed wearing appropriate personal protective equipment, which consisted of a powered purified air respirator or N95 mask, an impermeable Tyvek suit and boot covers, triple gloves, a head cover, and a shield).

Statistical Analysis

The GeneXpert software will not generate target Ct values when a test is valid but the targets are not detected. All instrument Ct values were entered into MS Excel for Mac and proportions and 95% confidence intervals were estimated using VassarStats: Website for Statistical Computation (Vassar College). Multiple logistic regression analyses were conducted using SigmaStat in SignaPlot version 13 for Windows (Systat Software) to determine the likelihood of a positive test over a range of nominal EBOV concentrations in blood and semen.

RESULTS

Analytical Sensitivity and Repeatability in Semen and Blood

The GeneXpert instrument uses the internal control and target gene Ct values to determine whether the assay is valid and which of the targets (GP, NP, or both) is detected [11]. Validation of blood was undertaken to allow for a direct comparison of the performance of the assay in detecting EBOV RNA in each of these fluids. On both instruments, the assay detected GP and/or NP for all concentrations for both sample types, with the exception of the 100 copies/mL (0 of 8 detected) that was tested for blood only. The analytical sensitivity and repeatability results for whole blood and semen are shown in Table 1. There was no discernible difference between the 2 instruments for repeatability and analytical sensitivity; therefore, all data points are combined for all analyses, with the exception of the direct instrument correlation data.

The analytical limit of detection (LOD) was estimated as the concentration corresponding to a 95% probability of a positive test result for each specimen tested on days 1 and 3 after spiking with dilutions of inactivated virus stock (Figure 1) [28]. In probit analysis, the 95% LOD for blood was 275 copies/mL (2.44 log copies/mL) on day 1 and 279 copies/mL (2.45 log copies/mL) on day 3. Probit analysis for semen showed that the 95% LOD was 1000 copies/mL (3.00 log copies/mL) on day 1 and 1259 copies/mL (3.10 log copies/mL) on day 3. Unspiked blood and semen donor samples (n = 40 and n = 50, respectively) were all undetected (Table 1).

Detectability With Increasing Time From Specimen Collection to Testing

Target detectability, in whole blood and semen, was determined by testing the EBOV-spiked samples on days 1, 2, and 3 (up to 72 hours after collection), and stability was determined by the presence of either gene target. At least 1 of the target genes (GP or NP) was detectable over all 3 days for all concentration replicates down to 1000 EBOV-spiked copies/mL. The NP gene was detected for all but 1 replicate of the 500 EBOV-spiked copies /mL in blood samples (15 of 16); for semen samples, NP was detected in 46 of 70 samples with 500 EBOV-spiked copies/mL. The 250 EBOV-spiked copies /mL sample replicate results showed a decrease in detectability for both sample types (Table 2). For blood, there is a slight decrease in target

Table 1. Analytical Sensitivity and Repeatability in Whole-Blood and Semen Samples

Blood						Semen					
Nominal Viral RNA Copies/mL	Total No.	Positive Results, No.	% Detected (95% CI)	Ct, Mean (SD)		Nominal Copies/mL	Total No.	Positive Results, No.	% Detected (95% CI)	Ct, Mean (SD)	
				GP	NP					GP	NP
1 000 000	8	8	100 (68–100)	33.9 (0.4)	29.5 (0.4)	1 000 000	30	30	100 (89–100)	36.2 (1.5)	31.7 (1.5)
100 000	8	8	100 (68–100)	37.3 (1.2)	33.1 (1.1)	100 000	30	30	100 (89–100)	37.7 (1.0)	32.9 (0.9)
10 000	8	8	100 (68–100)	39.8 (2.2)	36.3 (2.1)	10 000	30	30	100 (89–100)	40.8 (1.5)	36.1 (1.4)
1000	8	8	100 (68–100)	36.3 (1.2)	36.1 (4.5)	1000	70	66	94 (89–99)	39.2 (3.4)	38.2 (3.6)
500	16	15	94 (72–99)	NA	37.2 (3.6)	500	70	48	69 (57–78)	NA	NA
250	16	9	56 (33–77)	NA	NA	250	70	36	51 (40–63)	NA	NA
100	16	0	0 (0–32)	NA	NA	0	50	0	0 (0–13)	NA	NA
0	40	0	0 (0–16)	NA	NA	NA	NA	NA	NA	NA	NA

Abbreviations: CI, confidence interval; Ct, cycle threshold; GP, glycoprotein; NA, not applicable; NP, nucleoprotein.

detectability over 72 hours at the LOD, although the predicted difference in LOD of −0.01 log copies/mL was within ±0.25 log copies/mL. Similarly, for semen, the predicted difference in LOD was −0.10 log copies/mL, also within the acceptable range.

There was an observed difference in target gene detectability in both sample types for testing completed over the entire study. The NP gene was detected in 84% (95% confidence interval, 74%–91%) of blood samples (54 of 64) and 77% (72%–81%) of semen samples (231 of 300), and the GP gene was detected in 47% (35%–59%) of blood samples (30 of 64) and 51% (45%–56%) of semen samples (152 of 300).

