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Development of a panel of Recombinase Polymerase Amplification assays for the detection of biothreat agents

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Syndromic panels for infectious disease have been suggested to be of value in point of care diagnostics for developing countries and for biodefense. To test the performance of isothermal Recombinase Polymerase Amplification (RPA) assays we developed a panel of ten RPAs for biothreat agents. The panel included RPA assays for *Francisella tularensis*, *Yersinia pestis*, *Bacillus anthracis*, variola virus, and Recerse Transcriptase Recombinase Polymerase Amplification (RT-RPA) assays for Rift Valley fever virus, Ebola virus, Sudan virus and Marburg virus. Their analytical sensitivities ranged from 16-21 molecules detected (probit analysis) for the majority of RPA and RT-RPA assays. A magnetic bead based total nucleic acid extraction method was combined with the RPA assays and tested using inactivated whole organisms spiked into plasma. The RPA showed comparable sensitivities to real time RCR assays in these extracts.

The run times of the assays at 42°C ranged from 6-10 minutes and they showed no cross detection of any of target genomes of the panel nor of the human genome. The RPA assays therefore seem suitable for implementation of syndromic panels onto microfluidic platforms.
Introduction

Syndromic panels for infectious and emerging infectious diseases have been suggested to be of value in point-of-care (POC) diagnostics for developing countries and for biodefense (29). Since the introduction of molecular diagnostics and in particular real time PCR, ample proof of its sensitivity and specificity has been generated. Indeed molecular diagnostics are deemed superior to bacterial culture techniques or serological diagnostics (6, 34, 44). It has even been suggested to entirely eliminate the old methods in order to streamline centralised laboratories for molecular diagnostics (5, 13, 14).

In recent years alternative isothermal amplification methods which can be categorized into (i) T7 promotor driven amplifications (transcription mediated amplification (TMA), Nucleic Acid Sequence-Based Amplification (NASBA), Single primer isothermal amplification (SPIA)), (ii) strand displacement methods (Strand Displacement Amplification (SDA), Loop-mediated isothermal amplification (LAMP), Smart amplification (SmartAmp)), (iii) helicase dependent amplification (HDA), (iv) recombinase polymerase amplification (RPA), and (v) rolling circle amplification (RCA) methods (1, 3, 12, 17, 36) have been developed. Some were purposely designed for isothermal amplification starting from RNA (TMA, NASBA, SPIA), whereas others initially targeted DNA (SDA, LAMP, HDA, RPA, RCA) and were only later adapted for RNA targets. Nonspecific intercalating fluorophores or fluorescent primers have been used for real time detection in LAMP, SDA, HDA and RCA, and specific detection probe formats have been developed for NASBA, RCA, HDA and RPA (24, 28, 31, 37, 38).

In isothermal and exponential recombinase polymerase amplification (RPA) the phage recombinase UvsX and its co-factor UvsY form a nucleoprotein complex with
oligonucleotide primers to scan for homologous sequences in a DNA template. Recognition of a specific homologous sequence leads to the initiation of strand invasion of the complex and the opposing oligonucleotides are then extended by isothermal (42°C) strand displacement amplification via Sau polymerase (Staphylococcus aureus) yielding dsDNA amplificates very much like in PCR. Real time amplificate detection can be performed by using TwistAmp™ exo-probes. Exo-probes carry internal fluorophore and quencher linked to thymine bases and separated by an abasic site mimic (tetrahydrofuran) localized approximately 15 nucleotides upstream from the 3'end of the probe (45-55 nucleotides (nt)). Once the probe hybridizes to its target sequence the abasic site is recognized and cleaved by Exonuclease III. The smaller downstream probe section carrying the quencher is released and fluorescence develops proportionally to the RPA mediated amplification (31).

The second probe type for real time fluorescent detection is the TwistAmp™ fpg probe, a 30nt oligonucleotide, which carries a quencher at the 5’end and the fluorophore at an internal position 4-5nt downstream from the quencher via a C-O-C linker (or dR group). During hybridisation of the probe the linker is cleaved by the DNA glycosylase FPG (E. coli), thus causing separation of fluorophore and quencher and subsequently the proportional increase of fluorescence.

