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Research Article

Rapid Detection of *Ebola Viruses* Causing Ebola Hemorrhagic Fever by Recombinase-Aid Amplification

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Abstract

In this study, we established a rapid and accurate method for the detection of Ebola virus (EBOV) by a novel technology of isothermal amplification (recombinase-aid amplification, RAA). The specific primers and TaqMan probe used in the real-time RT-RAA were designed based on the conservative regions of the nucleoprotein (NP) gene in the Ebola-Zaire. The synthetic DNA of the nucleoprotein gene in the Ebola-Zaire was used as a template. RAA enable the detection of 1×10^8copies/μL in 5 min and of 1×10^2 copies/μL in 25 min. A rapid, specific, sensitive and low equipment requirements method of real-time RT-RAA was successfully established, laying the foundation for rapid diagnosis of Ebola hemorrhagic fever.

Keywords: Ebola Virus; RT-RAA; Isothermal Amplification; Rapid Detection.

Introduction

Ebola hemorrhagic fever (EHF) is an acute haemorrhagic and zoonotic disease, which was caused by Ebola virus (EBOV) infection. The virus causes systemic bleeding, and it was first found in the Ebola River region of Southern Sudan and Democratic Republic of the Cong (Old Zaire) in 1976 [1,2]. Ebola virus (EBOV), a member of the Filovirus, carries a negative-sense RNA genome in virions, and contains viral envelope, matrix, and nucleocapsid components [3]. The genus EBOV contains 5 genetically distinct filoviruses: Ebola-Zaire (EBOV-Z), Ebola-Sudan (EBOV-S), Ebola-Bundibugyo (EBOV-B), Ebola Reston (EBOV-R) and Ebola Coast (EBOV-C) [4,5]. Two of them, EBOV-Z and EBOV-S, have high pathogenicity and mortality rate in human and non-human primates, and the mortality rate of the EBOV-Z can reach high up to 90% [6]. Thus, a rapid diagnosis for the dangerous EBOV-Z is needed urgently.

Several methods have been established for the detection of EBOV, for instance, IgM and IgG antibody capture ELISAs, RT-PCR, nested RT-PCR, RT-LAMP and so on [7,8,9,10,11]. Recently, the molecular detection method has been developed for rapid and accurate diagnosis of EBOV in the early. In this study, we aim to use a novel technology of isothermal amplification (recombinase-aid amplification, RAA) to establish a rapid detection method for EBOV-Z, which is characterized by extremely rapid, high sensitivity, specificity and can be higher throughput.

RAA is a isothermal amplification system which enable the enzyme system work at the constant temperature of 37 degree, the recombinase (RecA) deriving from the *E. coli* combines with DNA primers to form a RecA-Primers complex. The complex then binds to the complementary sequence of the primer sites in DNA templates. The enzyme system will turn the double-stranded DNA template into a single-stranded DNA at the site complement with the primer. The single-stranded DNA will open as the single-stranded DNA which enables the target site bind with the primers by the single-stranded DNA-binding protein (SSB). The primer is lengthened to form a new complete DNA strand of the template by the DNA polymerase and next amplification cycle repeat as above (Figure 1). With this amplification method, the target amplification can be finished within 30 minutes compare to traditional real time PCR [12]. Several diagnostics test have been developed using various kinds of isothermal amplification technologies to detect disease-causing organisms such as, *Dengue virus*, *Salmonella enterica*, *Mycobacterium tuberculosis*, *Methicillin-resistant staphylococcus aureus,* avian influenza (H7N9, H5N1), *streptococcus pneumoniae, orf virus* [13,14,15,16,17,18,19,20]. In this study, we established a sensitive, specific and rapid way to diagnose EBOV-Zwithreal-time RT-RAA, a kind of isothermal amplification.

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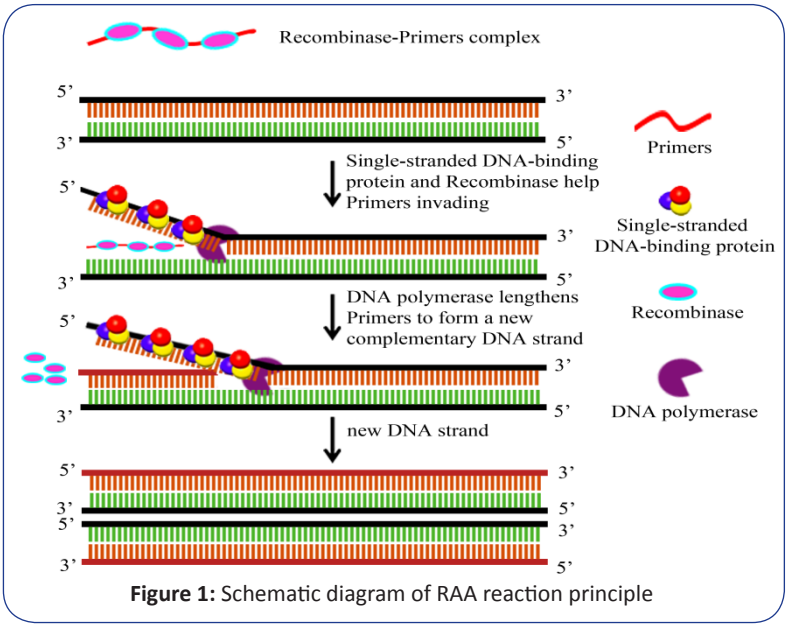

