Virus persistence after recovery from acute Lassa fever in Nigeria: a 2-year interim analysis of a prospective longitudinal cohort study

Anke Thielebein, Yemisi Ighodalo, Abubakar Taju, Thomas Okorok, Recheal Omiru, Rita Esumeh, Paulson Ebhodaghe, Aniene Ekanem, Ganjuwala Gbenganle, Rosemary Giwa, Annick Renevey, Julia Hinzmam, Jonas Müller, Elisa Pallach, Meke Pahlmann, Jeremie Guedj, Joy Nwatuzor, Okwosola Femi Babatunde, Donatus I Adomeh, Danny Asogun, Nosa Akpede, Sylvanus Okogbenin, Stephan Günther, Lisa Oestereich*, Sophie Duraffour*, Ephraim Ogbaini-Emovon*

Summary

Background There is anecdotal evidence for Lassa virus persistence in body fluids. We aimed to investigate various body fluids after recovery from acute Lassa fever, describe the dynamics of Lassa virus RNA load in seminal fluid, and assess the infectivity of seminal fluid.

Methods In this prospective, longitudinal, cohort study we collected plasma, urine, saliva, lacrimal fluid, vaginal fluid, and seminal fluid from Lassa fever survivors from Irrua Specialist Teaching Hospital in Edo State, Nigeria. Inclusion criteria for participants were RT-PCR-confirmed Lassa fever diagnosis and age 18 years or older. Samples were taken at discharge from hospital (month 0) and at months 0–5, 1, 3, 6, 9, 12, 18, and 24 after discharge. The primary objective of this study was to quantitatively describe virus persistence and clearance and assess the infectivity of seminal fluid. Lassa virus RNA was detected using real-time RT-PCR. Infectivity was tested in cell culture and immunosuppressed mice. We used a linear mixed-effect model to analyse the dynamics of virus persistence in seminal fluid over time.

Findings Between Jan 31, 2018, and Dec 11, 2019, 165 participants were enrolled in the study, of whom 159 were eligible for analysis (49 women and 110 men). Low amounts of Lassa virus RNA were detected at month 0 in plasma (49 [45%] of 110 participants), urine (37 [34%]), saliva (five [5%]), lacrimal fluid (ten [9%]), and vaginal fluid (seven [21%]) of 33 female participants. Virus RNA was cleared from these body fluids by month 3. However, 35 (80%) of 44 male participants had viral RNA in seminal fluid at month 0 with a median cycle threshold of 26·5. Lassa virus RNA remained detectable up to month 12 in seminal fluid. Biostatistical modelling estimated a clearance rate of 1·19 log10 viral RNA copies per month and predicted that 50% of male survivors remain Lassa virus RNA-positive in seminal fluid for 83 days after hospital discharge and 10% remain positive in seminal fluid for 193 days after discharge. Viral RNA persistence in seminal fluid for 3 months or more was associated with higher viraemia (p=0·006), more severe disease (p=0·0075), and longer hospitalisation during the acute phase of Lassa fever (p=0·0014). Infectious virus was isolated from 48 (52%) of 93 virus RNA-positive seminal fluid samples collected between month 0 and 12.

Interpretation Lassa virus RNA is shed in various body fluids after recovery from acute disease. The persistence of infectious virus in seminal fluid implies a risk of sexual transmission of Lassa fever.

Funding German Federal Ministry of Health, German Research Foundation, Leibniz Association.

Copyright © 2021 The Author(s). Published by Elsevier Ltd. This is an Open Access article under the CC BY 4.0 license.
Research in context

Evidence before this study
We searched PubMed for research articles published from database inception until Jan 4, 2021, with no language restrictions, using the terms "(Lassa virus OR Lassa fever) AND (persistence OR body fluid OR semen OR seminal fluid OR urine OR vaginal fluid OR lacrimal fluid OR saliva OR sexual transmission)". The search retrieved 107 entries, of which seven reported findings relevant to this study. Two publications (published in 2017 and 2020) reported four patients with Lassa virus RT-PCR-positive seminal fluid. Culture investigations yielded infectious virus in one seminal fluid specimen on day 20 of illness. Six publications reported detection of viral RNA or infectious virus in urine, saliva, or throat swabs during acute disease; prolonged shedding in urine was reported in three cases. We aimed to investigate Lassa virus persistence and clearance in various body fluids in a cohort of Lassa fever survivors. In this Article, we present the 2-year interim analysis.

Added value of this study
We investigated a cohort of 159 Lassa fever survivors over a period of 2 years following discharge from the hospital. Only four anecdotal case reports on detection of Lassa virus in seminal fluid of survivors. The virus was also found in urine, saliva, or throat swabs during acute disease; prolonged shedding in urine was observed in three cases. We aimed to investigate Lassa virus persistence and clearance in various body fluids in a cohort of Lassa fever survivors. In this Article, we present the 2-year interim analysis.

