



HHS Public Access

Author manuscript

Lancet Microbe. Author manuscript; available in PMC 2022 July 05.

Published in final edited form as:

Lancet Microbe. 2022 July ; 3(7): e533–e542. doi:10.1016/S2666-5247(22)00065-9.

Evaluation of viral load in patients with Ebola virus disease in Liberia: a retrospective observational study

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Contributors

MJM and ER wrote the original draft and designed figures. MJM, ER, DSC, and VJM searched the literature. MJM, ER, and FF formally analysed the data. MJM, ER, FF, DSC, and VJM designed the study methodology. MJM, ER, FF, KR, EDW, HF, DSC, and VJM curated data. MJM, ER, DSC, VJM accessed and verified all the data. MJM, FF, MM, AS, RG, JKE, KR, EdW, HF, DCS, and VJM contributed to the study investigation. FF, HF, DSC, and VJM supervised the study. MM, AS, RG, JKE, HF, DSC, and VJM provided study resources. FF, MM, AS, RG, JKE, KR, EDW, HF, DSC, VJM contributed to review and editing of the manuscript. All authors had full access to all the data in the study and had final responsibility for the decision to submit for publication.

Declaration of interests

We declare no competing interests.

Data sharing

The deidentified patient data that were used in this study will be made freely available upon request from the corresponding author.

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Summary

Background—Viral load in patients with Ebola virus disease affects case fatality rate and is an important parameter used for diagnostic cutoffs, stratification in randomised controlled trials, and epidemiological studies. However, viral load in Ebola virus disease is currently estimated using numerous different assays and protocols that were not developed or validated for this purpose. Here, our aim was to conduct a laboratory-based re-evaluation of the viral loads of a large cohort of Liberian patients with Ebola virus disease and analyse these data in the broader context of the west Africa epidemic.

Methods—In this retrospective observational study, whole blood samples from patients at the Eternal Love Winning Africa Ebola treatment unit (Monrovia, Liberia) were re-extracted with an optimised protocol and analysed by droplet digital PCR (ddPCR) using a novel semi-strand specific assay to measure viral load. To allow for more direct comparisons, the ddPCR viral loads were also back-calculated to cycle threshold (C_t) values. The new viral load data were then compared with the C_t values from the original diagnostic quantitative RT-PCR (qRT-PCR) testing to identify differing trends and discrepancies.

Findings—Between Aug 28 and Dec 18, 2014, 727 whole blood samples from 528 individuals were collected. 463 (64%) were first-draw samples and 409 (56%) were from patients positive for Ebola virus (EBOV), species *Zaire ebolavirus*. Of the 307 first-draw EBOV-positive samples, 127 (41%) were from survivors and 180 (59%) were from non-survivors; 155 (50%) were women, 145 (47%) were men, and seven (2%) were not recorded, and the mean age was 29·3 (SD 15·0) years for women and 31·8 (SD 14·8) years for men. Survivors had significantly lower mean viral loads at presentation than non-survivors in both the reanalysed dataset (5·61 [95% CI 5·34–5·87] vs 7·19 [6·99–7·38] \log_{10} EBOV RNA copies per mL; $p<0\cdot0001$) and diagnostic dataset (C_t value 28·72 [27·97–29·47] vs 26·26 [25·72–26·81]; $p<0\cdot0001$). However, the prognostic capacity of viral load increased with the reanalysed dataset (odds ratio [OR] of death 8·06 [95% CI 4·81–13·53], $p<0\cdot0001$ for viral loads above 6·71 \log_{10} EBOV RNA copies per mL vs OR of death 2·02 [1·27–3·20], $p=0\cdot0028$ for C_t values below 27·37). Diagnostic qRT-PCR significantly ($p<0\cdot0001$) underestimated viral load in both survivors and non-survivors (difference in diagnostic C_t value minus laboratory C_t value of 1·79 [95% CI 1·16–2·43] for survivors and 5·15 [4·43–5·87] for

non-survivors). Six samples that were reported negative by diagnostic testing were found to be positive upon reanalysis and had high viral loads.

Interpretation—Inaccurate viral load estimation from diagnostic C_t values is probably multifactorial; however, unaddressed PCR inhibition from tissue damage in patients with fulminant Ebola virus disease could largely account for the discrepancies observed in our study. Testing protocols for Ebola virus disease require further standardisation and validation to produce accurate viral load estimates, minimise false negatives, and allow for reliable epidemiological investigation.

Introduction

Ebola virus disease results from infection with Ebola virus (EBOV), species *Zaire ebolavirus*, a filovirus that is considered enzootic in central and west Africa.^{1,2} Patients with Ebola virus disease typically present with constitutional signs and symptoms, followed by voluminous diarrhoea and vomiting, coagulopathy, multiorgan failure, and shock, with an average case fatality rate (CFR) of 60–70%.^{1,2} From December, 2013, to June, 2016, the west African countries of Guinea, Liberia, and Sierra Leone suffered the largest and longest Ebola virus disease epidemic on record.³ Monrovia, Liberia, was an epicentre of the epidemic. The Eternal Love Winning Africa 3 (ELWA-3) Ebola treatment unit (ETU), operated by Médecins Sans Frontières and located on the outskirts of the city, provided care for more than 1800 patients with Ebola virus disease during the epidemic.^{4,5} West Africa remained Ebola virus disease-free following this epidemic until February, 2021, when new cases were once again detected in Guinea.⁶

Higher viral load, as estimated by cycle threshold (C_t) values from quantitative RT-PCR (qRT-PCR) analyses, has been shown to be associated with increased mortality from Ebola virus disease.^{7–16} Randomised controlled trials evaluating Ebola virus disease therapeutics have made use of viral load estimates (based on diagnostic C_t values) to stratify patients, and the results of those trials hinge on accurate quantification.^{17,18} However, different assays, platforms, and protocols were used across the many ETUs in operation during the 2013–16 west Africa epidemic, none of which were intended or validated for quantitative purposes, and gave a wide range of viral load estimates in varied units (eg, arbitrary units or RNA equivalents), with epidemiological investigations using these data yielding inconsistent and often confusing results.^{7–13,15} In this retrospective study, we aimed to reanalyse samples from a large cohort of patients, both positive and negative for EBOV, from an ETU in Liberia to allow for more accurate viral load quantification and comparison to field diagnostic data, and for evaluation of these data within the broader context of the west Africa Ebola virus disease epidemic

Methods

Study design and participants

Between Aug 28 and Dec 18, 2014, whole blood samples were collected in EDTA (edetic acid) tubes from patients at ELWA-3 ETU in Monrovia, Liberia for diagnostic purposes and allocated a unique sample identification number. Samples were evaluated for the presence

of EBOV RNA by qRT-PCR as previously described (appendix pp 1–2),⁵ and a subset of the remainder of the whole blood samples was transported to Rocky Mountain Laboratories, Hamilton, MT, USA.

