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Original article

MOLECULAR CHARACTERISATION OF LUMPY SKIN DISEASE VIRUS AND SHEEPPOX VIRUS BASED ON *P32* GENE

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Summary

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Lumpy skin disease virus (LSDV) and sheeppox virus (SPV) have a considerable economic impact on the cattle and small ruminant industry. They are listed in group A of contagious disease by the World Organization for Animal Health (OIE). This study addressed molecular characterisation of first LSDV outbreak and an endemic SPV in Kurdistan region of Iraq based on *P32* gene. The results indicated that *P32* gene can be successfully used for diagnosis of LSDV. The phylogenic and molecular analysis showed that there may be a new LSDV isolate circulating in Kurdistan which uniquely shared the same characteristic amino acid sequence with SPV and GPV, leucine at amino acid position 51 in *P32* gene as well as few genetically distinct SPV causing pox disease in Kurdistan sheep. This study provided sequence information of *P32* gene for several LSDV isolates, which positively affects the epidemiological study of *Capripoxvirus*

Key words: *Capripoxvirus*, Iraq, LSDV, *P32* gene, SPV

INTRODUCTION

Lumpy skin disease virus (LSDV), goatpox virus (GTPV) and sheeppox virus (SPPV) belong to the genus *Capripoxvirus*, family Poxviridae (Fauquet *et al.*, 2005). Capripoxviruses consist of large enveloped double strand DNA. The genome is about 151 kb. There are close genetic relations among the species of the *Capripoxvirus* genus. However, there are unique traits responsible for virulence and host range. This property makes LSDV a host-specific pathogen for cattle and SPPV – host-specific for sheep (El-Nahas *et al.*, 2011).

The lumpy skin disease is severe, highly contagious, characterised by nodules in the skin and enlarged superficial lymph nodes, sometimes fatal (Davies, 1991). Sheep pox and goat pox are characterised by pyraexia and pock lesions on the skin and internal organs causing high mortality, particularly in young animals

(Fulzele *et al.*, 2006). The diseases have a considerable economic impact on the cattle and small ruminant industry and are listed in group A of contagious diseased by the World Organization for Animal Health (OIE) (Kitching, 2000).

LSD is endemic in the African countries and has spread into most of the Middle East countries, Asia and there is a risk of further spread into Europe (Tuppurainen & Oura, 2012). In Iraq, LSD was recorded for the first time in the autumn of 2013, and it continues to spread to all of the Iraqi governments (Al-Salihi & Hassan, 2015). Classically, *Capripoxvirus* laboratory examination includes virus isolation, fluorescent antibody test (FAT), enzyme-linked immunosorbent assay (ELISA) (Chand *et al.*, 1994). However, the classical examinations are not readily available everywhere, and some of them have low specificity due to cross-reactions between *Parapoxvirus* and *Capripoxvirus* (Murphy *et al.*, 1999). Currently, sensitive and specific molecular methods are used for detection of *Capripoxvirus* targeting the *P32*, *GPCR* and *RPO30* genes (Zhou *et al.*, 2012).

The aims of this study were the molecular characterisation of *Capripoxvirus* in Kurdistan region of Iraq based on *P32* gene as well as to provide information on the sequence of *P32* gene of LSDV.

MATERIALS AND METHODS

Samples

Skin nodules were collected from 9 cows suspected of lumpy skin disease (Fig. 1). Scab samples also were obtained from 5 sheep suspected of sheep pox in various geographical regions of the Sulaimani Governorate in a period from November 2013 to December 2014. The samples were collected aseptically and transported in a cool box to the molecular diagnostic laboratory in the Sulaimani veterinary directorate.

