رقم البحث (29)

KELCH LIKE GENE FOR DIFFERENTIATION BETWEEN FIELD ISOLATE AND TISSUE CULTURE ADAPTED STRAIN OF LUMPY SKIN DISEASE VIRUS

BY

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ABSTRACT

Lumpy skin disease (LSD) is a trans-boundary viral disease of cattle caused by a virus (Neethling strain) in the genus Capripoxvirus of the family Poxviridae. Trial for differentiation between previously isolated lumpy skin disease virus (LSDV) from Egypt during summer, 2011 and tissue culture adapted strain of LSDV (Ismailyia 88) was done depending on Kelch like gene. This gene thought to play a role in capripoxvirus virulence in its host. Kelch like gene (569 bp) was amplified by PCR. . The amplified gene was sequenced then analysis of the nucleotides and deduced amino acids sequence was done. The sequence analysis revealed that LSDV isolate, LSDV Ismailia 88 strain and reference capripoxviruses retrieved from Gen bank showed significant homology at nucleotide level. The multiple alignment of the deduced amino acids sequence showed unique criteria in all tissue culture adapted strains of capripoxviruses including LSDV Ismailia 88 strain as they revealed presence of stop codons. This criterion not demonstrated in all capripoxviruses isolates including our LSDV isolate. The result revealed that sequence analysis Kelch like gene could be used in differentiation between field isolate LSDV and tissue culture adapted LSDV. Further study could be directed toward generation of genetically modified LSDV vaccine by deliberately deletion of Kelch-like gene from LSDV isolate to become irreversibly attenuated.

Key Words: Lumpy skin disease virus, polymerase chain reaction (PCR), dot blot hybridization (DBH), Kelch like gene, sequence analysis.

INTRODUCTION

Lumpy skin disease (LSD) is an acute, subacute or inapparent viral disease of cattle restricted to parts of Asia and Africa characterized by pyrexia, generalized skin and internal pox lesions, and generalized lymphadenopathy (**Hamoda et al.,2002, Tuppurainen, 2005 and Mercer et al., 2007**).

The genus Capripoxvirus of the family Poxiviridae comprises three closely related viruses, namely lumpy skin disease (LSD), sheeppox (SP) and goatpox (GTP) viruses. These viruses are the etiological agents of economically important diseases which collectively constitute the most serious poxvirus diseases of production animals **(Mercer et al., 2007 and Babiuk et al., 2008).**

In Egypt, Protection of cattle against LSD was carried out using a Roumanian sheep pox vaccine strain produced in sheep as reviewed in **Davies, 1991** and a Kenyan sheep pox tissue culture vaccine strain produced in Vero cell line (**Michael et al., 1994**). The live modified lumpy skin disease virus (LSDV) vaccine (Ismailyia strain) was produced and proved to be safe, potent and capable to protect cattle against challenge with virulent LSDV (**Daoud et al., 1998**).

LSDV circulate in some farms in Egypt, Israel and Oman although animals were vaccinated with sheep pox vaccine (**Fayed et al., 2006, Bahgat and Khalil, 2007, Stram et al., 2008 and Kumar, 2011)**.

LSDV genome is a linear dsDNA molecule of 151 kbp consists of a central coding region bounded by identical 2.4 inverted terminal repeats and contains 156 putative genes. The complete nucleotide sequences of LSDV and sheeppox virus (SPV) genomes are similar by 97% **(Tulman et al., 2001 and 2002).** Capripoxviruses (CaPVs) are antigenically indistinguishable from each other and able to induce heterologous cross protection (**Buller et al., 2005**). The very close antigenic relationship between CaPVs, with the existence of only one serotype have been used for the preparation of live attenuated vaccines that protect all ruminants against CaPV infection **(Kitching et al., 1986 and 1989).**

Kelch-like protein are encoded by CaPVs and thought to play arole in virus virulence and virus-host interaction **(Balinsky et al., 2007).**

The goal of the present study was using of Kelch like gene for differentiation of tissue culture adapted LSDV from field isolate LSDV as this could be of great value if LSDV vaccine prepared from LSDV would use in future.

MATERIALS AND METHODS

Virus strains:

- LSDV field isolate was previously isolated from skin nodules of LSD suspected cows in Egypt during summer, 2011 in Dakahlia Governorate on CAMs of ECEs after four passages in a P.h.D thesis **(El-Tholoth, 2012)**. The isolates were kept at -20 ºC until used.
- Lumpy skin disease virus (Ismailyia 88 strain): Tissue culture adapted LSDV/Ismailyia88 strain was kindly supplied from the Pox Vaccine Production and Research Department, Veterinary Serum and Vaccine Research Institute (VSVRI), Abbasia, Cairo, Egypt. It was prepared in Madien Darby Bovine Kidney (MDBK).

