

## Original Article

# Mixed infection of peste-des-petits ruminants and Capripox in goats in South Kivu, Democratic Republic of Congo

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## ABSTRACT

**Objective:** We aimed at determining the prevalence and characterizing the CaPV, determining the CaPV-PPRV coinfection prevalence and providing data about phylogenetic relationship between the fusion protein of PPRV and *P32* gene of CaPV.

**Materials and methods:** A total of 150 samples including animals swabs, tissues and blood were collected from unvaccinated goats in a PPR and/or Capripox outbreaks in South Kivu, Eastern of Democratic Republic of the Congo. Conventional PCR and reverse transcriptase (RT-PCR) were used respectively to amplify *P32*, *RPO30*, *GPCR* genes of Capripox virus and Fusion (F) protein of PPRV. Positive samples were sequenced for phylogenetic analysis.

**Results:** Out of 150 tested animals, 64.7% (n=97/150) were PPRV positive, 52.7% (n=79/150) were Capripox positive and 38.7% (n=58/150) were positive for both PPRV and CaPV. The pairwise comparison of *P32* gene of CaPV and *F* gene of PPRV showed 99.75% of identity percentage among goatpox virus sequences, 96.95% among PPRV sequences and 47.91% between CaPV and PPRV sequences.

**Conclusion:** The study has demonstrated high prevalence of CaP V-PPRV mixed infection in South Kivu. Lumpy skin virus disease (LSVD) is a lineage circulating which has a genetic relationship between its *P32* gene and the *F* gene of PPRV giving the challenge to differentiate the two diseases at the clinical farm level.

## KEYWORDS

Capripox; Coinfection; DR Congo; Goat; Phylogeny; PPRV

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## INTRODUCTION

Small ruminants are important livestock and have a significant contribution in the economic status of farmers from developing countries. Goats and sheep are considered as ‘poor man’s’ financial resources. The productivity of these animals are mostly affected by respiratory diseases caused by several types of pathogens including bacteria, virus, fungi and parasites in single or mixed infections (Ozmen et al., 2009; Chu et al., 2011; Saeed et al., 2015). The proper management in appropriate time of these diseases depend more on accurate identification and/or diagnostic of the suspected responsible pathogens(s). Several pathogens including capripoxvirus (CaPV), *Pasteurella multocida* (PM), peste des petits ruminants virus (PPRV), *Mycoplasma capricolum* and *Capripneumoniae* (Mccp) are responsible for major respiratory syndromes of goats and sheep in Africa and middle East (Thiaucourt et al., 1996; Harper et al., 2006; Odugbo et al., 2006; Valadan et al., 2014).

Peste-des-petits-ruminants virus (PPRV) is a negative-sense RNA virus; single stranded belonging to the genus of morbillivirus in a Paramyxoviridae family causes an acute infectious disease of small ruminants with high morbidity and mortality rates. Pox diseases in goats and sheep are caused respectively by goatpox virus (GTPV) and sheeppox virus (SPPV) of capripox virus (CaPV) genus. The clinical signs seem to be similar but they are two different diseases belonging to two different families. These diseases are characterized by skin pox lesions, conjunctivitis, mucous membranes, high fever and respiratory distress (Babiuk et al., 2008). PPR and CaP nearly the same endemic regions (Brown, 2011; Libeau et al., 2014).

Many authors and investigators such as Thonur et al. (2012) and Venkatesan et al. (2014) have developed conventional PCR and quantitative PCR (qPCR) based assays for detection, characterization and differentiation of closely related pathogens or pathogens.

However, for the viruses enclosed in this study, only conventional single plate PCR and reverse transcriptase PCR assays are described. The current study detects and characterized concurrently peste-des-petits-ruminants and capripox viruses in goats using a single tube respectively in two and one-step reaction.

## MATERIALS AND METHODS

This study was conducted from January 2016 to September 2017 in the molecular biology laboratories at the International Institute of Tropical Agriculture (IITA)

Hub in Bukavu, DR Congo and the “Université Evangélique en Afrique (UEA)” in DR Congo.

