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Abstract

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Reference


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Ebola virus disease diagnosis by real-time RT-PCR: A comparative study of 11 different procedures

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ABSTRACT

\textbf{Background:} The diagnosis of Ebola virus disease relies on the detection of viral RNA in blood by real-time reverse-transcription PCR. While several of these assays were developed during the unprecedented 2013–2015 Ebola virus disease outbreak in West Africa, few were applied in the field.

\textbf{Objectives:} To compare technical performances and practical aspects of 11 Ebola virus real-time reverse-transcription PCR procedures.

\textbf{Study design:} We selected the most promising assays using serial dilutions of culture-derived Ebola virus RNA and determined their analytical sensitivity and potential range of quantification using quantified \textit{in vitro} transcribed RNA; viral load values in the serum of an Ebola virus disease patient obtained with these assays were reported. Finally, ease of use and turnaround times of these kits were evaluated.

\textbf{Results:} Commercial assays were at least as sensitive as in-house tests. Five of the former (Altona, Roche, Fast-track Diagnostics, and Life Technologies) were selected for further evaluation. Despite differences in analytical sensitivity and limits of quantification, all of them were suitable for Ebola virus diagnosis and viral load estimation. The Lifetech Lyophilized Ebola Virus (Zaire) 2014 assay (Life Technologies) appeared particularly promising, displaying the highest analytical sensitivity and shortest turnaround time, in addition to requiring no reagent freezing.

\textbf{Conclusions:} Commercial kits were at least as sensitive as in-house tests and potentially easier to use in the field than the latter. This qualitative comparison of real-time reverse transcription PCR assays may serve as a basis for the design of future Ebola virus disease diagnostics.

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1. Background

As of September, 2015, more than 28,000 cases and over 11,000 deaths have occurred during the West African epidemic of Ebola virus disease (EVD) (http://apps.who.int/ebola/ebola-situation-reports), which is caused by the Makona variant of the \textit{Zaire ebolavirus} (EBOV) species. Its genome is a 19 kb long single-stranded, negative-sense RNA. Rapid diagnosis is a critical infection control measure, particularly in light of EVD’s early symptomatology, which is indistinguishable from that of other infections including malaria [1]. Ebola virus diagnostics have improved considerably following the development of real-time reverse transcription PCR (real-time RT-PCR) assays capable of rapidly detecting viral RNA in blood specimens [2–7]. Their ability to estimate viral loads in blood, which correlate with clinical outcome [8–11], as well as in various body fluids [12–20], makes them useful tools in the post-diagnostic phase. The recent implementation, though somewhat controversial, of a negative blood real-time RT-PCR result as a discharge criterion has only increased these assays’ importance [21,22].

Yet commercial molecular diagnostic assays were made available only in 2014. Validation results of real-time RT-PCR assays recently used in the field in West Africa and in other countries for screening are limited and not publicly available, and no assays have yet been compared systematically.
2. Objectives

We undertook the present comparative study to evaluate the performance of several commercial and in-house EBOV real-time RT-PCR assays in order to inform future selections and use of these tools both in the field and in high-resource settings.

3. Study design

The study flow chart is shown in Fig. 1.

3.1. EBOV RNAs

C5 (GenBank no. KJ660348) and C15 RNAs (GenBank no. KJ660346) extracted from vero-cell cultures after inoculation with serum from patient C5 in Guékédou and patient C15 in Kissidougou [23] were kindly provided by the Swiss Institute for NBC-Protection (Spiez Laboratory, Biology–Virology Group, Spiez, Switzerland). Alignment of the two sequences revealed 5 nucleotide changes (99% nucleotide sequence homology): two were located in the N gene at positions 2124 and 2185, one in the GP gene at position 6909, and two in the L gene at positions 13,856 and 15,660. None of these positions are targeted by any of the real-time RT-PCR assays tested in our study, except possibly for the position 13,856 nucleotide change, which could be involved in the target sequence of the RealStar® Filovirus Screen RT-PCR Kit 1.0, the Roche LightMix® Modular Ebola Virus Zaire assay, and/or the FTD® Ebola real-time RT-PCR.