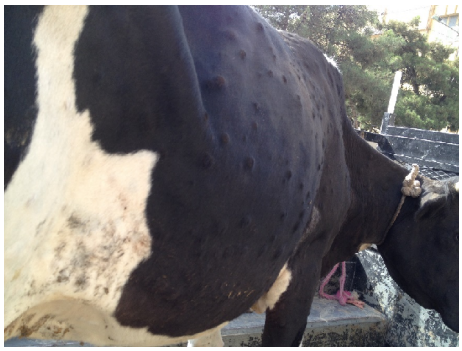

Fig. 1. Photograph of cow with severe LSD lesions in the skin.

DNA extraction

The skin samples were put in a sterile Petri dish then cut with a sterile surgical disposable blade to a smaller pieces, and pureed to become paste-like in consistency. Fifteen grammes of pureed sample were then used for DNA extraction using genomic DNA extraction kit (tissue) (Geneaid, Korea) according to the manufacturer's instructions. The purified DNAwas then stored at –20 °C for subsequent PCR assay.

PCR amplification

The PCR amplification reaction was done according to the manufacturer instructions using Bioneer AccuPower PCR Premix. The PCR primers were designed from *P32* gene that is specific for the *Capripoxvirus* genus with the following sequence: forward primer CTAAAATTAGAGAGCT ATACTTCTT and reverse primer CGATTTCCATAAACTAAAGTA to amplify 390 bp (Heine *et al.*, 1999). The primers were constructed by Macrogen (Korea). The PCR reaction was performed

with 5 µL of DNA template and 1 µL of 10 pmol forward and reverse primers, then the reaction was made up to a final volume of 20 µL with DEPC-H_2O. The thermal cycler (BIO-RAD, USA) parameters were: initial denaturation at 94 °C for 5 mins, 38 cycles denaturation at 94 °C for 30 s, annealing at 50 °C for 30 s, extension at 72 °C for 30s ; and a final extension phase run at 72 °C for 5 min.

Electrophoresis and gel analysis

Electrophoreses of PCR products were done in a 1.2 % agarose gel. Seven µL of PCR products were stained with 3 µL safe dyes then loaded on the gel and visualised by UV transilluminator and photographed (UVETIC, UK). The amplicon sizes were estimated according to the migration pattern of a 100 bp DNA ladder.

Direct sequencing

Thirty-five microliters of positive PCR products were sequenced in Macrogen sequencing service in South Korea. The identity of each nucleotide was verified twice. The coding sequences were submitted to Genbank database with accession numbers KF996498, KM047052, KM047053, KM047055, KM047056, KM047057, KM047058, KM047059, KP313621, KP313622, KF992798, KP313623, KP313624, KP313625.

Phylogenetic analysis

Partial amino acid sequences of *P32* gene of *Capripoxvirus* strains worldwide were obtained from the GenBank. Multiple alignments of these sequences were performed by MEGA 6 with ClustalW method (Thompson *et al.*, 1994). MEGA 6 was used to perform phylogenetic analysis with Neighbor-Joining (NJ). The bootstrap values were determined from 1000 replicates of the original data. Sufficient detail should be provided to allow the work to be repeated.

RESULTS

Capripoxvirus detection

The partial *P32* genes of *Capripoxvirus* were successfully amplified in all clinical samples. The expected amplicon size, 390 bp of *Capripoxvirus* was detected in the 9 cow samples and 5 sheep samples, the amplified fragments were confirmed by sequencing (Fig. 2).

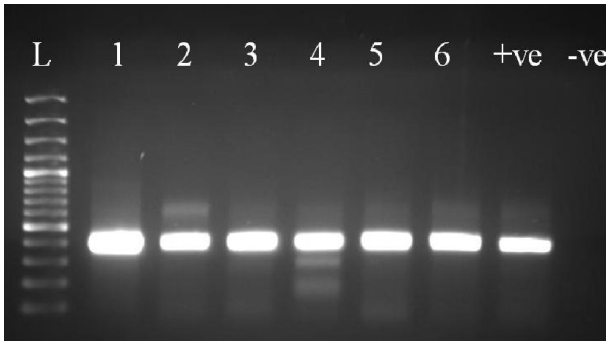

Fig. 2. Agarose gel electrophoresis pattern shows PCR amplification of 390 bp from the *P32* gene of Kurdistan *Capripoxvirus.* Lane L: 100 bp DNA ladder, lanes 1–3: LSDV field strain, lanes 4–6: SPV field strain, lane +ve: positive control (vaccine), lane –ve: negative control.