Procedures

For this study, we extracted RNA from a convenience sample of whole blood samples (n=727; appendix p 1) using an optimised protocol using TRIzol reagent (ten parts TRIzol to one part blood), Phasemaker tubes with chloroform phase separation, and PureLink RNA columns (Invitrogen, Thermo Fisher Scientific, Waltham, MA, USA; appendix pp 3–4). Elution was done with $3 \times 35 \mu\text{L}$ (105 μL total) 10 mmol/L Tris-HCl pH 7.5. Viral complementary DNA was generated from the RNA extract with SuperScript IV (Thermo Fisher, Waltham, MA, USA) using random hexamers according to the manufacturer's protocol. A novel, semi-strand specific intergenic assay was developed and used to quantify the viral load in each sample using a QX200 Droplet Digital PCR system (Bio Rad, Hercules, CA, USA; appendix pp 1–2). All repeat samples and samples that were EBOV-negative by diagnostic qRT-PCR were also reanalysed using ddPCR in the laboratory (appendix p 2).

To initially assess the quality of the extracted viral RNA from the stored samples, and to allow for additional comparisons to be made between the differing variables in the datasets (table 1), a subset of 75 of the TRIzol-extracted samples were randomly selected and analysed with exactly the same diagnostic L qRT-PCR assay and instruments used at ELWA-3 (appendix pp 3–4).⁵ 43 (57.3%) of the 75 samples were from non-survivors. Due to limited availability of patient samples, reanalysis of the entire sample set by laboratory qRT-PCR was not done; data from these 75 samples were instead used to calculate laboratory C_t values using a standard curve for the remainder of the samples from the \log_{10} EBOV RNA copies per mL to allow for more direct comparisons (ie, C_t value to C_t value) where appropriate (appendix pp 3–4).

Outcomes

The primary outcome in this study was the comparison between \log_{10} EBOV RNA copies per mL and diagnostic C_t values for retrospectively analysed blood samples. Secondary outcomes were viral load and days from symptom onset, survivorship, and length of ETU stay.

Statistical analysis

We used two-tailed unpaired *t*-tests with Welch's correction or ANOVA (Welch or Brown-Forsythe, depending on skewness) to compare the difference in outcomes by group (eg, survivorship or days from symptom onset). Paired *t*-tests were used to compare changes in diagnostic C_t values and reanalysed laboratory C_t values (both measured and calculated). Association between the outcomes and continuous variables of interest (eg, length of ETU stay) were assessed using Spearman's ρ . Slopes of lines of best fit were compared with ANCOVA.

We assessed the association between \log_{10} EBOV RNA copies per mL and multiple variables of interest including patient survivorship, time from symptom onset to ETU presentation, and time from ETU admission to death or discharge using multivariable linear regression, controlling for patient age, sex, and days from symptom onset to admission. To determine whether an optimal cutoff value for the \log_{10} EBOV RNA copies per mL and the diagnostic C_t values existed to predict patient survivorship we did a receiver operating characteristic curve analysis, minimising the absolute value of the difference between sensitivity and specificity using the OptimalCutpoints package. The change in case fatality rate over time was assessed by computing the average number of deaths per 3-week period from Aug 28 to Dec 11, 2014, according to date of ETU admission. Statistical analyses were done with R (version 3.6.1–3) and GraphPad Prism (version 9.3.1), and statistical significance was assessed at $p < 0.05$.

The deidentified samples and data used for this study received a determination of “not human subjects research” by the National Institutes of Health Office of Human Subjects Research Protection (reference number Exempt 12701) and the study was approved by the University of Liberia–Pacific Institute for Research and Evaluation Institute’s Review Board (reference number Protocol 17–02-025).

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.

Results

727 samples from 528 unique individuals were evaluated. 463 (64%) of these samples were first-draw samples obtained upon presentation to the ETU; the remainder were repeat samples obtained from convalescing patients to assess for viral clearance.⁵ The 463 first-draw samples included 156 (34%) from EBOV-negative patients and 307 (66%) from EBOV-positive patients. A total of 409 EBOV-positive samples were present, including both first-draw samples and repeat-draw samples. Of the 307 EBOV-positive patients, 155 (50%) were women with a median age of 29.3 (SD 15.0) years and an overall CFR of 58.6% (table 2). 102 repeat samples from patients with Ebola virus disease and 318 samples that were EBOV-negative (including 156 first-draw samples and 162 repeated confirmatory samples) by diagnostic qRT-PCR were reanalysed using ddPCR (appendix p 2).

Survivors spent a median of 11 (IQR 6) days in the ETU (appendix p 7); longer stays in these patients were associated with higher viral loads, probably due to increased disease severity, and later in the epidemic, probably due to increased bed and resource availability (appendix p 7). Non-survivors spent a median of 4 (IQR 5) days in the ETU, with shorter stays associated with higher viral loads; length of stay was consistent throughout the epidemic (appendix p 7). The mean viral load at admission for survivors ($5.61 \log_{10}$ EBOV RNA copies per mL [95% CI 5.34–5.87]) was significantly lower ($p < 0.0001$) than in non-survivors ($7.19 \log_{10}$ EBOV RNA copies per mL [6.99–7.38]; figure 1A), even when accounting for patient age, sex, and days since symptom onset. This association was also observed when comparing the diagnostic C_t values obtained by qRT-PCR at triage (mean

C_t value of 28.72 [27.97–29.47] for survivors vs 26.26 [25.72–26.81] for non-survivors; $p < 0.0001$), although there was markedly less separation between the means of the groups (figure 1A). When the viral loads of survivors and non-survivors were compared by day from symptom onset to sample collection, significant differences were also found for all timepoints compared (0–3 days, 4–7 days, and 8 days) for both the \log_{10} EBOV RNA copies per mL and diagnostic C_t value groups, although the differences were greater and had increased statistical significance for the \log_{10} EBOV RNA copies per mL group (figure 1B).