Methods
Study design and participants
We did a prospective, longitudinal, cohort study of Lassa fever survivors discharged from the Lassa fever ward of the Irrua Specialist Teaching Hospital in Edo State, Nigeria. Inclusion criteria were having a Lassa fever diagnosis confirmed by RT-PCR and being age 18 years or older. During the recruitment period, from Jan 31, 2018, to Dec 11, 2019, 165 participants were enrolled (appendix 1 p 4). This 2-year interim analysis includes all study visits from Jan 31, 2018, to July 29, 2020 (appendix 1 p 4).

All participants provided written informed consent. This study was approved by the Irrua Specialist Teaching Hospital Health Research Ethics Committee (number STH/HREC/20171208/44).

Procedures
Median duration of hospitalisation was 11 days. All participants received standard of care, including ribavirin, during their stay on the Lassa fever ward. Participants were discharged according to national guidelines: either a negative RT-PCR test at day 10 (ie, the tenth day in hospital; day 0 is day of admission) and the patient was afebrile, or RT-PCR was positive after treatment at day 10 but there were no symptoms suggestive of Lassa fever for 72 h after treatment. Participants were informed about the study while on the ward and recruited within 2 weeks after discharge, except for 16 participants who were recruited at month 1 or 3 after discharge (reason for late inclusion not recorded).

Participants were followed up for 24 months with nine study visits at the hospital day clinic: at discharge (+3 days) and 0-5 month (+3 days), 1 month (+5 days), 3 months (+2 weeks), 6 months (+2 weeks), 9 months (+4 weeks), 12 months (+4 weeks), 18 months (+4 weeks), and 24 months (+4 weeks) after discharge. Participants were called by phone about 1 week before each visit. If a visit could not be arranged within the specified timeframe, the participant was invited for the next scheduled visit. At each visit, a physical examination was done, laboratory results from the previous visit were communicated, and participants received counselling about safe sex practices.

Blood, urine, saliva, lacrimal fluid, seminal fluid, and vaginal fluid were collected at each visit. Blood was collected using an EDTA (edetic acid)-Monovette (Sarstedt; Newton, NC, USA); urine in a urine cup and filtered through a 0·22 µm filter (Sarstedt); saliva using a Pure-SAL collection device (Oasis Diagnostics; Vancouver, BC, USA); and lacrimal fluid using a flexible minitip swab (Copan Diagnostics; Murrieta, CA, USA) and stored in 1 mL universal transport medium (UTM). Male participants provided seminal fluid in urine cups; lubricant was not used because it could interfere with downstream laboratory analysis. Female participants collected vaginal fluid using a regular swab that was placed in 1 mL UTM (Copan Diagnostics). Specimens were kept onsite at −80°C.

Low amounts (cycle threshold \( C_t > 35 \)) of Lassa virus RNA were detected in plasma, urine, saliva, lacrimal fluid, and vaginal fluid. However, moderate to high (\( C_t 20–35 \)) amounts of viral RNA were found for up to 1 year in seminal fluid of male survivors. Testing in cell culture and mice showed viable virus in seminal fluid up to 9 months after discharge. This study provides clear evidence for persistence of infectious Lassa virus in seminal fluid. The number of participants and follow-up specimens allowed us to quantitatively describe the dynamics of virus shedding and factors associated with longer persistence.

Implications of all the available evidence
The persistence of infectious virus in seminal fluid implies a risk of sexual transmission of Lassa fever. Therefore, male survivors are recommended to use safe sex practices for 1 year after discharge from hospital. Testing seminal fluid could be implemented in endemic areas. Female survivors should use safe sex practices after discharge from hospital for 3 months. Further research on the potential of sexual transmission of Lassa fever are warranted.
Lassa virus RT-PCR analysis was done prospectively at the hospital. RNA was extracted from 140 µl saliva or lacrimal swab UTI; 70 µl plasma, urine, or vaginal swab UTM; or 50 µl seminal fluid using the QiAamp Viral RNA Kit (Qiagen; Hilden, Germany). The lower volumes were used for some sample matrices to reduce the risk of RT-PCR-inhibition. Lassa virus RNA was detected using the RealStar Lassa Virus real-time RT-PCR kits 1.0 and 2.0 (Altona Diagnostics; Hamburg, Germany) on a RotorGene Q thermocycler (Qiagen). The cycle threshold (Ct) values obtained with the assay targeting the GPC gene were used for this study.

