Persistence and clearance of Ebola virus RNA from seminal fluid of Ebola virus disease survivors: a longitudinal analysis and modelling study

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Summary

Background By January, 2016, all known transmission chains of the Ebola virus disease (EVD) outbreak in west Africa had been stopped. However, there is concern about persistence of Ebola virus in the reproductive tract of men who have survived EVD. We aimed to use biostatistical modelling to describe the dynamics of Ebola virus RNA load in seminal fluid, including clearance parameters.

Methods In this longitudinal study, we recruited men who had been discharged from three Ebola treatment units in Guinea between January and July, 2015. Participants provided samples of seminal fluid at follow-up every 3–6 weeks, which we tested for Ebola virus RNA using quantitative real-time RT-PCR. Representative specimens from eight participants were then inoculated into immunodeficient mice to test for infectivity. We used a linear mixed-effect model to analyse the dynamics of virus persistence in seminal fluid over time.

Findings We enrolled 26 participants and tested 130 seminal fluid specimens; median follow up was 197 days (IQR 187–209 days) after enrolment, which corresponded to 255 days (228–287) after disease onset. Ebola virus RNA was detected in 86 semen specimens from 19 (73%) participants. Median duration of Ebola virus RNA detection was 158 days after onset (73–181; maximum 407 days at end of follow-up). Mathematical modelling of the quantitative time-series data showed a mean clearance rate of Ebola virus RNA from seminal fluid of –0·58 log units per month, although the clearance kinetic varied greatly between participants. Using our biostatistical model, we predict that 50% and 90% of male survivors clear Ebola virus RNA from seminal fluid at 115 days (90% prediction interval 72–160) and 294 days (212–399) after disease onset, respectively. We also predicted that the number of men positive for Ebola virus RNA in affected countries would decrease from about 50 in January 2016, to fewer than 1 person by July, 2016. Infectious virus was detected in 15 of 26 (58%) specimens tested in mice.

Interpretation Time to clearance of Ebola virus RNA from seminal fluid varies greatly between individuals and could be more than 13 months. Our predictions will assist in decision-making about surveillance and preventive measures in EVD outbreaks.

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Introduction

The Ebola virus disease (EVD) outbreak in west Africa lasted more than 2 years, affected almost 29 000 people in Guinea, Liberia, and Sierra Leone, and resulted in more than 11 000 deaths.[1] On Jan 14, 2016, WHO declared the end of the EVD outbreak in Liberia, with all known chains of transmission stopped in west Africa.[2] However, there is concern about persistence of the Ebola virus in survivors, specifically in the male reproductive tract.[3,4] In conjunction with the discovery of filoviruses in 1967 in Marburg, Germany, Marburg virus has been shown to be sexually transmitted by men who shed the virus in seminal fluid after recovery from the disease.[5] Reports from EVD patients subsequently confirmed virus persistence in seminal fluid after recovery for Ebola and Sudan viruses.[6–11] A cross-sectional study[12] detected Ebola virus RNA in seminal fluid of EVD survivors of the 2014–16 epidemic up to 9 months after disease onset. However, there are no quantitative virus-load data for seminal fluid to facilitate mathematical modelling of virus clearance.

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Research in context

Evidence before this study

Before this study, reports from previous Ebola and Marburg virus disease outbreaks indicated that filoviruses may be sexually transmitted by male survivors and that seminal fluid of survivors may contain filovirus RNA or infectious particles. We did a PubMed search (1960 to March 31, 2015) using the search string (ebola virus OR ebola fever OR marburg virus OR marburg fever OR filovirus) AND (persistent OR persistence OR semen OR seminal fluid OR sexual transmission) [converted by PubMed into a longer string with 43 terms] and retrieved 83 citations. The search was complemented by gathering information from review articles identified through the PubMed search. We identified five original articles published between 1968 and 2007 that were relevant to the design of our study. These studies described sexual transmission of Marburg virus in one case, and detection of Marburg, Ebola, or Sudan virus or corresponding RNA in seminal fluid of seven of eight male survivors (88%) cumulatively tested. The longest time after the onset that a semen specimen tested positive was 101 and 82 days by RT-PCR and virus isolation, respectively. While these studies provided proof-of-concept for filovirus persistence in seminal fluid, we designed a larger longitudinal study to quantitatively describe the dynamics of Ebola virus RNA load in seminal fluid and estimate the parameters of the clearance kinetic using mathematical modelling. Before submission of this manuscript, we undertook another PubMed search using the same terms to consider all evidence that accumulated during implementation of our study (April 1, 2015, to Feb 21, 2016). The search retrieved 40 citations, of which three were relevant to the interpretation of our data. One cross-sectional study, which tested one seminal-fluid specimen per survivor, detected Ebola virus RNA in 46 of 93 specimens (49%). The longest time after the onset that a specimen tested positive was 284 days. Two articles described one case of sexual transmission of Ebola virus.

