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The evaluation of 7 commercial real-time PCR kits for *Zaire ebolavirus* using virus-like particle–encapsulated EBOV RNA

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ABSTRACT

Several Chinese commercial real-time PCR kits for *Zaire ebolavirus* have been developed after the Ebola outbreak and used by Chinese medical teams in West Africa. In order to know the essential performance indicators of these kits, analytical sensitivity and precision were evaluated with virus-like particle (VLP)–encapsulated EBOV RNA. The limit of detection (LOD) and the precision were completed with a series of VLPs. The maximum and minimum of LOD was acquired by ZJ BioTech and Daan gene, respectively. For precision, all of the detection results were <5% except the maximum 5.17%. Among them, Puruikang, Daan gene, Sansure, ZJ BioTech, and Huada demonstrated superior reproducibility. Overall, the requirements of LOD <1000 copies/mL and coefficient of variation <5% could be satisfied by all kits except Kehua. Meanwhile, it is feasible for VLPs as a substitute of positive samples in assay evaluation.

1. Introduction

Ebola virus belongs to the Filoviridae family and contains a single negative-sense, nearly 19 kb RNA strand which encodes 7 proteins: nucleoprotein (NP), phosphoprotein (VP35), matrix protein (VP40), glycoprotein (GP), replication-transcription protein (VP30), matrix protein (VP24), and RNA dependent RNA polymerase (L) (Fields et al., 2007). Among the 5 species (*Bundibugyo*, *Sudan*, *Zaire*, *Tai Forest*, and *Reston*), the *Zaire* species has the most frequent outbreaks and the highest mortality rate from 57% to 90% (McElroy et al., 2014) to human being. In 2014, viral isolation and sequence alignment were conducted and identified that the epidemic strain in West Africa was *Zaire ebolavirus* (EBOV) (Baize et al., 2014).

In early August 2014, the World Health Organization (WHO) had announced the outbreak of Ebola virus disease (EVD) in West Africa as "public health emergency of international concern." The number of both infectors and deaths of EVD in the recent epidemic was the highest and more than 22 prior outbreaks in total since the first identified in 1976 (WHO, 2014). The center for EBOV plague was Guinea, Liberia, and Sierra Leone, along with other countries in West Africa, such as Nigeria and Senegal. Europe and the United States had also been reported as sporadic affected regions (Ledgerwood et al., 2014).

Confronted with the situation above, China authority had provided supports to Africa since September 2014. Sierra Leone–China friendship hospital and a mobile biosafety level 3 laboratory were built; diagnostic reagents for EBOV, public health experts, and medical staff were all transported and dispatched 4 times to EBOV-affected areas (Gao and Feng, 2014). In response to the aiding West Africa, plenty of commercial kits for qualitative detecting *Zaire* Ebola virus had been independently developed in China. The ZJ BioTech kit had obtained the full approval of WHO in May 2015 and the certification from Conformité Européene (CE) since 2010. Five of them were approved by China Food and Drug Administration (CFDA), including Daan gene, ZJ BioTech, Puruikang, Tianlong, and HuaDa BioTech, and put into use in West Africa. The kit of Puruikang, which was applied by Chinese medical team, took the principal responsibility for testing Ebola virus in epidemic area. However, no evaluation for these commercial kits had been performed yet. In this circumstance, the National Center for Clinical Laboratories conducted the assessment of 7 Chinese commercial EBOV real-time reverse transcription PCR (RT-PCR) assays using virus-like particles (VLPs), concerning the analytical sensitivity and precision for the qualitative diagnostic reagents.

2. Materials and methods

2.1. Preparation of EBOV VLPs

The full-length sequences of 3′ long terminal repeat (LTR) (1–55 bp); NP (56–2340 bp); and parts of GP (6291–6500 bp and 7761–7850 bp), L (13051–13710 bp), and 5′ LTR (18,271–18,630 bp) of recently epidemic *Zaire* strain (GenBank: KJ660346, KJ660347, and KJ660348) were gene synthesized by Thermo Fisher Scientific (Beijing, China). The selected sequences were split into 2 parts, and each part

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was individually subcloned into the vector of pACYC-MS2. The recombinant plasmids of pACYC-MS2-NP and pACYC-MS2-GP/L were transformed into the *Escherichia coli strain BL21* (*DE3*). Two kinds of MS2 VLPs, respectively, contained the RNA of NP fragment along with 3′ LTR, and GP and L sequences along with 5′ LTR were prepared by inducible expressions of isopropyl-β-D-thiogalactopyranoside and purification by means of exclusion chromatography according to our previous protocols (Song et al., 2011; Sun et al., 2013).

The prepared VLPs were digested with DNase and RNase in order to eliminate the influence of nucleic acid out of the capsid of VLPs. Subsequently, the contents were extracted from the VLPs using the kit for viral nucleic acid extraction (Qiagen China, Shanghai, China). RT-PCR and ordinary PCR were conducted simultaneously to verify the target RNA (not DNA) encapsulated in VLPs, as shown in Fig. S1 (Supplemental material). After extracting the RNA in successfully prepared VLPs, the concentration was tested and estimated following the formula: (6.02×10^{23} copies/mol) × armored RNA mass (g/mL)/molar mass (g/mol) = concentration (copies/mL). Two kinds of VLPs were diluted individually by normal human serum as substitutes of clinical samples.

