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Recombinase polymerase amplification assay for rapid detection of Rift Valley fever virus

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ABSTRACT

Background: Detection of nucleic acids of Rift Valley fever virus (RVFV) has been shown to be useful in field diagnostics.
Objectives: To develop an isothermal 'recombinase polymerase amplification (RPA)' assay on an ESEquant tubescanner device.
Study design: RPA was adapted for RNA amplification by first developing a two-step and then a one-step-RT-RPA protocol. Several RT enzymes were tested and the best sensitivity was achieved using Transcriptor (Roche). Finally an RT-RPA pellet containing a recombinant MuLV was tested in RVFV one-step-RT-RPA.
Results: The one-step-RT-RPA assay showed a sensitivity of 19 molecules detected as determined by probit analysis of eight runs using a RVFV S-segment based quantitative RNA standard and detected 20 different RVFV strains. The assays showed no cross detection of the human genome and several agents of a typical biothreat panel. It performed almost as good as the assay using glycerol buffer based Transcriptor albeit at a cost of 1-$\log_{10}$ step in sensitivity. The presented combination of one-step-RT-RPA and portable fluorescence reading device could be a useful tool for field or point of care diagnostics.
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1. Background

Rift Valley fever (RVFV) is a zoonotic virus causing disease in ruminants and man. It is classed as a transboundary disease in Africa by the FAO and outbreaks of RVFV have been associates with high economic costs.[1,2] Several diagnostic real time PCRs for RVFV detection have been described[3–5] and shown to be of good value in mobile diagnostics.[6]

Recombinase polymerase amplification (RPA) is an isothermal exponential nucleic acid amplification and detection method. In this reaction the phage derived recombinase UvsX assisted by its co-factor UvsY forms a nucleoprotein complex with oligonucleotide primers to scan for homologous sequences in a DNA template. Upon identifying the specific homologous sequence strand invasion is initiated followed by strand displacement amplification via Sau polymerase (*Staphylococcus aureus*). Starting from opposite primers amplified dsDNA copies very much like in PCR are generated albeit at only one temperature (42 °C). Real time signal detection of the amplification event can be performed by using TwistAmp™ Exo probes. These synthetic oligonucleotides (45–55 nt) carry an internal fluorophore and suitable quencher in close proximity to each other, both linked to thymine bases separated by an abasic sitemimic (tetrahydrofurane). These internal modifications are localized approximately 15 nucleotides from the 3′ end of the probe. Upon binding to a target DNA molecule the abasic site is recognized and cleaved by Exonuclease III, prompting the release of the smaller downstream probe section carrying the quencher and thus the separation of flurophore and quencher. The resulting fluorescent signal accumulates proportionally to the RPA mediated amplification.[7]

2. Objective

To develop a highly sensitive and specific fluorescent real time RT-RPA assay for the detection of Rift Valley fever virus using an easily readable colorimetric system.[8,9]

3. Study design

3.1. Virus culture, RNA preparation

Virus strains are listed in Table 1 and were grown on VeroE6 cells and RNA extracted as described. A S-segment based RNA standard was used as described.[10]

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Table 1
Primers for standard and for RPA assay.

Name	Sequence 5′–3′
RVFV RPA FP	CATTTTCATCATCATCCTCCKGGGCTTRTTG
RVFV RPA RP	GARCTCYTAAAGCAGTATGGTGGGGCTGACT
RVFV RPA P	GGGAGAAGGATGCCAAGAAAATGATTGT (BHQ1-dT) (THF) (FAM-dT) GGCTCTRACTCGTG (phosphate)

RVFV RPA FP/RP RPA primer, RVFV RPA P: RPA Exo probe; BHQ1-dT: thymidine nucleotide carrying Blackhole quencher 1; THF: tetrahydrofuran spacer; FAM-dT: thymidine nucleotide carrying Fluorescein; phosphate: 3′ phosphate to block elongation.

3.2. Real time RT-RPA amplicon design

Primers were designed for the S-segment using sequences with the following accession numbers: RVFV: AF134530-41, AF134543, AF134545-51, NC_002045, Y53771, D0380152-81, EU574057-87. The RPA amplicon for the detection of RVFV was designed for East- and West-African strains as well as the attenuated strains MP12 and clone 13.[11,12] The RPA probe was synthesized by TIBMOLBIOL using an inverse arrangement of fluropohore and quencher (BHQ1-dT) (THF) (FAM-dT).

3.3. cDNA synthesis and RPA conditions

A fresh dilution range from 10^7 to 10 molecules/μl of the RNA standard was performed as described.[10] For two-step RT-RPA 2 μl of each dilution step, 100 nM M13 primer, 10 μM dNTPs, 20 U RNaseOut Inhibitor (Invitrogen, Darmstadt, Germany), 10 U Transcriptor Reverse Transcriptase and 1× RT reaction buffer (Roche, Mannheim, Germany), were mixed in a 20 μl volume and incubated at 55 °C for 30 min, inactivated at 85 °C for 5 min and cooled on ice.