In order to determine both the analytical sensitivity and the linear range of selected real-time RT-PCR assays, we used two RNA transcripts 990 bases long (TriLink BioTechnologies, San Diego, USA) at known concentrations. One spans the L gene (nt 12,792 to nt 13,781 of the genomic strand, GenBank ref: KM233117) and the other the NP gene (nt 374 to nt 1363 of the genomic strand, GenBank ref: KM233117); together these cover all real-time RT-PCR targets used in this study. We also used RNA extracted from the serum of an EVD patient managed in our institution [19]. All RNA in the same experiment, either extracted or transcribed in vitro, underwent the same freeze–thaw cycle.

3.2. RNA extraction

RNA extraction was performed with the EasyMag automate (NuclISENS® EasyMag, bioMérieux, Geneva, Switzerland) following the manufacturer’s instructions. 400 μl of specimen (Ebola Zaire positive serum) were inactivated by 1 ml of lysis buffer (EasyMag® Lysis Buffer), followed by nucleic acid extraction with a final elution volume of 50 μl.

3.3. rRT-PCR assays

The following six commercial assays were selected based on their availability on the market in Switzerland at the time of this study and their ability to be conducted on open platforms and adapted to the specifications of an average clinical microbiology Laboratory: RealStar® Filovirus Screen RT-PCR Kit 1.0 (ref: 441013, Altona, Hamburg, Germany) and the Zaire EbolaVirus assay from the RealStar® Filovirus Type RT-PCR Kit 1.0 (ref: 451003, Altona, Hamburg, Germany), Roche LightMix® Modular Ebola Virus Zaire (ref: 40-0666-96, Roche, Rotkreuz, Switzerland), FTD® Ebola (ref: FTD-71-64, Fast-track Diagnostics (FTD), Sliema, Malta), Lifetech Ebola Virus (Zaire 2014) and Lifetech Lyophilized Ebola Virus (Zaire 2014) (ref: 4489990, Life Technologies, Waltham, USA). These tests are referred to as Altona Screen, Altona Type, Roche, FTD, Lifetech and Lifetech L, respectively, throughout the manuscript.

According to the manufacturer’s instructions, the following real-time PCR platforms were suitable for each commercial kit:

Altona screen: Mx3005P™ QPCR System (Stratagene), VERSANT® kPCR Molecular System AD (Siemens), ABI Prism® 7500 SDS and 7500 Fast SDS (Applied Biosystems), LightCycler® 480 Instrument II (Roche), Rotor-Gene® 3000/6000 (Corbett Research), Rotor-Gene® Q5/6plex Platform (QIAGEN), and CFX96™/Dx Real-Time System (BIO-RAD).

Altona type: VERSANT® kPCR Molecular System AD (Siemens), ABI Prism® 7500 SDS and 7500 Fast SDS (Applied Biosystems), LightCycler® 480 Instrument II (Roche), Rotor-Gene® 3000/6000 (Corbett Research), Rotor-Gene® Q5/6plex Platform (QIAGEN), and CFX96™/Dx Real-Time System (BIO-RAD).

Roche: LightCycler® 480 II Instrument (Roche), and Cobas z480 (Roche).

FTD: ABI Prism® 7500 SDS, 7500 Fast SDS, and ABI ViiA7 (Applied Biosystems), CFX96™/Dx Real-Time System (BIO-RAD).

LifeTech and Lifetech L: 7500 Fast SDS, ABI ViiA7, and QuantStudio real-time PCR systems (Applied Biosystems).