2.2. The characteristics of 7 commercial kits

In all participating assays in this evaluation, the CFDA had approved the kits from Daan gene, ZJ BioTech, and Puruikang in green channel for direct application in West Africa, and the kit of Puruikang was appointed as the specialized detection assay for Chinese medical team. Subsequently, Huada and Tianlong acquired qualification from CFDA. Meanwhile, the manufacturers of Sansure and Kehua developed the EBOV detection kits for alternative productions. The characteristics of the 7 commercial EBOV real-time RT-PCR detection kits are summarized in Table 1. Among all of the manufacturers for *Zaire* species detection, only Sansure preferred the magnetic bead–based method for RNA extraction and provided related reagents by themselves. Others took the conventional approach of spin column, and QIAamp viral RNA mini kit (Qiagen China) was selected in the process for nucleic acid extraction. Meanwhile, Ebola virus type testing could merely be carried out by the kit of Sansure, while others focused on the specific detection of *Zaire* species. The manufacturer of Puruikang chose GP fragment as detection site, while others mainly concentrated on NP site, which was reflected in Fig. 1.

2.3. Limit of detection

Two kinds of EBOV VLPs were, respectively, diluted in a 10-fold series and tested preliminarily from 10^7 copies/mL to 10 copies/mL. Applied Biosystems 7500 fast real-time PCR system was used for evaluation of 7 commercial kits. After the general scope of effective detection was determined, the endpoints for limitation were set. A series of diluted endpoints (500 copies/mL, 250 copies/mL, 100 copies/mL, 50 copies/mL, 25 copies/mL, and 5 copies/mL) were used to compare the different concentrations for limit of detection (LOD) studies. Meanwhile, the logarithmic transformation for copies was conducted correspondingly. The experiments for LOD were performed in 5 operating days, and every endpoint tested for 3 replicates in each time. Totally, 15 results for LOD were obtained, and each data had only 2 outcomes: detected or undetected (Table 2). The data were processed, and Probit analysis was used for regression analysis by SPSS version 19.0.

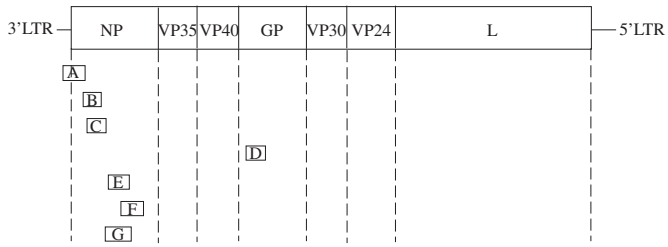

Fig. 1. Organization of Ebola virus genome and detected target regions for commercial assays. Ebola virus genome is arranged in the follow order: 3′LTR-NP-VP35-VP40-GP-VP30-VP24-L-5′LTR. The detection assays were substituted by letters. A: ZJ BioTech; B: Daan Gene; C: HuaDa BioTech; D: Puruikang; E: Tianlong; F: Sansure; G: Kehua. The detection regions for the 7 commercial kits were marked in the sketch map.

2.4. Precision

Combined with the results of LOD, the concentrations for precision detection were confirmed accordingly. The VLP-encapsulated GP fragment, with the concentrations of 10^5 copies/mL, 10^3 copies/mL, and 110 copies/mL, was used for the assessment of the kit of Puruikang. Meanwhile, the serial concentration of 8×10^4 copies/mL, 8×10^2 copies/mL, and 120 copies/mL for VLP-contained NP fragment was set to evaluate the remaining 6 kits. However, for the kit of ZJ Bio Tech, the lowest detection concentration was not included because of its high value of LOD. EBOV VLPs were diluted in advance and stored in 4 °C during the whole process for precision detection. The repeatability and reproducibility of the 7 commercial assays were individually completed by detecting the intra-assay and inter-assay variations for the crossing threshold (Ct) values. The intra-assay precision was determined from 20 replicates, while the inter-assay precision was calculated from 6 independent PCR runs with 3 replicates per run for each kit. Additionally, for the assay from Sansure Bio-Tech, the precision was only detected with the *Zaire* strain of Ebola virus instead of other 3 types. The data were analyzed, and the means, SDs, and coefficient of variation (CV) were calculated by SPSS version 19.0. The accredited range of CV for intra-assay and inter-assay variations of all evaluated assays was below 5%.

Table 1
Characteristics of 7 commercial EBOV real-time RT-PCR kits.

Commercial kits	Target region	Genotyping	Principle	Dye	IC (dye)	Quantitative	RNA extracted method	Approval agency
Daan gene	EBOV-NP	No	TaqMan	VIC	Yes (CY5)	No	Spin column	CFDA
ZJ BioTech	EBOV-NP, 3′ LTR	No	TaqMan	FAM	Yes (HEX)	No	Spin column	CE WHO CFDA
HuaDa BioTech	EBOV-NP	No	TaqMan	FAM	Yes (VIC)	No	Spin column	CFDA
KeHua	EBOV-NP	No	TaqMan	FAM	Yes (HEX or VIC)	No	Spin column	No
TianLong	EBOV-NP	No	TaqMan	FAM	Yes (HEX or VIC)	No	Spin column	CFDA
Sansure	EBOV-NP SUDV-NP TAFV-GP BDBV-NP	Yes	TaqMan	FAM	Yes (HEX)	No	Magnetic bead	No
Puruikang	EBOV-GP	No	TaqMan	FAM	Yes (HEX)	No	Spin column	CFDA

IC = internal control. Approval agency: CE = Conformite Europeenne; WHO = World Health Organization; CFDA = China Food and Drug Administration.