**Experimental animals and study locations:** Purposive selection of 150 animals (goats) presenting both Capripox and Peste-des-petits ruminants clinical signs including skin pox lesions, high fever, conjunctivitis, mucous membranes, respiratory distress, proliferative and self-resolving lesions around the muzzle and lips, serous mucopurulent nasal and ocular discharge, diarrhea, eye lids matting, skin nodules, lacrimation, gums and soft palate erosions and labored breathing were selected and sampled in four different agro-pastoral regions of South Kivu (Mwenga, Shabunda, Kalehe and Fizi) located in the Eastern part of the DR Congo (Figure 1).

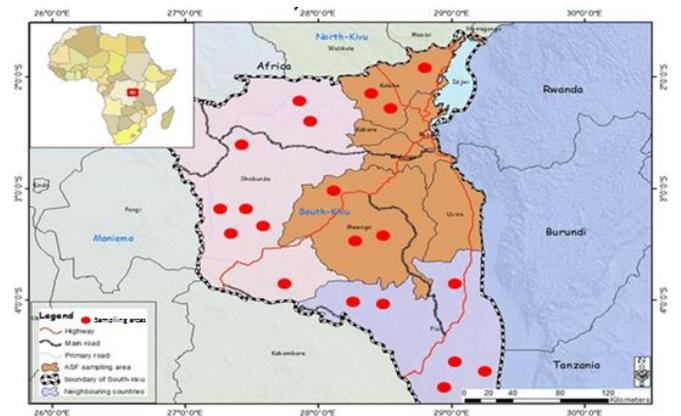


Figure 1: Study areas (indicated by red circles)

**Sample collection and preparation:** Whole blood, tissues and swabs were collected either from dead or live goats suspected of suffering from the disease. Approximately 6 ml blood sample was collected for each animal into vacutainer tubes containing anticoagulant, EDTA (BD Biosciences, Franklin Lakes, USA), using a sterilised needle after the jugular vein puncture. Each tube was labelled. The buffy coat was found after centrifuging of 500  $\mu$ L histopaque<sup>®</sup>-1077 (Aldrich) coated with 1 mL of blood at 400 x g for 30 minutes at 4°C. The upper part was discarded while the buffy coat was transferred into a sterile new microfuge tube and stored at -80°C until the extraction of DNA and RNA. Six (6) mL aliquots were put into two different tubes, 3 mls to each; one of the aliquots was used for genomic DNA extraction to detect CaPV and the other one for the total RNA extraction to detect PPRV.

Oculo-nasal discharges were collected from animals and stored in 3 mL BD transport medium for viruses (Franklin Lakes, USA). They were then vortexed to dislodge any cells from the swabs and centrifuged at 10,000xg for 5 min at room temperature. The upper part

was decanted into sterile microfuge tube and stored at  $-80^{\circ}\text{C}$  until RNA and DNA extractions were done. Tissues collected from dead animals were lymph nodes, lung and intestines. Samples issues from the same animal were pooled and processed together. Approximately 250 mg of tissue sample was homogenized in 2.5 mL of Gibco® Mixture (Carlsbad, USA) to make a tissue suspension of 10%, then the suspension was then centrifuged for five minutes at 10,000xg at room temperature before the storage in  $-80^{\circ}\text{C}$  until total RNA and genomic DNA extractions were done.

**Extraction of RNA and DNA:** Viral RNA was recuperated from the coat, homogenized tissue and oculo-nasal swabs using QIAamp viral RNA Mini extraction kit (Hilden, Germany) following the manufacturer's instructions as proposed by [Kgotlele et al. \(2014\)](#). Viral DNA was recovered from the samples using the DNeasy Qiagen extraction kit (Hilden, Model: 69506) respecting manufacturer's procedure at the IITA hub laboratory. Both DNA and RNA quality and concentration were tested by nanodrop 2000 and 1.0-1.5% agarose gel electrophoresis with the buffer TAE 0.5X to evaluate the degradation and integrity.

**Amplification:** Reverse transcription polymerase chain reaction (RT-PCR) was carried out in PCR System 9700 (Carlsbad, USA) with Bioneer pre master mixt and specific primers to amplify a 372-bp fragment of PPRV-fusion gene using a set of published primers: fusion forward (5'-GAG-ACT-GAG-TTT-GTG-ACC-TAC-AAG-C-3') and fusion reverse (5'-CAG-CAG-CAT-ATT-AAT-GTG-ACA-AGC-CCT-AG -3') as proposed by [Esmaelizad et al. \(2011\)](#) and [Forsyth and Barrett \(1995\)](#).