The purpose of the study to develop a panel of RPA assays for a POC microfluidic platform. We describe the development of highly sensitive and specific fluorescent real time RPA and RT-RPA assays for the detection of relevant Category A Bioterrorism Agents including gram+, gram- bacteria, and DNA and RNA viruses on the mobile ESEquant Tubescanner device. This mobile small footprint device collects fluorescence signals over time allowing for simultaneous real time documentation of increasing fluorescence signals in a 8 tube strip (15, 33).
Materials and Methods

Quantitative molecular standards

Bacteria: Quantitative pCRII backbone plasmid standards were generated for the pagA gene (Bacillus anthracis plasmid pX01), pla gene (Yersinia pestis) as described in (40). A capC gene carrying plasmid (B. anthracis, plasmid pX02) was provided by the Robert-Koch-Institut (7). DNA virus: The Variola virus (VARV) HA gene was synthesized and ligated into pMA-RQ by Geneart, Regensburg, Germany, the Vaccinia virus (VACV) plasmid carrying the LE gene was provided by the Robert-Koch-Institut (19). RNA Viruses: A quantitative Ebola virus (EBOV), Sudan virus (SUDV), Marburg virus (MARV) NP-gene RNA standards were used as described (41, 43). A new quantitative Sigma virus (SIGV) G gene based RNA standard was generated and transcribed as described (43).

Viral and bacterial material

Genomic DNA of Orthopox viruses (Vaccinia virus (Elstree 5), Camelpox virus (CP19), Monkeypox virus (MP4) and Orthopox virus (OPV 90/3) was provided by Hermann Meyer, Institute of Microbiology, German Armed Forces. Inactivated and gamma-irradiated bacteria and viruses were provided by the following institutes: B. anthracis spores (ATCC 14578), Y. pestis (03-1501) and F. tularensis (Ft 12) by Centre for Biosecurity 2, Robert Koch Institute, Berlin; Vaccinia virus NYCBH strain (VR-1536), and Rift Valley fever virus (ZH548) by Centre for Biosecurity 1, Robert Koch Institute, Berlin; Marburg virus (Musoke strain) and Ebola virus (Zaire strain) by Bernhard-Nocht Institute, Hamburg; and Sigma virus by Institute of Virology, Göttingen. The organisms were cultured in the donating institutions at biosafety 3 or 4 levels.
Real time PCR

The quantitative standards for *B. anthracis*, capC and pagA, *Y. pestis* pla, *F. tularensis* tul4, VACV, RVFV, EBOV, SUDV and MARV were tested using published real time PCR protocols (19, 41, 43). A new real time PCR amplicon was designed for the SIGV G gene and for the VARV HA gene. Real time PCR assays for DNA and RNA targets were performed using the LightCycler® Fast-Start DNA Master HybProbe kit and the LightCycler® 480 RNA Master Hydrolysis Probes respectively on a Light Cycler 2.0 (Roche, Mannheim, Germany) using the 2nd derivative method for analysis. All real time PCR assays showed the sensitivities reported in the original publications. The SIGV and the VARV assays showed analytical sensitivities of 10 molecules detected per reaction.

RPA-conditions

RPA was performed in a 50µl volume using the TwistAmp™ exo kit (TwistDX, Cambridge, UK) 420nM RPA primers and 120nM RPA-probe, 14mM 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 0.2 ml reaction tubes each containing a dried enzyme pellet. Mg–acetate was pipetted into the tube lids. Subsequently 1µl standard DNA or genomic DNA was added to the tubes. The lids were closed and the Mg-acetate centrifuged into the tubes using a minispin centrifuge and the tubes were immediately placed into a ESEquant tubescanner device (Qiagen Lake Constance, Stockach, Germany).

