Validation of the Cepheid GeneXpert for Detecting Ebola Virus in Semen

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On behalf of: Saturday Quellie, Kelly Chason, Emmanuel Sumo, Mason Toukolon, Yonnie Otieno, Heinzfried Ellerbrok, Marcia M. Hobbs, David Hoover, Karine Dube, David A. Wohl and William A. Fischer II

ASLM Meeting
03-08 December 2016
Cape Town, South Africa
Ebola Virus

First described in 1976 in Zaire, now the Democratic Republic of Congo (DRC)

- 5 Species: Zaire, Sudan, Bundibugyo, Tai Forest and Reston
- 2013-2016 outbreaks caused by Ebola Zaire
Validation Study for testing semen

**Background**: After the period of sustained Ebola virus (EBOV) transmission during the 2013-2016 epidemic, sporadic clusters of Ebola virus disease were reported in West Africa.

- A study led by the CDC, WHO, and the Liberian MOH suggests that the prevalence of viral persistence and shedding in semen may be **as high as 26%** among men who are seven to nine months out from recovery from acute EVD.
- 2 documented index cases of sexual transmission from EVD survivor to partner
- WHO has recommended that men who have survived EVD refrain from unprotected sex for at least 12 months following recovery or until their semen is confirmed to be EBOV-free.

*Despite this recommendation and the need for accurate testing for EBOV in this and other body fluids, no fully validated assay for EBOV detection in fluids other than blood is available.*

**Objective**: Validate the Cepheid Xpert Ebola assay for EBOV RNA detection in whole semen samples obtained from uninfected donors and spiked with inactivated EBOV.
Setting
Liberia

- **10,678 Total Cases** (suspected, probable & confirmed), **3,163 Laboratory-confirmed Cases** and **4,810 total deaths**
- **Estimated # of survivors**

Assay validation was performed at the Phebe Hospital PCR Laboratory in Suakoko, Bong County, Liberia.
The laboratory layout follows recommendations from the CDC and WHO.

- **High-risk Area**: contains an area to don and doff personal protective equipment (PPE), an area for sample reception and decontamination, and an area for specimen preparation and virus inactivation within a glove box.

- **Low-risk Area**: contains separate rooms for pre- and post-amplification.
Test Platform

• The Cepheid Xpert Ebola Assay was granted an EUA from the US FDA on 23-March-2015

• Rapid, automated test for qualitative detection of two gene targets of the Ebola Zaire virus - glycoprotein (GP) and nucleoprotein (NP)

• The Cepheid Xpert system offers a number of advantages compared to traditional PCR methods:
  » incorporation of internal controls for host species and virus
  » rapid processing time (≈90min)
  » reduced need for specimen handling
## Verification and/or Validation

<table>
<thead>
<tr>
<th>Performance Specification</th>
<th>Blood</th>
<th>Semen</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Analytical Repeatability</strong></td>
<td>FDA Approved or Cleared Test</td>
<td>GeneXpert</td>
</tr>
<tr>
<td>Verify</td>
<td>20-40 across AMR</td>
<td>40+/20-</td>
</tr>
<tr>
<td><strong>Analytical Sensitivity (LOD)</strong></td>
<td>Verify; literature or manufacturer documentation OK</td>
<td>3 points near low end, midpoint, and high end</td>
</tr>
<tr>
<td><strong>Analytical Specificity</strong></td>
<td>Verify; literature or manufacturer documentation OK</td>
<td>Adopted Manufacturer’s claims (package insert)</td>
</tr>
</tbody>
</table>
Samples

- Lyophilized Ebola virus stock that originated from a Guekedou isolate of the Ebola Zaire strain was obtained from RKI
  - The virus stock was heat-inactivated and subsequently gamma irradiated
  - Absence of infectivity was confirmed by cultivation experiments.
  - Virus stock was diluted by genomic equivalents in the sending lab.
  - The nucleic acid content of the heat-inactivated and gamma-irradiated virus stock was verified by three separate expert Ebola laboratories
- Whole Blood and Whole Semen was collected from uninfected individuals and spiked with reconstituted lyophilized Ebola virus stock that originated from a Guekedou isolate of the Ebola Zaire strain.
  - Blood: 2 x 4mL EDTA tubes from 4 donors, spiked immediately after collection to achieve a dilution series of samples containing $10^2$-$10^6$ copies/mL
  - Semen: self-collected in 3-ounce sterile collection cups from 6 donors and allowed to liquefy for 30-45min prior to spiking with the EBOV stock in the same manner as blood.
- The first set of the dilution series samples was tested the same day as collection and spiking; the remaining sets were stored at 2-8°C until testing, within 72hrs.
Assay

- All tests were performed using a single Xpert Ebola Test kit lot across all days of testing.
- Samples were tested on two GeneXpert Dx Instruments
- Testing rotated between two technicians each day.