Briefly, a volume of 20- $\mu\text{L}$  reaction containing 50 ng/ $\mu\text{L}$  of total RNA was used for reverse transcription. Each tube contained: 1  $\mu\text{L}$  of OligodT and/or 50–250 ng random hexamers, 1 ng to 5  $\mu\text{g}$  total RNA, 1  $\mu\text{L}$  dNTP Mix, 12  $\mu\text{L}$  of sterile/distilled water. The mixture was heated for 5minutes at  $65^{\circ}\text{C}$  before chilled on ice quickly and then centrifuged at full speed (14 000 rpm) for 30 Sec to collect the contents. A total of 4  $\mu\text{L}$  of 5 X First-Strand buffers, 2  $\mu\text{L}$  of 0.1 M DTT and 1  $\mu\text{L}$  of RNaseOUT were added for purity of the nucleic acid yield. The tube contents were mixed and incubated for 2 min at  $42^{\circ}\text{C}$  then 1  $\mu\text{L}$  of SuperScript™ II RT was then added and mixed gently by pipetting up and down then incubated for 50 min at  $42^{\circ}\text{C}$ . Finally, the reaction was inactivated by heating for 15 minutes at  $70^{\circ}\text{C}$ . The amplification was carried out at  $95^{\circ}\text{C}$  for 2 min for cDNA initial denaturation followed by 35 cycles in three steps: denaturation of 30 Sec at  $94^{\circ}\text{C}$ , 45 Sec at  $55^{\circ}\text{C}$  for

annealing and then elongation for 2 min at  $72^{\circ}\text{C}$ . A final extension at  $72^{\circ}\text{C}$  for 7 min was added.

The *P32*, *GPCR* and *RPO30* genes were amplified for capripox detection and genetic characterization using published sets of primers targeting different regions of capripox as suggested by [Zhou et al. \(2012\)](#). P32-forward (5' ATG-GCA-GAT-ATC-CCA-TT 3') and P32-reverse (5' TTA-CCA-CAG-GCT-ATT-AGA-AG 3') set of primers were used to amplify 1181 bp fragments. GPCR-forward (5' TTT-ATC-AGC-ACT-AGG-TCA-TTA-TCT 3') and GPCR-reverse (5' TAT-CAC-TCC-CTT CCA-TTT-TTA-T 3') set of specific primers amplified the 1684 bp fragments and RPO30-forward (5' CTC-TGT-TCC-AAA-CTA-AAT-CAT 3') and RPO30-reverse (5' TTT-TTG-TAT-TAC-CAA-TTT-CTG 3') amplified the 1385 bp fragments. A total volume of 100  $\mu\text{L}$  contained 20 ng of extracted DNA, 20  $\mu\text{L}$  5  $\times$  PS buffer, 1  $\mu\text{L}$  prime DNA polymerase, 8  $\mu\text{L}$  of dNTP, 63  $\mu\text{L}$  nuclease-free water and 0.20  $\mu\text{M}$  of each primer was used for PCR in a GeneAmp thermocycler following the cycling parameters bellow:  $98^{\circ}\text{C}$  for 10 Sec for initial denaturation, 35 cycles of denaturation for 10 Sec at  $98^{\circ}\text{C}$ , annealing for 20 Sec at  $47^{\circ}\text{C}$  and extension at  $72^{\circ}\text{C}$  for 120 Sec. A final extension followed for 20 min at  $72^{\circ}\text{C}$ .

The PCR product was then run on a 1.5% agarose gel electrophoresis pre-mixed with Gel Red nucleic acid stain (Candler, NC, USA). Visualization of PCR products was done using a BioDoc imaging. The PCR products were then purified using QIAquick Gel Extraction Kit (cat. nos. 28704 and 28706) before they were sequenced using Big Dye Terminator Kit Version 3.1.

**Data and phylogenetic analysis:** Microsoft Office Excel 2016 was used to calculate frequencies of PPRV and Capripox samples prevalence by using a formula of proportion as given by [Nei and Kumar \(2000\)](#).  $P = \frac{\text{\#positive samples}}{\text{Total sample}}$ , Where  $P$  is prevalence.