RT-PCR-positive semen samples were transferred on dry ice to the biosafety level 4 laboratory in Hamburg, Germany, and stored at −80°C. Because experimentation under biosafety level 4 conditions is time and resource consuming, only a subset of samples was assessed for infectivity in this interim analysis. Samples were subjectively selected by the investigator, with low Ct samples intentionally taken more frequently than high Ct samples. Vero 76 cells (ATCC CRL-1587) in a T10 flask were inoculated with a mix of 50 µl seminal fluid and 950 µl Dulbecco’s Modified Eagle Medium (DMEM [Pan Biotech; Aidenbach, Germany]) that had been filtered through a 0.1 µm filter. After incubation for 1 h at 37°C, the supernatant was removed and replaced with fresh DMEM containing 100 U/mL penicillin, 100 µg/mL streptomycin, 1 mM glutamine, 0.5 mM pyruvate, 1x non-essential amino acids, and 3% fetal calf serum (FCS). Cells were incubated for 10 days including passaging to T25 and T75 flasks. Virus growth was verified using three Lassa virus-specific assays, specifically immunofluorescence, immunofocus assay, and RT-PCR (appendix 1 pp 1–2; appendix 2).

In-vivo infectivity testing was done only if in-vitro testing was not successful. Severe combined immuno-deficiency (SCID) mice, which were previously successfully used to isolate Ebola virus from seminal fluid,26 were inoculated intraperitoneally with a mix of 50 µl seminal fluid and 50 µl phosphate-buffered saline containing 3% FCS. Two mice were used per specimen. The animals received 0.1% enrofloxacin in drinking water. At day 14 or 28, mice were euthanised and blood and organs were collected. Homogenised organs and blood were assessed for Lassa virus infection by RT-PCR (appendix 2). Animal experiments were approved by the Office for Food Safety and Veterinary Affairs (appendix 2). Animal experiments were approved by the Office for Food Safety and Veterinary Affairs (appendix 2). Animal experiments were approved by the Office for Food Safety and Veterinary Affairs (appendix 2). Animal experiments were approved by the Office for Food Safety and Veterinary Affairs (appendix 2). Animal experiments were approved by the Office for Food Safety and Veterinary Affairs (appendix 2). Animal experiments were approved by the Office for Food Safety and Veterinary Affairs (appendix 2). Animal experiments were approved by the Office for Food Safety and Veterinary Affairs (appendix 2). Animal experiments were approved by the Office for Food Safety and Veterinary Affairs (appendix 2). Animal experiments were approved by the Office for Food Safety and Veterinary Affairs (appendix 2). Animal experiments were approved by the Office for Food Safety and Veterinary Affairs (appendix 2). Animal experiments were approved by the Office for Food Safety and Veterinary Affairs (appendix 2). Animal experiments were approved by the Office for Food Safety and Veterinary Affairs (appendix 2). Animal experiments were approved by the Office for Food Safety and Veterinary Affairs (appendix 2).

Outcomes
The primary objective of this study was to quantitatively describe virus persistence and clearance in various bodily fluids, particularly seminal fluid, in survivors of Lassa fever following discharge from hospital. Secondary objectives were to assess the infectivity of the virus in seminal fluid and to determine whether virus persistence in seminal fluid is related to clinical, virological, or demographic variables (post-hoc analysis).

Statistical analysis
We used biostatistical modelling to characterise Lassa virus RNA persistence and clearance. The dynamics of Lassa virus RNA in seminal fluid was described by a linear mixed-effect model: $y_i = \theta + \beta y_{i-1} + \epsilon_i$ (equation 1) where $y_i$ is the Ct value observed in individual $i$ at time $t_i$ and $\theta = \exp(0.0*t_i)$ ($\epsilon_i$ is the random effect. A proportional error model best described the errors. We estimated model parameters using the stochastic approximation expectation maximisation algorithm in Monolix 2019R2 (Lixoft; Antony, France). Negative RT-PCR results were considered as right-censored data with a Ct of more than 40.3. This Ct value relates to the 50% limit of detection of the Lassa virus RT-PCR assay (appendix 1 p 1). The individual parameters were estimated as a mode of their conditional posterior parameter distribution using empirical Bayes estimates with 1000 simulated parameters per individual (appendix 1 p 5). Throughout the modelling, measured and hypothetical Ct values were taken as a proxy for the Lassa virus RNA concentration. Output Ct variables were converted into arbitrary concentration units using the equation: $\log_{10}$ (relative Lassa virus RNA concentration) = $-0.295 \times$ Ct (equation 3; appendix 1 p 1).

To estimate the chance of a male survivor being RT-PCR-positive at a given time after discharge, we used equation 2 with the individual values for $CT_0$ and $\alpha$ and calculated hypothetical Ct values for each participant and each day after discharge. We then calculated the likelihood of a positive test result for each Ct value using the cumulative Gaussian distribution describing the test outcome at the limit of detection of the RT-PCR assay (appendix 1 p 1). The mean of the individual likelihood values was calculated for each day after discharge.

