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Recombinase polymerase amplification assay for rapid detection of lumpy skin disease virus

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Abstract

Background: Lumpy skin disease virus (LSDV) is a *Capripoxvirus* infecting cattle and Buffalos. Lumpy skin disease (LSD) leads to significant economic losses due to hide damage, reduction of milk production, mastitis, infertility and mortalities (10 %). Early detection of the virus is crucial to start appropriate outbreak control measures. Veterinarians rely on the presence of the characteristic clinical signs of LSD. Laboratory diagnostics including virus isolation, sequencing and real-time polymerase chain reaction (PCR) are performed at well-equipped laboratories. In this study, a portable, simple, and rapid recombinase polymerase amplification (RPA) assay for the detection of LSDV-genome for the use on farms was developed.

Results: The LSDV RPA assay was performed at 42 °C and detected down to 179 DNA copies/reaction in a maximum of 15 min. Unspecific amplification was observed with neither LSDV-negative samples ($n = 12$) nor nucleic acid preparations from orf virus, bovine papular stomatitis virus, cowpoxvirus, Peste des petits ruminants and Blue tongue virus (serotypes 1, 6 and 8). The clinical sensitivity of the LSDV RPA assay matched 100 % ($n = 22$) to real-time PCR results. In addition, the LSDV RPA assay detected sheep and goat poxviruses.

Conclusion: The LSDV RPA assay is a rapid and sensitive test that could be implemented in field or at quarantine stations for the identification of LSDV infected case.

Keywords: Lumpy skin disease virus, Recombinase polymerase amplification assay, Point of need test, Cattle

Background

Lumpy skin disease (LSD) affects primarily cattle and occasionally buffalo [1, 2]. It causes pyrexia, generalized skin and pox lesions of internal organs, as well as generalized lymphadenopathy [3, 4]. The disease exists in three forms, acute, subacute or unapparent [5]. LSD is caused by an enveloped double-stranded DNA virus called LSD virus (LSDV), which together with sheep poxvirus (SPV) and goat poxvirus (GPV) constitutes the genus *Capripoxvirus* of the *Chordopoxvirinae* subfamily of the *Poxviridae* family [6, 7].

The origin of LSDV is unknown. It was reported for the first time in Zambia in 1929 as a hypersensitivity reaction of cattle to insect bites [8, 9]. In Egypt, LSDV was first reported in Suez and Ismailia Governorates in May and October 1988 and thereafter spread throughout Egypt leading to 50,000 infected cattle and 1,449 mortalities in 1998 [10, 11]. During epizootics LSDV is mainly transmitted mechanically by blood feeding insects e.g. *Aedes aegypti* [12]. Due to the rapid spread of LSDV and the severe economic losses caused, the Office International des Epizooties (OIE) includes LSDV in the listed notifiable disease of cattle [13].

Diagnosis of LSD depends initially on clinical signs. Definite diagnosis can be performed *via* virus isolation, electron microscopy, identification of antigen by immunofluorescence, serum neutralization, agar gel precipitation, antigen capture ELISA and Dot ELISA [3, 14]. In addition, conventional and real-time polymerase chain reactions (PCR) for the detection of the

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LSDV have been described [3, 15–18]. All the above-mentioned methods are not suitable for screening cattle under field conditions or at quarantine stations, as they require highly skilled staff and a well-equipped laboratory. Simple, portable, and rapid tests to detect LSDV at the point of need could improve initiation of control measures as early as possible. This study describes the development and evaluation of a real-time RPA assay for the detection of LSDV genome.