Added value of this study

This study provides longitudinal data that allows systematic investigation of the dynamics of Ebola virus RNA concentrations in seminal fluid in a cohort of male survivors. The quantitative time-series data revealed considerable variability between participants in the clearance of Ebola virus RNA from seminal fluid. Although rare, survivors can continuously shed Ebola virus RNA for more than 13 months after disease onset. We also describe the clearance kinetic as well as its variability using biostatistical modelling.

Implications of all the available evidence

The longitudinal data described in this study and cross-sectional data are complementary. Biostatistical modelling of these datasets facilitates reliable prediction of the proportion of men with seminal fluid positive for Ebola virus RNA over time after onset of disease and their prevalence during an epidemic. These predictions will assist in decision-making about surveillance and preventive measures in Ebola virus disease outbreaks.

In this longitudinal study, we measured viral load in seminal fluid from a cohort of men discharged from three Ebola treatment units (ETUs) in Guinea between January and July, 2015. We aimed to collect sequential samples of seminal fluid every 3–6 weeks from participants during follow-up to analyse patterns of intensity and duration of Ebola virus shedding in seminal fluid over time.

Methods

Study design and participants

We recruited men aged 18–65 years who had had a PCR-confirmed EVD diagnosis, and were discharged from the ETUs in Conakry-Gbessia, Forecariah, or Coyah, Guinea, between Jan 20, 2015, and July 6, 2015. We contacted patients who had been discharged between Jan 20 and May 5, 2015, by telephone; and prospectively enrolled patients who had been discharged between May 6 and July 6, 2015, during their routine 1 month follow-up visit.

We followed up participants at the study centre established at the ETU in Coyah. Participants provided a sample of seminal fluid through masturbation in a private environment. Follow-up visits were scheduled every 3–6 weeks until two consecutive samples were negative for Ebola virus RNA on PCR, as recommended by WHO.[3] We telephoned study participants to remind them of follow-up visits 3 days before appointments. At enrolment and each follow-up visit, participants were counselled about safe sexual practices, and received condoms and an expense allowance. The test result was communicated by phone to the participants in conjunction with safe sex counselling.

We collected clinical and demographic data, including date of EVD onset and treatment, from patients' case files. Most study participants had received favipiravir on a compassionate use basis during the acute illness in adherence to the protocol developed for the Jiki trial.[13]

Participants gave written informed consent and the study was approved by the National Committee for Ethics in Medical Research of Guinea (permit No. 64/CNERS/15).

Ebola virus-specific real-time RT-PCR

We processed samples of seminal fluid from participants immediately after collection at the European mobile laboratory (EMLab) unit in Coyah. Workers extracted RNA from 50 µL of seminal fluid using the QIAamp Viral RNA Mini Kit (Qiagen, Hilden, Germany) after addition of the internal control RNA for the PCR assay. We tested samples for Ebola virus using the RealStar Zaire Ebolavirus RT-PCR Kit (altona Diagnostics, Hamburg, Germany; this kit is derived from the RealStar

Filovirus Screen RT-PCR Kit and contains only the reagents for detection of Ebola virus) on a Rotor-Gene Q thermocycler (Qiagen).[14] We included only the runs with valid internal control in analysis and we used threshold cycle (Ct) as an inverse measure of Ebola virus RNA concentration in the specimen. The high precision of the kit facilitates pooling of Ct values from separate runs for biostatistical analysis (appendix p 1). Relative virus RNA concentration (c_{rel}, in arbitrary units) was estimated from the Ct values using an equation (equation 1) based on experimentally generated standard curves (appendix p 1).

$$\log(c_{rel}) = -0 \cdot 262 \times Ct. \quad (1)$$

Remaining specimens were kept on-site at –20°C, sent on dry ice by an air courier service to the Bernhard Nocht Institute in Hamburg, Germany, and then stored at –80°C in the biosafety level (BSL) 4 laboratory. Ct values for blood and urine were retrieved from laboratory records.

Factors associated with Ebola virus positivity

In an attempt to identify factors that might be associated with Ebola virus detection in seminal fluid, we used non-parametric tests to compare participants' age, time of admission after onset, Ct in blood, length of stay in the ETU, treatment with favipiravir, and the time after onset when the first seminal fluid sample was taken in participants who were negative in all samples and those who were positive in at least one sample.