To evaluate the quality of the viral load data following reanalysis, receiver operating characteristic analysis was done to assess the ability of the \log_{10} EBOV RNA copies per mL versus the C_t values from diagnostic qRT-PCR analysis at the ETU to prognosticate between survivors and non-survivors. Optimal cutoffs were chosen based on an equal balance of sensitivity and specificity. The ddPCR \log_{10} EBOV RNA copies per mL provided improved discrimination between survivors and non-survivors when compared with the diagnostic qRT-PCR C_t values (figure 2A). For the samples reanalysed by ddPCR, the optimal cutoff was 6.71 \log_{10} EBOV RNA copies per mL (figure 2A, B). Patients with a value above this cutoff had a substantially increased likelihood of death (odds ratio [OR] 8.06, 95% CI 4.81–13.53, $p < 0.0001$) and an overall CFR of 80.1%. For patients with viral loads below 6.71 \log_{10} EBOV RNA copies per mL, the CFR was 33.3%. Using diagnostic qRT-PCR C_t values, the optimal cutoff was 27.37 (figure 2A, C). Patients with a C_t value below 27.37 had a modestly increased likelihood of death (2.02, 1.27–3.20, $p = 0.0028$) and an overall CFR of 66.9%. For patients with a C_t of more than 27.37, the CFR was 50%. Area under the receiver operating characteristic curve analysis was substantially improved using the \log_{10} EBOV RNA copies per mL compared with the diagnostic C_t values (0.80 [95% CI 0.75–0.85] vs 0.66 [0.59–0.72]).

Diagnostic C_t values obtained by qRT-PCR at the ETU showed a strong association with the \log_{10} EBOV RNA copies per mL obtained by ddPCR upon laboratory reanalysis in surviving patients (Spearman's $\rho = 0.70$, $p < 0.0001$; figure 3A). For non-surviving patients, however, the association was considerably weaker (Spearman's $\rho = 0.17$, $p = 0.0229$), with increasing deviation as diagnostic C_t values increased. The slopes of the lines of best fit ($m = 0.25$ for survivors vs $m = 0.10$ for non-survivors) also differed significantly from one another ($p < 0.0001$). Similar deviation was also noted when comparing the diagnostic C_t values with the laboratory C_t values in the subset of 75 samples for which laboratory C_t values were measured (table 1) and the slopes of the lines of best fit (0.96 for survivors vs 0.47 for non-survivors) differed significantly ($p = 0.0368$), with a pronounced decrease from the expected value of 1 present only in the non-survivors (appendix pp 5–6). Upon comparison of laboratory C_t values (both measured and calculated) to diagnostic C_t values, significant differences ($p < 0.0001$) were found for both survivors and non-survivors using paired t -tests, although the mean difference (diagnostic C_t value minus laboratory C_t value) was substantially greater in non-survivors (5.15 [95% CI 4.43 to 5.87]) compared with survivors (1.79 [95% CI 1.16 to 2.43]; figure 3B). For non-survivors, the magnitude of the difference of diagnostic C_t values minus laboratory C_t value associated directly and significantly ($m = 1.4$, $p = 0.0103$; ANOVA $p = 0.0310$) with time from symptom onset to sample collection (figure 3C); likewise, C_t value differences in non-survivors increased

in samples collected closer to the time of death ($m=-0.30$, $p=0.0021$; figure 3D). Similar significant trends were not observed in survivors.

Six of the reanalysed samples that were EBOV-negative by diagnostic qRT-PCR were found to be positive upon laboratory reanalysis by both ddPCR and qRT-PCR (appendix p 8), yielding an overall false negative rate of 1.4% ($6/[409 + 6]$). Five of these were from patients that were never admitted to the ETU, as they tested negative at triage. The remaining patient was diagnosed with Ebola virus disease and admitted to the ETU but died following a second sample that was negative by diagnostic qRT-PCR.

The mean \log_{10} EBOV RNA copies per mL in non-survivors at admission decreased significantly over the course of the epidemic by $0.016 \log_{10}$ EBOV RNA copies per mL per day (95% CI -0.023 to -0.008), from means of $7.6 \log_{10}$ EBOV RNA copies per mL (7.3 to 7.9) during the first 3 weeks of the observation period to $6.5 \log_{10}$ EBOV RNA copies per mL (5.8 to 7.2) during the final 3 weeks ($p=0.0046$; figure 4A). No significant trend was observed in survivors. The time from symptom onset to ETU admission was also significantly shorter for non-survivors when comparing the means of the first 3 weeks of the observation period to the last 3 weeks of the observation period, decreasing from 6.8 days (5.7 to 7.9) to 3.7 days (2.4 to 5.0 ; $p=0.0006$; figure 4B). An overall downward trend was also present in the time from symptom onset to ETU admission for survivors but was not statistically significant (figure 4B). The case fatality rate did not significantly change (slope= 0.26 , $p=0.9066$) over the observation period when averaged over 3-week intervals from Aug 28, 2014, to Dec 11, 2014, by date of ETU admission (figure 4C), and remained steady around 60%.

Discussion

We did a laboratory-based re-evaluation of a cohort of patient samples from an ETU in Liberia using optimised extraction and processing protocols and a newly developed intergeneric ddPCR assay to quantify viral load more accurately and consistently for Ebola virus disease. Although both the viral load estimates from reanalysis (\log_{10} EBOV RNA copies per mL) and the ETU (diagnostic C_t values) were associated with patient outcomes, the reanalysed viral load estimates demonstrated significantly improved prognostic capacity, lending confidence in the validity of these data. Upon comparison of the \log_{10} EBOV RNA copies per mL with the diagnostic C_t values, the viral loads in non-survivors were significantly skewed; in survivors, however, they were similar. To investigate these results further, we then used laboratory C_t values from reanalysis of a subset of the samples and a standard curve conversion from \log_{10} EBOV RNA copies per mL to allow for a more direct comparison of the viral load measurements (diagnostic C_t value to laboratory C_t value). This comparison showed that the diagnostic qRT-PCR used at ELWA-3 consistently, substantially, and significantly underestimated the viral load in non-survivors. Moreover, this effect was time-dependent—ie, the magnitude of underestimation was greater both with increasing self-reported time from symptom onset to sample collection and with the more objective measurement of time from sample collection to death. Conversely, samples from patients that survived, and particularly samples obtained during convalescence, showed little or no comparable discrepancies. These systematic trends suggest that an underlying biological

process was responsible for the significant underestimation of viral load in non-survivors by diagnostic qRT-PCR.

It has been proposed that blood-based PCR assays for acute haemorrhagic fever viruses could be compromised by PCR inhibitors present at unusually high concentrations in samples from patients with fulminant illness, possibly due to the extensive cell and tissue death that can occur in such cases.¹⁹ In these scenarios, erroneously elevated C_t values could be reported, despite the presence of high viral loads. Such an effect was reported in a sample from a moribund patient infected with Sudan virus (an ebolavirus closely related to EBOV) in 2001.¹⁹ Another study during the 2013–16 west Africa Ebola virus disease outbreak reported that one-third of their observed non-surviving cohort died despite the presence of apparently declining viral loads (as estimated by diagnostic qRT-PCR C_t values), with comorbidities or irreparable tissue damage, or both, offered as explanations.⁹ Our findings here suggest that, rather than a true decline in viral load in such patients, excessive PCR inhibition in non-survivors might have yielded confounding data.