In addition, we tested 5 in-house real-time RT-PCR assays: The real-time RT-PCR designed by Gibb and colleagues in 2001 [2], as well as a modified version, which we adapted according to the viral circulating sequences in 2014; a modified version of the Ebola Zaire-TM assay designed in 2010 [7], which was adapted according to 2014 EBOV sequences by the Swiss Institute for NBC-Protection (Spiez Laboratory, Biology–Virology Group, Spiez, Switzerland); and two in-house real-time RT-PCR assays, which were designed according to publicly available Ebola sequences through October 2014, the “EBOV-GP-GE-14” and the “EBOV-L-GE-14” real-time RT-PCRs, targeting the GP and L genes respectively. These in-house assays are referred to as EBOGP-1D, EBOGP-1D14, Ebola Zaire-TM, EBOV-L-GE-14 and EBOV-GP-GE-14, respectively. Primers pairs and probes were designed using Primer Express software version 3.0.

The Roche, Altona Screen and Altona Type assays were used with the LightCycler 480 thermocycler (Roche Diagnostics, Rotkreuz, Switzerland) while the FTD, Lifetech and Lifetech L assays were run with the ViiA7 thermocycler (Life Technologies, Waltham, USA), according to the manufacturer’s instructions. All non-commercial real-time RT-PCRs were run using the StepOne Plus thermocycler (Life Technologies Waltham, USA). Characteristics of the 11 evaluated real-time RT-PCR assays, including cycling conditions and primers’ and probes’ sequences, when available, are summarized in Table S1.

3.4. Lower limits of detection and quantification, and EBOV RNA quantification

For each selected real-time RT-PCR assay, known concentrations of in vitro transcribed RNAs (see above) were run in triplicate in two separate experiments to establish standard curves and define the limit of detection (LOD) and the lower limit of quantification (LOQ). The LOD was defined as the lowest RNA concentration detected in all of the six replicates. The LOQ was defined based on both triplicate experiments as the lowest RNA concentration that could be plotted on a standard curve with a slope between −3.1 and −3.6 (corresponding to a PCR efficiency between 90% and 110%), an r² value above 0.95, and visually limited dispersion around the curve. For EBOV RNA quantification in the clinical specimen, a standard
4. Results

4.1. Analytical sensitivity comparison of six commercial and five in-house rRT-PCR assays

Each of the six commercial and five in-house assays was evaluated by end-point dilution using 5-fold dilutions of two culture-derived full-length EBOV genome RNAs, related to isolates collected in Guinea in March 2014 (named C5 and C15 RNAs) (Fig. 1 and Table S2).

While all 11 assays could detect RNA from both isolates, the commercial kits tended to be more sensitive. Since the in-house real-time RT-PCRs provided no advantages in terms of performance or ease of use, they were not selected for further evaluation. We did not retain the Lifetech Ebola Virus (Zaire 2014) assay for further testing either, as it was less sensitive than its lyophilized counterpart and lacked practical advantages. The LOD was determined for the five most promising assays (Altona Screen, Altona Type, Roche, FTD, and Lifetech L), using serial dilutions of quantified in vitro transcribed RNAs (Fig. 2, Table 1). As can be seen in Fig. 2, which represents two separate experiments performed seven months apart, the inter-assay variability is very small.

4.2. Lower limit of quantification

The five selected assays were further tested for their quantification abilities (Fig. 2, Table 1). Of note, the highest RNA concentration tested in our analysis was 6.25E9 RNA copies/ml, which did not reach the upper limit of quantification for any assay.

4.3. EBOV RNA quantification and end-point dilution using serum of an EVD patient

The five commercial real-time RT-PCRs were also run in duplicate on 3-fold dilutions of RNA extracted from a serum sample withdrawn from an EVD patient managed in our institution [19] (Table 1). The EBOV RNA quantification values obtained with the various assays in the undiluted serum were as follows: 1.16E6 RNA copies/ml (Altona Screen), 1.13E5 RNA copies/ml (Altona Type), 9.5E4 RNA copies/ml (Roche), 4.0E4 RNA copies/ml (FTD), and 2.5E4 RNA copies/ml (Lifetech L).