In vitro and in vivo infectivity assay

To show that the Ebola virus RNA detected in seminal fluid was indicative of infectious virus, we selected 26 samples from eight study participants for infectivity testing. We chose participants who had a large number of specimens sampled during follow up. Virus infectivity assays were done in the BSL-4 laboratory in Germany. We used severe combined immunodeficiency (SCID) mice for in vivo testing and had verified that the outbreak strain, Ebola virus Makona, efficiently replicates in SCID mice (appendix p 4). We diluted seminal fluid by 1:5 in SpermRinse (Vitrolife, Göteborg, Sweden) and centrifuged at 1500×g for 2 min. The supernatant was cleaned through an Ultrafree-MC HV centrifugal filter (Merck Millipore, Darmstadt, Germany) at 12 000×g for 4 min. We anaesthetised SCID mice with isoflurane and inoculated intraperitoneally with 200 µL of cleaned seminal fluid. Two mice were used per seminal fluid specimen. The animals received 1% enrofloxacin in their drinking water. We took blood samples from mice once per week. At 3–4 weeks from inoculation, mice were killed and their organs were collected. We tested blood and organs for the presence of Ebola virus RNA using the RealStar Zaire Ebolavirus RT-PCR Kit.

To further confirm the presence of infectious virus, we inoculated new SCID mice intraperitoneally with the homogenised lung tissue of selected seminal-fluid-inoculated SCID mice in 100 µL Glasgow's minimal essential medium (Life Technologies, Darmstadt, Germany) supplemented with 2% fetal calf serum. We inoculated three mice per sample; one mouse was killed at day 2 to generate baseline viral-load data and the remaining two were killed at day 30. We tested blood and organs for Ebola virus RNA as described elsewhere in the Methods. Mouse experimentation was approved by Behörde für Gesundheit und Verbraucherschutz, Freie und Hansestadt Hamburg, Germany (permit V1307/591-00.32).

See Online for appendix

In addition, Vero E6 and HepG2 cells were inoculated with 100 µL cleaned seminal fluid per well of a six-well plate in the presence of vancomycin, gentamicin, tetracycline, and amphotericin B. These drugs were required because the samples of seminal fluid were heavily contaminated with bacteria and fungi. Cells and supernatant were passaged several times. We verified virus growth in the cells on immunofluorescence using specific antibodies against Ebola virus or an increase in virus RNA concentrations in the cell culture supernatant of one order of magnitude or more, as measured on real-time RT-PCR. Before we did the virus isolation experiments, we optimised procedures and verified that centrifugal filtration and the antibiotics and antifungal administered did not affect Ebola virus titre and growth rate in cell culture. Pilot experiments that included samples taken early after recovery showed that SCID mice are more sensitive than Vero cell culture in detecting infectious virus (7/10 *vs* 1/10 samples tested positive; appendix p 5). Therefore, samples from later stages (more than 100 days after disease onset) were tested in SCID mice only.

Biostatistical modelling

We used a linear mixed-effect model to analyse the dynamics of virus persistence in seminal fluid over time (appendix p 1). The model had two parameters: a hypothetical baseline Ct value at the time of disease onset (Ct_0), and the clearance rate α. Both parameters were assumed to follow a log-normal distribution with a mean representing an average patient and a SD for exponential individual random effects allowing the parameters to vary across patients. An error model with errors varying proportionally to the predicted Ct was found to best describe residual errors. We estimated model parameters using the stochastic approximation expectation maximisation (SAEM) algorithm in Monolix v4.3 (Lixoft, Antony, France).[15] This approach is based on maximum-likelihood estimation, which takes into account the data under the limit of detection as left-censored data.[16] The Ct for negative PCR results was set at >37, which approximates the 95% detection limit of our PCR assay.[14] Because the SD for Ct_0 in seminal fluid could not be estimated, we used a value of 30%, which corresponded to the SD of the Ct values for Ebola virus RNA in plasma

at disease onset (t_0). For each parameter, we estimated the mean and SD of interindividual variability. The Ct-based clearance rate was transformed into an Ebola virus RNA clearance rate using equation 1; an increase in Ct by 3·8 cycles corresponded to a decrease in Ebola virus RNA concentration by 1 log unit. To estimate the proportion of male survivors with persisting viral RNA, we simulated 1000 replicate datasets for the 26 study participants using the distributions for Ct_0 and α and calculated the proportion of patients with Ebola virus detectable by PCR in seminal fluid for each day after disease onset (mean and 90% prediction interval [PI]). The fitted decay distribution and the previous incidence of EVD in Guinea, Liberia, and Sierra Leone from Jan 1, 2014, to Oct 14, 2015 were combined to estimate the absolute number of men who would still have Ebola virus RNA in their seminal fluid in each week.[17] We included confirmed and probable case numbers available from WHO,[1] and assumed complete reporting, 60% mortality from disease, and that 40% of survivors were men.[18]

Role of the funding source

The funders of the study had no role in study design, data collection, data analysis, data interpretation, or writing of the report. The corresponding author had full access to all the data in the study and had final responsibility for the decision to submit for publication.

