Reserch Article

RPO30 Gene based PCR for Detection and Differentiation of Lumpy Skin Disease Virus and Sheep Poxvirus Field and Vaccinal Strains

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Abstract | Lumpy skin disease virus (LSDV) and sheep poxvirus (SPPV) are Capripoxviruses that are considered as an emerging hazard to cattle, sheep and goat. Diseases caused by capripoxviruses are transboundary in nature and are regularly spread into neighboring, non-endemic regions with significant economic implication. Capripoxvirus isolates are extremely conserved with genome identities of at least 96% between SPPV, GTPV and LSDV. The LSDV is similar in antigenicity and in cultural characteristics to SPPV. The current study was delineated to identify capripoxviruses from different clinical samples and differentiate capripoxviruses without the need of gene sequencing. A total of 16 lumpy skin disease clinical samples (skin nodules, scabs, buffy coat, lymph aspirate and engorged ticks) and three sheep pox biopsy skin samples were subjected to DNA extraction followed by 30 kDa RNA polymerase subunit gene-based polymerase chain reaction (PCR) (RPO30) together with tissue culture-adapted cattle LSDV/Ismailyia88 strain and two Sheep poxvirus vaccinal strains as control. LSDV yield amplicon differed in length by 21 nucleotides from those produced from SPPV either field or vaccinal strain. Among different LSD clinical samples, skin nodules and scabs are proved excellent sample material as they yield clear DNA bands that reflect the high concentrations of the virus. The results of the current study confirm the suitability of RP030 gene in differentiation of lumpy skin disease virus and sheep poxvirus in a single PCR assay without the necessity of post processing steps.

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Introduction

Lumpy skin disease virus (LSDV), sheep pox (SPPV) and goat pox virus (GTPV), members of the Capripoxvirus genus of the Poxviridae family are causative agents of Lumpy skin disease (LSD), sheep pox and goat pox respectively (Buller et al., 2005; Diallo and Viljoen, 2007), which are important endemic diseases in Egypt. They are now expanding their territory. LSDV was first recognized in 1929 originating in sub-Saharan Africa. The endemic geographic range of LSDV was restricted to the continent of Africa (Woods, 1988), in the past decade, new incursion of LSDV have been reported in the Middle Eastern, European and west Asian regions (Wainwright et al., 2013; Tageldin et al., 2014; Al-Salihi and Hassan, 2015; Ripani and Pacholek, 2015; OIE, 2016). There are obvious differences between the geographical dis-

tribution of SPPV and LSDV. Sheep pox and goat pox are found in parts of Africa (north of the equator), Asia, the Middle East, and most of the Indian subcontinent (Asagba and Nawathe, 1981; Mondal et al., 2004; Bhanuprakash et al., 2005).

Pox lesions are considered the most characteristic features of Capripoxvirus infection. LSD is characterized by fever, nodules on the skin, mucous membranes and internal organs, enlargement of lymph nodes, edema of the skin, and sometimes death (Coetzer, 2004; Babiuk et al., 2008). The disease is associated with significant production losses as a result of decreased weight gain, reduced milk yield, damaged to hides and wool. LSDV is an occasionally fatal disease of cattle with a morbidity averaging 10% and usually ranges from 3% to 85% (Thomas and Mare, 1945) and mortality (1–3%) in affected herds but may sometimes reach 40% (Coetzer, 2004; Rouby, 2010) and over 75% as reported by Diesel (1949). In contrast sheep pox is considered a direct cause of death at any stage of the disease (Yeruham et al., 2007). The overall mortality is usually lower than 10% but case fatality rates can be reach 100% in some young animals (Rao and Bandyopadhyay, 2000; Bhanuprakash et al., 2006). In newly imported and highly susceptible flocks high morbidity and mortality rates approaching 100% can be occur and range from 1% to 75% in indigenous breeds. Sheep pox is characterized by fever, widespread pox lesions throughout the skin and mucous membranes, rhinitis, conjunctivitis, and respiratory distress. Production losses are similar to LSD with decreased weight gain and damage to hide and also due to increased abortion rates, and increased susceptibility to pneumonia and fly strike (Babiuk et al., 2008).