<table>
<thead>
<tr>
<th>Sample Type</th>
<th>Sample Inactivation Protocol</th>
</tr>
</thead>
</table>
| Blood         | 1. 100uL whole whole blood into the 2.5mL Xpert Lysis Buffer  
                2. Room temperature (15-28°C) for 10min  
                3. 1000uL of the sample/Lysis Buffer added to the Xpert Ebola Cartridge |
| Semen         | 1. 100uL whole semen into the 2.5mL Xpert Lysis Buffer  
                2. Room temperature (15-28°C) for 10min  
                3. **Add 100uL 1M DTT**  
                4. Room temperature (15-28°C) for 10min  
                5. 1000uL of the sample/Lysis Buffer added to the Xpert Ebola Cartridge |
| WHO Panel     | 1. 100uL RNase-, DNAse and Protease-free water added to lyophilized sample.  
                2. 100uL reconstituted panel sample added into the 2.5mL Xpert Lysis Buffer  
                3. Room temperature (15-28°C) for 10min  
                4. 1000uL of the sample/Lysis Buffer added to the Xpert Ebola Cartridge |
Controls

- Commercial controls (SeraCare Ebola Control) were obtained from Cepheid and tested each day for QA and test stability
  - Positive control - containing both GP and NP targets
  - Negative control - human serum
- Specimen-specific controls for validation of extraction and biosafety protocols
  - Negative: donor semen (undiluted), tested in triplicate over 5 days and in singlet over an additional 10 days on each instrument (N of 50)
  - Positive: 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 of 30).
Results

Analytical Sensitivity and Repeatability

At least one of the target genes (GP or NP) was detectable over all three days for all concentration replicates down to 1,000 EBOV-spiked cp/mL.

<table>
<thead>
<tr>
<th></th>
<th>Blood</th>
<th></th>
<th></th>
<th>Semen</th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Nominal cp/mL</td>
<td>N</td>
<td>Positive Results</td>
<td>Percent Detected (95% CI)</td>
<td>Mean Ct</td>
</tr>
<tr>
<td>---------</td>
<td>---------------</td>
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<td>------------------</td>
<td>--------------------------</td>
<td>---------</td>
</tr>
<tr>
<td></td>
<td>1,000,000</td>
<td>8</td>
<td>8</td>
<td>100 (68, 100)</td>
<td>33·9</td>
</tr>
<tr>
<td></td>
<td>100,000</td>
<td>8</td>
<td>8</td>
<td>100 (68, 100)</td>
<td>37·3</td>
</tr>
<tr>
<td></td>
<td>10,000</td>
<td>8</td>
<td>8</td>
<td>100 (68, 100)</td>
<td>39·8</td>
</tr>
<tr>
<td></td>
<td>1,000</td>
<td>8</td>
<td>8</td>
<td>100 (68, 100)</td>
<td>36·3</td>
</tr>
<tr>
<td></td>
<td>500</td>
<td>16</td>
<td>15</td>
<td>94 (72, 99)</td>
<td>37·2</td>
</tr>
<tr>
<td></td>
<td>250</td>
<td>16</td>
<td>9</td>
<td>56 (33, 77)</td>
<td></td>
</tr>
<tr>
<td></td>
<td>100</td>
<td>16</td>
<td>0</td>
<td>0 (0, 32)</td>
<td></td>
</tr>
<tr>
<td></td>
<td>0</td>
<td>40</td>
<td>0</td>
<td>0 (0, 16)</td>
<td></td>
</tr>
</tbody>
</table>

cp/mL = copies per milliliter. N = number. 95% CI = 95% Confidence Interval (Lower Limit, Upper Limit). Ct = Cycle threshold.

Table 1. Analytical sensitivity and repeatability in whole blood and semen.
# Results

## Statistical Limit of Detection

The limit of detection was estimated as the concentration corresponding to a 95% probability of a positive test result.

![Graph showing statistical limit of detection](image.png)

**Figure 1. Statistical limit of detection.** The solid line is blood Day 1, dotted line is blood Day 3; the dashed line is semen Day 1, and the dashed and dotted line is semen Day 3.

<table>
<thead>
<tr>
<th>Sample Type</th>
<th>Day 1 LoD</th>
<th>Day 3 LoD</th>
<th>Δ Day 1 to Day 3</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>cp/mL</td>
<td>Log_{10} cp/mL</td>
<td>cp/mL</td>
</tr>
<tr>
<td>Blood</td>
<td>275</td>
<td>2.44</td>
<td>279</td>
</tr>
<tr>
<td>Semen</td>
<td>1000</td>
<td>3.00</td>
<td>1259</td>
</tr>
</tbody>
</table>

Stability limits were defined as acceptable if the probit analysis of analytical limits of detection on Day 3 were within ±0.25 Log_{10} copies/mL (cp/mL) of those achieved on Day 1.
Results

Instrument Concordance and Specificity

- Verified by testing the WHO Proficiency Panel II (provided by the Robert Koch Institute) consisting of 11 blinded samples.
- The EBOV samples were detected while the Marburg Virus and Negative samples were not detected on both instruments.

