ORIGINAL ARTICLE

Ebola RNA Persistence in Semen of Ebola Virus Disease Survivors — Preliminary Report

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ABSTRACT

BACKGROUND

Ebola virus has been detected in the semen of men after their recovery from Ebola virus disease (EVD), but little information is available about its prevalence or the duration of its persistence. We report the initial findings of a pilot study involving survivors of EVD in Sierra Leone.

METHODS

We enrolled a convenience sample of 100 male survivors of EVD in Sierra Leone, at different times after their recovery from EVD, and recorded self-reported information about sociodemographic characteristics, the EVD episode, and health status. Semen specimens obtained at baseline were tested by means of a quantitative reverse-transcriptase–polymerase-chain-reaction (RT-PCR) assay with the use of the target-gene sequences of NP and VP40.

RESULTS

A total of 93 participants provided an initial semen specimen for analysis, of whom 46 (49%) had positive results on quantitative RT-PCR. Ebola virus RNA was detected in the semen of all 9 men who had a specimen obtained 2 to 3 months after the onset of EVD, in the semen of 26 of 40 (65%) who had a specimen obtained 4 to 6 months after onset, and in the semen of 11 of 43 (26%) who had a specimen obtained 7 to 9 months after onset; the results for 1 participant who had a specimen obtained at 10 months were indeterminate. The median cycle-threshold values (for which higher values indicate lower RNA levels) were 32.0 with the NP gene target and 31.1 with the VP40 gene target for specimens obtained at 2 to 3 months, 34.5 and 32.3, respectively, for specimens obtained at 4 to 6 months, and 37.0 and 35.6, respectively, for specimens obtained at 7 to 9 months.

CONCLUSIONS

These data showed the persistence of Ebola virus RNA in semen and declining persistence with increasing months since the onset of EVD. We do not yet have data on the extent to which positivity on RT-PCR is associated with virus infectivity. Although cases of suspected sexual transmission of Ebola have been reported, they are rare; hence the risk of sexual transmission of the Ebola virus is being investigated. (Funded by the World Health Organization and others.)

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The number of new cases of Ebola virus disease (EVD) in western Africa has declined from a peak of 1063 cases in the week of October 9, 2014, to fewer than 10 confirmed cases per week for 11 consecutive weeks as of October 7, 2015.[1]. The main mode of transmission is direct contact with the blood or body fluids of a person with EVD or from the body of a person who died from EVD.[2,3] However, Ebola virus can persist in the body fluids of survivors during convalescence,[4,5] which may result in transmission of the virus. The potential for the persistence of Ebola virus in the semen of male survivors raises concern regarding the possible transmission of the virus to sexual partners.[6]

Previously, survivors of EVD were told to practice sexual abstinence or to use a condom for 3 months after recovery. These recommendations were based on virus-isolation results from semen specimens obtained from eight survivors of EVD or Marburg virus disease in previous epidemics,[5,7-10] in which the longest period that infectious virus was found in semen after the onset of symptoms was 82 days.

In March 2015, a woman in Liberia received a diagnosis of EVD and her only potential exposure that could be ascertained was sexual contact with a male survivor of EVD. Further investigation found Ebola virus RNA in the survivor's semen 199 days after the onset of his symptoms, with a genetic sequence that matched the sequence from the case patient.[11] Although no infectious virus was detected in this semen specimen, the possibility that infectious Ebola virus could persist in the semen of survivors approximately 6 months after the onset of illness prompted the World Health Organization (WHO) and the Centers for Disease Control and Prevention (CDC) to revise their guidelines regarding the length of time that survivors of EVD should avoid unprotected sexual activity.[12,13]

There are thousands of survivors of EVD in western Africa, and many are sexually active men. Sexual transmission of the Ebola virus could possibly result in new outbreaks several weeks or months after all known chains of transmission in the region have stopped. Although the epidemiologic observations to date suggest that sexual transmission is a rare event, the Sierra Leone Ministry of Health and Sanitation, in collaboration with the Sierra Leone Ministry of Defense, the Sierra Leone Ministry of Social Welfare, Gender, and Children's Affairs, the WHO, and the CDC initiated a study of the duration of virus persistence in the body fluids of survivors in Sierra Leone. We report initial findings from the pilot phase of the study, which investigated the persistence and viability of Ebola virus in the semen of male survivors of EVD.