Since capripoxviruses cause heavy economic losses among their hosts, it is crucially important to develop fast and specific diagnostic tests. Capripoxviruses are double-stranded DNA viruses with genomes approximately 150 kbp in size (Tulman et al., 2002). Capripoxvirus isolates are extremely conserved with genome identities of at least 96% between LSDV, SPPV, and GTPV (Tulman et al., 2002). Using routine laboratory tests Capripoxviruses cannot be distinguished (Kitching, 1986; Davies and Otema, 1981). Polymerase chain reaction (PCR) offers a rapid and sensitive diagnostic technique for capripoxvirus genome detection (Ireland and Binepal, 1998; Heine et al., 1999). Several PCR assays are currently available for differentiation between LSDV and SPPV, however, they target only one viral species (Stram et al., 2008; Orlova et al., 2006) or prerequisite sequence analyses (Hosamani et al., 2004; Cao et al., 1995). RPO30 gene based PCR designated by (Lamien et al., 2011a) provides a simple and quick differentiation between LSDV and SPPV in one step and is cost effective PCR. The present study elucidates the use of RPO30 gene based PCR for identification of LSDV from different clinical samples and differentiation between LSDV and SPPV recovered from naturally infected animals in Egypt along with the most commonly used vaccine strains in Egypt.

Material and Methods

Sample collection

Between May 2015 and August 2016, a total of 16 different clinical samples were collected from clinically LSD diseased animals during different outbreaks in Beni-suef governorate, Egypt together with three scab samples collected from sheep pox suspected field cases. Data of samples collection are illustrated in Table 1.

Virus strains and vaccines

Tissue culture-adapted cattle LSDV/Ismailyia88 strain was kindly supplied from the Pox Vaccine Production and Research Department, Veterinary Serum and Vaccine Research Institute (VSVRI), Abbasia, Cairo, Egypt. This strain was used as a positive control. Sheep poxvirus vaccine was obtained from VSVRI, prepared in Vero cell line and has a titre of $10^{4.5}$ TCID50/ml. It was supplied in lyophilized ampoules; each of them contained 100 doses. Sheep pox vaccine (RM 65 strain): Sheep poxvirus vaccine (RM65 strain) produced by Jordan Bio- Industries Center (JOVAC) in Jordan prepared in lamb kidney cells and has a titer of $10^{3.5}$ TCID50/ml was used in this study.

DNA extraction

Skin nodules, scab samples and ticks were triturated and homogenized with 50% phosphate buffered saline and made into 10% suspension. Extraction of viral DNA was performed using a DNA Mini Kit (Thermo, Germany) according to manufacturer's instructions. DNA from cattle buffy coat, lymph aspirate and tissue culture-adapted cattle LSDV/Ismailyia88 strain were directly extracted using a DNA Mini Kit (Thermo, Germany) according to manufacturer's instructions. Lyophilized Sheep pox vaccines were

Table 1: *Data of samples collection*

NO	Animal description	Source	Samples nature	Samples collection
1	Cow, ,6y	Individual cases	Skin nodules	The nodules were surgically extirpated after the skin was locally anesthetized with 2% lidocaine then placed in glycerol saline and stored at –20°C for PCR analysis.
2	Heifer10M	Individual cases	Skin nodules	
3	Cow,2y	Individual cases	Skin nodules	
4	Cow,6y	Individual cases	Skin nodules	
5	Cow 3y	Private farm	Scabs	Directly detached from the lesion
6	Cow3y	Private farm	Scabs	
7	Cow2y	Private farm	Scabs	
8	Calf 2M	Individual cases	Skin nodules	
9	Cow 3y	Individual cases	Skin nodules	
10	Cow 5y	Individual cases	Skin nodules	
11	Cow 3y	Individual cases	Buffy coat	Collected from jugular vein during febrile stage
12	Cow, 3y	Individual cases	Lymph aspirate	Collected from superfacial lymph node
13	Cow 3y	Individual cases	Scabs	
14	Calf premature birth	Individual cases	Skin nodules	
15	Cow 3y	Individual cases	Tick adult& engorged	Directly picked up from animals and when possible from the nodules using pointed forceps. The ticks were identified according to (Walker et al., 2003).
16	Cow 5y	Individual cases	nymph	
1	Sheep	Flocks	scabs	Directly detached from the lesion
2	Sheep			
3	Sheep			