Phylogenetic analysis

A phylogenetic tree (Fig. 3) was constructed based on the partial protein alignment of the *P32* gene of Kurdistan *Capripoxvirus* and other *Capripoxvirus* published in NCBI.

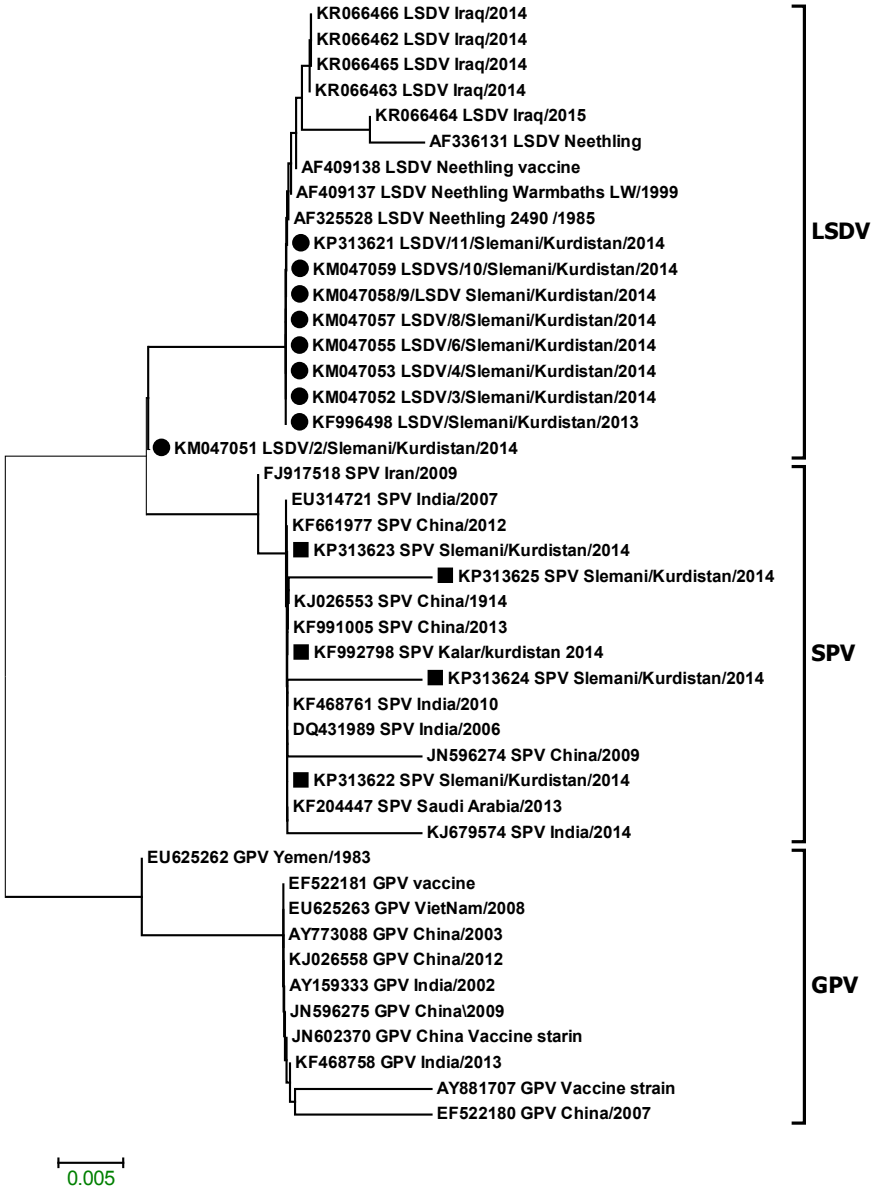

Fig. 3. Phylogenetic tree of Kurdistan *Capripoxvirus* isolates. Analyses of the phylogenetic tree according to partial *P32* amino acid sequence indicated 3 clusters. The circles show Kurdistan/LSDV field strains and squares – Kurdistan/SPV field strains.