Methods

Study Design and Oversight

The Sierra Leone Ministry of Health and Sanitation, the Sierra Leone Ministry of Social Welfare, Gender, and Children's Affairs, the WHO, and the CDC designed the study, and the Sierra Leone Ministry of Defense, the WHO, and the CDC gathered the data. The data analysis was performed and supervised by the CDC and the WHO. Manuscript planning and drafting were also overseen and performed by the Ministry of Health and Sanitation, the CDC, and the WHO. All these activities were performed in accordance with all applicable laws, regulations, and policies related to the protection of human participants and animals. A complete list of the members of the steering committee and the technical working group is provided in the Supplementary Appendix, available with the full text of this article at NEJM.org.

Study Population, Sampling, and Eligibility Criteria

We recruited a convenience sample of 100 male survivors from the Western Area District of Sierra Leone, which includes the capital of Freetown. We identified study participants who, at informational events that were held in conjunction with survivor associations, indicated interest in participation, as well as persons who were referred from Ebola treatment centers.

Participants were eligible for inclusion if they were men 18 years of age or older, could provide an official survivor certificate issued by the Ministry of Health and Sanitation (such certificates are provided to persons with laboratory-confirmed cases of EVD when they are discharged from an Ebola treatment center), and could provide written informed consent to participate in the study. We compensated participants for each visit to the study site. The research protocol was reviewed and approved by the Sierra Leone Ethical Review Board and the WHO Ethical Review Committee.

DATA COLLECTION

A member of the study team administered a questionnaire to all the participants at the time of enrollment to gather information about their EVD episode, self-reported health status, sexual behavior, and sociodemographic characteristics. The date of EVD onset was self-reported, and the date of discharge from the Ebola treatment center was ascertained from the participants' survivor certificates. We asked participants to provide a semen specimen in a private room and provided instructions to ensure that proper infection-control procedures were followed.

We gave participants pretest counseling at the time of enrollment and post-test counseling 2 weeks later when they received their individual reverse-transcriptase–polymerase-chain-reaction (RT-PCR) results. The counseling included information about the test performed, the meaning of the results, and education about sexual risk-reduction practices, including appropriate condom use and disposal. Trained counselors also offered participants a voluntary, confidential rapid test for the human immunodeficiency virus (HIV), according to the national testing algorithm. We referred participants to a clinic for survivors of EVD if it was needed, as determined by the trained medical staff of the study, or requested.

LABORATORY ANALYSES

After the semen specimens were collected, they were refrigerated (at 5 to 8°C) for no longer than 3 days and transported to the CDC field laboratory in Bo District, Sierra Leone. We performed quantitative RT-PCR testing using Ebola virus–specific gene targets (NP and VP40) and the human β_2-microglobulin (*B2M*) gene, as described previously.[14,15] We considered a specimen to be positive if the VP40 and NP gene targets were both detected within 40 cycles of replication. The specimen was considered to be negative if neither Ebola virus gene target was detected and the findings with respect to *B2M* status were positive. The findings were ruled to be indeterminate if either the VP40 or the NP gene target was detected but not both. Amplification of *B2M* served as an extraction control and RNA quality control.

The cycle-threshold value for each gene target is reported as the number of replication cycles that had occurred when the target was first detected. Cycle-threshold values have an inverse association with virus quantity, such that higher quantities of virus in given specimens have lower cycle-threshold values.[16]

STATISTICAL ANALYSIS

We report the number of participants who had a positive, indeterminate, or negative result on quantitative RT-PCR at enrollment according to the number of days between the self-reported onset of illness and the date that the semen specimen was obtained, rounded to the nearest whole month. Median cycle-threshold values, according to months after the onset of EVD, are reported, with the range of values observed for the NP and VP40 gene targets. Sociodemographic characteristics at baseline are also presented. Analysis of the data was performed with the use of Stata software, version 13.1 (StataCorp).

RESULTS

STUDY PARTICIPANTS

RT-PCR results were available for 93 of the 100 participants enrolled. Three participants were withdrawn from the study in accordance with the protocol after they were unable to provide a specimen at two consecutive visits. Four participants did not have diagnostic RT-PCR results from semen testing at baseline (i.e., the cycle-threshold value for *B2M* was above the cutoff value) and were excluded from this analysis.

The mean age of the 93 participants was 30 years (range, 18 to 58). A total of 15% of the participants had no formal education, 22% had less than 6 years of education, and 63% had 6 or more years. When the participants were asked about income, 43% reported not knowing their monthly income, 24% reported earning less than $100 (U.S.) per month, 13% reported earning in the range of $100 to $1,000 per month, 10% reported earning more than $1,000 per month, and 10% did not answer. No participant reported having received a diagnosis of HIV infection, tuberculosis, or diabetes. Among the 93 participants, the time from illness onset to study enrollment was 2 to 3 months (64 to 120 days) for 9 men (10%), 4 to 6 months (121 to 210 days) for 40 men (43%), 7 to 9 months (211 to 300 days) for 43 men (46%), and 10 months (306 days) for 1 man (1%).