Table 2: *Primer set for PCR*

Primer	annealing	target	Reference
F 5'-tctatgtcttgatatgtggtggtag-3'	55 °C	amplify the region containing a 21-nucleotide deletion in (SPPV) sequences	Lamien et al., (2011a)
R 5'-agtgattaggtggtgtattattttcc-3'			

reconstituted in 1 ml phosphate buffer saline (PBS) and were subjected to DNA extraction using a DNA Mini Kit (Thermo, Germany) according to manufacturer's instructions.

PCR amplification

PCR run was performed according to Lamien et al. (2011a) using primer set targeting RP030 gene (Table 2). PCR was carried out in a 25 µL reaction volume in a 200 µL capacity PCR tube containing 12.5 µL PCR Master Mix (Jena bioscience Gmbh, Germany), 1 µL of each primer (20 pmol/µL), 4 µL of extracted DNA and 6.5 µL of nuclease free water nuclease, and free water was used in negative control. The amplification was performed in Labnet^{R} Multigen Gradient thermal cycler, (Catalog TC9600-G- 230V), Labnet international, Inc. Edison, NJ, USA) after initial denaturation at 95 °C for 4 min, followed by 35 cycles of denaturation at 95 °C for 45 sec, annealing at 55 °C for 45 sec and extension at 72 °C for 45 sec and final extension at 72 °C for 7min. 72°C and final extension for seven minutes at 72°C. The PCR amplicons were analyzed by running 15 µl of the PCR products in 2% agarose gel stained with ethidium bromide (0.5µg/mL) in comparison with DNA ladder (50 bp and 100bp), (Biomatik R Code No. M7123 and M7508, Biomatik Corporation Ontario, Canada). Under UV illumination using gel documentation and analysis system the gel was photographed.

Results

Between May 2015 and august 2016 a total of 16 different clinical samples were collected from clinically diseased cows with typical clinical pictures of lumpy skin disease where skin nodules were shown scattered in all body (Figure 1) accompanied with edema in fore limbs (Figure 2) and enlargement of superficial lymph nodes (Figure 3). Regarding sheep, samples were collected from three flocks that were shown pox like signs where the infected sheep suffered from pyrexia accompanied with cutaneous papules especially

in wool-less areas of skin.

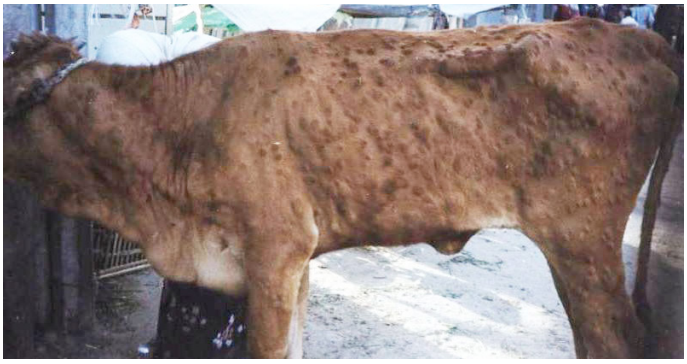

Figure 1: *Generalized skin nodules in LSD diseased animal*

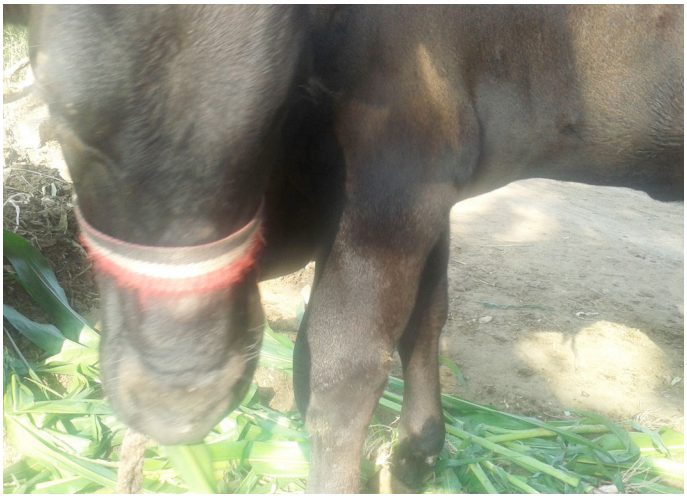

Figure 2: *Fore limb edema in LSD diseased calf*

Figure 3: *Enlargement of prescapular lymph node of a cow naturally infected with LSDV*