We identified six samples with possible false-negative diagnostic test results. Our observation that the diagnostic C_t values were consistently falsely elevated in non-survivors in our cohort suggests that the presence of occasional false negatives in our dataset is rational and expected. Nevertheless, we cannot rule out human error (eg, labelling mistakes or database errors) as an explanation for the potential false negatives identified, particularly considering the extensive handling, transportation, and storage of the samples. Regardless, false negatives are of particular concern with Ebola virus disease given the potential implications of erroneously releasing even a single positive patient, and any reasonable steps to improve diagnostic accuracy should be taken.

Although efforts were made to mitigate the possibility of inefficient extraction or PCR inhibition at ELWA-3 by simultaneously amplifying an endogenous extraction control (B2M), PCR inhibition might not affect all amplification targets equally, and sequence-specific effects have been observed; thus, successful amplification of this control does not necessarily preclude inhibition of the diagnostic EBOV targets.^{20–22} It has previously been suggested that this potential problem of PCR inhibition in samples from patients with severe viral haemorrhagic fevers could be overcome by incorporating additional control measures, including analysis of both an aliquot of the patient sample spiked with a small amount of viral RNA and a diluted aliquot of the patient sample, in addition to standard analysis of the naive patient sample.¹⁹ Although this would require additional time and labour, the combined benefit of ensuring accurate quantitative estimates (ie, C_t values) and safeguarding against false negatives is certainly sufficient justification, and such fail-safe measures could be implemented only in instances of high clinical suspicion or under other certain criteria, or both. It is also noteworthy that before the 2013–16 west Africa Ebola virus disease epidemic, no diagnostic EBOV assays were approved for use by any regulatory authority. Currently however, the US Food and Drug Administration and WHO have collectively approved nearly a dozen EBOV diagnostic assays that are PCR-based, although none have been validated for quantitative purposes using human Ebola virus disease samples.^{7,23} Rigorous reassessment of these assays in light of the findings reported here might therefore

be warranted to ensure both the accuracy of viral load estimates using diagnostic C_t values and to reduce false negatives.

The overall workflow employed here to generate the revised viral load estimates as \log_{10} EBOV RNA copies per mL is not practical for field diagnostic use. However, our finding that the subset of 75 samples that were reanalysed in the laboratory by qRT-PCR—which differed from the field diagnostic qRT-PCR only in the RNA extraction method used—yielded extremely similar results to those obtained from the laboratory ddPCR analysis suggests that simply optimising and standardising diagnostic extraction protocols can sufficiently safeguard against inaccurate viral load quantification in Ebola virus disease. Consideration should also be given to sample type (serum or plasma vs whole blood), as this has been shown to affect viral load quantification for other viruses with leukocyte tropism (eg, Epstein-Barr virus, hepatitis C virus, etc) similar to that of EBOV.^{24,25}

Using the improved viral load quantification, we were then able to confidently make important epidemiological observations with direct clinical implications. Previous studies from the 2013–16 west Africa Ebola virus disease epidemic reported conflicting and often puzzling trends in the relationships between viral load, time during epidemic, time from symptom onset, and CFR.^{8,9} In a cohort in Guinea, viral load and CFR both increased later in the epidemic, and sampling bias is offered as the most likely explanation.⁸ In another cohort from Sierra Leone, viral load and CFR both decreased as the epidemic progressed, and it was suggested that this was possibly due to either an increase in EBOV-specific IgG in the population or a reduced pathogenic phenotype of circulating EBOV.⁹ In our cohort, we observed that by the end of the observation period, non-survivors presented to ELWA-3 with more than a log-fold reduction in viral load compared with the beginning of the observation period (from 7.6 to 6.5 \log_{10} EBOV RNA copies per mL). This effect was likely attributable to the concomitant reduction in time from symptom onset to presentation to ELWA-3. By the end of the observation period, the non-survivors in our cohort presented to ELWA-3 over 3 days earlier (from 6.8 to 3.7 days), on average, following symptom onset, than at the beginning of the observation period. The viral kinetics differ in survivors and non-survivors of Ebola virus disease, with peaks in viral loads around day 5 in survivors and around day 7 in non-survivors; thus, the decrease in presentation time for non-survivors in our cohort was during the phase of exponential increase in viral load, thus offering a coherent explanation for the drop in viral load.^{2,10,11,14,16,26,27} The decrease in time from symptom onset to presentation to ELWA-3 might have resulted from heightened awareness of the Ebola virus disease epidemic in Liberia, strengthened by community outreach, educational campaigns, and improved access or acceptance to care in an ETU setting. Unfortunately, however, the earlier presentation was not associated with a decrease in CFR, which in our study remained steady at around 60% throughout the entire observation period. Although patient outcome in Ebola virus disease is multifactorial, this finding suggests that earlier initiation of the level of supportive care available at ELWA-3 (which generally consisted of oral rehydration, anti-diarrheal and antiemetic medications, and antibiotic and antimalarial treatment) did not improve survival. Patients with the most severe cases of Ebola virus disease often ultimately suffer from renal and respiratory failure, among other complications, and these issues were unable to be addressed at ELWA-3.

In light of the ongoing threat posed by EBOV, highlighted by the resurgence of Ebola virus disease in Guinea in 2021 and multiple outbreaks in the Democratic Republic of the Congo in 2021 and 2022, our findings support prioritising further assessment, standardisation, and broadscale implementation of Ebola virus disease diagnostic assays to ensure accurate viral load estimates, minimise the occurrence of false negative results, and facilitate meaningful epidemiological investigation using the most reliable data.^{6,28–30} The resources and infrastructure that are necessary to address outbreaks or epidemics of Ebola virus disease are still severely scarce in west and central Africa. Other filoviral diseases, such as Marburg virus disease, pose new threats to west Africa³¹ and perennial threats to central Africa, and diagnostic testing, vaccines, antivirals, and monoclonal antibodies remain underdeveloped or unavailable, despite the sobering lessons of the west Africa Ebola virus disease epidemic. Substantial and sustained investment must be made to improve patient care in the future.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

This research was supported by the Intramural Research Program of the National Institute of Allergy and Infectious Diseases, National Institutes of Health. Special thanks to the National Institutes of Health, Centers for Disease Control team that operated the diagnostic laboratory at ELWA-3 from August 2014 to March 2015 in Monrovia, Liberia, and to the Médecins Sans Frontières clinical partners and volunteers. We also are extremely grateful to the Liberian people for their support and cooperation during difficult times.