To test for associations between duration of Lassa virus RNA detection in seminal fluid and different variables, we stratified male participants post-hoc into two groups: group one were negative for Lassa virus RNA in seminal fluid or with RNA detected up to month 1 after discharge plus a subsequent RNA-negative specimen latest at month 3; and group two had RNA detected at month 3 or later. Variables tested included age, Ct value on admission to the Lassa fever ward, disease severity score during the acute phase using the levels of six clinical chemistry parameters, and duration of hospitalisation (appendix 1 p 3). Statistical significance of differences between the two groups was assessed with a univariate non-parametric test, the two-sided Mann-Whitney U test. Exact p values were calculated and p<0.0125 (Bonferroni-corrected.
significance threshold calculated as p<0.05 divided by 4) was considered significant. The 95% CI for prevalence was estimated using the Wilson score interval method. Missing data were not imputed.

Enrolled participants with only one timepoint and less than four specimens sampled and individuals who did not fulfil the inclusion criteria upon retrospective investigation were excluded from the analysis. Study visits and data analysis will continue until all participants have reached 24 months of follow-up.

Role of the funding source
The funder 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
We enrolled 165 participants, of whom 159 were eligible for analysis (figure 1; appendix 1 p 4). By the cutoff date of the interim analysis (July 29, 2020), 1160 (86%) of 1358 of all scheduled study visits were completed with a visit attendance rate of 997 (85%) of 1160 completed visits (appendix 1 p 4). A total of 319 plasma, 995 urine, 995 saliva, 995 lacrimal fluid, 284 vaginal fluid, and 555 seminal fluid samples were collected, tested by Lassa virus-specific real-time RT-PCR, and included in the analysis (figure 1).

49 of 110 participants (45%, 95% CI 36–54) tested directly after discharge from the Lassa fever ward still had detectable Lassa virus RNA in plasma (figure 2A; appendix 1 p 6). Additionally, Lassa virus RNA was found in the urine of 37 of 110 participants (34%, 26–43), in saliva of five of 110 participants (5%, 2–10), in lacrimal fluid of ten of 109 participants (9%, 5–19), and in vaginal fluid of seven of 33 female participants (21%, 11–38; appendix 1 p 6). The virus RNA concentration in these body fluids was low (Ct>35) with the exception of some urine samples (Ct 25–35) and decreased below the RT-PCR detection limit by month 3.

The highest Lassa virus RNA detection rate at any point in time was found with seminal fluid. 35 of 44 male participants (80%, 66–89) tested positive at month 0 and 63 of 81 male participants (78%, 68–86) tested positive at month 0·5 (appendix 1 p 6). The median Ct at these timepoints was 26·5 (IQR 23·3–31·3) for month 0 and 26·4 (23·3–30·8) for month 0·5, corresponding to viral RNA concentrations of approximately 5×10⁶ RNA copies per mL. The proportion of positives dropped to 45% (35 of 77; 95% CI 35–57) of RT-PCR results (median Ct 33·2 [IQR 30·6–35·3]) by month 3 and to 2%...
(0–10; one of 55) of RT-PCR results (Ct 38·3) by month 12. By month 18, all men had cleared the virus RNA below the assay’s detection limit.

To explore if duration of virus RNA shedding in seminal fluid was associated with age and variables of the acute phase of the disease, we categorised male participants into two groups: group 1 negative for Lassa virus RNA or with Lassa virus RNA detected up to month 1 after discharge, and group 2 with Lassa virus RNA detected at month 3 or later. Group 2 (participants with Lassa virus RNA detected at month 3 or later) was older (median 38·0 years [IQR 29·0–46·0] vs 33·5 [26·7–42·2], p=0·12), had higher viraemia on admission to the Lassa fever ward (median Ct 29·9

Figure 2: Lassa virus RNA clearance from body fluids

RNA was extracted from (A) plasma, (B) urine, (C) saliva, (D) lacrimal fluid, (E) vaginal fluid, and (F) seminal fluid. The Ct value is an inverse measure of the viral load in the body fluids; the estimated Lassa virus RNA concentration is shown on the right y-axis. The RNA concentration refers to the actual body fluid (graphs A, B, C, and F) or the swab medium (graphs D and E). Each dot represents a sample, with samples from the same patient connected by a line. Negative PCR results are shown below the dotted horizontal line. The number of participants tested and the absolute and relative fraction of RNA positives is shown below the graphs for each visit in the month after discharge. Ct=cycle threshold.

