Establishment of an in-house one-step real-time RT-PCR assay for detection of Zaire ebolavirus

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Abstract:
Ebola virus is a deadly causative agent with a high mortality rate of up to 90%, therefore it has been classified by the Center for Disease Control and Prevention (CDC) as a category A biological agent. The World Health Organization (WHO) recommended using RT-PCR based assays to rapidly detect the virus. In the present study, we established an in-house assay for detection of Zaire ebolavirus via real-time RT-PCR. The nucleotide sequence of the Zaire ebolavirus nucleoprotein (NP) gene was retrieved from the Genbank for designing primer pairs and probes using Primer Express 3.0 software. The RNA positive control was generated by in vitro RNA transcript synthesis. The optimal components in the 20 μl final volume of the real-time RT-PCR assay were 10 μl 2X QuantiTect Probe RT-PCR master mix, 0.6 μM of each primer, 0.1 μM of the probe, 0.2 μl RT mix and 5 μl of RNA template. The thermal cycle conditions were as follows: 50°C for 30 min, 95°C for 15 min, then 45 cycles of 15 s at 94°C, 60 s at 60°C. The limit of detection of the assay was 100 copies/reaction and 1414 FFU/ml with the positive RNA panel and sample panel of RNA extracted from cell culture supernatants of cells infected with Zaire ebolavirus 2014/Gueckedou-C05, respectively. The specificity of this assay was 100% when tested with the positive RNA panel of Ebola virus and other haemorrhagic fever viruses. In conclusion, we successfully established an in-house real-time RT-PCR assay for detection of Zaire ebolavirus in Vietnam with a limit of detection of 1414 FFU/ml and specificity of 100%.

Keywords: ebola virus, real-time RT-PCR, Vietnam, Zaire ebolavirus.

Classification number: 3.2, 3.5

Introduction
Ebola virus (EBOV) is a fetal causative agent of severe hemorrhagic fever epidemic with a high mortality rate of up to 90%. The virus was firstly discovered in 1976 when it caused two simultaneous outbreaks in Sudan and Zaire (now Democracy Republic of Congo) [1]. The recent Ebola outbreak in Western Africa was the largest in history with more than 28,602 suspected cases and 11,301 deaths. The cause of this outbreak was then identified as a Makona variant of Zaireebola virus [2]. The WHO declared the outbreak of EBOV disease in West Africa as a “Public health emergency of international concern” and called for a substantial global response in order to control this epidemic [3].

EBOV belongs to the Filoviridae family consisting of the five species: Zaire ebolavirus (ZEBOV), Sudan ebolavirus (SEBOV), Reston ebolavirus (REBOV), Bundibugyo ebolavirus (BEBOV) and Tai Forest ebolavirus (TEBOV) [1] EBOV is an enveloped, negative-sense, and single-strand RNA virus with its genome (19 kb in length) encoding for 7 proteins including nucleoprotein (NP), viral protein (VP35), matrix protein (VP40), glycoprotein (GP), replication-transcription protein (VP30), matrix protein (VP24), and RNA dependent RNA polymerase (L). No available vaccines or antiviral drugs exist for prevention and treatment of the EBOV disease. Therefore, early detection of suspected cases is critical for the management, surveillance and control of this deadly epidemic. Real-time RT-PCR assays were used routinely in the laboratory of clinical virology due to high sensitivity, specificity and rapid results, therefore the WHO recommended the use of a real-time RT-PCR assay as the first choice for detection of EBOV in clinical virology laboratories [4]. However, commercial real-time RT-PCR kits approved by the FDA were not available before the arrival of the epidemic in late 2013. Other relevant assays including ELISA, require a Bio safety level 4 (BSL-4) facility for isolation and viral culture [5]. Therefore, a simple, sensitive, and accurate assay based on real-time PCR, which is affordable in countries of limited resources, is essential for

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early detection of EBOV in inactivated specimens [6]. This study aims to establish and evaluate a real-time RT-PCR assay for detection of ZEBOV.