DNA extraction

DNA extraction from virus strains was done as described by **Sambrook and Russell (2001) and Viljoen et al., (2005**).

Differentiation of tissue culture adapted LSDV from LSDV isolate by sequence analysis of Kelch-like gene:

Kelch like gene was thought to play a role in capripoxvirus virulence in its host **(Balinisky et al., 2007)**. Sequencing analysis of Kelch-like gene of field skin LSDV isolate and tissue culture adapted strain of LSDV Ismailyia 88 strain was done according to **Sambrook and Russell (2001), Viljoen et al., (2005) and Balinisky et al., (2007)** as following: PCR amplification of Kelch-like gene from extracted DNA from field skin LSDV isolate and tissue culture adapted strain of LSDV Ismailyia 88 strain using specific primers for amplification of Kelch-like gene (569bp) of capripoxviruses. The primers sequences were as follows: forward primer (A95), 5′- CTATGTCTAATGCTCCTATAACGTTC-3′ and reverse primer (B7), 5′- TGTATAGTTAATAAAGTTGAAAGTTGGTC -3′followed by gel electrophoresis of the PCR products. The amplified bands of corrected size (569 bp) on the gel were cut with help of razor and the DNA in the gel slices was extracted by DNA gel

extraction kit (MONTAGE, MILLIPORE) and sequenced by JenaGen GmbH Biotechnologie-Gentechnik-Diagnostik (Jena,Germany). The obtained sequence data were analysed using ClustalW (http://www.ebi.ac.uk/clustalw/), and then the alignment *.aln output file was used for performing the phylogenetic Neighbor- Joining (N-J) analysis. Sequence divergence and identity percents werecalculated by MegAlign (DNASTAR, Lasergene ®), then analysis of the deuced amino acid sequence was done by Bioedit software version 7.0.0.

RESULTS

Sequence analysis of Kelch-like gene of tissue culture adapted LSDV Ismailia strain and LSDV cattle isolate:

Kelch like gene was thought to play a role in capripoxvirus virulence in its host. Sequencing analysis of Kelch-like gene of field skin LSDV isolate and tissue culture adapted strain of LSDV Ismailyia88 strain was done as following:

PCR amplification of Kelch-like gene and gel electrophoresis:

Gel electrophoresis of the PCR products obtained from amplification reaction of extracted DNA from field skin LSDV isolate and tissue culture adapted strain of LSDV Ismailyia 88 strain using specific primers that amplify Kelch-like gene revealed the amplification of the gene with correct size (569 bp) and **figure (1)** represents the obtained bands.

Sequencing of amplified Kelch-like gene and analysis of sequencing data:

The amplified bands of corrected size (569 bp) on the gel were cut with help of razor and the DNA in the gel slices was extracted by DNA gel extraction kit (MONTAGE, MILLIPORE) and sequenced by JenaGen GmbH Biotechnologie-Gentechnik-Diagnostik (Jena,Germany). The obtained nucleotide sequences of 569 bp PCR fragments representing the whole Kelch-like gene of cattle field skin LSDV isolate and tissue culture adapted strain of LSDV Ismailyia88 strain were recorded. The multiple alignment of the obtained sequences along with reference capripoxviruses available in Gene bank **(table 1)** was created using ClustalW (http://www.ebi.ac.uk/clustalw/). Percent identity of the nucleotide sequence of the Kelch-like gene (569 bp) of Capripoxviruses is tabulated in **table (2).** The Phylogenetic tree pattern for the alignment of the sequenced viruses (cattle LSDV isolate and tissue culture adapted LSDVIsmailyia88 strain) and references capripoxviruses was shown in **figure (2).**