Several capripox P32, RPO30 and GPCR nucleotide sequences of other capripoxviruses and the fusion gene of peste des petits ruminant virus isolated from different countries in different years were retrieved from NCBI gene bank using the online basic local alignment search tool (BLAST). Molecular Evolutionary Genetics Analysis (MEGA 6) software helped to perform the multiple alignments of the downloaded sequences using ClustalW method, Neighbor-Joining (NJ), Maximum Likelihood (MJ) tests and Hasegawa-Kishino-Yano (HKY) model for *RPO30* gene, Tamura 3-parameter (T92) model for fusion *PPRV* gene, *GPCR* and *P32* genes to perform phylogenetic analysis as suggested by [Nei and Kumar](#)

(2000) and Tamura et al. (2013). A total of 1000 replicates bootstrap value was considered to test the phylogenetic tree reliability (Tamura et al., 2013).

## RESULTS

**Prevalence of CaPV and PPRV in unvaccinated goats and sheep:** Among a total of 150 unvaccinated goats and/or sheep samples studied, 97(64.7%) were PPRV positive, 79(52.7%) were CaPV positive; while 58(38.7%) were both PPRV and CaPV positive (Table 1). High prevalence of PPRV and CaPV coinfection was found in Mwenga and Shabunda regions with respectively 19(54.3%) and 21(53.5%), then followed by Fizi region 18(45%). No coinfection was found in Kalehe region despite the high Capripox prevalence (37.1%) found in this region.

**Phylogenetic analysis of the CaPV and PPRV:** Studying the phylogenetic analysis of nucleotide sequences of *RPO30*, *P32* and *GPCR* genes. It was found that all the samples from South Kivu region in DR Congo were clustered under LSDV lineage as per their host origin (Figures 2-4).

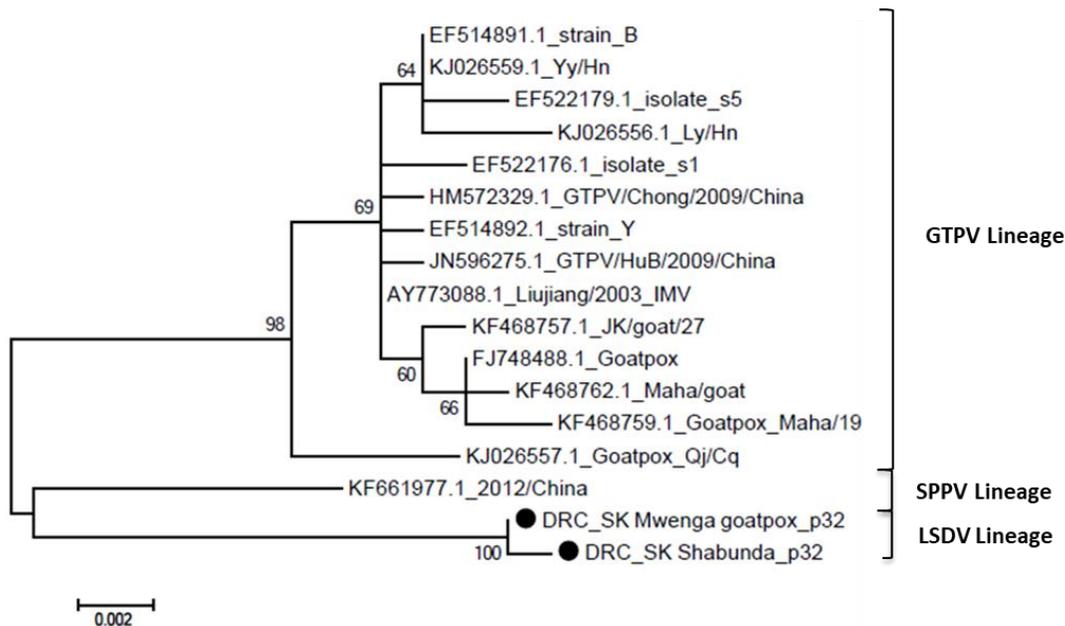
Note that the south Kivu Capripox sequences of the *RPO30* gene is almost similar at 99% with lumpy skin diseases virus RSA/54 Haden RNA polymerase isolate in South Africa with the accession number of GU119937.1 in NCBI gene Bank which are clustered into the lineage of lumpy skin diseases virus.