4.4. Ease of use and turnaround time

The complexity of technical manipulations was comparable between the five assays, except for the Lifetech Lyophilized system, which does not require any real-time RT-PCR mix preparation. For each assay, we estimated separately the handling time from RNA extraction to real-time RT-PCR and the real-time RT-PCR cycling time, and the total turnaround time (Table 1).
Fig. 2. Graphical representation of standard curve, linearity range, lower limit of quantification (LOQ) and limit of detection (LOD) for each EBOV rRT-PCR assay. Each plot shows values obtained from two separate experiments in which serial dilutions of in vitro transcribed EBOV RNA were run in triplicate. Red diamonds represent the first experiment and blue diamonds the second. The lowest RNA concentration for which the line passes through represents the LOQ. The vertical grey dashed line represents the LOD. For each assay, the cycling number recommended by each manufacturer is represented by the highest number indicated on the y axis. Diamonds situated above the maximum cycle line represent RNA dilutions that were not detected. Slope and $r^2$ values are indicated for each curve. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

Table 1
Comparison of five selected EBOV rRT-PCR assays.

<table>
<thead>
<tr>
<th>Assay</th>
<th>LOD (in vitro transcribed RNA copies/ml)</th>
<th>LOQ (in vitro transcribed RNA copies/ml)</th>
<th>Highest patient RNA dilution detected</th>
<th>Handling time from extraction to rRT-PCR$^a$ (min)</th>
<th>rRT-PCR cycling time (min)</th>
<th>Total turnaround time (min)</th>
<th>Storage temperature</th>
</tr>
</thead>
<tbody>
<tr>
<td>Altona Screen</td>
<td>1250</td>
<td>12,500</td>
<td>27×</td>
<td>70–100</td>
<td>120</td>
<td>190–220</td>
<td>−20 °C</td>
</tr>
<tr>
<td>Altona Type</td>
<td>6250</td>
<td>625,000</td>
<td>27×</td>
<td>70–100</td>
<td>120</td>
<td>190–220</td>
<td>−20 °C</td>
</tr>
<tr>
<td>Roche</td>
<td>1250</td>
<td>6250</td>
<td>27×</td>
<td>70–105</td>
<td>80</td>
<td>150–185</td>
<td>−20 °C</td>
</tr>
<tr>
<td>FTD</td>
<td>625</td>
<td>1250</td>
<td>81×</td>
<td>70–100</td>
<td>75</td>
<td>145–175</td>
<td>−20 °C</td>
</tr>
<tr>
<td>Lifetech L</td>
<td>62.5</td>
<td>625</td>
<td>243×</td>
<td>55–80</td>
<td>50</td>
<td>105–130</td>
<td>−2–8 °C</td>
</tr>
</tbody>
</table>

$^a$ Nucleic acid extraction, rRT-PCR mix preparation, plate pipetting LOD: limit of detection; LOQ: lower limit of quantification.
5. Discussion

We provide the first systematic comparison of several EBOV real-time RT-PCR assays, six of which are commercially available, using serial dilutions of full-length EBOV RNAs from the 2013–2015 EVD outbreak. In-house assays did not match the performance of the commercial kits even after primer adaptations in line with recently isolated EBOV sequences. This could be explained in part by the lack of extensive reagent optimization.

The five most promising assays, all commercially available (Altona Screen, Altona Type, Roche, FTD, and Lifetech L), were selected for further evaluation using quantified RNA transcripts.

The Lifetech Lyophilized assay displayed the best analytical sensitivity with an LOD of 62.5 RNA copies/ml and the lowest LOQ (625 RNA copies/ml), which may be explained in part by the kit’s relatively high RNA eluate volume. Indeed, 25 µl of eluate is required; this is 2.5 to 5 times more than that required by the Roche, FTD and both Altona assays.

Turnaround times ranged from two (Lifetech Lyophilized) to 3.5 h (both Altona assays). The differences are relatively small, however, and should have little impact on patient management. Complexity of use and reagent storage constraints are likely to be more significant issues in the field.

