

Essentials of filoviral load quantification



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Quantitative measurement of viral load is an important parameter in the management of filovirus disease outbreaks because viral load correlates with severity of disease, survival, and infectivity. During the ongoing Ebola virus disease outbreak in parts of Western Africa, most assays used in the detection of Ebola virus disease by more than 44 diagnostic laboratories yielded qualitative results. Regulatory hurdles involved in validating quantitative assays and the urgent need for a rapid Ebola virus disease diagnosis precluded development of validated quantitative assays during the outbreak. Because of sparse quantitative data obtained from these outbreaks, opportunities for study of correlations between patient outcome, changes in viral load during the course of an outbreak, disease course in asymptomatic individuals, and the potential for virus transmission between infected patients and contacts have been limited. We strongly urge the continued development of quantitative viral load assays to carefully evaluate these parameters in future outbreaks of filovirus disease.

Introduction

The mononegaviral family *Filoviridae* currently has eight members, six of which are known to cause human disease. Of these six, the two marburgviruses, Marburg virus and Ravn virus, cause Marburg virus disease (International Classification of Diseases-10 [ICD-10] A98.3); and four ebolaviruses, Bundibugyo virus, Ebola virus, Sudan virus, and Taï Forest virus, cause Ebola virus disease (ICD-10 A98.4).¹ Patients typically present with a range of non-specific signs and symptoms, including fever, headache, weakness, malaise, myalgia, conjunctival injection, gastrointestinal disturbances (eg, abdominal pain, nausea, vomiting, and diarrhoea), and, less frequently, bleeding.¹ Marburg virus disease and Ebola virus disease are impossible to differentiate from each other on the basis of clinical observation alone.² In the early phases of disease and in the absence of a recognised outbreak, readily distinguishing filovirus disease from a host of more common causes of systemic febrile disease—for example malaria, typhoid fever, bacterial gastroenteritis, and other viral haemorrhagic fevers such as severe dengue or Lassa fever—is very difficult.¹ Thus, rapid and safe laboratory diagnosis of patients with suspected Marburg virus disease and Ebola virus disease is imperative, and should not rely on filovirus culture, which requires specialised biosafety level 4 facilities.

In recent years, the development of field-deployable molecular assays, especially RT-PCR, for the diagnosis of filovirus infection has proved to be an invaluable tool for case identification and management, and for general outbreak control. At the peak of the massive and ongoing outbreak of Ebola virus disease in Western Africa, more than 44 laboratories provided such diagnostic services.³ Serological tests are not particularly useful in diagnosing acute filovirus infection, since the presence of IgG might mean little in a filovirus-endemic area and IgM can represent different stages of filoviral disease. Therefore, diagnosing recent filovirus infection might require sequential blood draws to ascertain increasing IgM

titres.⁴ Several rapid antigen detection tests (RDTs) have been developed, such as ReEBOV, SD Q Line, and OraQuick.^{5–7} However, RDTs have low sensitivity and specificity. Additionally, results obtained with RDTs still require confirmation by PCR, and at best are semi-quantitative. Nucleic acid detection is thus the most common procedure for diagnosing viral diseases, including filovirus disease, because of its unsurpassed specificity and sensitivity, and its ability to detect acute infection. Additionally, the virus does not need to be viable at the time of testing.⁸

Importance of filovirus load determination

Real-time RT-PCR provides not only a qualitative diagnosis, but also a surrogate measure of the virus burden in a sample by determining the cycle threshold, which varies inversely with viral load. Measurement of viral load is an important parameter in Marburg virus disease and Ebola virus disease, because viral load correlates with severity of disease, survival, and infectivity.^{9–16} Assessing the viral load, and thus the potential infectivity of a patient, can guide triage and admission placement to minimise risk of interperson transmission. Viral load measurements are also important to better understand the clinical presentation and pathogenesis of filovirus disease, and to interpret the efficacy of candidate therapies and vaccines in animal models and human beings.^{17–19} For instance, the interim analysis of a favipiravir monotherapy trial in Guinea revealed that the product might be efficacious against Ebola virus when the cycle threshold is 20 or higher, but not when it is less than 20.²⁰ For the final analysis, Sissoko and colleagues retested all samples with quantitative RT-PCR in a reference laboratory in France. Although the investigators observed a good correlation between cycle threshold values and RNA viral loads, they pointed out that the measured cycle threshold values might not be universally replicable, because they could vary depending on technique and technician experience, and that more robust standards are required.²¹