Results

During the enrolment period, 48 men met eligibility criteria, and 39 of these were able to be contacted (81%); 27 men agreed to participate (56%). One study participant was retrospectively excluded because laboratory evidence for EVD in this patient was ambiguous. Median age of the 26 participants was 31 years (IQR 26–40 years) and median Ct value in the diagnostic Ebola virus RT-PCR on blood at ETU admission was 28·7 (21·7–30·2). Participants had been admitted to an ETU a median of 4·5 days (IQR 2·2–6 days) after onset of symptoms, and were discharged after a median of 12·5 days (IQR 10–14 days) (table).

	Age (years)	Acute EVD phase				Seminal fluid samples						
		Admission to ETU (days after onset)	Ct in blood at admission	Duration of ETU stay (days)	Treatment	Samples (n)	Timing of first sample (days after onset)	Ct of first sample	Last EBOV-RNA positive sample (days after onset)	Ct of last positive sample	Last sample (days after onset)	Ct of last sample
1	40	9	29·4	8	FVP	12	40	23·8	254	31·5	338	-ve
2	45	2	31·5	13	FVP	9	35	28·8	168	36·6	270	-ve
3	35	7	29·2	14	FVP	11	37	25·3	251	33·0	336	-ve
4	18	2	20·0	12	FVP	8	28	24·2	177	34·8	255	-ve
5	33	6	19·9	14	FVP + CP	13	80	27·6	407	35·5	407	35·5
6	26	5	30·1	10	FVP	9	45	25·2	233	39·2	255	-ve
7	30	5	23·0	12	FVP + CP	7	39	22·7	158	33·6	180	-ve
8	46	2	22·0	10	FVP + CP	8	49	28·5	168	30·9	236	-ve
9	19	4	20·0	15	FVP	8	61	29·2	184	29·6	254	-ve
10	28	1	29·7*	9	None	5	85	22·4	177	30·2	282	-ve
11	27	4	20·7	14	FVP	4	47	26·5	57	34·6	243	-ve
12	32	6	25·0	14	FVP	4	85	31·8	94	30·2	274	-ve
13	32	2	17·0	14	FVP + CP	4	63	31·0	72	32·0	268	-ve
14	28	5	19·7	13	None	4	47	35·1	103	36·2	242	-ve
15	40	7	31·6	8	FVP	3	38	26·0	38	26·0	225	-ve
16	35	4	31·7	14	FVP + CP	3	61	33·1	61	33·1	118	-ve
17	18	5	21·8	7	None	2	72	29·2	72	29·2	289	-ve
18	55	7	32·3*	12	FVP + ZMapp	1	73	26·0	73	26·0	73	26·0
19	25	1	21·7	14	FVP	1	95	36·3	95	36·3	95	36·3
20	28	10	29·8	7	FVP	2	48	-ve	NA	NA	249	-ve
21	15	4	31·5	9	FVP	2	49	-ve	NA	NA	190	-ve
22	26	4	23·0	14	FVP	2	46	-ve	NA	NA	241	-ve
23	40	2	38·8	10	FVP	2	98	-ve	NA	NA	201	-ve
24	18	6	28·3*	13	None	2	91	-ve	NA	NA	295	-ve
25	47	6	29·6	12	None	2	109	-ve	NA	NA	314	-ve
26	40	3	30·3	15	None	2	127	-ve	NA	NA	329	-ve

EVD= Ebola virus disease; EBOV=Ebola virus; ETU=Ebola treatment unit; Ct=threshold cycle of EBOV RT-PCR; FVP=favipiravir; CP=convalescent plasma; NA=not applicable. *Measured after admission (3 days for patient 10; 1 day for patient 18; 7 days for patient 24).