Funding

Intramural Research Program of the National Institute of Allergy and Infectious Diseases, National Institutes of Health.

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Research in context

Evidence before this study

We searched PubMed using the search terms “Ebola virus”, “EBOV”, “viral load”, “PCR”, “copy number”, and “genome copies” for studies published in English between Jan 1, 1976, and Jan 1, 2022. Reanalysis of samples from patients with Ebola virus disease is very rarely undertaken. There are other studies which made use of large sets of Ebola virus disease samples that had been exported from Africa, but these were mostly all sequencing or phylogenetic studies. One study conducted a laboratory-based quantitative RT-PCT (qRT-PCR) reanalysis of 99 patient samples positive for Ebola virus (EBOV), species *Zaire ebolavirus*, to estimate viral load using a standard curve. Diagnostic cycle threshold (C_t) values was present for 84 (85%) samples; the discrepancies between the remaining 15 (15%) samples were not addressed. In another case report, a sample from a patient with fulminant Ebola virus disease was shown to be repeatedly falsely negative due to substantial PCR inhibition that could be overcome only by dilution (1:100) of the original sample before amplification. Most studies that have focused on estimating viral load in Ebola virus disease directly from original diagnostic C_t values by various means, which has resulted in inconsistent viral load estimates with differing units of measurement (eg, arbitrary units or RNA equivalents) that cannot be reliably compared. Furthermore, epidemiological investigations using these viral load data have produced highly divergent results.

Added value of this study

We used an optimised extraction and processing protocol together with a novel semi-strand-specific droplet digital PCR assay to reanalyse 727 patient samples, both EBOV-positive and EBOV-negative, collected in Liberia in 2014. This produced absolute quantification of viral load in patients with Ebola virus disease without reliance on standard curves, allowed for objective evaluation diagnostic qRT-PCR accuracy and viral load estimates, and provided a consistent, reliable dataset for epidemiological investigation. We report compelling evidence that the diagnostic qRT-PCR testing had underestimated the viral load in non-surviving patients, possibly due to the presence of PCR inhibitors from extensive tissue damage in fulminant Ebola virus disease. Moreover, this is the first study to our knowledge that has systematically re-evaluated presumably EBOV-negative samples for diagnostic accuracy, and our finding that six samples were likely falsely negative by diagnostic qRT-PCR provides added evidence that current protocols might require further optimisation and validation. Our epidemiological investigation of this patient cohort using this improved viral load dataset produced logical, consistent results. Most notably, we found that as the epidemic progressed non-surviving patients presented significantly earlier following symptom onset, with significantly lower viral loads. Nevertheless, the case fatality rate remained unchanged, suggesting earlier initiation of supportive care measures did not significantly decrease mortality.

Implications of all the available evidence

Current diagnostic qRT-PCR protocols for Ebola virus disease require further standardisation and validation to ensure viral load estimates are accurate and to minimise the occurrence of false negatives, which could be devastating for infection control during Ebola virus disease outbreaks. The possibility for potent PCR inhibition, particularly in fulminant cases, should be accounted for. Extant epidemiological investigations addressing viral load in Ebola virus disease as determined by estimates from diagnostic C_t values should be evaluated cautiously.

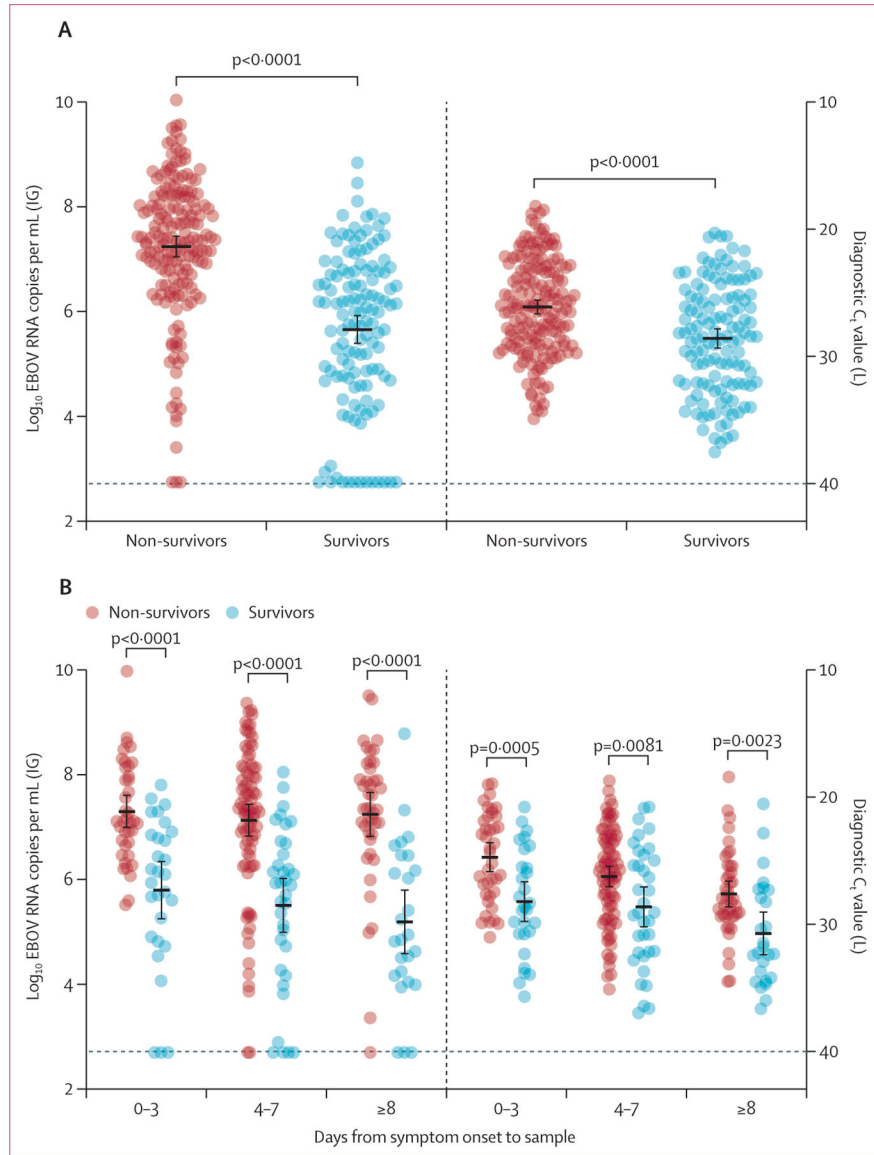


Figure 1: Correlation of patient outcome with viral load measurements and days from symptom onset