| Month | Plasma | Urine | Saliva | Lacrimal fluid | Vaginal fluid | Seminal fluid |
|-------|--------|-------|--------|----------------|---------------|----------------|
| 0     |        |       |        |                |               |                |
| 0.5   |        |       |        |                |               |                |
| 1     |        |       |        |                |               |                |
| 3     |        |       |        |                |               |                |
| 6     |        |       |        |                |               |                |
| 9     |        |       |        |                |               |                |
| 12    |        |       |        |                |               |                |
| 18    |        |       |        |                |               |                |
| 24    |        |       |        |                |               |                |

| Months | Plasma | Urine | Saliva | Lacrimal fluid | Vaginal fluid | Seminal fluid |
|--------|--------|-------|--------|----------------|---------------|----------------|
| Test (n) | 110 | 110 | 110 | 110 | 110 | 110 |
| Positive (n) | 49 | 49 | 49 | 49 | 49 | 49 |
| Positive (%) | 45% | 45% | 45% | 45% | 45% | 45% |

www.thelancet.com/microbe Vol 3 January 2022
between the two groups was assessed with two-sided Mann-Whitney test; \( p < 0.0125 \) was considered significant.

Male participants were stratified post-hoc into group 1 with short-term virus persistence (RNA negative or RNA positive at month 0, 0.5, or 1 with a subsequent RNA-negative specimen latest at month 3 after discharge) and group 2 with long-term virus persistence (RNA-positive at month 3, 6, 9, or 12). Group 1 (green circles) had 50 male participants and group 2 (orange circles) 35 male participants. Statistical significance of differences in the number of participants with available data for each variable and group. (A) Age of participants. (B) Cycle threshold value on admission to the Lassa fever ward. (C) Disease severity score during the acute disease.

Figure 3: Association of duration of Lassa virus RNA detection in seminal fluid with age and characteristics of the acute disease

We used biostatistical modelling to quantitatively describe the kinetics of Lassa virus RNA clearance from seminal fluid and estimate the proportion of men with Lassa virus RNA-positive seminal fluid over time. The linear mixed-effect model fitted well with the virus RNA data of the individual participants (appendix 1 p 5). The hypothetical baseline Ct value at the time of discharge (parameter \( C_{T0} \)) was estimated at 28.7 (SE 1.0) and the mean increase in \( C_t \) value per day of follow-up (parameter \( \alpha \)) was estimated at 0.135 (0.013), which corresponds to a mean decrease of 1.19 \( \log_{10} \) Lassa virus RNA copies in seminal fluid per month. Using the model parameters for the \( C_t \) value kinetics, we estimated that 50% of male survivors had detectable Lassa virus RNA in seminal fluid 83 days after hospital discharge and 10% of male survivors had detectable Lassa virus RNA in seminal fluid 193 days after discharge (figure 4).

To test whether detection of Lassa virus RNA in seminal fluid showed presence of infectious virus, we attempted to isolate virus from RT-PCR positive samples in vitro and in vivo. Vero 76 cells were inoculated with 93 (42%) of the 221 Lassa virus RNA-positive seminal fluid specimens collected from 42 participants up to 12 months after hospital discharge. The samples were selected semi-randomly with a bias toward low Ct values to increase the chance of virus isolation. 31 (33%) of the 93 specimens, which had failed the isolation attempt in cell culture, were additionally inoculated into SCID mice.

Infectious virus was detected in 48 (52%) of 93 specimens tested, specifically in 26 (28%) that were assayed in cell culture and in 22 (71%) of the 31 culture negatives assayed in mice (figure 5; appendix 2). The virus isolation rate was 14 (67%) of 21 seminal fluid samples collected at month 0, ten (38%) of 26 samples at month 0–5, 11 (39%) of 28 samples at month 1, eight (80%) of 10 samples at month 3, four (100%) of four samples at month 6, one (33%) of three samples at month 9, and zero (0%) of one sample at month 12. The samples with the lowest virus RNA concentration from which infectious virus was isolated in cell culture and mice had Ct values of 31.4 (approximately 1.8 \( \times \) 10⁴ RNA copies per mL) and 35.1 (1.5 \( \times \) 10⁵ RNA copies per mL), respectively. The longest period of detection of infectious Lassa virus in seminal fluid was 281 days after hospital discharge for a sample collected at the month 9 visit.

Discussion

This prospective longitudinal study shows presence of Lassa virus RNA in various body fluids following resolution of acute Lassa fever. The body fluid with the largest proportion of positive RT-PCR results, the highest virus RNA concentration, and longest duration of shedding was seminal fluid. Infectious Lassa virus was isolated from a substantial proportion of seminal fluid samples collected from study participants.
samples that had viral RNA concentrations greater than 10^4 copies per mL and were collected up to 9 months after discharge.

