Development of two-step recombinase polymerase amplification assays for incorporation into a multiplexed platform facilitating the identification of the causative agents of viral hemorrhagic fever

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Background

- Viral hemorrhagic fevers (VHFs) are a group of infectious diseases that interfere with the body's ability to clot blood, often leading to serious complications including death.
- The clinical presentation of VHF's are non-specific, making it challenging to distinguish by symptoms alone, especially from other infections like Malaria that may be co-circulating.
- There is a need to correctly detect and differentiate these infections, to ensure the correct treatments and control measures are implemented.
- Multiplex nucleic acid tests enable the screening of multiple targets at once, with techniques like recombinase polymerase amplification (RPA) allowing for rapid detection without the need for complex instrumentation.
- However, RPA assays are often constricted by the degree of multiplexing that can be carried out concurrently per reaction, while the complex RNA secondary structure has been known to reduce the sensitivity of a one-step RT-RPA reaction. We explored developing a platform incorporating a two-step RPA system, whereby RNA is first reverse transcribed separately, enabling more efficient cDNA generation subsequently amplified by a panel of assays.

Methods

Development of Two- step multiplex assay

- Five previously described one-step RPA assay assays for the detection of Malaria[1,2], Dengue[3] and Ebola[4] were modified to for a two step RT-RPA system. The optimum conditions for cDNA generation were determined using the Dengue and Ebola assay,
- The optimum cDNA conditions (Table 1) were then applied to all 5 assays and sensitivity and specificity of the two-step system evaluated as compared to one- and two-step qRT-PCR and one-step RT-RPA
- Serial dilutions from 0-500 copies per RPA reaction tested in triplicate

Development of RPA assay for detection of Lassa virus

- Conserved regions within the glycoprotein precursor (GPC) gene and the L gene were chosen for the design of candidate RPA primers and internal probes for two respective assays.
- Various Primer combinations screened for the ability to amplify 500 cDNA from a Lassa virus isolate in real-time.
- Negative controls were included.

Results

- Using the dengue and Ebola RPA assays we determined the optimum conditions for reverse transcription to make it compatible with RPA (Table 1).

Condition (per reaction)	Range evaluated	Dengue		Ebola		Recommended for both assays
		Optimum for max cDNA generation	Minimum for RT to proceed	Optimum for max cDNA generation	Minimum for RT to proceed	
Reverse primer	Random hexamers, Oligo(dT) Primers, assay specific reverse primers	Random hexamers	Random hexamers	Random hexamers	Random hexamers or Oligo dt primer	Random hexamers
Reverse primer concentration	1.25µM, 2.5µM, 3.75µM, 5µM	2.5µM	1.25-5µM	5µM	1.25-5µM	3.75µM
Incubation Temperature	37°C, 42°C, 55°C	42°C	37-55°C	42°C	37-55°C	
Incubation time	5 mins, 15 mins, 30 mins, 60 mins	30 mins		60 mins	>5 mins	≥ 30 mins
Total dNTP concentration	1mM, 2.5mM, 5mM, 10mM	1mM	1-5mM	5mM	1-5mM	2.5mM
Affinity Script Reverse transcriptase concentraion	5U, 10U, 25U, 50U	10U	5-50U	5U	5-50U	10U
Addition of DTT	With vs Without	With DTT	Either	With DTT	Either	With DTT
Reaction Buffer	Affinity script buffer vs RPA Rehydration buffer	Affinity Script	Either Buffer	Affinity Script	Either Buffer	Affinity Script
Optimum volume to add to RPA reaction per 20µL RT reaction	1µL, 2.5µL, 5µL, 10µL	10µL	1-10µL	10µL	1-10µL	10µL

Table 1. Optimum Reverse transcription assay conditions for generating cDNA suitable for two step-RPA

- The two-step RT-RPA system was found to be compatible with all assays used, showing comparable results to the one-step system (Table 2).
- Detection was generally earlier as cDNA was immediately available for amplification, with a stronger fluorescent output noted which may be beneficial at lower concentrations.
- qRT-PCR was found to be more sensitive for most assays apart from the pan plasmodium assay which performed best with the two step RT-RPA system, however the RT-RPA assays were all faster than the qRT-PCR assays.
- For the Lassa virus assay, seven forward and seven reverse primers were designed for both an assay targeting the GPC gene and one targeting the L gene, with 49 different primer combinations tested.
- Only one primer combination with the L gene generated a positive result, however not all replicates amplified.
- Amplification was noted with the GPC primer set, however the NTC's also generated a positive result indicating that the amplification was not specific to Lassa virus.

Dengue (Serotype 2)	One-step RPA	Two-step RPA	One-step qRT-PCR	Two-step qRT-PCR
500C	2/3	3/3	3/3	3/3
250C	2/3	3/3	3/3	3/3
100c	1/3	1/3	3/3	3/3
50c	1/3	2/3	3/3	0/3
25c	1/3	0/3	3/3	1/3
10c	1/3	0/3	1/3	0/3
5c	1/3	0/3	1/3	0/3
0	0/3	0/3	0/3	0/3
Ebola (Sudan)	**One-step RPA**	**Two-step RPA**	**One-step qRT-PCR**	**Two-step qRT-PCR**
500C	3/3	3/3	ND#	ND#
250C	2/3	3/3	ND#	ND#
100c	3/3	3/3	ND#	ND#
50c	2/3	3/3	ND#	ND#
25c	3/3	3/3	ND#	ND#
10c	1/3	3/3	ND#	ND#
5c	1/3	3/3	ND#	ND#
0	0/3	0/3	0/3	0/3
Ebola (Zaire)	**One-step RPA**	**Two-step RPA**	**One-step qRT-PCR**	**Two-step qRT-PCR**
500C	0/3	3/3	3/3	3/3
250C	0/3	3/3	3/3	3/3
100c	0/3	2/3	3/3	3/3
50c	0/3	2/3	3/3	3/3
25c	0/3	0/3	3/3	3/3
10c	0/3	0/3	3/3	2/3
5c	0/3	0/3	2/3	1/3
0	0/3	0/3	0/3	0/3
Malaria pan-plasmodium	**One-step RPA**	**Two-step RPA**	**One-step qRT-PCR**	**Two-step qRT-PCR**
500C	3/3	3/3	3/3	3/3
250C	3/3	3/3	3/3	3/3
100c	3/3	3/3	3/3	3/3
50c	3/3	3/3	3/3	2/3
25c	3/3	3/3	3/3	1/3
10c	3/3	3/3	1/3	0/3
5c	2/3	3/3	0/3	0/3
0	0/3	0/3	0/3	0/3
Malaria *P. falciparum*	**One-step RPA**	**Two-step RPA**	**One-step qRT-PCR**	**Two-step qRT-PCR**
500C	3/3	3/3	3/3	3/3
250C	3/3	3/3	3/3	3/3
100c	2/3	3/3	3/3	3/3
50c	0/3	2/3	3/3	3/3
25c	1/3	2/3	3/3	3/3
10c	1/3	0/3	3/3	3/3
5c	0/3	1/3	0/3	2/3
0	0/3	0/3	0/3	0/3

Table 2. Performance of each assay with serial dilutions of RNA

qRT-PCR assay used as reference was not specific for this particular isolate

Conclusions and Recommendations

Figure 1. Prototype platform design currently under development

- Two step detection offers improved sensitivity over one step RT-RPA
- One step assays can be easily converted to two step format using conditions describe
- Development of Lassa virus RPA challenging. Further assessment with new primer designs required
- Working with Redbud labs to integrate a near point-of-care platform that integrates sample preparation with amplification and detection (Figure 1).

References

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