Figure 1: Schematic diagram of RAA reaction principle

Materials and Methods

Materials

The gene segment of nucleoprotein (NP) of EBOV is provided by the National institutes for Food and Drug Control, China.

Instrument and Reagent

RNA purification Kit (Invitrogen), Twista portable real-time fluorescent quantitative detector (TwistDx, UK), Dry Bath, Reverse transcription recombinase-aid amplification (RT-RAA Kit, RAA-Basic Kit, RAA-exo Kit (Zhejiang Taijing Bio-Sci & Tech Co. Ltd, China).

Primers and Probe

Three pairs of primers (three forward primers and three reverse primers) and one probe (table 3) were designed referring to twenty nucleoprotein sequences of EBOV-Z published in GenBank and used in determining the feasibility of the detection of EBOV-Z by real-time RT-RAA.

One-step Reverse Transcription-RAA (RT-RAA)

The RNA of rice and *bombyx mori* were extracted by the RNA purification Kit and kept at -20°C. The target sequence was amplified using the RT-RAA Kit with the mRNA as template (the mechanism is demonstrated in Figure 2. The formulation of the reaction system was shown in Table 1. The components in Table 1 were mixed vertically in 1 min and 47.5μL reaction mixture was added into lypholizated tube with RAA enzyme system. The dry powder of RAA and solution were mixed uniformly with a pipette. 2.5μL of 280 mM Magnesium Acetate was added in the tube and the reaction was incubated at 37°C for 40 min. After the amplification was completed, the tube was centrifuged at 12000g for 1 minute. 5μL of the supernatant was used to detect the present of specific amplicon by agarose electrophoresis.

Amplification of the Target Sequence

The RAA-Basic Kit was used to amplify the target sequence with 50μL reaction system formulated according to table 1. The steps were the same with above One-step RT-RAA.

Table 1: Formulation of the reaction system

Component	Volume/μL
Primer A (10μM)	2
Primer B (10μM)	2
Buffer (4×)	12.5
RNA/DNA template	1
dH_2O	30
Total	47.5

Fluorescent RT-RAA

The RAA-exo Kit was used for the fluorescent RT-RAA. 50μL reaction system formulated according to table 1 added with fluoresce probe (0.6μL) was used with RNase Free dH_2O as the negative control. The reaction was placed in the Twista portable real-time fluorescent quantitative detector and the reaction was performed at 37°C for 30 min. The fluorescent signal was measured per 20 seconds.

Result

One-Step RT-RAA for the Amplification of Target DNA Sequence

To determine the feasibility of the EBOV detection with RT-RAA, genes in rice (reference genes: HSP and Actin) and *bombyx mori* (reference genes: cytoplasmic actin A3and BmGAPDH) are selected