Table: **Participants' clinical and demographic data**

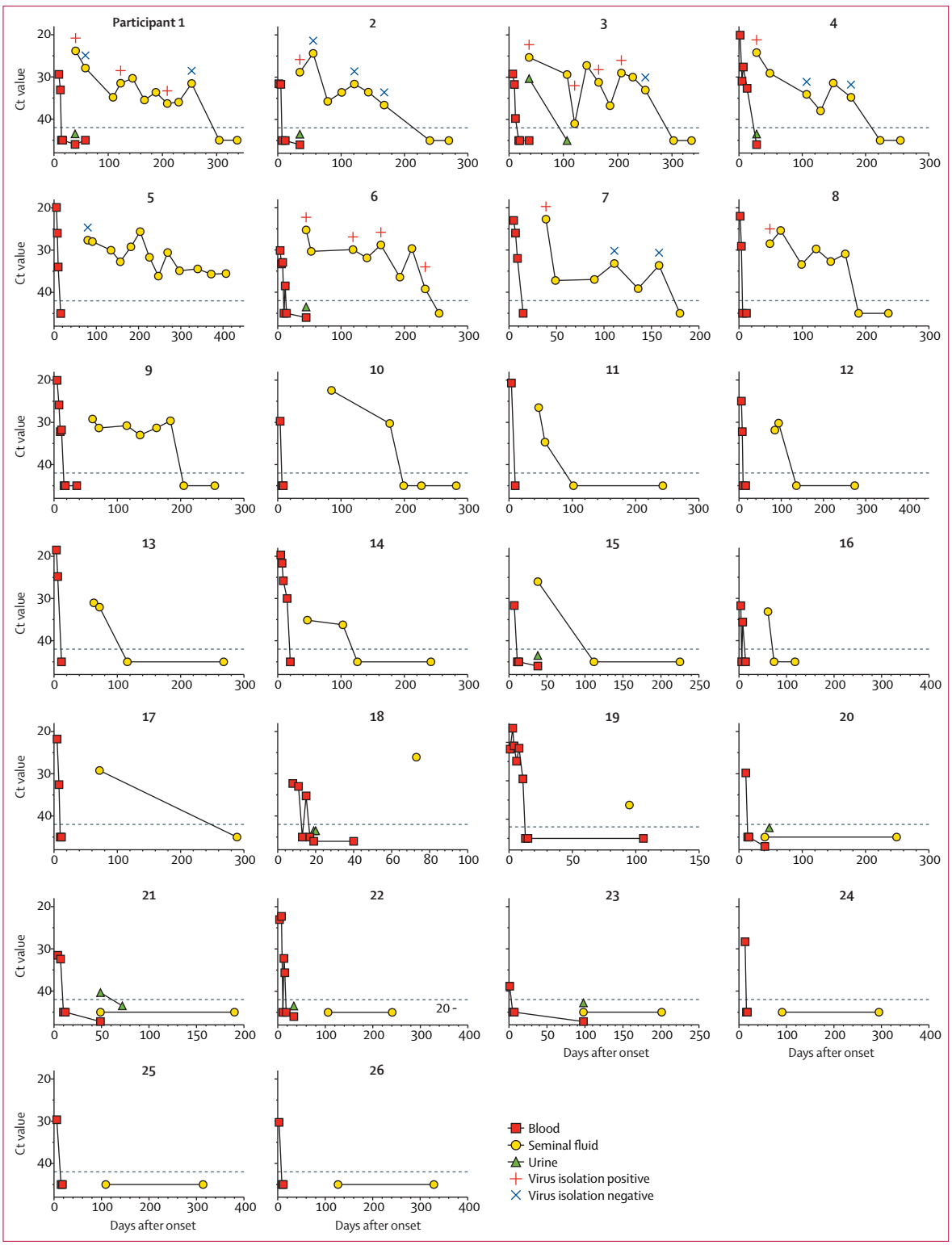

***Figure 1*: Kinetics of Ebola virus RNA clearance from blood, seminal fluid, and urine**
Ct=threshold cycle. Ct value is an inverse measure of the viral load in the body fluids. Negative PCR results are shown below the dotted horizontal lines drawn around the maximum Ct that can technically be measured. For the modelling, the Ct for negative PCR results was set at >37 cycles, which approximates the 95%-detection limit of the PCR assay. Patient identification numbers are shown in bold above each plot. Red and blue crosses show positive and negative results, respectively, of virus infectivity testing in SCID mice using seminal fluid samples from patients 1–8.

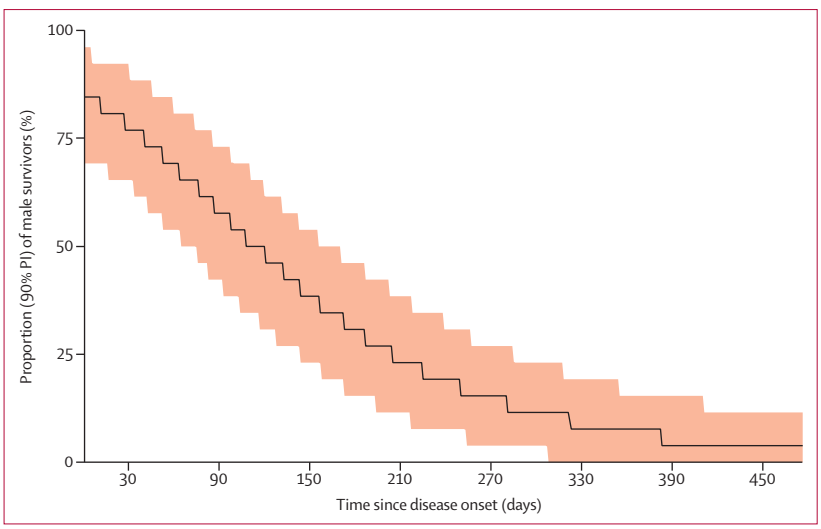

Figure 2: **Proportion, over time, of male survivors of Ebola virus disease who shed PCR-detectable Ebola virus RNA in seminal fluid**
Data are means and shaded area shows 90% prediction interval [PI].