DETECTION OF EBOLA RNA IN SEMEN

A total of 46 of the 93 men (49%) had a specimen that was positive on quantitative RT-PCR. Ebola

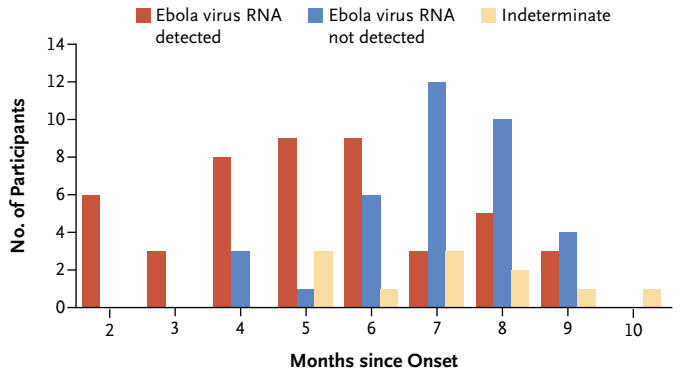

Figure 1. Results on Quantitative RT-PCR in Initial Semen Specimens Obtained from Survivors of Ebola Virus Disease, According to Time after Symptom Onset.

We performed quantitative reverse transcriptase–polymerase chain reaction (RT-PCR) testing using Ebola virus–specific gene targets (NP and VP40) and the human β_2-microglobulin (*B2M*) gene, as described previously.[14,15] We considered the findings to be positive if the VP40 and the NP gene targets were both detected within 40 cycles of replication. The findings were considered to be negative if neither Ebola virus gene target was detected and the findings regarding *B2M* status were positive. The findings were ruled to be indeterminate if either the VP40 or the NP gene target was detected but not both.

Table 1. Proportion of Positive Findings on Quantitative RT-PCR and Cycle-Threshold Values in the Semen of Survivors of Ebola Virus Disease, According to Time after Symptom Onset.*

Time after Symptom Onset	Positive Result	Cycle-Threshold Value	
		NP Target	VP40 Target
	no./total no. (%)	*median (range)*	
2–3 mo	9/9 (100)	32.0 (20.1–36.4)	31.1 (19.4 – 35.0)
4–6 mo	26/40 (65)	34.5 (25.7–38.4)	32.3 (24.6–37.9)
7–9 mo	11/43 (26)	37.0 (28.1–38.9)	35.6 (27.9–37.9)

* We performed quantitative reverse-transcriptase–polymerase-chain-reaction (RT-PCR) assays using Ebola virus–specific gene targets (NP and VP40) and the human β_2-microglobulin (*B2M*) gene, as described previously.[14,15] We considered the findings to be positive if the VP40 and the NP gene targets were both detected within 40 cycles of replication. Higher cycle-threshold values indicate lower RNA levels. The results for one participant who had a specimen obtained at 10 months were indeterminate.

virus RNA was detected in the semen of all 9 men from whom a specimen was obtained during the first 3 months after the onset of illness, in the semen of 26 of 40 men (65%) from whom a specimen was obtained at 4 to 6 months, and in the semen of 11 of 43 men (26%) from whom a specimen was obtained at 7 to 9 months (Fig. 1). The results for 1 participant who had a specimen obtained at 10 months were indeterminate. The proportions of men with semen samples that were negative or indeterminate on quantitative RT-PCR were higher with increasing months after the onset of EVD.

The median cycle-threshold values increased as the months after the onset of EVD increased. For specimens obtained at 2 to 3 months, the values were 32.0 with the NP gene target and 31.1 with the VP40 gene target; for those obtained at 4 to 6 months, the values were 34.5 and 32.3, respectively; and for those obtained at 7 to 9 months, the values were 37.0 and 35.6, respectively (Table 1).

The longest time after the onset of a participant's EVD symptoms that a semen specimen obtained at baseline remained positive on quantitative RT-PCR was 284 days (9 months). Conversely, the shortest time after symptom onset in a participant that an initial semen specimen was negative on quantitative RT-PCR was 128 days (4 months). Indeterminate results were encountered in 10 initial specimens in the range of 152 to 273 days after the onset of symptoms.

When we considered the number of days after a participant's discharge from the Ebola treatment center, the longest time that an initial semen specimen remained positive on quantitative RT-PCR was 272 days (9 months). The shortest time after a participant's discharge that an initial semen specimen was negative on quantitative RT-PCR was 100 days (3 months).