The left y-axes indicate the \log_{10} EBOV RNA copies per mL and correspond with the data to the left of the vertical grey lines in each panel; the right y-axes indicate diagnostic C_t value and correspond with the data to the right of the vertical grey lines in each panel. Note that the right y-axes are reversed. The horizontal black dotted lines indicate the limit of detection for the laboratory ddPCR assay ($2.7 \log_{10}$ EBOV RNA copies per mL) and the cycling limit used for the diagnostic qRT-PCR assay (40 cycles). Thick bars represent means, and error bars represent 95% CIs. Comparisons between the means of the groups were made using a *t*-test with Welch's correction. (A) Overall relationship between patient outcome with viral load measurements. Mean \log_{10} EBOV RNA copies per mL: non-survivors $7.19 \log_{10}$ EBOV RNA copies per mL (95% CI $6.99-7.38$), survivors $5.61 \log_{10}$ EBOV RNA copies per mL ($5.34-5.87$). Mean diagnostic C_t values are: 26.26 ($25.72-26.81$) for non-survivors and 28.72 ($27.97-29.47$) for survivors. (B) Patient outcomes with

viral load measurements by days from symptom onset to sample collection. The p values for all three comparisons of the \log_{10} EBOV RNA copies per mL data (left portion of figure) were <0.0001 . C_t =cycle threshold. ddPCR=droplet digital PCR. EBOV=Ebola virus, species *Zaire ebolavirus*. IG=intergenic assay. qRT-PCR=quantitative RT-PCR.

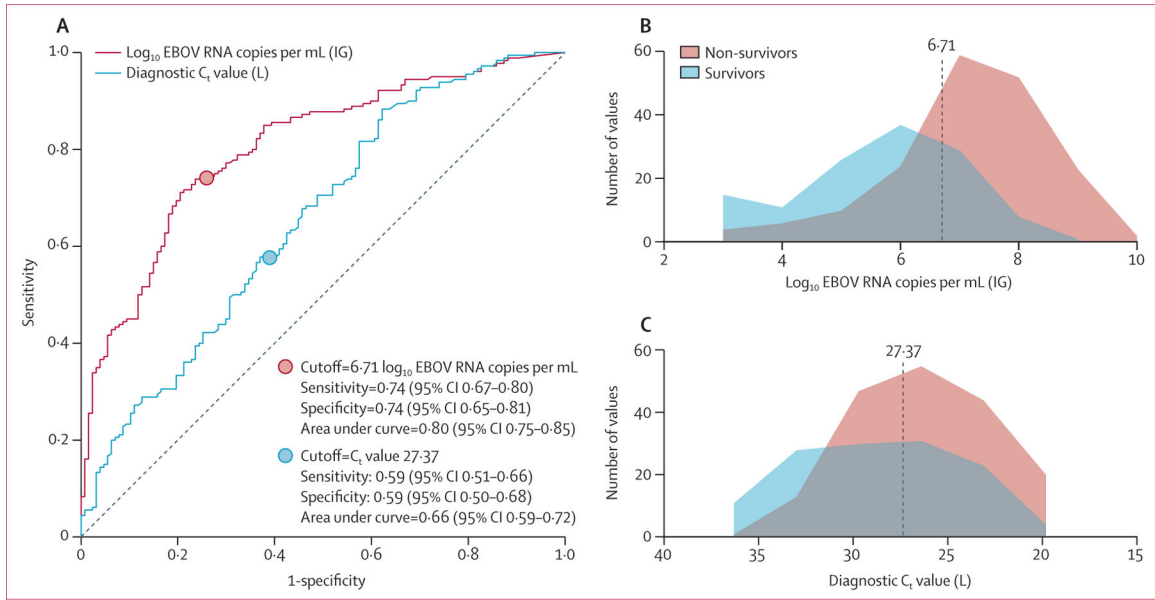


Figure 2: Capacity of viral load measurements to discriminate between survivors and non-survivors

(A) Receiver operating characteristic curve, with dots indicating the chosen location of the chosen cutoffs for both non-survivors and survivors. (B) Number of log₁₀ EBOV RNA copies per mL values, separated into survivors and non-survivors, with chosen cutoff indicated by vertical black dotted line. (C) Number of diagnostic C_t values, separated into survivors and non-survivors, with chosen cutoff indicated by vertical black dotted line. C_t=cycle threshold. EBOV=Ebola virus, species *Zaire ebolavirus*. IG=intergenic assay.