The virus RNA concentration in plasma, saliva, lacrimal fluid, and vaginal fluid was low with Ct values close to the detection limit of the PCR and usually dropped below the level of detection within 3 months. However, lacrimal and vaginal fluid were collected using a swab, therefore, the dilution of the primary specimen in the swab medium leads to an underestimate of the actual virus concentration. Ct values in urine were somewhat lower than other body fluids, although we could not obtain evidence for; the presence of infectious virus (RT-PCR-positive urine samples taken after discharge from five participants tested negative in cell culture). It is likely that the viral RNA in these body fluids reflects residual virus replication during convalescence. Detection of viral RNA in lacrimal fluid, saliva, and vaginal fluid might suggest replication in glandular tissues or mucosal epithelium; the detection of viral RNA in urine might suggest replication in kidney cells.

The most relevant finding of the study is the detection of viral RNA in seminal fluid of the majority of male survivors after clinical recovery. Duration and magnitude of viral RNA shedding clearly exceeds that of the other body fluids. This finding suggests that testis or glandular tissue in the prostate are relevant sites for persistence of tissue in the prostate are relevant sites for persistence of body fluids. This finding suggests that testis or glandular tissue in the prostate are relevant sites for persistence of Lassa virus. Using mathematical modelling, we estimated a clearance kinetics of 1.9-1.90 log_{10} Lassa virus RNA copies per month, which is four times faster than the clearance of Ebola virus RNA from seminal fluid of survivors of Ebola virus disease (0.58 log_{10} Ebola virus RNA copies per month). The overall percentage of male survivors who tested positive after discharge (80%) is comparable with the values measured for patients with Ebola virus disease. The time after discharge during which 50% of male survivors cleared Lassa virus RNA from seminal fluid was estimated to be day 83 and 90% of male survivors cleared Lassa virus RNA from seminal fluid was estimated to be 193 days. Consistent with the faster clearance kinetics of Lassa virus RNA compared with Ebola virus RNA, the time to RT-PCR negativity in male survivors is also shorter than estimated for Ebola virus disease (50% clearance after 115 days and 90% clearance after 294 days). Lassa virus RNA persistence in seminal fluid for 3 months or longer was statistically significantly associated with higher viraemia, severe disease, and longer hospitalisation during the acute phase of the disease. These findings could suggest that tissue damage, inflammation, or a high rate of virus replication over prolonged periods of time promote virus replication in the male reproductive tract. However, it cannot be excluded that, vice versa, increased virus replication in the reproductive tract contributes to the pathogenesis during the acute phase. The clinical relevance of prolonged virus replication in the male reproductive tract after recovery is also unclear. During the follow-up visits, male participants did not report severe sequelae or had recurrence of Lassa fever symptoms, although systematic clinical studies are needed.

Our extensive in vitro and in vivo infectivity studies using 93 RT-PCR positive seminal fluid samples showed the presence of infectious virus particles in 48 samples. Up to 9 months after discharge, infectious virus could be isolated in immunocompromised mice from samples with an estimated virus RNA concentration as low as 10^4 copies per mL. Thus, even the detection of low concentrations of Lassa virus RNA in seminal fluid could suggest the presence of infectious virus. However, detection of viral RNA does not generally suggest infectious virus. In our experimental systems, we have not been able to detect infectious virus in all viral RNA-positive samples tested, even in those with a high RNA concentration around 10^8 copies per mL. Whether this reflects presence of anti-Lassa virus components in seminal fluid, predominance of Lassa virus with a replication-deficient phenotype, or technical reasons, remains speculative.

The shedding of infectious virus in seminal fluid implies a risk of sexual transmission of Lassa virus. The extent of this risk depends on unknown factors, most importantly whether Lassa virus is contagious and stable in the specific environment (ie, seminal plus vaginal fluid) and whether the amount shed in seminal fluid reaches the minimum infectious dose required to initiate an infection via the mucosa. It is not known if the infectious virus detected in our experimental systems meets these criteria. So far, there is no epidemiological data supporting sexual transmission. Sequencing of Lassa virus in patients in hospital suggests that human-to-human transmission is a rare event. However, there are no systematic studies...
on the potential sexual transmission of Lassa virus. A study on Argentine haemorrhagic fever showed that spouses of male survivors could be at risk to contract the disease from their partners. Epidemiological and molecular investigation of human-to-human transmission of Lassa virus in communities is challenging due to the mild or asymptomatic course of most infections and the confounding exposure of humans to the rodent reservoir.