RPA was performed in a 50 μl volume using the TwistAmp™ exo kit (TwistDx, Cambridge, UK) 420 nM RPA primers and 120 nM RPA-probe, 2 μM DTT, 14 mM Mg acetate and TwistAmp™ rehydration buffer. All reagents except for the template or sample DNA and Mg-acetate were prepared in a mastermix, which was distributed into each tube of the 0.2 ml reaction tube strip containing a dried enzyme pellet. Mg-acetate was pipetted into the tube lids. Subsequently 1 μl cDNA was added to the tubes. The lids were closed and the Mg-acetate centrifuged into the tubes using a minispin centrifuge and the tubes immediately placed into the tubescanner device (Qiagen Lake Constance, Stockach, Germany). For one-step-RT-RPA the following ingredients were added to the RPA mastermix: 10 U Transcriptor (Roche, Mannheim, Germany), 20 U RNaseOut and 19 mM DTT. Other RT enzymes tested were Sensiscript (Qiagen, Hilden, Germany), MuLV (TwistDx, Babraham, UK). A TwistDx prototype RT-RPA kit containing 4 different concentrations of MuLV in the dried pellets was also used.

Fluorescence measurements were performed in an ESEquant tubescanner at 42 °C for 20 min. It contains a mobile sophisticated fluorescence sensor, which slides back and forth under a set of 8 tubes collecting fluorescence signals over time allowing for real time documentation of increasing fluorescence signals. A combined threshold and signal slope analysis is used for signal interpretation which can be confirmed by 2nd derivative analysis.[8,9]

3.4. Determination of sensitivity and specificity

The 2-step-RPA and the 1-step-RT-RPA were tested using the quantitative RNA standard in 8 replicates, the threshold time was plotted against molecules detected and a semi-log regression was calculated. For exact determination a probit regression was performed using the Statistica software (StatSoft, Hamburg, Germany). For cross detection studies we tesetd human genomic DNA, infectious agents of a biothreat panel (DNA: *Yersinia pestis*, *Francisella tularensis*, *Bacillus anthracis* and of Vaccinia virus; RNA: Ebola virus, Sudan virus, Marburg virus), RNA of Nairoviruses (Crimean-Congo Hemorrhagic Fever virus, Erve virus) and Phleboviruses (Sandfly Sicillian virus, Sandfly Turkey virus, Toscana virus, Sandfly Naples virus).

4. Results

4.1. Sensitivity of RT-RPA

In order to determine if RPA can be used for RNA targets cDNA produced from a RVFV S-segment based RNA standard using the Transcriptor enzyme was subjected to RPA. Using this approach an analytical sensitivity of 10 molecules was achieved (Fig. 1A). Then a one-step RT-RPA was performed by adding Transcriptor to the RPA mix. We tested several additives and found that the addition of DTT improved the RT-RPA leading to the same analytical sensitivity as in two-step-RT-RPA (Fig. 1A). The standard deviation of the threshold time values for 10^7 down to 10 molecules, ranged from 0.13 to 2.64 min in 2-step-RT-RPA and improved to 0.21–0.84 min in 1-step-RT-RPA indicating better reproducibility in RT-RPA. The slopes of the standard regression lines were 0.69 and 0.31, respectively. A probit analysis using the results of 8 runs of the one-step-RT-RPA predicted that in 95% of cases a minimum of 19 molecules are detected (Fig. 2). Additionally the RPA assay was tested with the RNA of 18 different RVFV strains representing East-, Central- and West-Africa (Table 2). All RVFV strains tested were detected but the limit of dilution of the individual strains was not tested.

4.2. Specificity

In cross detection studies the RPA was tested with human genomic DNA, DNA and RNA of organisms of a biothreat panel, 2 Nairoviruses and 4 Phleboviruses. The RVFV RT-RPA did not detect any of the tested nucleic acids.

Table 2
RVFV strains tested.

No.	Strain	Source	Country	RPA detection
1	73 HB 1230	Human	Central African Rep.	+
2	Ar B 1986	Mosquito	Central African Rep.	+
3	Ar Mg 811	Mosquito	Madagascar	+
4	Ar Mg 992	Mosquito	Madagascar	+
5	An Mg 990	Bovine	Madagascar	+
6	An K 6087	Bat	Guinea	+
7	H D 48,169	Human	Mauritania	+
8	H D 48,188	Human	Mauritania	+
9	H D 47,380	Human	Mauritania	+
10	H D 48,263	Human	Mauritania	+
11	An D 133,723	Animal	Mauritania	+
12	Ar D 141,896	Mosquito	Mauritania	+
13	SH M 169,867	Human	Mauritania	+
14	SH M 169,868	Human	Mauritania	+
15	SH M 169,885	Human	Mauritania	+
16	SH M 169,898	Human	Mauritania	+
17	SH M 172,776	Human	Mauritania	+
18	SH M 182,805	Human	Mauritania	+

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