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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

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

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 *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.

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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 Guéckédou 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:

Mx 3005P™ 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® Q5/6 plex 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® Q5/6 plex Platform (QIAGEN), and CFX96™/Dx Real-Time System (BIORAD).

Roche:

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

FTD:

ABI Prism® 7500 SDS, 7500 Fast SDS, and ABI ViiA7 (Applied Biosystems), CFX96™/Dx Real-Time System (BIORAD), LightCycler® 480 II Instrument (Roche), Rotor-Gene® 3000/6000 (Corbett Research), and SmartCycler® (Cepheid).

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 r^2 value above 0.95, and visually limited dispersion around the curve. For EBOV RNA quantification in the clinical specimen, a standard