Methods

DNA molecular standards

To produce a molecular LSDV DNA standard, a 910 nt fragment of the G-protein-coupled chemokine receptor (GPCR) gene (6981-7891 of the Genbank accession number: AF325528.1) of the LSDV reference strain (Neethling strain provided by the Pirbright Institute to the Friedrich-Loeffler-Institute, Greifswald-Insel Riems, Germany) was amplified using the in-house designed forward primer (FP): 5′-CATAGTCGATATCCCACATT G-3′, the reverse primer (RP): 5′- GCTAATACTACCA GCACTAC-3′ and the Taq DNA Polymerase (5 PRIME GmbH, Hilden, Germany). The PCR temperature profile was as follows: initial activation at 95 °C/3 min, 30 cycles of 94 °C/60 s, 55 °C/60 s and 72 °C/60 s and a final extension step of 72 °C/5 min. The amplified fragment was ligated into pCR®II using the TA-cloning kit dual promoter and transformed into One shot® chemically competent *E.coli* (Invitrogen, Darmstadt, Germany). Purified plasmids were verified by sequencing (Seqlab, Goettingen, Germany). The plasmid was linearized using the FastDigest HindIII (Fisher Scientific GmbH, Schwerte, Germany). The number of DNA molecules per microliter was measured by the Quant-iT™ PicoGreen® dsDNA Assay Kit (Fisher Scientific GmbH, Schwerte, Germany). Then the DNA standard was diluted to achieve a concentration range of 10^7–10^1 DNA molecules/μl. The standard was tested by applying primers and a modified probe (FP, 5′-GATAGTATCGCTAAACAATGG-3; RP, 5′-ATCCAAACCACCATACTAAG-3′; P, 5′-FAM-AC CTAGCTGTAGTTCACCCAGTAAA-TAMRA-3′) of a published real-time PCR protocol [16] using the Light Cycler 2.0 and the FastStart DNA Master HybProbe kit (Roche, Manheim, Germany).

LSDV RPA oligonucleotides and conditions

RPA primers and exo probe (Fig. 1) were synthesized by TIB MOLBIOL (Berlin, Germany). The LSDV RPA was performed in a 50 μl volume using the TwistAmp™ exo lyophilized kit (TwistDx, Cambridge, UK), using 420 nM RPA primers and 120 nM RPA exo-probe, 29.5 μl of rehydration buffer, 12.2 of molecular biology grade water. A mastermix was added directly to the lyophilized pellet provided in the tubes of a 8-tubes strip. Thereafter, 2.5 μl of Mg acetate (1 mM) were added to each lid. Finally, one microliter of DNA template was added to the pellet. The tube was closed, centrifuged, mixed well and centrifuged again before placed into the tubescanner (Twista, TwistDx, Cambridge, UK) for 15 min at 42 °C. After 230 s, the strip was retrieved, vortexed, centrifuged and placed again into the tubescanner (Twista, TwistDx, Cambridge, UK). The fluorescence signal was measured each 20 s using the FAM channel. In each run positive and negative controls were included. A combined threshold and 1st derivative analysis was used for signal interpretation. Samples produced an exponential amplification curve above the threshold of the negative control were consider positive.

LSDV assay cross reactivity

The Friedrich-Loeffler-Institute, Greifswald-Insel Riems, Germany provided reference nucleic acids for LSDV, SPV, GPV, orf virus, bovine papular stomatitis virus, cowpoxvirus, Peste des petits ruminants and Blue tongue virus (serotypes 1, 6 and 8) (Table 1). All samples contained a high concentration of viral nucleic acid as determined by the respective real-time PCRs recommended by the OIE (CT: 12-20).

Clinical sensitivity and specificity of the LSDV RPA assay

Twenty-two skin nodules of suspected LSDV-infected cattle were collected during the summer of 2012 in Dakahlia Governorate, Egypt. Diseased cattle exhibited either localized or generalized multiple skin nodules with or without systemic signs.