**Phylogenetic relationship between CaPV and PPRV:** With respect to the same study region, using trimmed sequences of 720-bp, when the three gene sequences of

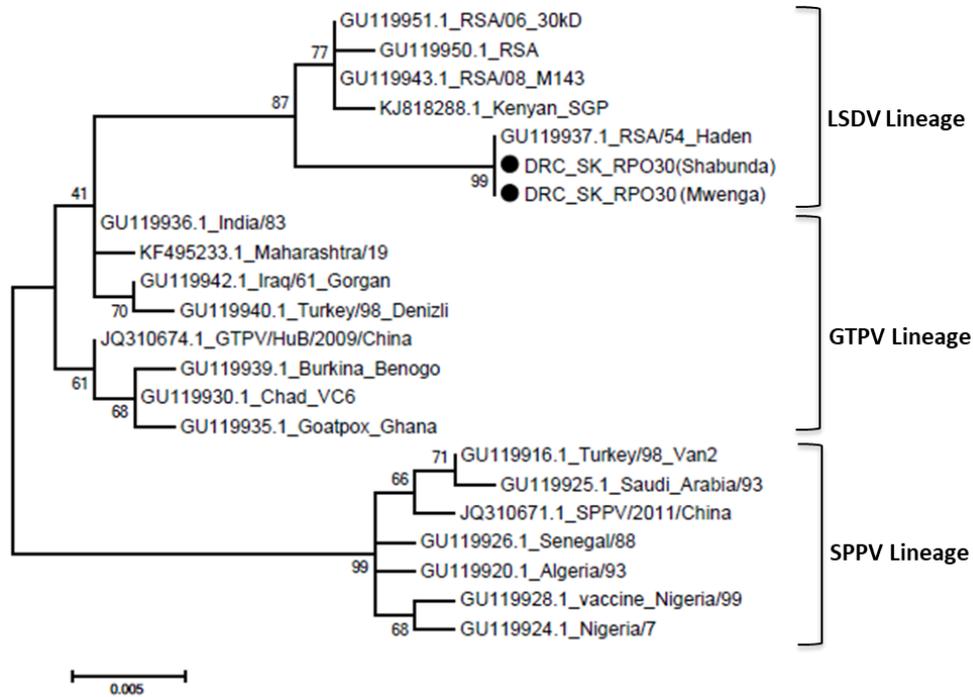
**Table 1.** CaPV and PPRV single and coinfection prevalence

Location	Tested (N)	PPRV +Ve	PPRV (%)	CaPV +Ve	CaPV +Ve (%)	PPRV-CaPV +Ve	PPRV-CaPV +Ve (%)
Shabunda	40	35	87.5	24	60	21	52.5
Mwenga	35	27	77.1	20	57	19	54.3
Fizi	40	28	70	22	55	18	45
Kalehe	35	0	0	13	37.1	0	0
<b>Total</b>	<b>150</b>	<b>97</b>	<b>64.7</b>	<b>79</b>	<b>52.7</b>	<b>58</b>	<b>38.7</b>

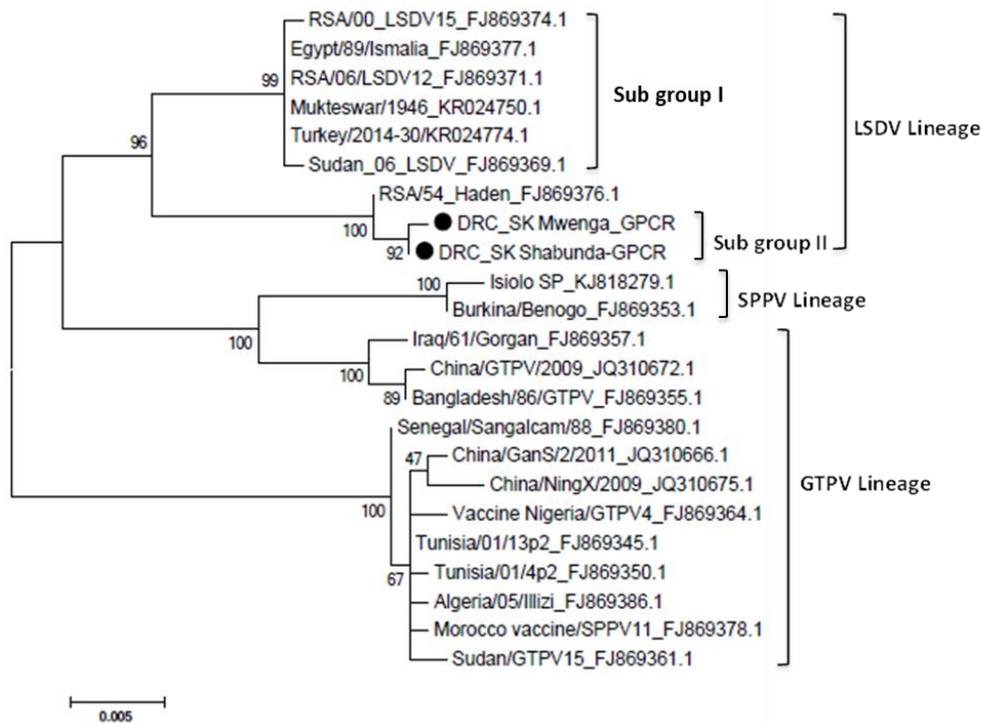
CaPV=Capripox, PPRV=Peste des petits ruminants, +Ve=Positive.