Instrument Concordance and Biosafety Procedure Validation

The WHO proficiency panel consisted of 11 blinded samples. The EBOV samples were detected, whereas while Marburg virus and negative samples were not detected on both instruments (Table 3).

The commercial controls were tested each day throughout the duration of the validation study on each instrument. The Ct values were plotted as part of quality control and assurance for stability of the instrument and test for each target, GP and NP, for the positive control (Figure 2). All positive and negative controls were valid, and there were no false-positives (negative controls with positive results) or false-negatives (positive controls with negative results) for either instrument (Table 1).

Validation of the specimen biosafety procedure was completed, using the prepared specimen-specific controls to ensure that specimen handling and sample inactivation practices would not inhibit PCR and result in false-negatives and also to ensure proper handling to minimize contamination resulting in false-positives. The semen positive control was spiked to contain 10^6 copies/mL sample, and the negative control was undiluted donor sample. No false-negative or false-positive results were observed for either instrument (all 30 positive semen controls had positive results, compared with 0 of 50 negative semen controls) (Table 1).

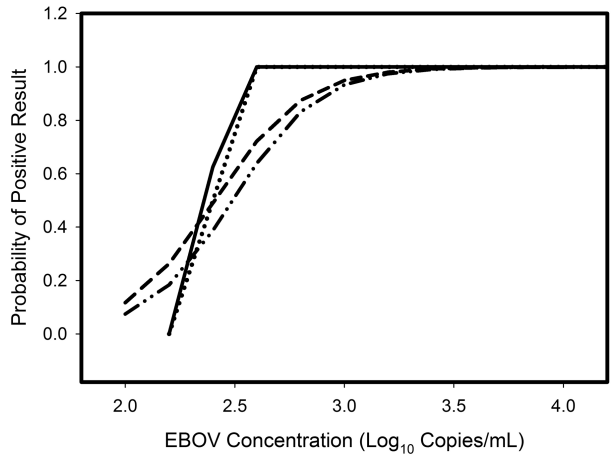

Figure 1. Statistical limits of detection. Solid line represents blood samples on day 1; dotted line, blood samples on day 3; dashed line, semen samples on day 1; and dashed and dotted line, semen samples on day 3. Abbreviation: EBOV, Ebola virus.

Discussion

Diagnostics are critical to emerging infectious diseases response. Throughout the 2013–2016 Ebola epidemic, diagnostic molecular-based platforms have been used to triage suspect cases, determine discharge eligibility, and, more recently, determine the duration of asymptomatic EBOV shedding in the semen of male survivors [2, 29–31]. However, interpretation of results from tests that have not been validated pose a challenge, because the limits of detection and accuracy of these assays under different conditions have not been defined. Given the threat of viral persistence in body fluids, including semen, and the recognition of sexual contact as a means of transmission of EBOV, a validated test to detect the presence of EBOV RNA in semen is essential to determine the duration of viral shedding and support public health recommendations to prevent sexual transmission of this virus.

In this first validation of EBOV PCR testing for semen we found the Cepheid Xpert Ebola assay to be accurate, precise and stable for detecting EBOV in semen and comparable to the testing of whole blood, with limits of detection of approximately 1000 and 275 copies/mL, respectively. An important finding

Table 2. Target Detectability for Blood and Semen Samples Over Time

		No. Detected /No. Tested					
		Blood Samples			Semen Samples		
Nominal Copies/mL	Target Gene	Day 1	Day 2	Day 3	Day 1	Day 2	Day 3
1.00×10^6	GP	4/4	2/2	2/2	10/10	10/10	10/10
	NP	4/4	2/2	2/2	10/10	10/10	10/10
1.00×10^5	GP	4/4	2/2	2/2	12/12	12/12	6/6
	NP	4/4	2/2	2/2	12/12	12/12	6/6
1.00×10^4	GP	3/4	0/2	0/2	5/12	10/12	5/6
	NP	4/4	2/2	2/2	12/12	12/12	6/6
1.00×10^3	GP	2/4	0/2	0/2	6/24	7/24	7/22
	NP	4/4	2/2	2/2	21/24	20/24	21/22
5.00×10^2	GP	4/8	0/4	0/4	13/24	12/24	6/22
	NP	8/8	3/4	4/4	18/24	15/24	13/22
2.50×10^2	GP	5/8	0/4	1/4	5/24	11/24	5/22
	NP	4/8	2/4	1/4	12/24	13/24	9/22

Abbreviations: GP, glycoprotein. NP, nucleoprotein.

was the effect of time from collection to testing on assay performance. Based on our data, consistent results for semen and whole blood can be achieved when testing is performed within 72 hours of specimen collection, with only a slight decrease in sensitivity (1259 and 279 copies/mL in semen and blood at day 3, respectively). In addition to the performance of the Cepheid Xpert platform in detecting EBOV RNA in semen, the lack of false-positive and false-negatives in this fluid also validates the specimen biosafety procedure. Quality control is paramount, and extraction controls should be tested along with the patient samples to assure that accurate results are achieved and reported.