DNA was extracted from the twenty-two skin nodules and twelve skin samples from apparently healthy cows using QIAamp DNA Mini Kit (Qiagen, Hilden, Germany). All samples were screened simultaneously by the LSDV RPA assay and real-time PCR as
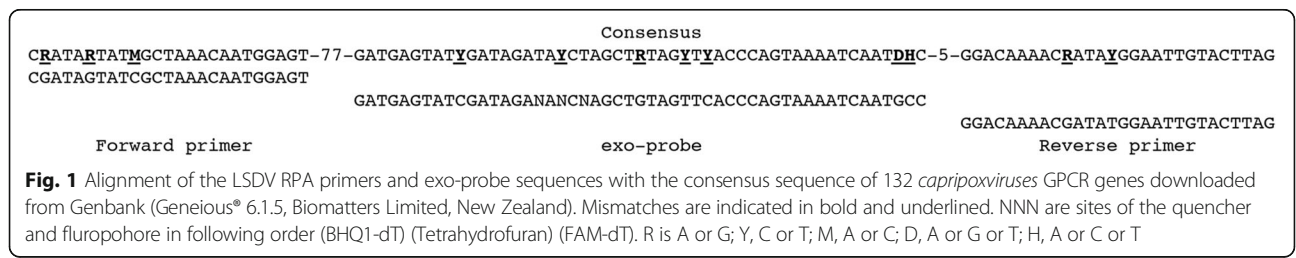


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                                    Consensus
CRATARTATMGCTAAACAATGGAGT–77–GATGAGTATYGATAGATAYCTAGCTRTAGYTYACCCAGTAAAATCAATDHC–5–GGACAAAACRATAYGGAATTGTACTTAG
CGATAGTATCGCTAAACAATGGAGT

                        GATGAGTATCGATAGANANCNAGCTGTAGTTCACCCAGTAAAATCAATGCC

                                                                        GGACAAAACGATATGGAATTGTACTTAG
        Forward primer                         exo-probe                        Reverse primer
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Fig. 1 Alignment of the LSDV RPA primers and exo-probe sequences with the consensus sequence of 132 *capripoxviruses* GPCR genes downloaded from Genbank (Geneious® 6.1.5, Biomatters Limited, New Zealand). Mismatches are indicated in bold and underlined. NNN are sites of the quencher and fluropophore in following order (BHQ1-dT) (Tetrahydrofuran) (FAM-dT). R is A or G; Y, C or T; M, A or C; D, A or G or T; H, A or C or T

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Table 1 List of reference viral strains

Virus	Strain	Reference
Lumpy skin disease virus	Neethling strain	[40]
Sheep poxvirus	Russia	NA
Goat poxvirus	Indian	NA
Cowpoxvirus	2	[41]
Orf Virus	Burgheßler	[42]
Bovine papular stomatitis virus	M1	[43]
Peste de petite Ruminant Virus	lineage IV_Kurdistan2011	[44]
Blue tongue virus	Serotypes 1, 6 and 8	[45]

NA is non-applicable

described above. The real time PCR CT values for these samples ranged from CT 18 to 35 (Additional file 1: Table S1).

Statistical methods

For the determination of the LSDV RPA assay analytical sensitivity by the molecular DNA standard, a semi-log regression analysis (PRISM, Graphpad Software Inc., San Diego, California) and a probit analysis (STATISTICA, StatSoft, Hamburg, Germany) were performed by plotting the RPA threshold time against the number of molecules detected. Clinical sensitivity and specificity values were calculated using standard formulas.

Results

The LSDV GPCR gene plasmid standard was used to determine the analytical sensitivity of the assay using a dilution range between 10^7–10^1/µl (Fig. 2). The LSDV RPA assay was performed eight times on the molecular standard, in which 10^7–10^3 DNA molecules were detected in 8/8 runs, 10^2, 7/8 and 10^1, 2/8 (Fig. 3a, Additional file 2: Table S2). Due to the inconsistency in the results, a probit regression analysis was applied, in which the sensitivity in 95 % of cases was determined at 179 DNA molecules/

reaction (Fig. 3b). While the real-time PCR analytical sensitivity was 37 DNA copies/reaction [16].

The LSDV RPA assay showed no cross detection with orf virus, bovine papular stomatitis virus, cowpoxvirus, Peste des petits ruminants and Blue tongue virus (serotypes 1, 6 and 8) genome. SPV and GPV, however, were detected by the LSDV RPA assay as well. The LSDV RPA assay was validated using 12 negative skin samples and 22 LSDV positive skin nodule samples. In comparison to real-time PCR, clinical sensitivity and specificity of the LSDV RPA assay was 100 % (Additional file 1: Table S1).