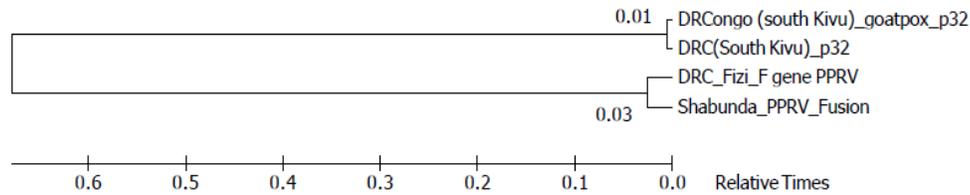
**Figure 2.** Phylogenetic relationship of *P32* gene of Capripox virus (CaPV). A Maximum-Likelihood phylogenetic tree depicting the relationship of DR Congo CaPV obtained from this study (indicated with circles) with other CaPV belonging to the three CaPV Lineages (GTPV, SPPV and LSDV). DR Congo CaPV from Eastern DR Congo in South Kivu region including Shabunda and Mwenga are identical and are clustered within LSDV Lineage. Phylogeny was inferred following 1000 bootstrap replications and values <50% were not shown.



**Figure 3.** Phylogenetic relationship of *RPO30* gene of Capripox virus (CaPV). A Maximum-Likelihood phylogenetic tree depicting the relationship of DR Congo CaPV obtained from this study (indicated with circles) with other CaPV belonging to the three CaPV Lineages (GTPV, SPPV and LSDV). DR Congo CaPV from Eastern DR Congo in South Kivu region including Shabunda and Mwenga are not 100% identical and are clustered within LSDV Lineage. Phylogeny was inferred following 1000 bootstrap replications.



**Figure 4.** Phylogenetic relationship of *GPCR* gene of Capripox virus (CaPV). A Maximum-Likelihood phylogenetic tree depicting the relationship of DR Congo CaPV obtained from this study (indicated with circles) with other CaPV belonging to the three CaPV Lineages (GTPV, SPPV and LSDV). DR Congo CaPV from Eastern DR Congo in South Kivu region including Shabunda and Mwenga are not 100% identical and are clustered within LSDV Lineage. Phylogeny was inferred following 1000 bootstrap replications.



**Figure 5.** Phylogenetic relationship of *P32* gene of Goats pox virus, Capripox virus (CaPV) and Fusion gene of Peste-des-petits-ruminants virus (PPRV). A Maximum-Likelihood phylogenetic

**Table 2.** Pairwise comparison showing percentage of identity between Capripox and peste-des-petits-ruminants viruses.

	1	2	3	4
DRC_GPV_Mwenga_p32gene		99.75	47.17	48.40
DRC_GPV_Shabunda_p32gene	99.75		47.42	48.66
DRC_Mwenga_PPRV Fgene	47.17	47.42		96.95
DRC_Shabunda_PPRV Fgene	48.40	48.65	96.95	

1=GPV\_Mwenga\_P32 gene; 2= GPV\_Shabunda\_p32 gene, 3=Mwenga\_PPRV F gene; 4=Shabunda\_PPRV F gene

the isolated Capri pox viruses were aligned with the one coding for the Fusion protein of PPRV isolates, the percent identical index for the F-gene of PPRV virus and the *P32* gene of CaPV isolates was 47.17% for Mwenga region and 48.65% for Shabunda (**Table 2; Figure 5**).