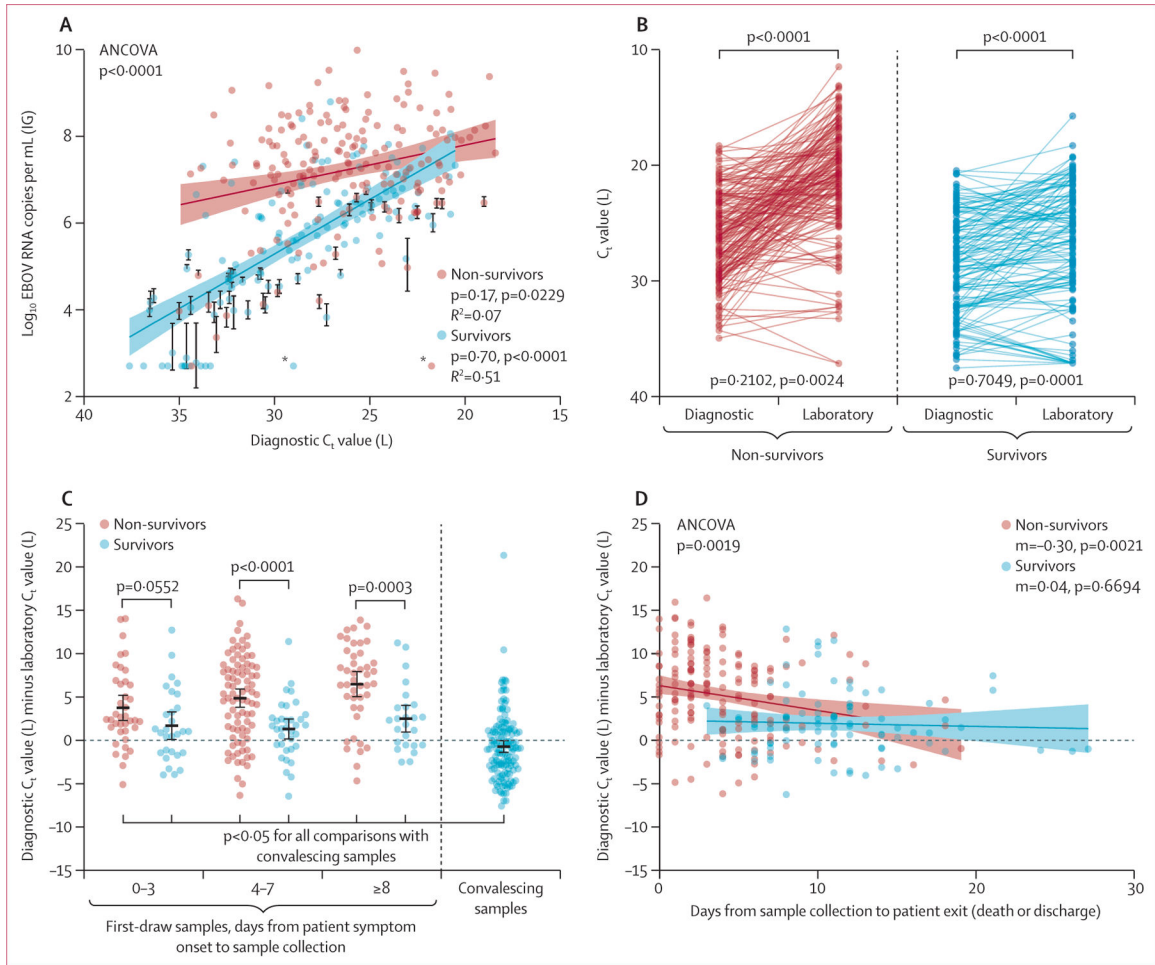


Figure 3: Underestimation of viral load in non-survivors by diagnostic qRT-PCR

(A) \log_{10} EBOV RNA copies per mL with diagnostic C_t values, separated by patient outcome, for the 307 first-draw EBOV-positive patient samples. Lines of best fit with 95% CIs (shaded area) are shown. Error bars visible on some individual data points indicate 95% Poisson CIs, but most are too small to be plotted. Horizontal dotted black line indicates the limit of detection ($2.7 \log_{10}$ EBOV RNA copies per mL) for the ddPCR assay. R^2 is the coefficient of determination and ρ is Spearman’s correlation coefficient with corresponding p values shown. Slopes of the lines include 95% CIs (shaded area) and slopes were compared with ANCOVA. Data points indicated by asterisks might represent samples that were mislabelled during transport or storage, as sample degradation is unlikely to offer a sufficient explanation for the negative results obtained upon laboratory reanalysis by ddPCR. The x-axis is reversed. (B) Laboratory C_t values compared with diagnostic C_t values per sample, separated by patient outcome. Data from the 75 samples reanalysed by laboratory qRT-PCR (table 1; appendix pp 5–6) was used to calculate laboratory C_t values for the remainder of the dataset, and the laboratory C_t values (both the 75 measured and the remainder calculated) are shown in comparison with the diagnostic C_t values by patient outcome. Comparisons were made using paired t -tests. The mean differences between the groups (diagnostic C_t value minus laboratory C_t value) were 5.15 (95% CI 4.43 to 5.87)

for non-survivors and 1.79 (95% CI 1.16 to 2.43) for survivors. ρ is Spearman's correlation coefficient and corresponding p values are shown. The y-axis is reversed. (C) Differences between diagnostic C_t values and laboratory C_t values (either measured or calculated, as above) by self-reported time from symptom onset for first-draw samples (non-survivors and survivors; left side of figure) and convalescing samples (survivors; right side of figure). Mean values of the survivor and non-survivor first-draw samples for the given timeframes (0–3 days, 4–7 days, or 8 days following symptom onset) were compared using t -tests with Welch's correction, and within each group (survivors or non-survivors) means were compared using Welch's ANOVA; p values are shown. Slopes of the lines include 95% CIs (shaded area) and slopes and p values are shown; slopes of the lines were compared with ANCOVA. Comparisons between means of (survivor) convalescent samples and (survivor or non-survivor) first-draw samples by days from symptom onset to sample were made using t -tests with Welch's correction, and p values for each comparison relative to the convalescing samples are as follows (from left to right): non-survivor, 0–3 days: $p < 0.0001$; survivor, 0–3 days: $p = 0.0068$; non-survivor, 4–7 days: $p < 0.0001$; survivor, 4–7 days: $p = 0.0033$; non-survivor, 8 days: $p < 0.0001$; survivor, 8 days: $p = 0.0004$. For the convalescing samples, only those with at least one (laboratory or diagnostic) C_t value of less than 40 were used for analysis; many samples had both laboratory and diagnostic C_t values greater than 40 and thus could not be meaningfully compared, so were excluded from this analysis. (D) Differences between diagnostic C_t values and laboratory C_t values (either measured or calculated, as above) for first-draw samples by days from sample collection to exit from the Ebola treatment unit (either death or discharge). The y-axis indicates the difference between the diagnostic C_t value and the laboratory C_t value (measured or calculated); the x-axis indicates the number of days from the collection of the sample (which is the day of admission) until death (for non-survivors) or discharge (for survivors). Lines of best fit include 95% CIs (shaded area) and slopes and p values are shown; slopes of the lines were compared with ANCOVA. Note that although the underlying comparison in this panel is similar in concept to that of (C), the same approach and x-axis could not be used for both since insufficient sample sizes would be present for the grouped timeframes (ie, survivors were never discharged earlier than 3 days from initial sample collection, and most stayed for more than 10 days). C_t =cycle threshold. ddPCR=droplet digital PCR. EBOV=Ebola virus, species *Zaire ebolavirus*. IG=intergenic assay. qRT-PCR=quantitative RT-PCR.