Discussion

Veterinarians rely mainly on the appearance of clinical signs for LSD diagnosis [8]. Establishment of a rapid diagnostic test to identify early stages of an LSD outbreak would allow rapid execution of control measures.

The developed LSDV RPA assay was highly sensitive (179 DNA copies detected/reaction) and rapid (total time: 2–15 min). In addition, we were able to obtain the same clinical sensitivity and specificity as the well-established real-time PCR assay [16] when testing 22 skin samples. The RPA assay is a technique for the isothermal amplification of DNA using enzymes and proteins to replace the repetitive cycles of three temperatures used for PCR [19]. Thus, RPA can be operated by portable more simple heating and detection devices instead of using thermal cycler devices. Moreover, RPA reagents are cold chain independent [20–22], which make them ideal for point of need testing.

The LSDV RPA exo-probe was designed to detect LSDV by placing two mismatches to the sequences of SPV and GPV at its 3′ prime end (Fig. 1 and Additional file 3: Figure S1) as well as the primers amplified 186 of the most variable gene in the *capripoxviruses*, GPCR
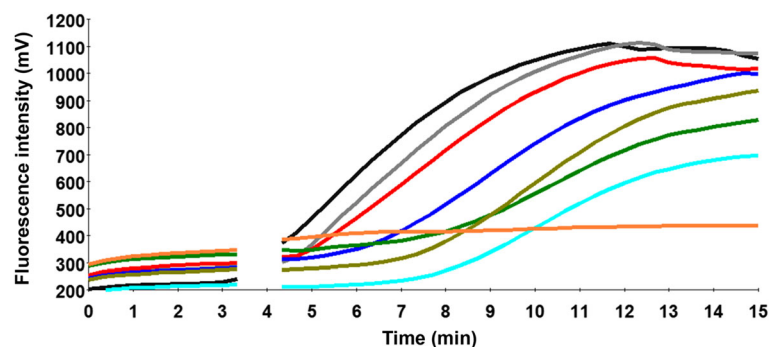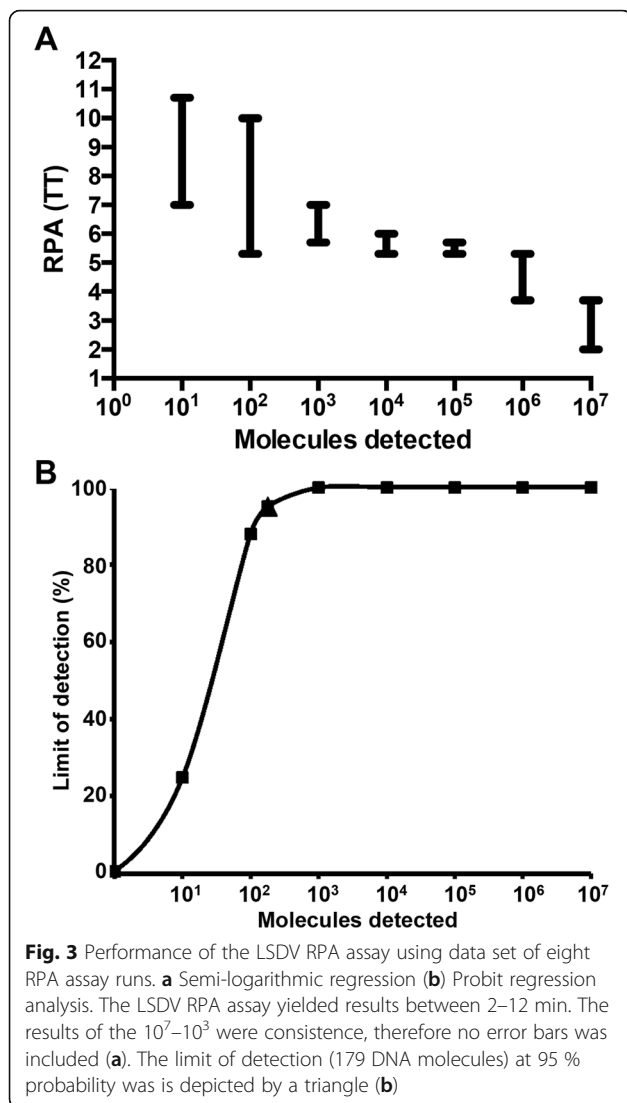