Materials and methods

Preparation of ZEBOV RNA positive standard

The 1306 bp nucleotide sequence of a partial NP gene and 3’ untranslated region (3’UTR) of recently epidemic ZEBOV strain (GenBank: KJ660348) was chemically synthesized and inserted into the pIDTBlue vector (4 μg) by IDT (USA). This plasmid was linearized by digestion with PciI restriction enzyme for in vitro RNA transcription with a Transcript Aid T7 High Yield Transcription Kit (Thermo Scientific), and the synthetic viral RNA transcripts were purified using a GeneJET RNA Purification Kit (Thermo Scientific) according to the manufacturer’s instructions. The RNA level was measured by a Nanodrop ND1000 spectrophotometer (Thermo Fisher Scientific) and then converted to the number of copies per μl. The RNA transcript was stored at -80°C for further use.

RNA extraction

RNA samples were extracted from 140 μl of clinical samples collected from patients in recently Ebola stricken Guinea and from cell culture supernatant of cells infected with ZEBOV2014/ Gueckedou-C05 and other haemorrhagic virus species including SEBOV, REBOV, TEOBO and the Marburg virus [Leiden-BNI 2008], and plasma of patients infected with dengue virus, Zika virus and chikungunya virus for assay cross-reactivity and specificity evaluation using QIAamp Viral RNA Mini Kit (Qiagen GmbH, Hilden, Germany) according to the manufacturer’s instructions. All clinical samples were inactivated before doing extraction by using an AVL buffer and absolute ethanol; then samples were incubated at 60°C for 60 minutes under BSL-4 conditions in the department of virology at Bernhard Notch of Tropical Medicine (BNITM), Hamburg, Germany. Extracted RNA samples were prepared at a concentration of 10^6 copies/μl. The transcribed ZEBOV RNA was evaluated by using our previously developed one-step real-time RT-PCR assay for EBOV detection. The RT-PCR product of the ZEBOV RNA in a 10^6 copies/μl concentration is a specific and thick band 830 bp in length (RT mix (+)), whereas there is no band for RT mix (-) RT-PCR (Lane 2 and 3, Fig. 1). Positive RT-PCR product was confirmed exactly by direct sequencing (Data not shown).

Results

ZEBOV RNA positive standard

The transcribed ZEBOV RNA was yielded with a high concentration of 1,400.3 ng/μl (1.44 x 10^2 copies/μl) and 2.01 A260/A280 ratio. Moreover, the RNA transcript was determined by specific size 1806 base in gel agarose electrophoresis (Data not shown). Additionally, the quality of RNA transcript was evaluated by using our previously developed one-step real-time RT-PCR assay for EBOV detection. The RT-PCR product of the ZEBOV RNA in a 10^6 copies/μl concentration is a specific and thick band 830 bp in length (RT mix (+)), whereas there is no band for RT mix (-) RT-PCR (Lane 2 and 3, Fig. 1). Positive RT-PCR product was confirmed exactly by direct sequencing (Data not shown).
Optimization of the one-step real-time RT-PCR assay: Concentrations of primers and probes were optimized in a final volume of 20 μl reaction mixture containing 5 μl of RNA template to obtain minimal Ct. Primer concentrations were tested from 0.1 to 0.6 μM and probe concentrations were tested from 0.05 to 0.4 μM. The optimal reaction was obtained at a primer concentration of 0.6 μM (for both primers) and a probe concentration of 0.1 μM.

Limit of detection and specificity of one-step real-time RT-PCR assay

The analytical sensitivity of the real-time RT-PCR assay was evaluated in triplicates on a sample panel ranging from 10^0 to 10^6 copies/μl which was created by serial dilutions of the synthetic viral stock RNAs. The threshold line was chosen at 0.1 during analysis and the data collected were analyzed by linear regression (r^2 = 0.99). The results showed that the one-step real-time RT-PCR assays could detect in samples at the concentration of 10^2 copies/reaction (Table 1).

Additionally, the diagnostic sensitivity of the assay was assessed by determination of the LoD, defined as the last dilution at which all replicates were positive. The results have shown the diagnostic sensitivity was 1.65 x 10^1 FFU/reaction, mean 1414 FFU/ml equivalent, indicating a good sensitivity (Fig. 3 and Table 2).