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The extent of viral load might be the key factor explaining the large variation in lethality—which is obviously among the most important metrics monitored in clinical trials—reported between different filovirus disease outbreaks, treatment units, and times during outbreaks. Variability in lethality might also relate to factors such as time from disease onset to presentation for care, quality of care available, patient demographics, or variant of infecting virus.^{14,22–24} Accurate viral load measurement is also important in interpreting Ebola virus persistence in and transmission risk from immune-privileged body compartments and fluids,²⁵ including the male gonads or semen,^{25–28} eyes,²⁹ CNS,³⁰ breast milk,³¹ and the intrauterine space in pregnant women.³² Additionally, Ebola virus has occasionally been found in sweat and urine,³³ and in atypical or asymptomatic cases.³² Similar reports note the persistence of Marburg virus in the eye,³⁴ semen,³⁵ and breast milk,³⁶ and in environmental samples. Viral load measurement could even be useful in assessing environmental decontamination practices.³⁷

Variability of filovirus load determination

Viral load assay results are subject to substantial interassay, intra-assay, inter-run, and interindividual variability.^{38,39} Additionally, interpretation of viral load is further confounded by the fact that viral load does not

necessarily correlate with viable replicating filovirus.⁴⁰ Slight differences between assays or genomic templates do not substantially affect diagnostic performance under controlled conditions.⁴¹ However, in general, data gathered over a long period from multiple sites—eg, within and between laboratories—cannot be compared, because each site used distinct and specific assays under variable conditions.⁴² For instance, a 1–2 log₁₀ difference in Ebola virus viral load, which may be within the margin of error of RT-PCR testing within and between many laboratories and assays, might correlate with significant differences in lethality.^{10,12–14} Standard curves, generated by assessing multiple samples with known quantities of filoviral RNA, can be used to measure the variability of results and offer a better understanding of the meaning of results across laboratories and time points. However, standard curves have rarely been generated under outbreak conditions, probably because of the high numbers of samples processed and to obtain and provide results rapidly.

Several real-time RT-PCR tests for Ebola virus are commercially available. Eight of these tests recently received emergency use authorisation from the US Food and Drug Administration (FDA),⁴³ and one received emergency use assessment and listing procedure from WHO,⁴⁴ and are commonly used in the field for diagnostic

Manufacturer	Viruses detected	Target gene	EUA approval from the FDA	EUAL procedure approval from the WHO	Qualitative assay	Quantitative assay	Limit of detection from manufacturer	Limit of detection from independent evaluation	Sensitivity* (%)	Specificity* (%)	
EZ1 Real-time RT-PCR Assay	US Department of Defense	Ebola virus	Glycoprotein	Yes	No	Yes	Yes†	1000–5000 PFU/mL	NA	100%	100%
Ebola Virus NP Real-time RT-PCR Assay	US Centers for Disease Control and Prevention	Ebola virus	Nucleoprotein	Yes	No	Yes	No	30 TCID ₅₀ /reaction	400 TCID ₅₀ /mL ⁴⁵	98–100%	100%
Ebola Virus VP40 Real-time RT-PCR Assay	US Centers for Disease Control and Prevention	Ebola virus	Viral protein 40	Yes	No	Yes	No	30 TCID ₅₀ /reaction	400 TCID ₅₀ /mL ⁴⁵	100%	94–100%
FilmArray NGDS BT-E Assay	Biofire Defense	Ebola virus	Unknown	Yes	No	Yes	No	10 000 PFU/mL	400 TCID ₅₀ /mL ⁴⁵	87–92%	100%
FilmArray Biothreat-E Test	Biofire Defense	Ebola virus	Unknown	Yes	Yes	Yes	No	600 000 PFU/mL	400 TCID ₅₀ /mL ⁴⁵	96%	100%
RealStar Ebolavirus RT-PCR Kit 1.0	Altona Diagnostics GmbH	Ebolaviruses or Marburg virus	RNA-dependent RNA polymerase	Yes	Yes	Yes	No	116–675 copies per µL	1250 copies per mL ⁴⁶	NA	NA
LightMix Ebola Zaire rRT-PCR Test	Roche Molecular Systems	Ebola virus	RNA-dependent RNA polymerase	Yes	No	Yes	No	4781 PFU/mL	1250 copies per mL ⁴⁶	97–8%	100%
Xpert Ebola Assay	Cepheid	Ebola virus	Glycoprotein + nucleoprotein	Yes	Yes	Yes	No	232–4 copies per mL or 0.13–1 PFU/mL	232 copies per mL or 1 PFU/mL ⁴⁷	90–100%	100%
Liferiver Ebola Virus Real-time RT-PCR Kit	Shanghai ZJ BioTech	Ebola virus	Unknown	No	Yes	Yes	Yes‡	1000 copies per mL	23.9 copies per reaction ⁴⁸ or 677 copies/mL ⁴⁹	NA	NA