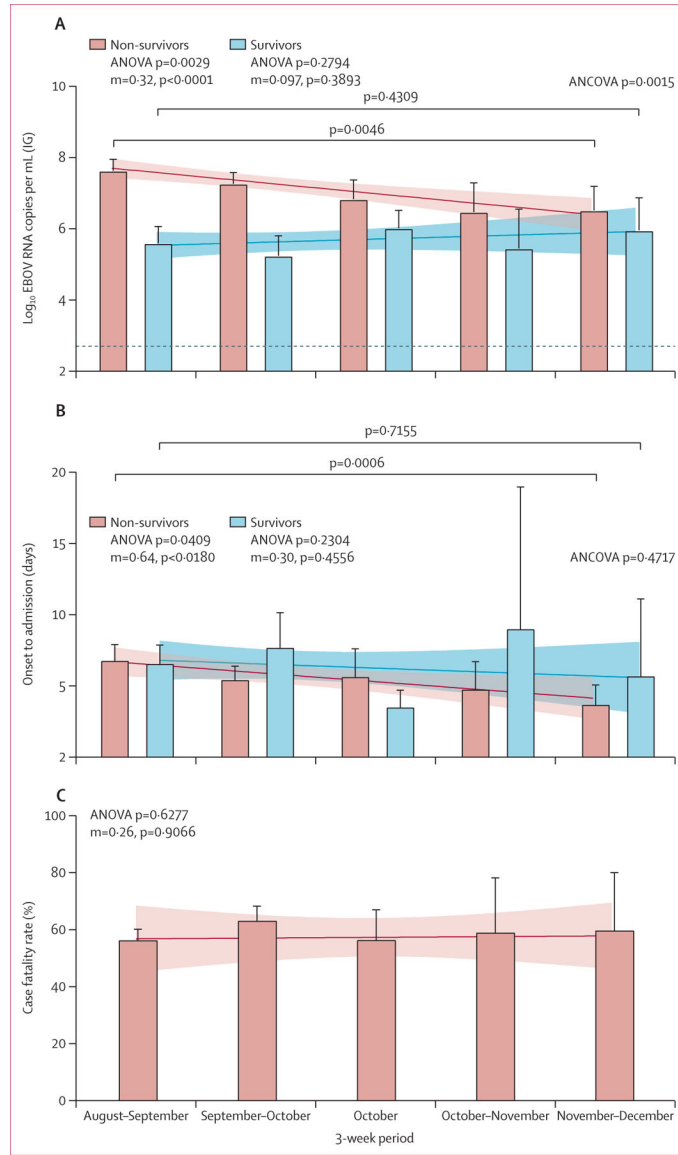


Figure 4: Changes in viral load (log₁₀ EBOV RNA copies per mL), time from symptom onset to presentation, and case fatality rate by 3-week intervals over the observation period

Means are shown, and error bars represent 95% CIs. Lines of best fit include 95% CIs (shaded area) and slopes and p values are shown; slopes of the lines were compared with ANCOVA. (A) Viral load (log₁₀ EBOV RNA copies per mL) at time of admission by 3-week interval during the observation period, separated into survivors and non-survivors. Horizontal dotted black line indicates the limit of detection (2.7 log₁₀ EBOV RNA copies per mL). When comparing the first 3-week period to the final 3-week period, mean viral loads were as follows: non-survivors 7.6 log₁₀ EBOV RNA copies per mL (95% CI 7.3–7.9) for first 3 weeks, 6.5 log₁₀ EBOV RNA copies per mL (5.8–7.2) for final 3 weeks (p=0.0046); survivors 5.6 log₁₀ EBOV RNA copies per mL (5.2–6.1) for first 3 weeks, 5.9 log₁₀ EBOV RNA copies per mL (5.0 to 6.9) for final 3 weeks (p=0.4309). (B) Days from self-reported symptom onset to presentation by 3-week interval during the observation period, separated into survivors and non-survivors. When comparing the first

3-week period to the final 3-week period, mean days from self-reported symptom onset to patient presentation at ELWA-3 were as follows: non-survivors 6·8 days (95% CI 5·7–7·9) for the first 3 weeks, 3·7 days (2·4–5·0) for the final three weeks ($p=0\cdot0006$); survivors 6·6 days (5·3–7·9) for the first 3 weeks, 5·7 days (0·4–11·0) for the final three weeks ($p=0\cdot7155$). (C) Case fatality rate by admission date averaged over 3-week intervals during the observation period, starting with the date of admission for the first patient in the dataset (Aug 27, 2014). EBOV=Ebola virus, species *Zaire ebolavirus*. IG=intergenic assay.

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Comparison of datasets

Table 1:

Sample size	Sample type	Extraction method	Assay	PCR target	Instrument
Diagnostic C _t values 727 (409 EBOV-positive; * 318 EBOV-negative [‡])	Fresh whole blood in EDTA	QIAGEN QIAamp Viral RNA Mini Kit	One-step	Polymerase gene (L) [‡]	Cepheid SmartCycler and Roche LightCycler
Laboratory log ₁₀ EBOV RNA copies per mL 727 (409 EBOV-positive; * 318 EBOV-negative [‡])	Stored whole blood in EDTA	TRIZol, Phasemaker tubes, PureLink columns	Two-step	Intergenic (VP30-VP24)	Bio Rad QX200 Droplet Digital PCR
Laboratory C _t values 727 (409 EBOV-positive; * 318 EBOV-negative [‡]); 75 first-draw EBOV-positive samples were reanalysed by qRT-PCR with C _t values for remaining samples calculated from standard curves	Stored whole blood in EDTA	TRIZol, Phasemaker tubes, PureLink columns	One-step	Polymerase gene (L)	Cepheid SmartCycler and Roche LightCycler

C_t=cycle threshold. EBOV=Ebola virus, species *Zaire ebolavirus*. EDTA=edetic acid. qRT-PCR=quantitative RT-PCR.

* 307 first-draw, 102 repeat-draw.

[‡] 156 first-draw, 162 repeat-draw.

[‡]For samples that were EBOV-positive by diagnostic qRT-PCR; diagnostic samples that were EBOV-negative by L qRT-PCR were also assessed by a one-step qRT-PCR targeting the viral protein 40 gene (VP40) on a separate Bio-Rad platform in the Ebola treatment unit.⁵

Characteristics and viral load of patients with first-draw samples that were positive for Ebola virus

Table 2:

	Proportion of participants (n=307)	Viral load
Sex		
Female	155 (51%)	6.40 (1.55)
Male	145 (47%)	6.64 (1.67)
Unknown	7 (2%)	7.17 (1.43)
Age, years		
<5	3 (1%)	6.04 (1.66)
5–14	36 (12%)	6.57 (1.75)
15–40	181 (59%)	6.44 (1.58)
>40	78 (25%)	6.74 (1.56)
Unknown	9 (3%)	5.76 (1.88)
Onset to admission, days *		
<3	29 (13%)	6.34 (1.67)
3–4	100 (33%)	6.65 (1.49)
5–6	45 (15%)	6.93 (1.52)
7–14	75 (24%)	6.48 (1.70)
15	12 (4%)	6.14 (1.87)
Unknown	46 (15%)	6.02 (1.63)
Outcome		
Survivor	127 (41%)	5.61 (1.50)
Non-survivor	180 (59%)	7.19 (1.34)

Data are n (%) or mean (SD).

* Self-reported.