The topology of the phylogenetic tree indicated three clusters; namely LSDV, GPV and SPV. All SPV strains of Kurdistan clustered in SPV lineage. The GPV was divided into two sub-clusters; one of them included only the Yemen strain (EU625262 GPV Yemen/1983), the other included GPV from China, India, and Vietnam. All Kurdistan LSDV strains in this study clustered in one lineage. KM047051 LSDV/2/Slemani/Kurdistan/2014 was separated from the other LSDV isolates and it formed a different subcluster.

Analysis of P32 protein of Kurdistan Capripoxvirus

Partial amino acid sequences of *P32* protein of Kurdistan LSDV and SPV were compared with *Capripoxvirus* sequences published in Genbank (Table 1). The homology between Kurdistan *Capripoxvirus* was 96–100%. The results showed 15 variable amino acids from the first 100 amino acids. The GPV lineage had unique characteristic residues 26G, 48K and 95V compared with other *Capripoxvirus* strains 26D, 48N, and 95A, respectively. The LSDV had an exclusive 51F amino acid residue as compared with 51L in other *Capripoxvirus* isolates. The SPV lineage had an exclusive inserted 57D amino acid residue. SPV also showed L64F substitution compared with LSDV and GPV lineages. All Kurdistan LSDV isolates presented in this study shared the same amino acid residue with other LSDV strains except for LSDV/2/Slemani/Kurdistan/2014 isolate accession No. (KM047051) that showed amino acid L51F substitution. There were only two amino acid substitutions in Kurdistan SPV isolates as compared with other *Capripoxvirus* strains – D18E in strains with accession No. (KP313625) and D46H in strains with accession No (KP313624).

DISCUSSION

In Iraq, the *Capripoxvirus* was isolated for the first time in Kurdistan, the northern part of Iraq and identified as GPV Sersenik strain (Tantawi & Al Falluji, 1979). Sheep pox and goat pox are endemic in Iraq (Zangana & Abdullah, 2013). The first natural outbreaks of LSD occurred in Iraq in 2013 (Al-Salihi & Hassan, 2015). In this study, molecular detection and phylogenic analysis of *Capripoxvirus* were performed in Sulaimani province in Kurdistan region of Iraq to analyse the genetic relationship with the other *Capripoxvirus* worldwide and to understand the epidemiology of the new LSDV outbreaks in Iraq. Several researches used the *P32* gene to detect SPV and GPV because it contains a most significant antigenic determinant present in all species of Capripoxvirus genus (Heine *et al.*, 1999; Tian *et al.*, 2010). Nevertheless, in this study the *P32* gene was used for identification of LSDV to detriment the validity of the primers to detect LSDV in cattle and to provide *P32* gene sequence information in LSDV because there were limited data descriptions of *P32* gene in LSDV as compared to *GPCR* gene and *RPO30* gene in the Genbank (Zhou *et al.*, 2012). The phylogenic analysis of the *Capripoxvirus* indicated three lineages; SPV, GPV, and LSDV. All *Capripoxvirus* isolates from sheep in this study clustered in SPV lineage. This outcome proved that SPV is host specific (Beard *et al.*, 2010). This finding also indicates that despite communal herding of sheep and goat, there was no clinical pox infection in goats in the Sulaimani province in 2013–2014.