EUA=emergency use authorisation. EUAL=emergency use assessment and listing. FDA=US Food and Drug Administration. NA=not applicable. PFU=plaque forming units. TCID₅₀=50% tissue culture infectious dose. *Provided by the manufacturers. †Quantitative ability was established by the use of synthetic RNA standard curves but this feature is not part of the FDA EUA version. ‡Not part of EUAL approval from WHO, only mentioned in the manual of the manufacturer.

Table: Overview of RT-PCR assays for filoviruses and their status with the FDA and WHO

purposes (table).⁸ However, none of these assays are validated for quantitative viral load assessment, and user manuals explicitly state that these assays are for qualitative purposes only. The reported sensitivities of these assays vary substantially, depending on the reagents and other materials used to assess the limits of detection. In addition to the commercial assays, various in-house quantitative assays for viral load of Ebola virus have been described, with limits of detection of approximately 1000 RNA copies per mL, but generally these assays have not been externally standardised or validated.^{11,49–51} Limits of detection depend on the PCR platform used and are not uniformly reported—for instance, they can be expressed as plaque forming units, 50% tissue culture infectious dose, or copies per mL. Therefore, Cherpillod and colleagues recommended that limits of detection values be expressed in IU/mL.⁴⁶

Availability of quantitative filovirus load assays

RT-PCR assays that provide rapid detection and RNA quantification were described for several haemorrhagic fever viruses, including Ebola virus, Marburg virus, Crimean–Congo haemorrhagic fever, Lassa virus, Rift Valley fever virus, yellow fever virus, and dengue viruses 1–4.⁵⁰ The EZ1 Real-time RT-PCR Assay (US Department of Defense, USA) for Ebola virus, which uses a synthetic RNA standard, was validated by good laboratory practices for quantitative measurement of viral load in non-human primate plasma. However, this feature was omitted in the emergency use authorisation version because of increased regulatory hurdles. Although this and other such assays could potentially be validated and approved for use on human samples, the added labour and complexity of validating a quantitative assay relative to a qualitative one have substantial impediments. The Liferiver Ebola Virus Real-time RT-PCR Kit (Shanghai ZJ BioTech, China) also offers the possibility for quantification with standard dilutions prepared from a prequantified positive control. However, to our knowledge, this assay is not widely used in the field, although inclusion in the recent WHO emergency use assessment and listing procedure might make this assay a more popular choice. Unfortunately, only the RealStar Ebolavirus RT-PCR Kit 1.0 (Altona Diagnostics GmbH, Germany) exists for filoviruses other than Ebola virus (table).

Conclusions

Quantitative assessment of viral load and valid comparison of viral loads detected by various PCR platforms and laboratories are important. We advocate for the development and evaluation of standardised reagents and validated assays for filovirus RNA quantification that are rapid, precise, easy to implement in resource-limited settings, and sufficiently robust to operate under outbreak conditions. We recognise that validation of quantitative assays is labour intensive,

requiring availability and testing of numerous predefined standards, and is difficult to implement under field conditions. A path forward might be gleaned from recent concerted international efforts to develop standardised quantitative assays and reference materials for other pathogens. For instance, high quality viral load clinical and analytical assessments are now possible for DNA and other RNA viruses.^{52,53} These assessments include three major commercial methods that are approved by the FDA for the measurement of HIV-1 RNA in plasma: Amplicor Monitor, Versant HIV RNA Kit, and NucliSens HIV-1 QT System. The limits of detection for these assays range from 10–40 genome copies per mL.⁵³ In the field of HIV/AIDS, viral load determination has become a routine test and is the basis of clinical patient management.⁵⁴ Although the commutability of some materials might need additional work to yield consensus,⁵⁵ clearly these efforts have a positive effect on comparability between assays.⁵⁶ Efforts towards standardising filovirus assays hold the promise of similar effects and should be vigorously pursued.

Contributors

LC, DGB, AG, JHK, and KKA conceived and wrote the first version of the manuscript. LC, ANH, and KKA collated the data in the table. All authors contributed to the literature search, data interpretation, discussions, consensus forming, and final version of the manuscript.

Conflicts of Interests

We declare no competing interests.

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