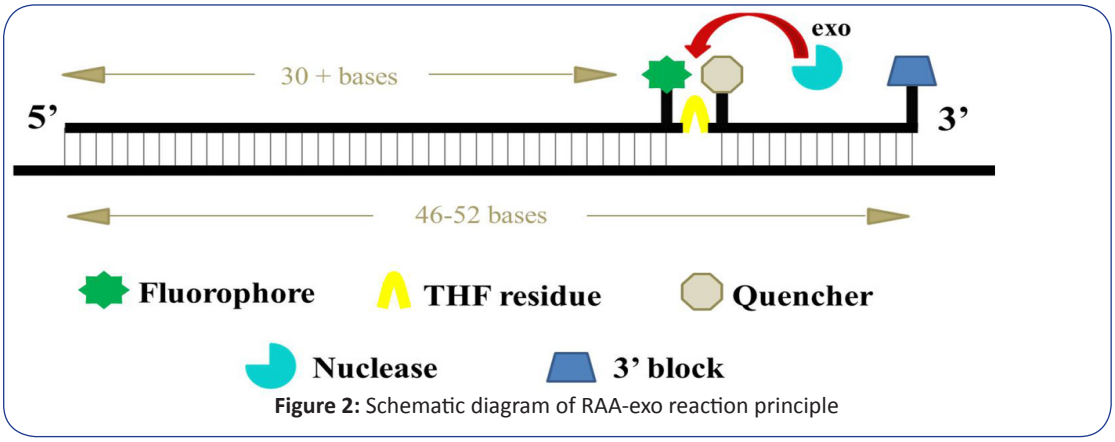

Figure 2: Schematic diagram of RAA-exo reaction principle

to be amplified by One-step RT-RAA (primer sequences are listed in table 3. Figure 3 and Figure 4 show the reverse transcription amplification of the target DNA sequence in rice and *bombyx mori*, respectively. The results show that the RT-RAA Kit can amplify the target DNA sequence in rice and *bombyx mori*.

Screening of Primers

The NP gene of EBOV was selected as the target gene in this study.

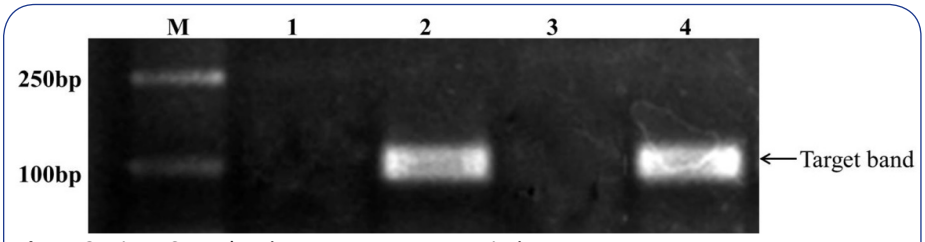

Figure 3: Rice HSP and actin gene reverse transcription

Lane:M, Marker; 1,3, negative; 2, rice HSP gene; 4, rice actin gene

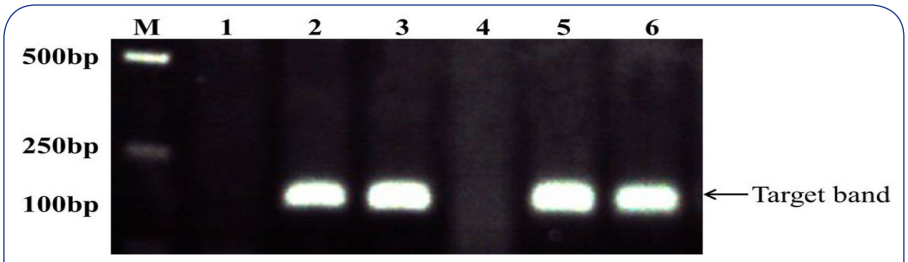

Figure 4: *Bombyx mori* cytoplasmic actin A3and BmGAPDH gene reverse transcription

Lane: M, Marker; 1,4, negative; 2,3, *Bombyx mori* cytoplasmic actin A3 gene; 5,6, *Bombyx mori* BmGAPDH gene

Table 2: Primers of the reference genes

Primer	Sequence (5'-3')	Size /bp
HSP-F	CTCAACATCTCACGTGAGATGCTCCAGCAG	120
HSP-R	GTTGTAGTCTTCCTTGTTCTCAGCGATCTC	
ACT-F	GCTATGTACGTCGCCATCCAGGCCGTCCTCTC	121
ACT-R	GAGCATATCCTTCATAGATGGGGACAGTGTG	
CAA3-F	GCCATCAGGGCGTGATGGTCGGCATGGGACAGAAG	176
CAA3-R	GGCGACACGCAGCTCATTGTAGAAGGTATGATGCC	
BmG-F	CATCATTCCTGCCTCTACTGGTGCTGCCAAAG	172
BmG-R	GACCTTTTGCTTGATGGCTTCATAGCTTGCAG	

As many internal studies indicated that the result and sensitivity of the isothermal amplification are impacted by primers. Three pairs of primers (NP-1, NP-2 and NP-3) were designed in this study (table 3) to test the sensitivities of the EBOV assay. Figure 5 shows that the products of the amplifications with different primers are different. A non-specificity band was presented with NP-1. Preferable results were obtained with using primer pairs NP-2 and NP-3, hence we used NP-2 and NP-3 for further experiments.