For RT-RPA 10U Transcriptor (Roche, Mannheim, Germany), 20U RNaseOut, 2mM DTT and 22.4 mM Mg acetate were added to the DNA-RPA mix described above. The same amount of primers and TwistAmp™ fpg probe were used with the rehydration buffer and the enzyme pellets of the TwistAmpTM fpg kit. Subsequently
1µl cDNA was added to the tubes. Fluorescence measurements (Excitation 470nm, Detection 520nm (FAM channel)) were performed at 42°C for 20 minutes. This reaction temperature was determined as optimal in terms of sensitivity from a temperature range of 39°C to 42°C. The Tubescanner software permits evaluation of the increase of fluorescence above three standard deviations over the background determined in minute one (adaptable) i.e. threshold validation. Additionally the slope of the curve as mV/time can be used (slope adaptable) i.e. slope validation. For confirmation the calculation of the 2nd derivative of the turning point of the upward fluorescence development can be applied to individual fluorescence curves with a very low slope (15, 33).

**Determination of sensitivity and specificity**

All quantitative DNA and RNA standards were tested by RPA in 8 replicates, the threshold time (in minutes) was plotted against molecules detected and a semi-log regression was calculated. For exact determination probit regression (35) was performed and the sensitivity at 95% calculated using the Statistica software (StatSoft, Hamburg, Germany).

In order to assay the sensitivity of extraction and detection in samples containing representative whole organisms of each category in the panel, inactivated, *B. anthracis* spores (gram positive), *Y. pestis* (gram negative), VACV (DNA virus), and RVFV (RNA virus) were diluted in 10-fold steps in PBS and spiked into plasma to achieve a final concentration of $10^1$ - $10^4$ genomic copies / ml. Additionally, 2 µl of Sigma virus in a concentration of $10^5$ genome genomic copies / ml were added to the prepared spiked plasma dilutions to monitor the performance of the extraction procedure.
Total nucleic acids from all bacterial and viral pathogens were prepared spiked plasma samples using the single innuPREP MP Basic Kit A (Jena Analytik, Jena, Germany) a magnetic bead separation rack combined with proteinase K treatment according to the manufacturer’s instructions. The nucleic acids were eluted in 100µl of nuclease-free distilled H₂O and 5µl were subjected to PCR or RPA.

Results

Amplicon design

The design of the RPA primers differs from PCR primers, as the minimum length of 30-35 nt rather than the TM guides design. Since it is not clear which features of the 5-prime end sequence of the primer actually supports the initiation of strand invasion typically several primer pairs have to be tested. On average 3 and at maximum 8 primer pairs were tested and the final amplicon lengths in general ranged from 107nt to 164nt (table 1). Due to the high homology among the Orthopoxvirus sequences the most challenging design was that for VARV. In the final design the RPA probe overlaps the upstream primer sequences by four nucleotides and covers a gap in the Variola sequence, which is not present in the other Orthopoxvirus sequences. Additionally the downstream primer mismatched all other Orthopoxvirus sequences at position 3 down from the 3-prime end to specifically select for the VARV sequences according to the ARMS principle (27). A RPA assay for VACV was designed for the same region for use in the extraction experiments.

Assay development steps for RT-RPA

The detailed development of DNA-RPA and RT-RPA was described for the assays for F. tularensis assay and RVFV respectively elsewhere (9, 10).
We here additionally describe comparison of the performance of the fluorescent TwistAmp™ exo-probe and the TwistAmp™ fpg probe in RT-RPA. We designed exo- and fpg-probes for the same target regions for EBOV, MARV and SIGV and tested them on the respective quantitative RNA standards. The sensitivities for the TwistAmp™ fpg probe assays were respectively 3-, 6-, and 3-log_{10}-steps lower than the sensitivities of the TwistAmp™ exo-probe assays. The results for EBOV are shown in Fig 2A.