There are three limitations we have identified with our study. First, there might be an over-representation of men. Knowledge on the persistence of other pathogens in seminal fluid, such as Ebola virus, might have led to a specific interest of men to obtain information on their health status through the study, which could explain why about twice as many men than women participated in this study. Second, essentially all patients have been treated with ribavirin during the acute phase of the disease. The effect of ribavirin on viral load in humans is unknown and the data from studies of Lassa virus in non-human primates and mice are conflicting. Additionally, ribavirin showed cell-protective effects in vivo that might modulate virus persistence. Thus, we do not know if and how this intervention could bias our results, which also potentially restricts the generalisability of our data to populations of patients with Lassa fever who are not treated with ribavirin or treated with other (future) drugs. Finally, as our study included only acutely ill patients in hospital, it remains speculative whether patients with mild or subclinical infections, who do not attend the hospital, also shed virus in seminal fluid. The risk of long-term persistence in mild or subclinical infections might be lower given that duration of shedding is associated with viraemia and disease severity in our participants. However, when extrapolating this association to non-hospitalised patients, the absence of ribavirin treatment in this group must be considered as a confounding factor.

Overall, our study has implications for patient management. We have shown that male survivors can have Lassa virus present in their seminal fluid following discharge from hospital; therefore, we recommend the use of safe sex practices for at least 1 year after discharge to prevent transmission. Testing seminal fluid should be offered on discharge and at follow-up appointments. Female survivors should also use safe sex practices for 3 months after discharge. Although the risk of transmission via urine, saliva, and lacrimal fluid is considered extremely low, survivors should be counselled on the hypothetical risk of infection. From a diagnostics perspective, testing for Lassa virus RNA in other body fluids might detect a recent infection (ie, an infection from the past 9 months), if a plasma sample is negative.

In conclusion, we describe the dynamics of persistence and clearance of Lassa virus in body fluids after acute disease. Male survivors shed infectious Lassa virus via the seminal fluid with the longest documented period of 281 days after discharge. These data imply a risk of sexual transmission of Lassa fever but further research is needed.

Contributors
ATH, SD, LO, SG, SO, DaA, and EO-E conceptualised the study and the methodology; ATa, YI, AE, RO, RE, and NA ran the day clinic and collected the sample and medical data; ATa, YI, TO, RO, RE, PE, CI, KG, JH, JM, EP, JN, OFB, and DoA did the laboratory analysis; ATH, YI, and MP did the project administration and logistics. ATH, SD, MP, LO, SG, and EO-E analysed the data. AR and JG did the biostatistical modelling. ATH, SD, LO, SG, and EO-E supervised the study; ATH, AR, SD, LO, and SG wrote the manuscript. All authors reviewed and edited the manuscript.

Declaration of interests
SG is member of the Scientific Advisory Group to advise WHO on the implementation of the WHO R&D Blueprint for action to prevent epidemics. All other authors declare no competing interests.

Data sharing
Anonymised individual participant data that underlie the results reported in this Article will be made available for 5 years once the study has been completed, and the data have been analysed and published. Researchers who provide a sound proposal will be given access. Proposals should be directed to Stephan Günther (guenther@bni.uni-hamburg.de) and Ephraim Ogbanai-Emwen (epogbanai@yahoo.com). Data requestors will need to sign a data access agreement.

Acknowledgments
We thank the staff of the Irrua Specialist Teaching Hospital who were involved in the care and management of patients with Lassa fever. This study was supported by the German Federal Ministry of Health through support of the WHO Collaborating Centre for Arbovirus and Haemorrhagic Fever Reference and Research at the Bernhard Nocht Institute for Tropical Medicine (agreements ZMV 11-2517/W1/0005) and through the Global Health Protection Program (agreement ZMV 11-2517/GHP/704), and the German Research Foundation to SG (GU 883/4-1 and GU 883/5-1).

References
1. Asogun DA, Gunther S, Akpede GO, Ihekwezu C, Zumla A. Lassa fever: epidemiology, clinical features, diagnosis, management and prevention. Infect Dis Clin North Am 2019; 33: 933–51.
2. Okokhere P, Colubi A, Azuhike C, et al. Clinical and laboratory predictors of Lassa fever outcome in a dedicated treatment facility in Nigeria: a retrospective, observational cohort study. Lancet Infect Dis 2018; 18: 684–95.
3. Akpede GO, Asogun DA, Okogbenin SA, et al. Caseload and case fatality of Lassa fever in Nigeria, 2001–2018: a specialist center’s experience and its implications. Front Public Health 2019; 7: 170.
4. Andersen KG, Shapiro BJ, Matranga CB, et al. Clinical sequencing uncovers origins and evolution of Lassa virus. Cell 2015; 162: 738–50.
5. Siddle KJ, Eronom P, Barnes KG, et al. Genomic analysis of Lassa virus during an Increase in cases in Nigeria in 2018. N Engl J Med 2018; 379: 1745–53.
6. Kafetzopoulou LE, Pullan ST, Lemey P, et al. Metagenomic sequencing at the epicenter of the Nigeria 2018 Lassa fever outbreak. Science 2019; 363: 74–77.
7. Dan-Nwrafo CC, Ipadeola O, Smout E, et al. A cluster of nosocomial Lassa fever cases in a tertiary health facility in Nigeria: description and lessons learned. 2018. Int J Infect Dis 2019; 83: 88–94.
8. Brigger A, Enria D, Fessilade MR, Maiztegui J. Contagio interhumano e infección clínica con virus junín en matrimonios residentes en el área endémica de fiebre hemorrágica argentina. Medicina (Buenos Aires) 1987; 47: 565.
9. Uyeki TM, Erickson BR, Brown S, et al. Ebola Virus persistence in semen of male survivors. Clin Infect Dis 2016; 62: 1552–55.
10. Sissoko D, Duraffour S, Kerber R, et al. Persistence and clearance of Ebola virus RNA from seminal fluid of Ebola virus disease survivors: a longitudinal analysis and modelling study. Lancet Glob Health 2017; 5: e80–88.
11. Deen GF, Broutet N, Xu W, et al. Ebola RNA persistence in semen of Ebola virus disease survivors—final report. N Engl J Med 2017; 377: 1428–37.
12 Mate SE, Kugelman JR, Nyenswah TG, et al. Molecular evidence of sexual transmission of Ebola virus. *N Engl J Med* 2015; 373: 2448–54.