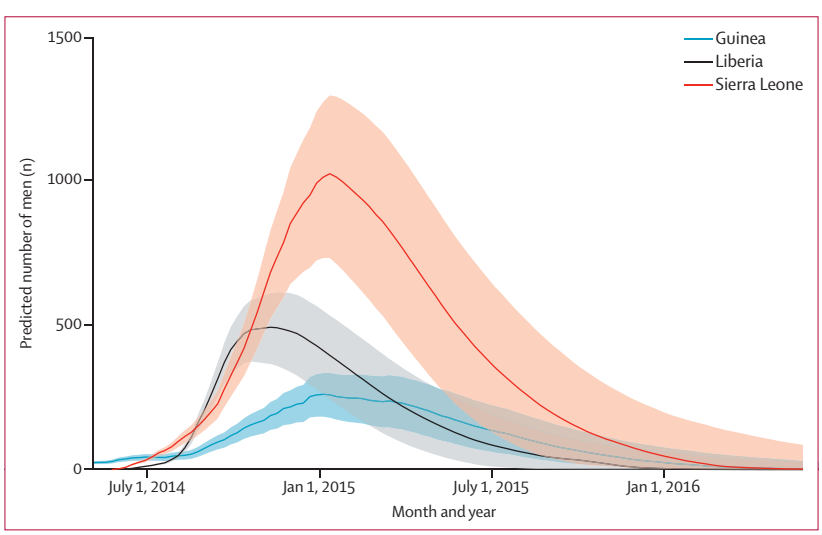

Figure 3: **Predicted number of men with PCR-detectable Ebola virus RNA in seminal fluid, July 2014 to January 2016**
Shading shows 95% CI. Predicted values rely not only on accurate EVD incidence data, but also on assumptions of constant survival rates, as well as statistical uncertainty in the fitting procedure. Therefore, point estimates should be interpreted with care, and attention given to the wide CIs.

14 patients (54%) had received compassionate treatment with favipiravir, five (19%) were treated with favipiravir plus convalescent plasma, and one (4%) was treated with favipiravir in combination with ZMapp. Six participants (23%) had received supportive standard of care only.

Collection of seminal fluid samples began a median of 55 days (IQR 45–84) after onset of disease. Participants were then followed up for a median of 197 days (IQR 187–209), which corresponds to a median period of 255 days (228–287) after disease onset. We collected a total of 131 seminal fluid samples and a valid Ebola virus RT-PCR result was obtained from 130 samples; one sample was excluded from further analysis because the internal assay control failed. Median number of samples per patient was 4 (IQR 2–8) (table).

Seminal fluid from 19 of the 26 participants (73%) was positive for Ebola virus on RT-PCR in the initial sample (figure 1). We analysed 116 samples during follow-up of these 19 Ebola-virus-positive participants, of which 86 samples (74%) tested positive. The last Ebola virus-positive sample from these 19 participants was taken a median of 158 days (IQR 73–181) after disease onset. 16 of the 19 (84%) participants subsequently cleared virus RNA from seminal fluid. Of the three participants with Ebola virus still detectable at the end of the observation period, two were lost to follow-up. The remaining patient continued to shed Ebola virus RNA in seminal fluid for at least 407 days after onset (patient 5, figure 1). 21 follow-up samples were taken from all patients who had previously tested negative. We did not observe re-appearance of Ebola virus RNA in any of these samples.

In our analysis of factors that might be associated with Ebola virus detection in seminal fluid, comparing participants who were negative in all samples (n=7) and those who were positive (n=19) in at least one sample, we did not find any statistically significant associations, including for treatment with favipiravir, although of note is the borderline significance of the time of collection of the first sample (median 49 days for Ebola virus-positive participants *vs* 91 days for Ebola virus-negative participants, two-tailed p=0·049, Mann-Whitney test).

In our biostatistical modelling, the linear mixed-effect model fitted well with the data of the 26 patients (appendix p 2). Mean increase in Ct (α) was estimated at 0·0729 per day of follow-up (SD of the between-participant variability=0·386), which corresponds to a mean decrease of 0·58 log units in the Ebola virus RNA concentration in seminal fluid per month. Using the model parameters for the Ct kinetics, we estimated that 50% of male survivors have PCR-detectable Ebola virus RNA in seminal fluid at 115 days after onset of disease (90% prediction interval 72–160) and 10% have detectable virus at 294 days (90% PI 212–399) (figure 2).