Altogether, despite differences in analytical sensitivities and LOQs, the Altona Screen, Roche, FTD, and Lifetech L assays all displayed analytical sensitivities high enough to ensure accurate EVD diagnosis and linear quantification ranges sufficient to assess viral kinetics, at least when dealing with high RNA concentrations.

The Zaire Ebolavirus strain from the RealStar® Filovirus Type RT-PCR Kit 1.0 was the least sensitive and the least suited for EBOV RNA quantification. This is a minor issue, however, if the assay is used primarily, as its name implies, to type the Zaire ebolavirus species.

Nevertheless, the different sensitivities exhibited by the real-time RT-PCR kits can have considerable clinical impact. For instance, as the viral RNA load decreases during the recovery phase, the various real-time RT-PCR assays may yield undetectable results at different time points, potentially resulting in prolonging or shortening of patient isolation time. This issue was exemplified during the management of an EVD infected patient in our center, as we obtained different real-time RT-PCR results with the Roche and Altona assays run on the same samples during the convalescent phase [19]. However, one can argue that when a patient shows signs of clinical cure, the low-level detection of viral RNA probably reflects genomic remnants rather than residual infectious virus [24]. Therefore, high Ct values obtained during the recovery phase should probably not be overemphasized.

EBOV RNA quantification by real-time RT-PCR is clinically useful, since high viremia is correlated with a higher mortality [8–11]. Precise EBOV RNA quantification will also be a valuable tool in trials evaluating antiviral agents.

It should be noted, however, that in this study we did not perform a complete real-time RT-PCR quantification validation, but rather assessed their respective quantification potential. A thorough validation according to the Minimum Information for Publication of Quantitative Real-time PCR Experiments (MIQE) guidelines [25] would be essential for any formal assessment of EBOV-RNA quantification performance. Moreover, as shown with the quantification of EBOV RNA in the serum of an EVD patient, RNA copies/ml values varied considerably according to the real-time RT-PCR assay, from 4.41 to 6.07 log_{10} RNA copies/ml. This variability can have an impact on viral load quantification results in clinical investigations, and hence on the conclusions drawn by such studies. Therefore, in order to compare quantification values obtained with different tests, they should be expressed in international units (IU) per milliliter using an international quantification standard, as has been done for other viruses.

The Lifetech L and FTD assays displayed remarkably low LOQs, 625 and 1250 RNA copies/ml, respectively, which could be interesting in EBOV RNA quantification in a research context. The Lifetech L assay, which to the best of our knowledge did not have the opportunity to be tested in the field during the 2013–2015 EVD outbreak, displays many interesting features. The absence of real-time RT-PCR mix preparation and freezer requirements, short turnaround time, high sensitivity and low LOQ render this assay particularly suitable in the context of an outbreak in limited-resource settings, in which cold chain management can be challenging.

Our study has several limitations. The first is the lack of sequence data regarding the primers and probes included in commercial assays, which is a consequence of the restrictive data-sharing policies applied by various agencies. Second, our study is not exhaustive, as we made a selection of available assays at a given time and at a specific place, and that were adapted to our laboratory. Third, it should be noted that we used a nucleic acid extraction procedure (EasyMag) not typically used in West Africa, and three different thermocyclers according to the different assays. Finally, the present analysis is focused on the 2013–2015 EVD epidemic in West Africa, and our conclusions are thus relevant to the Makona EBOV variant currently circulating. Indeed, while the Altona Screen and Roche assays performed well on RNA from the 2001 EBOV epidemic in Gabon, the same was not true for the FTD assay (data not shown). This illustrates the need to adapt real-time RT-PCR assays according to potential viral sequence evolution, as stressed by Sozhannan et al. [26].

In conclusion, among 11 EBOV real-time RT-PCR assays, commercial kits were superior to in-house assays; among the former, the Lifetech L assay displayed the highest sensitivity and ease of use as well as the lowest turnaround time.

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Competing interests

None declared.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.jcv.2016.01.017

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