## DISCUSSION

Peste-des-petits-ruminants (PPR) and capripox (CaP) are now mentioned as endemic diseases and major threat to livestock production and goat farming in DR Congo. Several authors including [Babiuk et al. \(2008\)](#), [Esmaelizad et al. \(2011\)](#), [Kgotlele et al. \(2014\)](#) and [Venkatesan et al. \(2014\)](#) reported on different aspects of PPRV and CaPV in goats focusing on disease diagnostic, characterization and vaccines development in several countries. [Babiuk et al. \(2008\)](#) confirmed that the prevalence of capripox has reduced considerably during the past 50 years but are now expanding in to many new areas which did not report the disease before with recent goats and sheeppox (GTP and SPP) outbreaks recorded in Greece, Vietnam and Mongolia and few lumpy skin disease (LSD) outbreaks recently reported in Israel and Ethiopia. LSD, GTP and SPP are classified by the World Organisation for Animal Health (OIE) as notifiable diseases due to their geographical coverage and economic loss impact to farmers. The PPRV and CaPV coinfection prevalence of 38.7% obtained in South Kivu region, in Eastern of Democratic Republic of Congo was high compared to other countries.

Between the years 2007 to 2016, several authors including [Saravanan et al. \(2007\)](#), [Kul et al. \(2008\)](#), [Mondal et al. \(2009\)](#), [Ozmen et al. \(2009\)](#), [Malik et al. \(2011\)](#) and [Karim et al. \(2016\)](#) reported goatpox as mixed infection with PPR by in many countries. The dual or multiple

infections caused by several pathogens increase the morbidity and mortality rates within animals in flocks or between animals individually. However, data are limited regarding the specific interactions during the dual occurrence of PPRV-CaPV in specific animals ([Malik et al., 2011](#)). Peste-des-petits-ruminants (PPR) is considered as a disease which reduce the activation of the immune response to capripox in goats and sheep in a mixed infection resulting in increasing of the mortality and morbidity rates and causing economic loss to farmers ([Rajak et al., 2005](#)).

This study has shown that the capripox virus that was circulating in South Kivu DR Congo was Lumpy Skin Disease Virus (LSDV) associated with goat pox and sheeppox viruses as referred to **Figure 2-4**. This is in contrast to results of previous studies carried-out in India, which indicated presence of GTPV and SPPV ([Santhamani et al., 2014](#)). However, LSDV lineage, host specificity and susceptibility should be documented through molecular diagnostic and characterization (CaPV whole genome sequence analysis) because reports are very limited on *RPO30* and *GPCR* and *P32* genes of CaPV. There is, therefore, the danger of increased uncontrolled trade of live animals resulting the risk for further spread from infected regions to non-infected areas especially in Asia and Africa.

Several authors have confirmed that the *P32*, *GPCR* and *RPO30* genes have specific molecular markers for LSD, GTP and SPP viruses and can be useful for genetic characterization of different strains/lineages ([LeGoffe et al., 2009](#); [Lamien et al., 2011](#)). The similarity between *P32* gene of Capri pox virus and *F*-gene of PPRV has diagnostic importance since it may precipitate cross-reactions and misdiagnoses. Pock lesions may distinguish

CaP from PPR. Proper distinction between the two diseases is important for respective accurate and successful vaccination/control strategies.

## CONCLUSION

CaPV and PPR are endemic in goat's populations in South Kivu region of Democratic Republic of Congo but the CaPV prevalence, species characterization and coinfection with PPR have been under-reported. This paper reports occurrence of high mortalities in goats due to mixed infection of PPR and Capripox. Lymph skin virus disease was diagnosed and characterized in goats. The prevalence of mixed infection is detected and reporting for the first time in Shabunda and Mwenga in the Eastern of DR Congo. Since the two diseases are of economic importance, appropriate control measures need to be put in place. Continuous surveillance for the coinfections also needs to be effected in order to establish the respective dynamics.

## ACKNOWLEDGEMENT

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## CONFLICT OF INTEREST

The authors declare that they have no conflict of interest.

## AUTHORS' CONTRIBUTION

BAB planned the study, analyzed the data and made the draft of the current manuscript. BPN collected the data and also assisted in the preparation of the manuscript. OC, CLB and GCG helped in preparing, drafting and correcting of this manuscript.

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