The multiple alignment of the deduced amino acids of Kelch-like gene of cattle LSDV isolate and tissue culture adapted strain of LSDV Ismailyia88 strain beside reference capripoxviruses available in Gene bank was revealed in **figure (3).** The multiple alignment of the kelch like gene from LSDV isolate, LSDV Ismailia 88 strain and reference capripoxviruses retrieved from Gen bank showed significant homology at nucleotide level. Percent identity of the nucleotide sequence of the Kelch-like gene showed that cattle LSDV isolate showed 96.0%, 94.9%, 96.7%and 96.0% identity with tissue culture adapted LSDV (Ismailyia88 strain), LSDV-59 vaccine, SPV-NK vaccine and GPV/G20 vaccine, respectively. These cattle LSDV isolate showed identity of 98.9%, 98.2% and 97.5% with LSDV-99 isolate, SPV-SA isolate and GPV-PL isolate, respectively. The Phylogenetic tree pattern for the alignment of the Kelch-like gene revealed that cattle LSDV, LSDV-99, SPV-SA and GPV-PL isolates were grouped together, while, SPV-NK vaccine and GPV/G20 vaccine were grouped in one cluster and LSDV-59 vaccine and tissue culture adapted LSDVIsmailyia88 strain were in another cluster. The multiple alignment of the deduced amino acids sequence kelch like gene from LSDV isolate, LSDV Ismailia 88 strain and reference capripoxviruses showed unique criteria in all tissue culture adapted strains of capripoxviruses including LSDV Ismailia 88 strain as they revealed presence of stop codons. This criterion not demonstrated in all capripoxviruses isolates including our LSDV isolate. The positions of these stop codons were recorded at different positions as following; tissue culture adapted LSDV-Ismailyia 88 strain showed stop codons at 7 positions: 8, 13, 30, 107, 123, 167 and 182, while in LSDV-59 vaccine stop codons were at 6 positions: 30, 106, 107, 123, 167 and 182. SPV-NK vaccine showed stop codons at 6 positions: 12, 17, 36, 107, 124 and 180, while in GPV/G20 vaccine stop codons were at 5 positions: 12, 33, 36, 107 and 180.

DISCUSSION

In the present study, conventional PCR and DBH were used for confirmative identification of LSDV isolate and tissue culture adapted LSDV/Ismailyia88. Viral DNAs were detected in LSDV isolate and tissue culture adapted LSDV/Ismailyia88 by both techniques. This indicates that both PCR and DBH could serve as a rapid, effective and specific method for laboratory confirmation of CaPVs. This result is in agreement with **Tuppurainen, 2005** who recorded that PCR could be used in detection of LSDV in biopsy samples, tissue culture and semen**, Awad et al., 2010** who detected CaPVs by PCR in skin

and blood samples and **El-Kenawy and El-Tholoth, 2011** who identified LSDV in CAMs of ECEs by PCR. Also, the result is in concurrence with **Awad et al., 2010** who succeeded in identification of LSDV in skin samples by DBH.

Differentiation between LSDV isolate and tissue culture adapted LSD-Ismailyia 88 strain could be of great value if LSDV vaccine prepared from LSDV would use in future. We depend on sequence analysis of Kelch like gene. Kelch like gene not documented in virus families outside Poxviridae. The gene present in all known poxviruses with exceptions of Parapoxviruses and Molluscum contagiosum **(Senkevich et al., 1997 and Delhon et al., 2004)** and it believed that this gene play a role in capripoxvirus virulence **(Balinsky et al., 2007).** The multiple alignment of the deduced amino acids sequences along with kelch like gene sequences of capripoxviruses available in Gene bank was created using ClustalW (http://www.ebi.ac.uk/clustalw/). The multiple alignment of the inferred amino acids showed that tissue culture adapted capripoxviruses strains including LSDV-Ismailia 88 strain revealed presence of stop codons that were absent in capripoxviruses isolates including our LSDV isolate. Tissue culture adapted LSDV-Ismailyia88 strain showed stop codons at 7 positions: 8, 13, 30, 107, 123, 167 and 182, while in LSDV-59 vaccine stop codons were at 6 positions: 30, 106, 107, 123, 167 and 182. SPV-NK vaccine showed stop codons at 6 positions: 12, 17, 36, 107, 124 and 180, while in GPV/G20 vaccine stop codons were at 5 positions: 12, 33, 36, 107 and 180. The result of Kelch-like gene sequencing revealed that its sequence analysis could be used in differentiation between virulent LSDV and tissue culture adapted LSDV.

This finding is in concurrence with **Balinsky et al., (2007)** who reported that deletion of Kelch-like gene from sheep poxvirus not cause any growth defects in lamb kidney cell culture but attenuated in vivo after intranasal or intradermal and inoculated lambs displayed a reduction in mortality, fever, viraemia, virus shedding and the appearance of lesions.

Further study could be directed toward generation of genetically modified LSDV vaccine by deliberately deletion of Kelch-like gene from LSDV isolate to become irreversibly attenuated and may be able to protect against infection with virulent LSDV. This vaccine will facilitate differentiation of virulent LSDV from LSDV vaccine as presence of antibodies against Kelch-like protein will indicate infection by virulent LSDV.