Analysis of the PCR products by agarose gel electrophoresis revealed the positive amplification of the RPO30 gene with correct size for LSDV (172bp) while field skin isolate of SPPV, tissue culture adapted SPPV vaccinal strains were shorter (151 bp) and easily distinguishable, relative to the LSDV amplicons (172 bp) (Figure 4). All DNA extracted from different LSD clinical samples yield a clear band in the gel after amplification of the target gene except that recovered from lymph aspirate. Skin nodules and scabs gave more obvious bands than that from buffy coat and ticks (Figure 4).

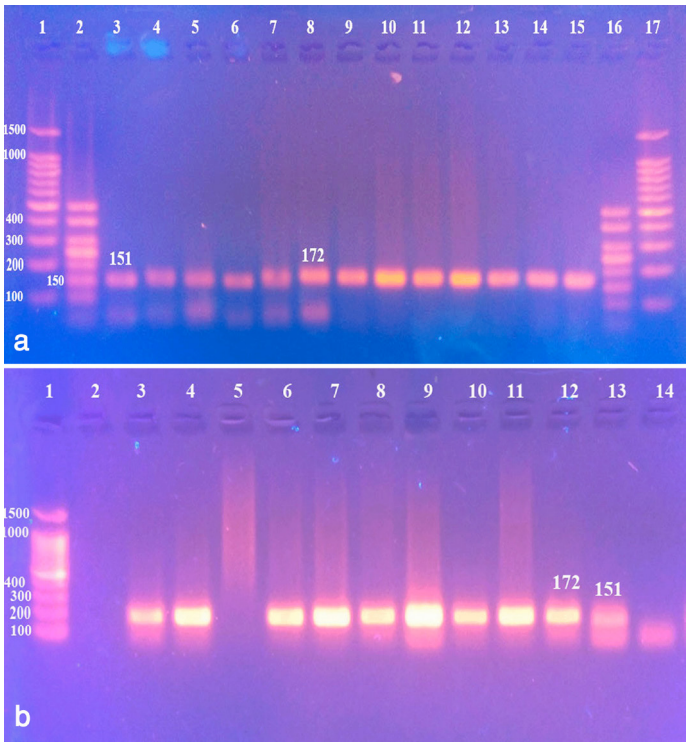

Figure 4: *Gel electrophoresis of RPO30 gene based PCR assay for detection and differentiation of LSDV and SPPV.*

a: *Lane 1: 100bp DNA ladder, Lane 2: 50bp DNA ladder, Lane 3: SPPV (VSVRI), Lane 4: SPPV (RM 65 strain), Lane 5, 6, 7: SPPV (field strains), Lane 8, 9: LSDV (Ticks), Lane10, 11, 12, 13: LSDV (scabs), Lane 14: LSDV (buffy coat), Lane 15: LSDV (Ismailia strain), Lane 1: 50bp DNA ladder, Lane 2: 100bp DNA ladder*

b: *Lane 1: 100bp DNA ladder, Lane 3: LSDV (Ismailia strain), Lane 4: LSDV (skin nodules), Lane 5: LSDV (lymph aspirate), Lane 6:12: LSDV (skin nodules), Lane 13: SPPV (VSVRI), Lane 14: control negative*

Discussion

Classification within CaPV genus is relies upon the host from which the virus is isolated with the hypothesis that CaPVs are strongly host-specific (Babiuk et al., 2008). SPPV and GTPV cannot be distinguished from each other with routine serological techniques