DISCUSSION

We gathered evidence showing that an Ebola virus RNA signal on quantitative RT-PCR was found in the semen of male survivors of EVD at least 9 months after the onset of symptoms. Because the data in this report are cross sectional, we are limited to reporting only the point prevalence among participants rather than describing individual-level persistence and clearance of the RT-PCR signal over time. Among this cross-sectional group of participants, all 9 male survivors who provided a sample during the first 3 months after the onset of illness had positive results on quantitative RT-PCR. During months 4 to 6, more than half the enrolled survivors had positive results on quantitative RT-PCR. The per-

centage of male survivors with positive results continued to decline over time, with approximately one quarter of the participants having positive findings on quantitative RT-PCR at 7 to 9 months after onset.

We observed that the median cycle-threshold values for the NP and VP40 gene targets increased over time, which indicated that the median quantity of viral RNA in the semen decreased over time. Our study cohort included only survivors whose onset of illness was 10 months or less before enrollment, so we do not yet know how long survivors of EVD may have Ebola RNA detectable on quantitative RT-PCR in semen. Follow-up of this cohort is ongoing, and this report will be finalized when additional data to address the issues of infectivity are available.

The detection of Ebola virus RNA by quantitative RT-PCR does not necessarily indicate that infectious virus is present. The quantitative RT-PCR assay used in this study is highly sensitive, with a detection limit per reaction of 30 median tissue-culture–infective doses ($TCID_{50}$) for the NP and VP40 gene targets in blood and urine samples to which a known quantity of live virus was added.[14,15] However, the targeted RNA sequences detected by quantitative RT-PCR could be detecting the presence of the full genome from an intact replicating virus or from smaller fragments that are unable to replicate and infect a host cell. Virus-isolation assays are under way, in which the specimens will be inoculated onto mammalian cells and the cell cultures will be observed for cytopathic effect as the virus replicates, which is the best available standard to approximate infectivity.

The cycle-threshold value for Ebola RNA has been shown to be a good approximation of the viral load in blood,[16] with an increasing cycle-threshold value indicating a decrease in the viral load. A limited study that examined the relationship between cycle-threshold values and virus isolation did not detect infectious virus in blood specimens from patients with EVD when cycle-threshold values were greater than 35.5 with the NP gene target.[17] However, experiments have not yet been performed to predict the cycle-threshold value at which viable virus can no longer be cultured in semen. It is possible that even if men provide samples that are positive on quantitative RT-PCR several months after the onset of illness, the higher cycle-threshold values (such as the median values of 37.0 value with the NP gene target and 35.6 with the VP40 gene target at 7 to 9 months observed in the current study) may indicate that their semen is no longer infectious. Ongoing serial testing until the men in this study cohort have two consecutive negative results on quantitative RT-PCR, as defined above, and performing viral culture of the RT-PCR–positive specimens will enable us to address the question of the duration of persistence of potentially infectious virus in semen.

Our cross-sectional analysis of baseline data describes the preliminary results in this cohort. Follow-up of this preliminary report continues so that we may investigate the presence and persistence of virus in the semen of survivors of EVD, including studying the relationship among cycle-threshold values, viral isolation, and genome sequencing; assessing how long semen from a survivor of EVD will remain positive; and exploring risk factors for the persistence of Ebola virus in semen.

Although our findings are based on a cohort of 100 male survivors of EVD, the public health implications are still uncertain. The ongoing study of quantitative RT-PCR positivity and virus isolation in semen will provide better estimates of the duration of viral persistence and related probabilities of persistence at various points in time.

We do not yet have sufficient information to assess the risk of transmission through sexual intercourse, oral sex, or other sex acts from men with viable virus in their semen. Before the Ebola epidemic in western Africa, a single case of Marburg virus disease and one case of EVD had been linked to sexual contact with survivors of Marburg virus disease and EVD, respectively.[7,10] In western Africa, cases that have been linked to sexual contact with survivors of EVD have not been systematically documented, and fewer than 20 in total have been suspected (Knust B, CDC; Formenty P, WHO: personal communication).

Although the potential contribution of sexual transmission to the scale of the epidemic is largely unknown, the unprecedented number of more than 16,000 survivors of EVD across Sierra Leone, Guinea, and Liberia, roughly half of whom are male, creates the potential for transmission and initiation of new chains of transmission, even months after the outbreak has ended. Even though there have been only rare cases of EVD

linked to sexual transmission, research is needed to investigate whether infectious virus may be present in vaginal fluid or other body fluids after recovery, and the testing of additional body fluids in both male and female survivors is planned.