<table>
<thead>
<tr>
<th>WHO Panel II</th>
<th>#3</th>
<th>#6</th>
<th>#9</th>
<th>#5</th>
<th>#4</th>
<th>#8</th>
<th>#7</th>
<th>#2</th>
<th>#10</th>
<th>#1</th>
<th>#11</th>
</tr>
</thead>
<tbody>
<tr>
<td>Virus Dilution</td>
<td>1.00E-02</td>
<td>1.00E-03</td>
<td>1.00E-04</td>
<td>1.00E-05</td>
<td>1.00E-06</td>
<td>1.00E-03</td>
<td>1.00E-04</td>
<td>1.00E-04</td>
<td>1.00E-03</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Instrument A</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>27.0</td>
<td>30.8</td>
<td>34.6</td>
<td>36.3</td>
<td>36.7</td>
<td>26.2</td>
<td>28.9</td>
<td>29.3</td>
<td>ND</td>
<td>ND</td>
<td>NP</td>
</tr>
<tr>
<td></td>
<td>31.8</td>
<td>35.7</td>
<td>41.5</td>
<td>ND</td>
<td>39.6</td>
<td>31.0</td>
<td>33.7</td>
<td>34.0</td>
<td>ND</td>
<td>ND</td>
<td>GP</td>
</tr>
<tr>
<td>Instrument B</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>26.2</td>
<td>30.8</td>
<td>35.2</td>
<td>38.2</td>
<td>37.0</td>
<td>26.0</td>
<td>29.7</td>
<td>29.4</td>
<td>ND</td>
<td>ND</td>
<td>NP</td>
</tr>
<tr>
<td></td>
<td>30.9</td>
<td>35.6</td>
<td>38.6</td>
<td>ND</td>
<td>ND</td>
<td>30.4</td>
<td>34.3</td>
<td>34.0</td>
<td>ND</td>
<td>ND</td>
<td>GP</td>
</tr>
<tr>
<td>Mean Ct (SD)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>26.6</td>
<td>30.8</td>
<td>34.9</td>
<td>37.3</td>
<td>36.9</td>
<td>26.1</td>
<td>29.3</td>
<td>29.4</td>
<td>ND</td>
<td></td>
<td>NP</td>
</tr>
<tr>
<td></td>
<td>(±0.6)</td>
<td>(±0.4)</td>
<td>(±1.3)</td>
<td>(±0.2)</td>
<td>(±0.1)</td>
<td>(±0.6)</td>
<td>(±0.1)</td>
<td></td>
<td></td>
<td></td>
<td>GP</td>
</tr>
<tr>
<td></td>
<td>31.4</td>
<td>35.7</td>
<td>40.1</td>
<td>30.7</td>
<td>34.0</td>
<td>34.0</td>
<td>(±0.4)</td>
<td>(±0.4)</td>
<td>(±0.0)</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>(±0.6)</td>
<td>(±0.1)</td>
<td>(±2.1)</td>
<td>(±0.4)</td>
<td>(±0.4)</td>
<td>(±0.0)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

ND = Not Detected. Ct = Cycle threshold. SD = Standard Deviation.

Table 3. Instrument concordance.
Conclusions

• The Cepheid Xpert Ebola assay had a limit of detection of 1,000 cp/mL in semen and 275 cp/mL in blood. Limits of detection increased with longer intervals between collection and testing. However, acceptable results were obtained up to 72 hours after specimen collection.

• A validated assay for EBOV RNA detection in semen is critical for the care of male survivors of Ebola and for the generation of sound public health recommendations.

Where to from here: The diagnostics have not evolved to meet the needs of this virus, we must push the field forward so that patient management is based on quality results.
Prevention of sexual transmission of Ebola virus disease in Liberia through a national semen testing and counselling programme for survivors: an analysis of Ebola virus RNA results and behavioural data — Does NOT mention having validated the test for this specimen type.
Thank You

• Phebe Hospital Compound, including Dr. Jefferson Sibley (Medical Director) and the Phebe Hospital PCR Lab
• UNC Center for AIDS Research (CFAR) Lab
• UNC CFAR Virology, Immunology and Microbiology Lab
• Drs. Susan Fiscus, Melissa Miller, and Julie Nelson for guidance and manuscript review.
• Funding was provided by the Bill and Melinda Gates Foundation.
• Clinical Research Management for logistical support.
• Lisa Hensley, PhD, shared the NIH semen protocol.
Design it…

Build it…

Fire! Fire!

Teaching ≈ Learning

…then they named her, Nenikolay

Honorary Tar Heels