Modelling predicted that at Jan 31, 2016, there would have been 16 men (95% CI 2–65) in Guinea, one man (0–53) in Liberia, and 27 men (95% CI 1–168) in Sierra Leone who had PCR-detectable Ebola virus RNA in seminal fluid (figure 3). The mean date by which all men should have cleared Ebola virus RNA below the PCR detection limit was predicted to be July, 2016, (95% CI March, 2016, to April, 2017; defined as the first month with <1 positive man) in Guinea; February, 2016, (October 2015–November, 2016) in Liberia; and June, 2016, (February, 2016–March, 2017) in Sierra Leone.

None of the SCID mouse organs collected at day 2 contained virus RNA, whereas organs of animals inoculated with the same inoculum but killed at day 30

contained virus RNA (appendix p 5). Thus, baseline viral load in organs is below the detection limit of the RT-PCR assay and, so we took the presence of RT-PCR-detectable Ebola virus RNA in organs at 3–4 weeks after inoculation as evidence of virus replication.

We obtained evidence for infectious virus in 15 (58%) of 26 samples from seven of eight participants selected for infectivity testing (figure 1, patients 1–4 and 6–8). In mice inoculated with seven of these samples (Ct range 22·7–29·9; taken 28–119 days post onset), Ebola virus RNA appeared in blood 1–2 weeks post inoculation and was detected at the end of the experiment in spleen, kidney, liver, lung, heart, and brain (appendix p 4). In mice inoculated with the remaining eight samples (Ct range 28·5–42·7; taken 49–233 days post onset), virus was not detected in blood, but was detected in organs, mostly in lung or heart. Inoculation of new SCID mice with Ebola virus RNA-positive lung homogenate from first-passage mice—including from mice that had had virus detected only in the lung—led to appearance of virus RNA in blood and presence of virus RNA in organs (appendix p 5). The ability to passage the virus further confirms the replication competence of Ebola virus detected in the SCID mice.

Discussion

Although earlier studies provided proof-of-concept for filovirus persistence in seminal fluid, we designed a larger longitudinal study to quantitatively describe the dynamics of Ebola virus RNA load in seminal fluid and estimate the parameters of the clearance kinetic using mathematical modelling. The findings from our longitudinal study not only show the persistence of Ebola virus in seminal fluid, but also describe great variability in the duration of Ebola virus RNA shedding in seminal fluid of men who survive EVD. Further, our results of infectivity testing in SCID mice showed that Ebola virus RNA detected by PCR assay in seminal fluid is indeed indicative of infectious virus particles in a large proportion of patients.

Our data set suggests a mean clearance rate of Ebola virus RNA from seminal fluid of about half a log unit per month. From cohort data, we predicted that about 10% of male survivors still shed Ebola virus RNA above the detection limit of our PCR assay almost 10 months after disease onset. Our data could be considered in the design of post-outbreak surveillance strategies and preventive measures to reduce the potential risk of sexual transmission of persisting virus.[3]

The longitudinal setting of our study facilitates modelling of the individual virus clearance rate. Indeed, we observed a wide variation between participants in clearance kinetics. Some men (about a quarter of participants in our study) either do not shed virus RNA from seminal fluid at all, or clear the virus from this compartment rapidly. However, this proportion might be over-represented in our cohort since sampling in men who never tested positive was delayed compared with men who had at least one positive finding (difference between median times to testing=40 days, p=0·049). Nevertheless, our mathematical modelling takes this bias into account. All but one man available for follow-up had cleared Ebola virus RNA between 50 and 300 days after onset. The survivor who did not clear Ebola virus RNA remained positive for at least 407 days after disease onset. Others, who have also investigated virus persistence in the 2014–16 EVD outbreak, have reported the presence of virus RNA in seminal fluid up to 565 days after discharge.[19,20] Such a long period of virus persistence is consistent with our population clearance model.

In agreement with our kinetic model, there were no subsequent positive tests from the 21 follow-up specimens from patients who had previously tested negative, suggesting that clearance is a non-reversible process. However, the intervals between sampling were too long to detect short-term recurrence. That there was no Ebola virus RNA detected in two initial samples taken at around day 50 after disease onset (participants 20 and 21) is consistent with an early study[7,8] that reported the semen of one of five male survivors as Ebola virus PCR-negative at day 62, but contrasts somewhat with a 2015 cross-sectional study[12] that did not observe Ebola -virus-negative men within a period of 3 months after onset. However, with the exception of this very early phase, the fitted decay distributions describing the proportion of men with PCR-positive seminal fluid over time as modelled from our cohort as well as from the previous cross-sectional data correspond surprisingly well (appendix p 3).[17] That longitudinal and cross-sectional data from two different countries lead to essentially the same conclusions is reassuring and underlines the validity of the data of both studies.