**RPA sensitivity**

Quantitative molecular plasmid and RNA standards were generated and verified by real time PCR (data not shown) and used to test the analytical sensitivity of all final RPA assays (Figure 3AB, table 3). The analytical sensitivity of the DNA-RPA assays was about 10 molecules detected (md) or as per probit analysis 16-19 md per reaction. Only the assay for the capC gene of *B. anthracis* plasmid pX02 showed a lower sensitivity of 100-1000 md or as per probit analysis 778 md per reaction (Figure 3A, Table 3). The standard deviation of the standard curve threshold time values ranged from 0.1 at 10^7 molecules to 2.6 at 10 md. The slopes of the semi-log standard regression lines (SRL) ranged from -0.33 to -0.96 indicating an efficiency of 1072 to 11 if using the formula \( E = 10^{1/\text{slope}} \) used for real time PCR assays which at an idealised exponential efficiency have an efficiency of 1.

RT-RPA was performed, by adding Transcriptor RT enzyme (Roche, Mannheim) to the RPA mix. Optimal performance was observed at 22.4mM Mg acetate and 2mMDTT and the analytical sensitivities of the RPA assays ranged from 10-100 md or as per probit analysis from 16-21 md per reaction (Figure 3B, Table 3). The standard deviation of the standard curve threshold time values ranged from 0.2 at 10^7.
molecules to 2.6 at 10 md. SRL slopes ranged from $0.9 \times 10^{-6}$ to 0.56 indicating an efficiency ($E=10^{1/slope}$) of $10^8$ to 61.

**Sensitivity of RPA assays in whole organism nucleic acid extracts**

Extraction efficiency of the innuPREP MP basic kit was tested with plasma spiked with whole organisms of each organism category encountered in the biothreat panel using real time PCR assays. Results of the performance of the innuPREP MP basic kit tested by real time PCR analysis are illustrated in Fig. 4. Pathogens spiked in plasma at a range of $10^4$-$10^7$ md per reaction were efficiently extracted by the innuPREP MP basic kit and were detected at high sensitivity by real-time PCR methods. Additionally, the internal control was efficiently detected in extracts of all the spiked samples as determined by real time PCR (CT $22.56 \pm 0.51$, n= 64). The results here demonstrate that this total nucleic acid extraction method is robust and highly reproducible. The same extracts were used for detection by the respective RPA assays. The results of RPA performance are summarized in table 4. The (RT)-RPA assays for *F. tularensis* and RVFV each specifically detected strains of respective strain panels as described in (9, 10).

**Specificity**

The specificity of all RPA assays was determined by cross testing human genome DNA, and the nucleic acids of all the other biothreat agents in the panel i.e. genomic DNA of *Y. pestis* (strain 03-1501), *F. tularensis ssp. holarctica* (strain LVS), *B. anthracis* (strain 3007), Vaccinia virus (VR-1536), the VARV plasmid, the genomic RNA of Ebola virus, Sudan virus, Marburg virus and Sigma virus. Only specific detection was observed. Additionally the RPA assay for VARV did not detect the genomic DNA of tested Orthopox viruses. These were all detected by the VACV RPA
assay, which did not detect the VARV plasmid. The RPA assays for the detection of bacteria were tested against a panel of bacterial genomes as described earlier (Table 1 in (9), table S1) and showed exclusively specific detection.

To assay the influence of human genomic DNA on the RPA assays, we determined the concentration of human DNA in the eluates of negative sera extracted with the RNeasy kit (Qiagen). We then added the determined average amount of 70 ng/µl human genomic DNA to RPA and RT-RPA reactions. The added background DNA delayed threshold time points negligibly for RPA and up to 3 min for one-step-RT-RPA. It had no effect on the sensitivity (Fig. 2 B, C).

5. Discussion

In order to develop a panel of isothermal detection assays for Category A Bioterrorism Agents we assessed recombinase polymerase amplification (RPA) for the following reasons: (i) it is an exponential amplification with specific amplificate confirmation using a fluorescent probe, (ii) it contains GP32, a single strand binding protein and a good enhancer for the amplification of RNA molecules with complicated secondary structures (42), (iii) it needs only three conserved regions for oligonucleotide design, (iv) available dried pellet reagents facilitate field use or point of care applications.