The LoD of each test was determined to be the lowest concentration resulting in 95% positive detection of 20 replicates. Furthermore, we also evaluated the sensitivity of the assays on several clinical specimens with different viral loads measured with a Realstar Ebola PCR kit in BNITM. Therefore, the diagnostic sensitivity of the assay was confirmed at the 1.65 x 10^1 FFU/reaction, it was also set as the LoD for the assay. End-point real-time RT-PCR products also showed specific bands with a length of 103 bp on agarose gel (Fig. 4).

Table 1. Results of analytical sensitivity.

<table>
<thead>
<tr>
<th>Concentration</th>
<th>1st Exp Ct</th>
<th>2nd Exp Ct</th>
<th>3rd Exp Ct</th>
<th>Mean Ct</th>
<th>SD</th>
<th>CV</th>
</tr>
</thead>
<tbody>
<tr>
<td>E6</td>
<td>26.39</td>
<td>26.01</td>
<td>27.1</td>
<td>26.5</td>
<td>0.55</td>
<td>0.30</td>
</tr>
<tr>
<td>E5</td>
<td>30.16</td>
<td>29.75</td>
<td>32.26</td>
<td>30.7</td>
<td>1.34</td>
<td>1.81</td>
</tr>
<tr>
<td>E4</td>
<td>34.67</td>
<td>34.09</td>
<td>36.24</td>
<td>35.0</td>
<td>1.11</td>
<td>1.23</td>
</tr>
<tr>
<td>E3</td>
<td>38.84</td>
<td>38.23</td>
<td>40.54</td>
<td>39.2</td>
<td>1.19</td>
<td>1.43</td>
</tr>
<tr>
<td>E2</td>
<td>40.41</td>
<td>39.7</td>
<td>43.71</td>
<td>41.2</td>
<td>2.13</td>
<td>4.57</td>
</tr>
<tr>
<td>E1</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
</tbody>
</table>

SD: standard deviation, CV: coefficient of variation.
The cross-reactivity and specificity of the assay were tested with RNAs extracted from the supernatant of cell-cultures infected with other EBOV species: SEBOV, REBOV, TEBOV, and Marburg virus [Leiden-BNI 2008], dengue virus, Zika virus and chikungunya virus. There was no cross-reaction of the assay with any of the other EBOV species which were observed. The diagnostic specificity was 100% of all tested samples which were negative for ZEBOV and closely other hemorrhagic fever viruses.

**Discussions**

EBOV disease is a major public health issue in the world. Among five EBOV species, ZEBOV caused a majority of the outbreaks in Africa with the highest case-mortality rate of up to 90%. After the three week period of incubation, EBOV disease presents with unspecific symptoms and is usually difficult to differentiate from other tropical diseases [7]. Therefore, diagnostic laboratory assays play an important role in confirming or excluding suspected cases [5]. In recent years, several methods for detecting EBOV have been developed for use in clinical virology laboratories, including the use of several assays under Emergency Use Authorization, and others evaluated in a field setting. Due to the fact that EBOV is categorized as a high-hazard pathogen, diagnostic methods including viral culture and isolation require it to be handled in a BSL-4 facility. However, in resource-limited countries, the WHO and CDC have advised that EBOV can be tested in BSL-2 conditions by nucleic acid testing if specimens are inactivated by appropriate methods.

The first real-time PCR assay was developed by Gibb, et al. to detect and differentiate between ZEBOV and SEBOV in patient samples collected during the 2000 Gulu outbreak [8] sensitive, and specific laboratory diagnostic test is needed to confirm outbreaks of Ebola virus infection and to distinguish it from other diseases that can cause similar clinical symptoms. A one-

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**Table 2. The diagnostic sensitivity and specificity of real-time RT-PCR.**

<table>
<thead>
<tr>
<th>Sample panel</th>
<th>ZEBOV RNA Quantity (FFU/reaction)</th>
<th>Mean Ct value</th>
<th>Replicates</th>
<th>Assay results</th>
</tr>
</thead>
<tbody>
<tr>
<td>Diluted E-1</td>
<td>$1.65 \times 10^5$</td>
<td>22.3</td>
<td>6</td>
<td>100% Positive</td>
</tr>
<tr>
<td>Diluted E-2</td>
<td>$1.65 \times 10^4$</td>
<td>26.31</td>
<td>6</td>
<td>100% Positive</td>
</tr>
<tr>
<td>Diluted E-3</td>
<td>$1.65 \times 10^3$</td>
<td>29.68</td>
<td>6</td>
<td>100% Positive</td>
</tr>
<tr>
<td>Diluted E-4</td>
<td>$1.65 \times 10^2$</td>
<td>33.62</td>
<td>9</td>
<td>100% Positive</td>
</tr>
<tr>
<td>Diluted E-5</td>
<td>$1.65 \times 10^1$</td>
<td>34.72</td>
<td>20</td>
<td>100% Positive</td>
</tr>
<tr>
<td>Diluted E-6</td>
<td>$1.65 \times 10^0$</td>
<td>-</td>
<td>6</td>
<td>100% Negative</td>
</tr>
</tbody>
</table>