Most of our patients had been treated with favipiravir during the acute phase, in some instances in combination with other specific therapies. The clinical trial for evaluation of favipiravir did not show statistically significant effects on EVD outcome.[13] Consistent with this result, we did not note any evidence that favipiravir treatment during the acute phase prevents persistence of Ebola virus in seminal fluid. However, our group size was not large enough to detect small differences with statistical significance.

Another potential bias in our study might stem from the refusal of about 40% of eligible and reachable survivors to participate. We assume that the personal nature of the study (for example, the topic of masturbation) and its implications for men's sexual life is a matter of concern for potential participants. Men might also fear stigma associated with participation within their families or communities. However, there is no reason to assume that these social and psychological aspects in the selection of the participants affected the outcome of the study.

The precise body compartment where Ebola virus persists and from which the Ebola virus RNA found in

seminal fluid is shed is not known. It might be the testes, which is an accepted immunologically privileged site where foreign antigens are less well recognised by the immune system. However, the prostate gland or other accessory glands, which contribute to seminal fluid, are also plausible sites (although not immunologically privileged). There are examples of Ebola virus persistence in various body compartments; some of them such as the placenta,[21] eye,[22,23] and central nervous system[24] are also immunologically privileged. However, the detection of Ebola virus RNA in saliva,[9,25] sweat,[26] and breast milk[9] may point to a certain tropism of the virus to glandular tissue.

Our experiments in mice indicate shedding of infectious virus particles in seminal fluid for up to 200 days after disease onset (patients 3 and 6), which suggests active virus replication at the site of persistence. Experimental data are supported by the report[20] of a probable sexual transmission of the virus from a male survivor more than 500 days after onset of disease. The clinical relevance of ongoing low-level virus replication in the male reproductive tract is unclear. Although we have not conducted an in-depth systematic clinical investigation at the follow-up visits, none of our study participants presented with symptoms that required further evaluation.

The relevant operational question arising from this study is about the risk of sexual transmission of Ebola virus. The data presented here should be interpreted with caution. Our models are based on PCR data generated with a detection limit of about 3·5 log Ebola virus RNA copies/mL and whether this cut-off also discriminates infectious from non-infectious individuals is unclear. We found evidence for infectious virus in samples with a wide Ct range, although about 40% of all PCR-positive specimens tested negative in SCID mice. Thus, PCR-positive samples have to be considered potentially infectious; however, we did not establish a clear correlation between virus RNA concentration and infectivity in our study.

From a technical point of view, the low recovery rate in cell culture is worth mentioning. It may be that the sample matrix (seminal fluid) and the specific strain had a role in this finding. The Vero E6 and HepG2 cells we used for the experiments might be less permissive for Ebola virus Makona in clinical material.

Another limitation of our study is the possible bias in selection of the study participants: recruitment was mainly from patients who had received drug therapy rather than from the larger survivors' community, which may have affected the outcome of the study. Further, enrolment for some participants was up to 2–3 months after disease onset, resulting in fewer data points than would be ideal. Therefore, the clearance kinetics might be more complex than our linear models predict. To identify more complex patterns, future studies should sample more frequently, especially at time points closer to disease onset. Another study limitation is that given the great variability in the clearance kinetics, the confidence intervals for estimates are quite large. There is also uncertainty in the assumptions made and, thus, in the final predictions of our models. Finally, it remains unclear whether survivors with Ebola virus RNA in seminal fluid are generally infectious. Because we could not relate virus RNA concentration to infectivity and transmissibility, the predictions indicate only the potential for sexual transmission of EVD by survivors.

Despite these uncertainties, the data presented here might assist in decision-making about post-outbreak surveillance and preventive measures. Great care is needed in the use of the data to prevent further stigmatisation of men who survive EVD.

Contributors

DS, SD, RK, DM, MWC, XA, and SG designed the study. DS, JSK, AHB, A-MC, and GC did the clinical investigations in Guinea. SD, JAB, FRK, MC-C, LLC, LEK, EK, JM, LVP, NYR, and KS performed the laboratory investigations in Guinea. RK, TR, LO, BP, SW, MR, AL, EP, and SB did experiments in the biosafety level 4 laboratory. ER provided essential components for the experiments. DS, SD, RK, DM, MWC, XA, and SG analysed clinical and laboratory data. JG, THTN, RME, CHW, and WJE did the mathematical modelling. ADC, RW, MG, CG, PF, SK, DM, and XA coordinated the fieldwork. DS, SD, RK, JG, THTN, RME, DM, MWC, XA, and SG wrote and edited the manuscript. All authors reviewed the final draft.

Declaration of interests

We declare no competing interests.

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