Table 1. Partial amino acid alignment of *P32* gene of Kurdistan *Capripoxvirus* isolates. Variable amino acids are highlighted in gray, and identical nucleotides – indicated with dots

Name of isolates	*Species*	*Amino acids*	*No.*
KM047052 LSDV/3/Slemani/Kurdistan/2014	*LSDV*	*MAIPLYVIPIVGREISDVVPELKSDNDIFYKK--VDTVKDFKNSDVNFF*	*[50]*
KM047051 LSDV/2/Slemani/Kurdistan/2014	*LSDV*	................................--...............	*[50]*
KM047057 LSDV/8/Slemani/Kurdistan/2014	*LSDV*	................................--...............	*[50]*
KM047055 LSDV/6 /Slemani/Kurdistan/2014	*LSDV*	................................--...............	*[50]*
KM047059: LSDVSlemani/Kurdistan/2014	*LSDV*	................................--...............	*[50]*
KP313621 LSDV/11/Slemani/Kurdistan/2014	*LSDV*	................................--...............	*[50]*
KF996498 LSDV/Slemani/Kurdistan/2013	*LSDV*	................................--...............	*[50]*
KM047053 LSDV/4/Slemani/Kurdistan/2014	*LSDV*	................................--...............	*[50]*
KM047058 LSDV/9/Slemani/Kurdistan/2014	*LSDV*	................................--...............	*[50]*
KR066466 LSDV Iraq/2014	*LSDV*	-----------.....................--...............	*[50]*
KR066465 LSDV Iraq/2014	*LSDV*	-----------.....................--...............	*[50]*
KR066464 LSDV Iraq/2015	*LSDV*	-------------...................--...............	*[50]*
KR066463 LSDV Iraq/2014	*LSDV*	----------......................--...............	*[50]*
KR066462 LSDV Iraq/2014	*LSDV*	-----------.....................--...............	*[50]*
AF325528 LSDV Neethling 2490 /1985	*LSDV*	................................--...............	*[50]*
AF336131 LSDV Neethling	*LSDV*	................................--...............	*[50]*
AF409137 LSDV Neethling Warmbaths LW/1999	*LSDV*	................................--...............	*[50]*
AF409138 LSDV Neethling vaccine	*LSDV*	................................--...............	*[50]*
EU625262 GPV Yemen/1983	*GPV*	........................G.......--...............	*[50]*
EF522181 GPV vaccine	*GPV*	........................G.......KK............K..	*[50]*
KJ026558 GPV China/2012	*GPV*	........................G.......--............K..	*[50]*
EU625263 GPV VietNam/2008	*GPV*	........................G.......--............K..	*[50]*
JN596275 GPV China\2009	*GPV*	........................G.......--............K..	*[50]*
EF522180 GPV China/2007	*GPV*	........................G.......--.G..........K..	*[50]*
AY773088 GPV China/2003	*GPV*	........................G.......--............K..	*[50]*
JN602370 GPV China Vaccine strain	*GPV*	........................G.......--............K..	*[50]*
KF468758 GPV India/2013	*GPV*	........................G.......--............K..	*[50]*
AY159333 GPV India/2002	*GPV*	........................G.......--............K..	*[50]*
AY881707 GPV Vaccine strain	*GPV*	........................G.......--............K..	*[50]*
FJ917518 SPV Iran/2009	*SPV*	................................--...............	*[50]*

Table 1 (cont'd). Partial amino acid alignment of *P32* gene of Kurdistan *Capripoxvirus* isolates. Variable amino acids are highlighted in gray, and identical nucleotides – indicated with dots