**Other viruses**

- Sudan EBOV Gulu: 3, 100% Negative
- Reston EBOV: 3, 100% Negative
- Tai Forest EBOV: 3, 100% Negative
- Marburgvirus Leiden: 3, 100% Negative
- Marburgvirus Popp: 3, 100% Negative
- Dengue virus: 3, 100% Negative
- Chikungunya virus S27: 3, 100% Negative
- Zika virus: 3, 100% Negative

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**Fig. 3. Concentration dilutions from $1.65 \times 10^5$ to $1.65 \times 10^0$ FFU/reaction.**

**Fig. 4. Representative agarose gel 2% of end-point products of one-step real-time RT-PCR ZEBOV RNA from $1.65 \times 10^5$ to $1.65 \times 10^0$ FFU/reaction.** M: marker 50 bp (thermo scientific), NC: negative control; 1-6: $1.65 \times 10^5$ - $1.65 \times 10^0$ FFU.
tube reverse transcription-PCR assay for the identification of Ebola virus subtype Zaire (Ebola Zaire). In addition, the real-time PCR assay measured the viral load in the patients’ plasma, which has been shown to be associated with the outcome of the disease. Recent studies have shown that most patients in Western Africa with high viral load associated with a poor prognosis and higher mortality rate [9]. However, there was not a commercial real-time PCR assay approved by the FDA for use upon emergence of the EBOV outbreak in Western Africa, whereas, various laboratory-developed assays have demonstrated significant variability in regards to their sensitivity of detection as well as their reliability [4, 10, 11].

In this study, we established an in-house assay for detection of recent ZEBOV by one-step real-time RT-PCR. Ideally, optimization of assays needs to be performed on EBOV-RNA samples extracted from the stock viral strains, but it is very difficult to acquire this material in Vietnam because there have yet to be any reported cases of EBOV infection. Therefore we used RNA transcribed in vitro from a plasmid containing the NP gene of EBOV to generate both the acceptable standards for the optimization of components and appropriate reaction conditions, as well as for the evaluation of the analytical sensitivity of the assay. Furthermore, we validated the established assay with an RNA sample extracted from inactivated cell culture supernatant of infected cells with ZEBOV 2014/Gueckedou-C05 and several clinical samples to determine the LoD and diagnostic sensitivity at the BNITM in Hamburg, Germany. Results showed that the analytical sensitivity of the assay obtained was at a concentration of $10^6$ copies/reaction, whereas specificity was 100% as tested with RNA extracted from other EBOV species and close other hemorrhagic fever viruses. When tested on RNA extracted from the supernatant of infected cells with ZEBOV 2014/Gueckedou-C05 indicated the LoD at a concentration of 1414 FFU/ml and 100% of positive clinical samples. Importantly, we also optimized one-step real-time RT-PCR using a total volume of 20 μl per reaction, making this assay save more reagents. One notable point, the established assay performed on both the Rotor-Gene Q and LightCycler instrument showed a similar performance. Compared with previous studies, the established assay in this study had higher sensitivity and specificity. When comparing this assay to others it can be said to be affordable in cost and to provides accurate results in a short period of time. In addition, the volume of RNA template and related requirements should be considered when comparing this assay to others. Therefore, it is very important to standardize and optimize with more extensive reagents and then validate these assays further in regards to international WHO reference materials.

In conclusion, we developed a highly specific, sensitive assay for the detection of ZEBOV by one-step real-time RT-PCR with the LoD concentration of 1414 FFU/ml, and specificity of 100%. This assay could be used to detect ZEBOV in samples taken from subjects suspected of infection, after returning from travel in infected regions.

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