(Davies and Otema, 1981) and most isolates are host specific. SPPV mainly causes disease in sheep and GTPV mostly affects goats however, some isolates can cause serious disease in both species these strains usually have intermediate host specificity (Davies, 1976; Kitching, 1986; Babiuk et al., 2008). Although LSDV is closely related to SPPV and GTPV, natural infection of sheep and goats with LSDV has not been reported. However latest molecular studies have described a close relationship between LSDV and the Kenya sheep-1 (KS-1), proposing that KS-1 is actually LSDV (Tulman et al., 2002). The KS-1 strain is obtained from the attenuated Kenyan sheep and goat pox vaccine virus (KSGP) O-240 (Gershon and Black, 1989; Chand et al., 1994). Finally, Lamien et al. (2011b) and Tuppurainen et al. (2014) confirmed that the commonly used KSGP O-240 is not SPPV but is actually LSDV.

The definitive confirmatory diagnosis of any causative agent is achieved by sequencing a part or the genome as a whole; however, it is time consuming, needs special reagents and is not a cost effective approach in the development countries. RPO30 gene based PCR assay according to Lamien et al. (2011a) provides an easily approach for CaPV classification and helps in the quick differentiation between SPPV and GTPV/LSDV without the requisite of DNA sequencing.

In the current study, different clinical samples (n: 16) (Table 1) from naturally infected cattle that exhibited numerous skin lesions and enlargement of superficial lymph nodes with edema in one or more limbs were collected. All these signs account for LSD and came in accordance with that previously reported by (Coetzer, 2004). Samples (n: 3) were also collected from field cases among sheep flocks suffered from pyrexia, oculonasal discharges and characteristic skin pox lesions suggesting sheep pox as recorded by (Yune and Abdela, 2017; Babiuk et al., 2008).

Polymerase chain reaction assay based on 30 kDa RNA polymerase subunit gene- was performed according to Lamien et al. (2011a) on DNA extracted from clinical samples and from tissue culture-adapted cattle LSDV/Ismailyia88 strain as a positive control as well as Sheep poxvirus vaccine (Romanian strain) and Sheep pox vaccine (RM 65 strain). Within RPO30 gene of SPPV there are a 21-nucleotide deletion in the 5' end compared to that of GTPV and LSDV. Primers employed in the current study bind and amplify the partial RPO30 gene containing the 21-nucleotide deletion. As a result SPPV yields the product size (151 bp) which is lesser than the LSDV product size (172 bp). The same PCR was also used by Yan et al., (2012) for CaPV species identification.

DNA extracts of collected clinical samples yield clear bands in the gel after amplification of the target gene, however; skin nodules and scabs gave more obvious bands than that from buffy coat which reflect their high virus concentration. The results came in accordance with Carn and Kitching (1995) who stated that virus concentration in the nodules is higher than that present in blood during viremia. Therefore skin nodules are considered as better sample for LSDV detection. Scabs also are the best sample material as they are easy to collect without the use of local anaesthesia and withstand long transport in different temperatures. Collected ticks were identified as *Rhipicephalus annulatus* ticks (nymph and adult) according to (Walker et al., 2003). Recently the role of ticks in the transmission of LSDV was confirmed by Tuppurainen et al. (2011); Tuppurainen and Oura (2012); Tuppurainen et al. (2013); Lubinga et al. (2015); Rouby et al. (2017) suggesting them as excellent sample for LSDV detection. In the current study *R. annulatus* tick (nymph and adult) yield a clear band in the gel (Figure 4). Unfortunately lymph aspirate failed to give a positive result; it may be related to absence of the virus in the sample.

Owing to cross-protection within the Capripoxvirus genus, SPPV vaccines have been broadly used for cattle against LSDV (Kitching, 2003). Sheep poxvirus vaccines were used in the current study as in Egypt both vaccines are used for cattle to control the spread of LSDV.

Conclusions

RPO30 gene-based PCR is a cost effective, accurate, and simple for detection and differentiation of SPPV and LSDV in a single PCR. The 21-nucleotide deletion in the 5' end in the RPO30 gene of SPPV makes it a reliable signature for SPPV. Buffy coat and ticks are considered good samples for LSDV detection with skin nodules and scabs are preferred.

Conflict of interest statement

The author declares that she has no conflict of interest.

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