Programs such as semen testing and preventive behavioral counseling are needed in order to help survivors of EVD appreciate and mitigate the possible risk of sexual transmission. Such programs would help men and women understand their individual risk and take appropriate measures to protect their sexual partners, specifically in regard to condom use and disposal, and could provide links to care and counseling programs for survivors. Because semen-testing programs are not yet universally available, outreach activities are needed to provide education regarding recommendations and risks to survivor communities and sexual partners of survivors in a way that does not further stigmatize the community of survivors of EVD.

Persons who survive EVD face myriad challenges. Many survivors have family members and friends who died from EVD. Many are unemployed, face stigma from their communities, and have lingering sequelae in addition to the risk of persisting virus in semen. Due respect and continuing efforts that have strong sustainable support from within the local communities are crucial in mitigating negative effects in terms of further stigma attached to survivors.

The views expressed in this article are those of the authors and do not necessarily represent the official positions of the World Health Organization (WHO) or the Centers for Disease Control and Prevention (CDC).

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Disclosure forms provided by the authors are available with the full text of this article at NEJM.org.

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APPENDIX

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REFERENCES

1. World Health Organization. Ebola situation report — 7 October 2015 (http://apps.who.int/ebola/current-situation/ebola-situation-report-7-october-2015).
2. Dowell SF, Mukunu R, Ksiazek TG, Khan AS, Rollin PE, Peters CJ. Transmission of Ebola hemorrhagic fever: a study of risk factors in family members, Kikwit, Democratic Republic of the Congo, 1995. J Infect Dis 1999;179:Suppl 1:S87-S91.
3. Dietz P, Jambai A, Paweska JT, Yoti Z, Ksaizek TG. Epidemiology and risk factors for Ebola virus infection in Sierra Leone — May 23, 2014–January 31, 2015. Clin Infect Dis 2015 July 15 (Epub ahead of print).
4. Kreuels B, Addo MM, Schmiedel S. Severe Ebola virus infection complicated by gram-negative septicemia. N Engl J Med 2015;372:1377.
5. Rodriguez LL, De Roo A, Guimard Y, et al. Persistence and genetic stability of Ebola virus during the outbreak in Kikwit, Democratic Republic of the Congo, 1995. J Infect Dis 1999;179:Suppl 1:S170-S176.
6. Rogstad KE, Tunbridge A. Ebola virus as a sexually transmitted infection. Curr Opin Infect Dis 2015;28:83-5.
7. Rowe AK, Bertolli J, Khan AS, et al. Clinical, virologic, and immunologic follow-up of convalescent Ebola hemorrhagic fever patients and their household contacts, Kikwit, Democratic Republic of the Congo. J Infect Dis 1999;179:Suppl 1:S28-S35.
8. Bausch DG, Towner JS, Dowell SF, et al. Assessment of the risk of Ebola virus transmission from bodily fluids and fomites. J Infect Dis 2007;196:Suppl 2:S142-S147.
9. Emond RT, Evans B, Bowen ET, Lloyd G. A case of Ebola virus infection. Br Med J 1977;2:541-4.
10. Martini GA, Schmidt HA. Spermatogenic transmission of the "Marburg virus": causes of "Marburg simian disease." Klin Wochenschr 1968;46:398-400. (In German.)
11. Possible sexual transmission of Ebola virus — Liberia, 2015. MMWR Morb Mortal Wkly Rep 2015;64:479-81.
12. World Health Organization. Interim advice on the sexual transmission of the Ebola virus disease. 2015 (http://www.who.int/reproductivehealth/topics/rtis/ebola-virus-semen/en/).
13. Centers for Disease Control and Prevention. Ebola virus disease — transmission. 2015 (http://www.cdc.gov/vhf/ebola/transmission/index.html).
14. Centers for Disease Control and Prevention. Ebola virus NP real-time RT-PCR assay. 2014 (http://www.fda.gov/downloads/MedicalDevices/Safety/EmergencySituations/UCM418810.pdf).

15. Centers for Disease Control and Prevention. Ebola virus VP40 real-time RT-PCR assay, 2014 (http://www.fda.gov/downloads/MedicalDevices/Safety/EmergencySituations/UCM418815.pdf).
16. Towner JS, Rollin PE, Bausch DG, et al. Rapid diagnosis of Ebola hemorrhagic fever by reverse transcription-PCR in an outbreak setting and assessment of patient viral load as a predictor of outcome. J Virol 2004;78:4330-41.
17. Spengler JR, McElroy AK, Harmon JR, Ströher U, Nichol ST, Spiropoulou CF. Relationship between Ebola virus real-time quantitative polymerase chain reaction-based threshold cycle value and virus isolation from human plasma. J Infect Dis 2015;212:Suppl 2:S346-S349.