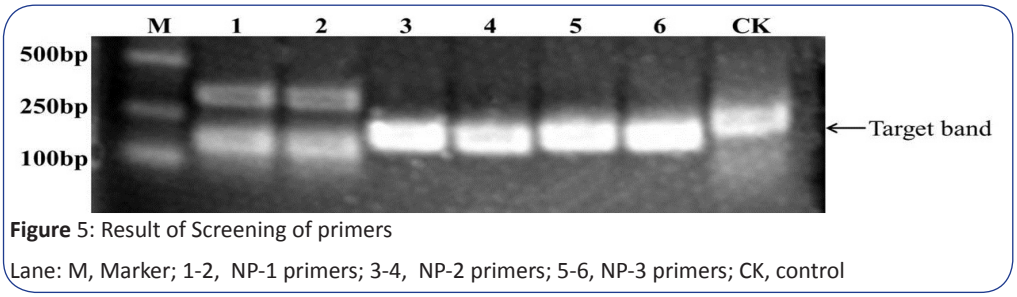

Figure 5: Result of Screening of primers

Lane: M, Marker; 1-2, NP-1 primers; 3-4, NP-2 primers; 5-6, NP-3 primers; CK, control

To determine the sensitivity of NP-2 and NP-3 for RT-RAA detection, amplifications with these two sets of primers are operated with templates diluted with 10 fold from 30pg. Results showed that the target band amplified with NP-3 was brighter than that of NP-2, even at a low template concentration (see Figure 6).T amplification with primer NP-3 for NP gene in EBOV-Z is more sensitive than that of primer NP-2. We then selected primer NP-3 for further experiments.

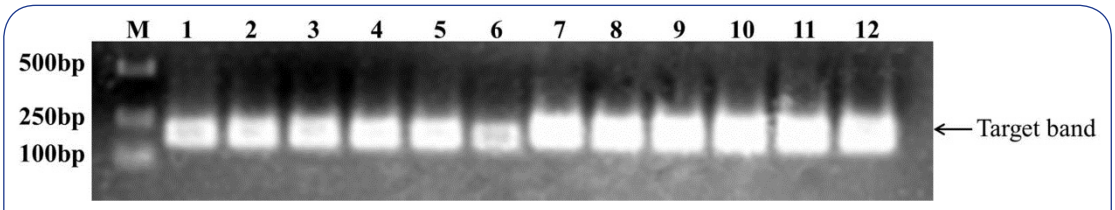

Figure 6: Sensitivity result of RAA amplification

Lane: M, Marker; 1-6, amplification with different concentrations of NP-2 primer (1-2, 30 pg; 3-4, 3pg; 5-6, 0.3pg); 7-12, amplification with different concentrations of NP-3 (7-8, 30 pg; 9-10, 3pg; 11-12, 0.3pg);

Based on the aforesaid results, NP-3 was selected as the primer of RT-RAA detection. A TaqMan fluorescent NP-3-Probe was designed according to the sequence in EBOV corresponding to NP-3amplicon (the sequence of the probe was showed in table 3).

RAA-Exo Fluorescent Detection for NP Gene Inebov-Z

The synthetic NP gene templates were diluted ten-times from 10^8copies/μL to 10^2copies/μL by RNase Free dH_2O and detected with RAA-Exo fluorescent kit as described in section 2.6. The positive result was considered if the fluorescence curve showed increasing fluorescent signal within 30 min. As shown in Figure 7, our assay was able to detection good signal at 10^2copies/μL DNA templates in 22 minutes of reaction. With high concentrate level of DNA template (10^8copies/μL), results can be detected as soon as 4 minutes with the negative control is kept at a low level of fluorescent signal.

Discussion

Researches on the rapid detection of EBOV have been performed through fluorescent RT-PCR with highly conversed sequences of EBOV nucleoprotein and glycoprotein (NP and GP) [21,22]. In

Table 3: Primers and probe of the NP gene in EBOV-Z

primer	Sequence (5'-3')	size/bp
NP-1-F	GAAATAGTTTAAAGACAAATTGCTCGGAATC	195
NP-1-R	CTCAAGATTGTTTACTTGATACACTGGGATG	
NP-2-F	GAACGAAATCAGCTTCCAGCAAACAAACGCG	193
NP-2-R	GTCGGATCATCATCTTGATGGCCAGGATTGTC	
NP-3-F	CATATGATGAAGGATGAGCCTGTAGTTTTCAG	201
NP-3-R	CAGGATTGCCATGAATTTATTCCTGTGATTC	
NP-3-Probe	TCCACCATGGCTCACTGAAAAAGAGGCCA(F)G(H)A(B)GATGAGAATA-GATTTG—Spacer C3*	

*(F) represents Fluorophore, (H) represents THF residue, (B) represents Quencher.