As to analytical sensitivity and specificity, the RPA assays developed showed a performance equal to PCR (table 3) and showed no cross detection amongst their respective targets. Compared to PCR, RPA reaction time however was much shorter and surprisingly one-step-RT-RPA assays were quicker (LOD reached at 4-8 minutes) than RPA assays (LOD reached 7-10 minutes).

The SRLs of the RT-RPA assays showed even lower slope values indicating very
fast reaction kinetics. We assume that this might be due to an additive effect of the fluorogenic detection of (i) RNA templates, (ii) the initially generated cDNA (ssDNA) as is generic in T7 promotor driven isothermal assays such as TMA or NASBA, and (iii) the RPA products (dsDNA). Alternatively the initiation of RPA may be facilitated by single stranded cDNA.

The published $K_m$ values for Exonuclease III ($K_m= 6.3 \times 10^{-9}$ M (nicks/minute), (18), and FPG ($K_m= 7 \times 10^{-9}$ M (excisions/min), (4)) range in the same order of magnitude implying comparable activity levels. Nevertheless, the assays using cleavage of fpg-probes showed a significantly reduced sensitivity than the exo-probe assays (Fig. 2A), suggesting that in RPA the FPG enzyme kinetics are not as favourable to real time detection as those of Exonuclease III.

The results of the whole organism extraction experiments indicate that the magnetic bead based total nucleic acid extraction kit used showed efficient extraction of DNA and RNA for all tested organism categories. Moreover, it was demonstrated that real time PCR and RPA show comparable detection sensitivities when in these extracts (table 4).

LAMP assays may also be considered as a good option for isothermal detection and miniaturisation (2). In general LAMP assays need 4-6 primers leading to longer amplicons and possibly more difficult design in the case of highly variable RNA viruses, whereas the RPA design with three oligonucleotides offers almost the same flexibility as real time PCR. However the longer TwistAmp™ exo-probes can be a design obstacle, which can be partly circumvented by allowing probe and primer to overlap. The use of LNA nucleotides might help to reduce probe length as has been shown for TaqMan probes (11, 39).

In comparison published LAMP assays for *B. anthracis*, Monkeypox, RVFV, MARV and EBOV (table 3) have longer run times (18 – 60 minutes) at 60-63°C than the
RPA assays but show about the same sensitivity (Table 3). However, not all LAMP assays have been adapted for real-time fluorescence as some of them use turbidity index for readout.

The current advantage of RPA is that the reaction mixture containing enzymes, nucleotides and buffer are provided in dried pellets, which is very well amenable to POC or field use. This is now also possible for RT-RPA (10). The only ingredients that need to be added are primers, probe and sample.

With a small footprint of 17.4 x 18.8 cm and a weight of 1 kg (including the labtop) the ESEquant tubescanner system is significantly lighter and smaller than all other available state of the art mobile PCR cyclers such as SmartCycler, R.A.P.I.D., RAZOR (5-35 kg), or the Loopamp® Realtime Turbidimeter 2.0 for LAMP assays (5 kg). At 4000 Euro the ESEquant tubescanner is also considerably cheaper than any of the mobile PCR devices. In combination with the ESEquant tubescanner RPA is therefore a very attractive nucleic acid detection method that could easily be installed in hospitals or laboratories, which cannot afford real-time PCR cyclers.

The only constraints of isothermal amplification methods are enzyme activity rates since there is no dependency on rapid temperature ramping as in PCR. This feature makes them more amenable to engineer microfluidic lab-on-chip devices than PCR.

A recent review on miniaturisation efforts for NASBA, LAMP, HDA, SDA, RCA and RPA pointed out that low-temperature isothermal methods such as SDA, NASBA, RCA and RPA show an advantage for miniaturisation as they need much less energy input and are therefore better candidates for battery-driven handheld devices than high-temperature isothermal reactions (LAMP, SmartAmp, HDA) (2).