Name of isolates	*Species*	*Amino acids*	*No.*
KF991005 SPV China/2013	*SPV*	. . .. - -	*[50]*
EU314721 SPV India/2007	*SPV*	. . .. - -	*[50]*
KF468761 SPV India/2010	*SPV*	. . .. - -	*[50]*
JN596274 SPV China/2009	*SPV*	. . .. - -	*[50]*
KF204447 SPV Saudi Arabia/2013	*SPV*	. . .. - -	*[50]*
KJ026553 SPV China/1914	*SPV*	. . .. - -	*[50]*
KJ679574 SPV India/2014	*SPV*	. . .. T . . . . - -	*[50]*
KF661977 SPV China/2012	*SPV*	. . .. - -	*[50]*
DQ431989 SPV India/2006	*SPV*	. . .. - -	*[50]*
KP313622 SPV Slemani/Kurdistan/2014	*SPV*	. . .. - -	*[50]*
KF992798 SPV Kalar/Kurdistan 2014	*SPV*	. . .. - -	*[50]*
KP313623 SPV Slemani/Kurdistan/2014	*SPV*	. . .. - -	*[50]*
KP313624 SPV Slemani/Kurdistan/2014	*SPV*	. . .. - - . . . . H	*[50]*
KP313625 SPV Slemani/Kurdistan/2014	*SPV*	- - - - . . . . E . . . . - -	*[50]*
KM047052 LSDV/3/Slemani/Kurdistan/2014	*LSDV*	*F K D K K D - I S L S Y K F L I W E K V E K S G G V E N F T E Y F S G L C N A L C T K E A K S S I A*	*[100]*
KM047051 LSDV/2/Slemani/Kurdistan/2014	*LSDV*	L . . . . . -	*[100]*
KM047057 LSDV/8/Slemani/Kurdistan/2014	*LSDV*	. . . . . . -	*[100]*
KM047055 LSDV/6 /Slemani/Kurdistan/2014	*LSDV*	. . . . . . -	*[100]*
KM047059: LSDVS lSlemani/Kurdistan/2014	*LSDV*	. . . . . . -	*[100]*
KP313621 LSDV/11/Slemani/Kurdistan/2014	*LSDV*	. . . . . . -	*[100]*
KF996498 LSDV/Slemani/Kurdistan/2013	*LSDV*	. . . . . . -	*[100]*
KM047053 LSDV/4/Slemani/Kurdistan/2014	*LSDV*	. . . . . . -	*[100]*
KM047058 LSDV/9/Slemani/Kurdistan/2014	*LSDV*	. . . . . . -	*[100]*
KR066466 LSDV Iraq/2014	*LSDV*	. . . . . . -	*[100]*
KR066465 LSDV Iraq/2014	*LSDV*	. . . . . . -	*[100]*
KR066464 LSDV Iraq/2015	*LSDV*	. . . . . . - . . . . - -	*[100]*
KR066463 LSDV Iraq/2014	*LSDV*	. . . . . . -	*[100]*
KR066462 LSDV Iraq/2014	*LSDV*	. . . . . . -	*[100]*
AF325528 LSDV Neethling 2490 /1985	*LSDV*	. . . . . . -	*[100]*
AF336131 LSDV Neethling	*LSDV*	. . . . . . - V	*[100]*
AF409137 LSDV Neethling Warmbaths LW/1999	*LSDV*	. . . . . . -	*[100]*

Table 1 (cont'd). Partial amino acid alignment of *P32* gene of Kurdistan *Capripoxvirus* isolates. Variable amino acids are highlighted in gray, and identical nucleotides – indicated with dots