Fig. 2 The results layout of one LSDV RPA assay run. Fluorescence development over time using a dilution range of 10^7–10^1 molecules/µl of the DNA molecular standard (Graph generated by ESEquant tubescanner software). 10^7 represented by black line; 10^6, gray; 10^5, red; 10^4, blue; 10^3, green; 10^2, cyan; 10^1, dark khaki; negative control, orange. The LSDV RPA assay detected down to 10 DNA molecules/reaction. After 230 s, the strip was taken out of the tubescanner for mixing, therefore, no fluorescence signals were recorded

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Fig. 3 Performance of the LSDV RPA assay using data set of eight RPA assay runs. **a** Semi-logarithmic regression (**b**) Probit regression analysis. The LSDV RPA assay yielded results between 2–12 min. The results of the 10^7–10^3 were consistence, therefore no error bars was included (**a**). The limit of detection (179 DNA molecules) at 95 % probability was is depicted by a triangle (**b**)

gene [23, 24]. Nevertheless, SPV and GPV were detected. This might be due to the fact that the length (48–52 bp) of the RPA exo probe compensates for the presence of nucleotide mismatches [25, 26]. Also, the position of mismatches appear not to affect RPA oligonucleotide binding [27], but have a great influence on the PCR primes and probe [28]. However, the assay will be also useful for detecting SPV and GPV, unfortunately, no clinical samples were available to validate the assay for both viruses.

Several conventional and real-time PCRs have been established to identify the *capripoxviruses* [18, 29–34], but none are able to distinguish between various species [35]. One PCR assay was established for the differentiation between these viruses; however, many GPVs were identified as SPV [16]. This is due to the high homology of up to 96 % between the members of this genus [23], which also affected the LSDV RPA

assay. The OIE recommends sequencing and phylogenetic analysis to differentiate between LSDV, SPV and GPV. For a point of need field test the LSDV-RPA described here can still be of help as SPV and GPV are not known to infect cattle and using it on cattle samples, therefore, provides the specificity needed especially as the panel of other cattle infecting viruses tested all scored negative in the cross detection assessment.

Several loop-mediated isothermal amplification (LAMP) assays have been established to identify *capripoxviruses* [36–38]. The design of LAMP assays requires four to six oligonucleotides and a minimum of four binding sites. The LAMP results can be read by naked eye, if turbidity read out is used [39] after about 60 min. In contrast, the RPA assay developed here was very fast (15 min) and required two primers and one probe.

Conclusion

In conclusion, LSDV RPA yielded similar results as the corresponding real-time PCR assay, but RPA was quicker and much easier to handle. Furthermore, combination with a simple extraction method will allow its employment at low resource settings, quarantine stations or farms.

Additional files

Additional file 1: Table S1. Screening 22 skin nodules samples with real-time PCR and RPA assays. (DOCX 57 kb)

Additional file 2: Table S2. Reproducibility of LSDV RPA assay using data sets of eight RPA assay runs using the DNA molecular standards. 10^7–10^3 DNA molecules were detected 8 out of 8 runs; 10^2, 7/8 and 10^1, 2/8. (DOCX 54 kb)

Additional file 3: Figure S1. Mapping 132 nucleotide sequences derived by BLAST nucleotide search to the LSDV RPA amplicon as well as RPA primers and probe. The alignment was performed by using Geneious (V: 9.0.5. Biomatters Limited. New Zealand). The Genbank accession number and name were given. Grey represents the identical sequence. A. C. G. T were highlighted in red. violet. yellow. green. respectively. whenever a mismatch to the LSDV RPA amplicon was recorded. (DOCX 515 kb)

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