13 Diallo B, Sissoko D, Loman NJ, et al. Resurgence of Ebola virus disease in Guinea linked to a survivor with virus persistence in seminal fluid for more than 500 days. *Clin Infect Dis* 2016; 63: 1353–56.

14 Raabe VN, Kann G, Röbler BS, et al. Favipiravir and ribavirin treatment of epidemiologically linked cases of Lassa fever. *Clin Infect Dis* 2017; 65: 855–59.

15 Diallo B, Amoo OS, Shaibu JO, et al. Monitoring of Lassa virus infection in suspected and confirmed cases in Ondo State, Nigeria. *Pan Afr Med J* 2020; 36: 253.

16 Leifer E, Gocke DJ, Bourne H. Lassa fever, a new virus disease of man from West Africa. Report of a laboratory-acquired infection treated with plasma from a person recently recovered from the disease. *Am J Trop Med Hyg* 1970; 19: 677–79.

17 Emound RT, Bannister B, Lloyd G, Southee TJ, Bowen ET. A case of Lassa fever: clinical and virological findings. *BMJ* 1982; 285: 1001–02.

18 Lunkenheimer K, Hufert FT, Schmitz H. Detection of Lassa virus RNA in specimens from patients with Lassa fever by using the polymerase chain reaction. *J Clin Microbiol* 1990; 28: 2689–92.

19 Johnson KM, McCormick JB, Webb PA, Smith ES, Elliott LH, King JJ. Clinical virology of Lassa fever in hospitalized patients. *J Infect Dis* 1987; 155: 436–64.

20 Choi MJ, Worku S, Knust B, et al. A case of Lassa fever diagnosed at a community hospital—Minnesota 2014. *Open Forum Infect Dis* 2018; 5: ofy131.

21 Asogun DA, Adomeh DI, Ehmeju J, et al. Molecular diagnostics for lassa fever at Irrua specialist teaching hospital, Nigeria: lessons learnt from two years of laboratory operation. *Plos Negl Trop Dis* 2012; 6: e1839.

22 Nigeria Centre for Disease Control. National guidelines for Lassa fever case management November 2018. https://ncdc.gov.ng/themes/common/docs/protocols/92_3547068332.pdf (accessed Oct 28, 2021).

23 Chen R, Zhang W, Gong M, et al. Characterization of an antiviral component in human seminal plasma. *Front Immunol* 2021; 12: 580454.

24 Lippold S, Braun B, Kruger F, et al. Natural inhibitor of human cytomegalovirus in human seminal plasma. *J Virol* 2019; 96: e01855–18.

25 Meyer BJ, Southern PJ. A novel type of defective viral genome suggests a unique strategy to establish and maintain persistent lymphocytic choriomeningitis virus infections. *J Virol* 1997; 71: 6757–64.

26 Lingas G, Rosenke K, Safronezt D, Guedj J. Lassa viral dynamics in non-human primates treated with favipiravir or ribavirin. *PLoS Comput Biol* 2021; 17: e1008535.

27 Oestereich L, Rieger T, Ludtke A, et al. Efficacy of favipiravir alone and in combination with ribavirin in a lethal, immunocompetent mouse model of Lassa fever. *J Infect Dis* 2016; 213: 334–38.

28 Carrillo-Bustamante P, Nguyen THT, Oestereich L, Gunther S, Guedj J, Graw F. Determining ribavirin’s mechanism of action against Lassa virus infection. *Sci Rep* 2017; 7: 11693.