Name of isolates	*Species*	*Amino acids*	*No.*
AF409138 LSDV Neethling vaccine	*LSDV*	. . . . . . - V	*[100]*
EU625262 GPV Yemen/1983	*GPV*	L . . . . . - . . . . V	*[100]*
EF522181 GPV vaccine	*GPV*	L . . . . . - . . . . V	*[100]*
KJ026558 GPV China/2012	*GPV*	L . . . . . - . . . . V	*[100]*
EU625263 GPV VietNam/2008	*GPV*	L . . . . . - . . . . V	*[100]*
JN596275 GPV China\2009	*GPV*	L . . . . . - . . . . V	*[100]*
EF522180 GPV China/2007	*GPV*	L . . . . . - . . . . V	*[100]*
AY773088 GPV China/2003	*GPV*	L . . . . . - . . . . V	*[100]*
JN602370 GPV China Vaccine starin	*GPV*	L . . . . . - . . . . V	*[100]*
KF468758 GPV India/2013	*GPV*	L . . . . . - . . . . V	*[100]*
AY159333 GPV India/2002	*GPV*	L . . . . . - . . . . V	*[100]*
AY881707 GPV Vaccine strain	*GPV*	L . . . . . - . . . . . R . . . . V	*[100]*
FJ917518 SPV Iran/2009	*SPV*	L . . . . . D . . . . . . L . . . . - - - - - -	*[100]*
KF991005 SPV China/2013	*SPV*	L . . . . . D . . . . . . L	*[100]*
EU314721 SPV India/2007	*SPV*	L . . . . . D . . . . . . L	*[100]*
KF468761 SPV India/2010	*SPV*	L . . . . . D . . . . . . L	*[100]*
JN596274 SPV China/2009	*SPV*	L . . . . . D . . . . . . L . . . . E	*[100]*
KF204447 SPV Saudi Arabia/2013	*SPV*	L . . . . . D . . . . . . L	*[100]*
KJ026553 SPV China/1914	*SPV*	L . . . . . D . . . . . . L	*[100]*
KJ679574 SPV India/2014	*SPV*	L . . . . . D . . . . . . L	*[100]*
KF661977 SPV China/2012	*SPV*	L . . . . . D . . . . . . L	*[100]*
DQ431989 SPV India/2006	*SPV*	L . . . . . D . . . . . . L	*[100]*
KP313622 SPV Slemani/Kurdistan/2014	*SPV*	L . . . . . D . . . . . . L	*[100]*
KF992798 SPV Kalar/Kurdistan 2014	*SPV*	L . . . . . D . . . . . . L	*[100]*
KP313623 SPV Slemani/Kurdistan/2014	*SPV*	L . . . . . D . . . . . . L	*[100]*
KP313624 SPV Slemani/Kurdistan/2014	*SPV*	L . . . . . D . . . . . . L	*[100]*
KP313625 SPV Slemani/Kurdistan/2014	*SPV*	L . . . . . D . . . . . . L	*[100]*

All Kurdistan LSDV isolates in this study clustered in LSDV lineage. However, LSDV/2/Slemani/Kurdistan/2014 isolate, accession No. (KM047051) is separated into a different sub cluster. LSDV/2/ Slemani/Kurdistan/2014 had an exclusive amino acid substitution L51F as compares to other LSDV in the Genebank. As the L51F substitution is the sequence characteristic for SPV, there may be new circulating LSDV isolates in Kurdistan. The homology between Kurdistan *Capripoxvirus* was 96–100%. This finding justifies a previous publication that despite the close relationship between *Capripoxvirus,* they are phylogenetically distinct (Tulman *et al.*, 2002). The topology of the phylogenetic tree based on amino acid sequence indicated that SPV and LSDV had a closer genetic relationship than goat GPV (Fig. 3). This result is similar to a phylogenetic analysis done by Beard *et al.* (2010). Conversely, the phylogenetic analysis based on nucleotide sequence of the same gene (*P32*) indicated that LSD and GPV had a closer genetic relationship than SPV (Zhu *et al.*, 2013).

In conclusion, the *P32* gene is efficient for the detection and characterisation of LSDV. The analysis of the *P32* genes of Kurdistan *Capripoxvirus* strains collected at different geographical locations in 2013–2014 suggests that few genetically distinct SPV circulated in the country. There may be a new LSDV isolate in Kurdistan which uniquely shared the same characteristic sequence of SPV at amino acid 51L in *P32* gene. These findings provide new information on the epidemiology of *Capripoxvirus* in Iraq.

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