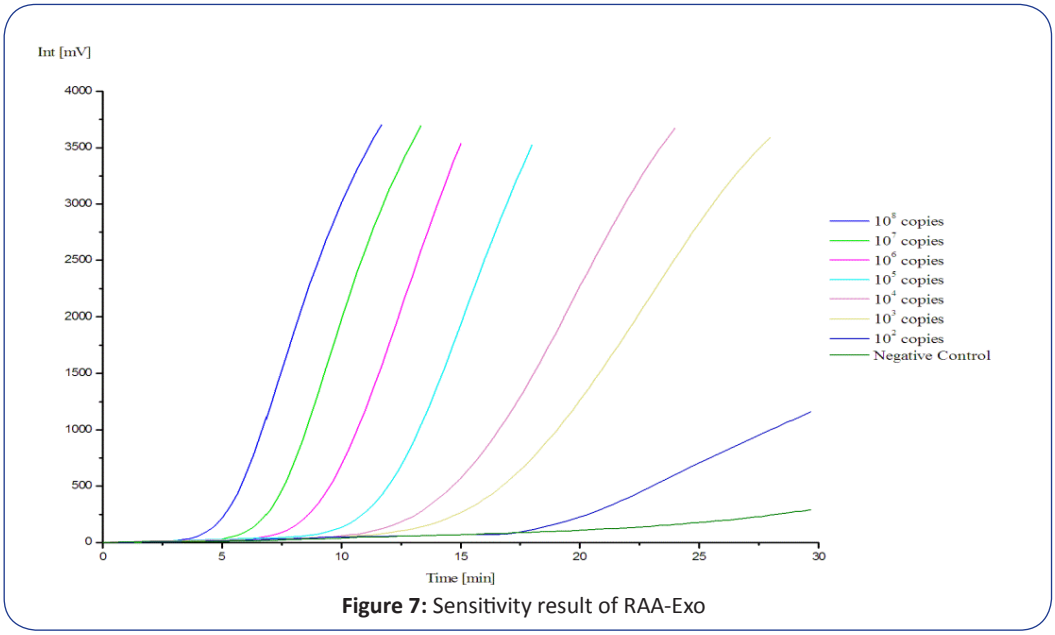

Figure 7: Sensitivity result of RAA-Exo

this study, we screened out a conversed region with high homology in NP gene sequence according to the 20 gene sequences of EBOV published on Gen Bank, designed specific primers and probes according to this region, and successfully established a rapid detection method for EBOV-Z by using fluorescent RT-RAA.

Recombinase-aid amplification (RAA), a novel technology of isothermal amplification of nucleic acid was employed in this study, which can lead to a rapid and accurate quantitative analysis to the target sequence. Furthermore, this technology shows high sensitivity and specificity. Compared to traditional PCR, the reaction time required for RAA is much shorter, and the instruments required by RAA are simpler. Only a small thermostat such as a water-bath or a metal-bath, even a constant temperature incubator, is sufficient to perform RAA reaction. Additionally, RAA also has following benefits: specific bands obtained are consistent with PCR product and the amplicon can be sequenced directly or linked to a vector; the primer designing is simple, which is identical to that of PCR except the length of primers; the amplification is performed at a constant low temperature, such as 37°C; the amplification is completed rapidly. Real Time quantitative analysis can be achieved through RAA using fluorescently-labeled probe; and the product also can be detected using biotin labeled primer combining with colloidal gold test strip.

In this study, the measurement of EBOV-Z through fluorescent RAA reverse transcription kit resulted in a rapid and accurate quantitative analysis for NP gene of EBOV-Z. This measurement has high sensitivity and specificity. EBOV-Z can be detected rapidly at a low constant temperature by using the RAA fluorescence detection kit. RAA fluorescence detection kit can be more sensitive than traditional PCR in EBOV-Z detection [22]. Particularly, the kit can detect as little as 10^2copies. The reaction time is also shorter, e.g. 10^2copies can be detected within 19 min. Further, this detection method is simple and convenient, which even doesn't need precise instrument and professional skills. It only requires a simple portable device for fluorescent signal detection.

Due to the specificity of the Ebola hemorrhagic fever sample, the experiments in the current manuscript did not use real strains of Ebola to avoid accidental release/contamination, however, through experiments of one-step RT-RAA amplification of HSP and actin gene of rice and cytoplasmic actin A3 and BmGAPDH of *bombyx mori*, we were able to fully demonstrate that RAA is feasible metnod to amplify RNA in an efficient and rapid manner. In sum, we have established a diagnostic method of Ebola virus by RAA fluorescence reverse transcription method rapid, sensitive and specific. It is very suitable to apply to the testing of DNA/RNA in a need of rapid diagnosis such as Ebola hemorrhagic fever clinically.

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