The implementation of RPA on centrifugational LabDisks was recently described (25). This type of cartridge could fulfill the requirements for simple benchtop devices if sample preparation were included. It would come closest to a lab on a
cartridge in contrast to the majority of systems for miniaturized molecular assays currently developed which have aptly been described as ‘chip in lab’ rather than ‘lab on chip’ platforms (2).

In summary we have developed a panel of very rapid and highly sensitive isothermal real time RPA assays for the detection of Category A Bioterrorism Agents covering gram negative, gram positive bacteria, DNA viruses and RNA viruses. We also showed that a commercially available magnetic bead based total nucleic extraction kit, which could be used in resource-poor settings can be efficiently combined with RPA. We now aim at integrating all assays onto a microfluidic POC device and testing this syndromic panel of RPA assays on clinical samples.

Acknowledgements

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33. Qiagen. 2011. ESEQuant Tube Scanner

http://www.qiagen.com/Products/ESEQuantTubeScanner.aspx?r=603


**Figure Legends**

Figure 1. Details of the RPA amplicon for VARV. All nucleotides in the alignment matching in the VARV sequence are presented as dots. Primer sequences are presented as full sequences. Gaps are presented as hyphens. VARV RPA FP is presented in sense and VARV RPA P and RP are presented as reverse complement sequences. Grey fields: VARF RPA FP: Degenerated IUB code positions, VARV RPA P: The TTT triplet used for the attachment of BTF (table 2), VARF RPA RP: nucleotide at position 3 of the 3-prime end matching all other orthopoxviruses. Sequences: cowpox AY902252, camelpox AF438165, monkeypox AF380138, vaccinia virus M35027, variola virus X69198.

Figure 2. Real time (RT-)RPA assay performance. A: Comparison of exo-probe and fpg-probe performance in RT-RPA. Standard regression lines (SRLs) for EBOV one-step-RT-RPA were generated from eight data sets (exo-probe, black squares) and 3 data sets (fpg-probe, white squares). B: Influence of background DNA on EBOV one-step-RT-RPA, black squares: SRL as above, white squares: SRL of the same assay with 70 ng human genome DNA background. C: Influence of background DNA on RPA, black squares: SRL derived of 8 data sets of *B. anthracis* RPA, white squares: SRL of the same assay with 70 ng human genome DNA background.

Figure 3. Standard regression lines (SRL) of all developed assays including assays for *Francisella tularensis* and Rift Valley fever virus already described in (9, 10). SRLs were derived from 8 data sets each. A: DNA-RPA assays. B: one-step-RT-RPA assays.
Figure 4: Extraction efficiency of the innuPREP MP basic kit. Plasma was spiked with whole organisms (range: $10^4$ - $10^5$/ml) and nucleic acids were extracted. Eluates were tested by respective quantitative real time PCR assays in triplicate. The amount of measured md per reaction is plotted against the 10-fold serial dilution of pathogens in plasma. The dotted line represents calculated 100% efficiency of extraction. A: *B. anthracis* (gram positive) extracts tested with pag-PCR, B: *Y.pestis* (gram negative) tested with pla-PCR, C Vaccinia virus tested with LE-PCR, D Marburg virus tested with NP-PCR.
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Table 2. Primers and probes

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Table 3 Sensitivity of RPA assays

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<th>Infectious agent and (target gene)</th>
<th>Sensitivity Real-time-PCR 3 Runs (source)</th>
<th>Sensitivity RPA 8 Runs</th>
<th>Sensitivity RPA Probit 95% 8 Runs</th>
<th>RPA threshold time to sensitivity limit (min)</th>
<th>Sensitivity LAMP (source)</th>
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<td>B. anthracis (pagA)</td>
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<td>$10^9$ (21)</td>
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<td><strong>RNA assays</strong></td>
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* calculated from fg given in original publication, **monkeypox LAMP assay
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* pagA assays used for detection
Figure 2

A

B

C
Figure 3

A

- VARV
- B. anthracis (cap)
- B. anthracis (oas)
- Y. pestis
- F. tularensis

B

- RVFV
- SEBOV
- ZEBOV
- MARV
- SIGV
Figure 4

A

B

C

D