Our findings are concordant with a recent report by Pettit and colleagues [32], who compared the performance of a quantitative RT-PCR assay for detecting EBOV in blood and semen. In contrast to that study, we also completed method validation for the Cepheid Xpert system for detecting EBOV RNA in semen by defining the limits of detection and reproducibility. The Cepheid Xpert system offers a number of advantages over standard PCR methods, including incorporation of internal controls for host species and virus, rapid processing time, and reduced need for specimen handling. That the system was able to also perform well in detecting EBOV RNA in semen greatly expands its research and clinical utility, given the evidence of seminal persistence and sexual transmission of this virus. At present, testing of semen for EBOV is largely inaccessible to men in West Africa, and the limited testing that is being conducted has not, to our knowledge, been validated for this body fluid. Our validation of the Cepheid Xpert Ebola assay removes at least one obstacle to its being more widely available to men who have recovered from EVD.

However, despite the performance characteristics of the Cepheid Xpert for Ebola in semen, much remains unknown about the results of this and any other EBOV PCR technique in this body fluid. Because the test detects RNA targets, it is not clear whether a detectable result indicates the presence of infectious virus. Similarly, an undetectable result cannot be interpreted to indicate the complete absence of virus or risk of infectiousness, because there is no established threshold for the concentration of EBOV in the semen below which transmission is not possible. Therefore, counseling and clinical decision making should incorporate messaging regarding the limitations of the assay when it is used.

Our study had several limitations. We were unable to assess clinical samples, particularly those with high virus titers that would be seen in an outbreak; therefore, additional assessment of the biosafety procedure and complete inactivation of live EBOV using the Xpert Ebola assay was not performed. The inclusion of commercial controls and extraction controls requires a stable environment monitored by trained laboratory staff with access to consistent power supply in a space that is temperature controlled (for the instrument and reagents), not easily implemented in the field during an outbreak. In addition, we chose to use precision pipettes for sample inactivation and addition to the cartridge; we did not use the swab or Pasteur pipettes included in the kit. It is worth noting, however, that these instruments are widely used in Africa for tuberculosis testing, in various settings, and the Xpert Ebola assay could be added to the test menu if needed.

Importantly, EBOV used for this validation study was first heat-inactivated and gamma-irradiated. This process can limit the sensitivity of PCR diagnostic platforms by introducing strand brakes into the genomic RNA. However, it has been previously demonstrated that in comparison with infectious virus, PCR detection of gamma-irradiated EBOV differed by a 10–100-fold dilution that seems to be strain dependent [33]. Therefore, the impact of the inactivation process is probably modest, and

Table 3. Instrument concordance using WHO Proficiency Panel II samples.

Virus Strain:	Ebola-Zaire					Ebola-Zaire / Gueckedou			Marburg	Negative
Virus Dilution:	**1x10-2**	**1x10-3**	**1x10-4**	**1x10-5**	**1x10-6**	**1x10-3**	**1x10-4**	**1x10-4**	**1x10-3**	
Ct Values by Target Gene										
Instrument A										
GP	27.0	30.8	34.6	36.3	36.7	26.2	28.9	29.3	ND	ND ND
NP	31.8	35.7	41.5	ND	39.6	31.0	33.7	34.0	ND	ND ND
Instrument B										
GP	26.2	30.8	35.2	38.2	37.0	26.0	29.7	29.4	ND	ND ND
NP	30.9	35.6	38.6	ND	ND	30.4	34.3	34.0	ND	ND ND
Both Instruments, mean Ct (SD)										
GP	26.6 (0.6)	30.8 (0.0)	34.9 (0.4)	373 (1.3)	36.9 (0.2)	26.1 (0.1)	29.3 (0.6)	29.4 (0.1)	ND	ND ND
NP	31.4 (0.6)	35.7 (0.1)	40.1 (2.1)			30.7 (0.4)	34.0 (0.4)	34.0 (0.0)	ND	ND ND

Abbreviations: WHO, World Health Organization; NA, not applicable; Ct, cycle threshold; GP, glycoprotein; ND, not detected; NP, nucleoprotein; SD, standard deviation.

Figure 2. Xpert Ebola test and instrument stability. Cycle threshold (Ct) values are shown for rEbolaGP-NP (positive control). Gray-filled symbols represent glycoprotein; white-filled symbols, nucleoprotein; squares, instrument A; and circles, instrument B.

the process would produce true limits of assay detection that are more conservative than obtained with infectious virus. Finally, the Xpert Ebola assay is a qualitative test proved to detect the presence of the EBOV virus in the blood, and we have shown that this study also performs well for semen. The platform cannot provide quantitative testing and therefore may have limited application to patient care (for informing treatment) or for determining infectiousness, and/or transmission.

In conclusion, detection of EBOV in semen with the Cepheid Xpert Ebola assay was found to be accurate, with a lower LOD of approximately 1000 copies/mL. These results support the expanded use of this assay to detect Ebola in semen and blood, to guide individual and public health responses during and after an outbreak of this virus.

Notes

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