Table (1): List of Capripoxviruses sequences used for the sequence analysis of Kelch-like gene:

Isolation country	Virus isolate	Isolation year	Genbank accession no.	Reference
Egypt	Cattle LSDV 011	2011	In process	**This paper**
	Ismailia 88 LSDV	1990	In process	**This paper**
South Africa	LSDV-Neethling vaccine LW 1959 (LSDV-59)	1959	AF409138	**Kara et al., (2003)**
	LSDV- Neethling Warmbaths LW (LSDV-99)	1999	AF409137	**Kara et al., (2003)**
Kazakhstan	SPV-SA	1987	AY077833	**Tulman et al., (2002)**
	SPV-strain NISKHI (SPV-NK)	1994	AY077834	
	GPV-Pellor strain(GPV-PL)	2000	AY077835	
	GPV-strain G20-LKV (GPV-G20)	2000	AY077836	

Table (2): Identity percent of Kelch gene nucleotide sequences of cattle LSDV isolate and tissue culture adapted LSDV-Ismailia88 strain compared with reference sheep poxviruses (isolate and vaccine strains), goat poxviruses (isolate and vaccine strains) and LSDV (isolate and vaccine strains).

Percent Identity (upper triangle); Divergence (lower triangle)

	1	2	3	4	5	6	7	8		
1		96.0	98.9	94.9	98.2	96.7	97.5	96.0	1	CattleLSDV isolate
2	4.2		95.1	98.4	94.2	95.3	93.5	94.6	2	LSDV Ismailia88 strain
3	1.1	5.1		94.0	97.2	95.8	96.5	95.1	3	LSDV-99 isolate
4	5.3	1.6	6.2		93.1	94.2	92.8	93.5	4	LSDV-59 vaccine
5	1.8	6.0	2.9	7.2		94.9	99.1	94.2	5	SPV-SA isolate
6	3.4	4.9	4.3	6.0	5.3		94.2	99.3	6	SPV-NK vaccine
7	2.5	6.8	3.6	7.6	0.9	6.0		93.5	7	GPV-PL isolate
8	4.2	5.7	5.1	6.8	6.0	0.7	6.8		8	GPV/G20 vaccine
	1	2	3	4	5	6	7	8		

Figures

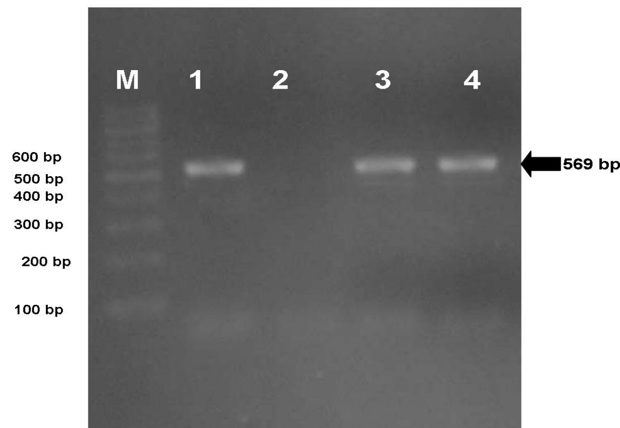

Figure (1): Gel electrophoresis of PCR products of Kelch-like gene (569 bp) of LSDV isolate and tissue culture adapted strain of LSDV Ismailyia 88 in agarose gel.

- **M:** DNA marker.
- **Lane 1 & 4:** The amplified products prepared from tissue culture adapted strain of LSDV Ismailyia 88 strain.
- **Lane 2:** Control non-infected skin sample.
- **Lane 3:** The amplified products prepared from LSDV isolate.

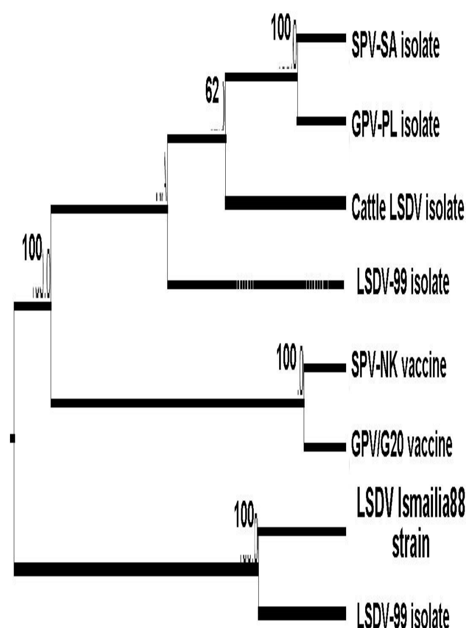

Figure (2): Phylogenetic tree of cattle LSDV isolate and LSDV (Ismailyia88 vaccinal strain) with others that were taken from the GenBank database based on Kelch like-gene sequence. Numbers show bootstrap report for the branches.

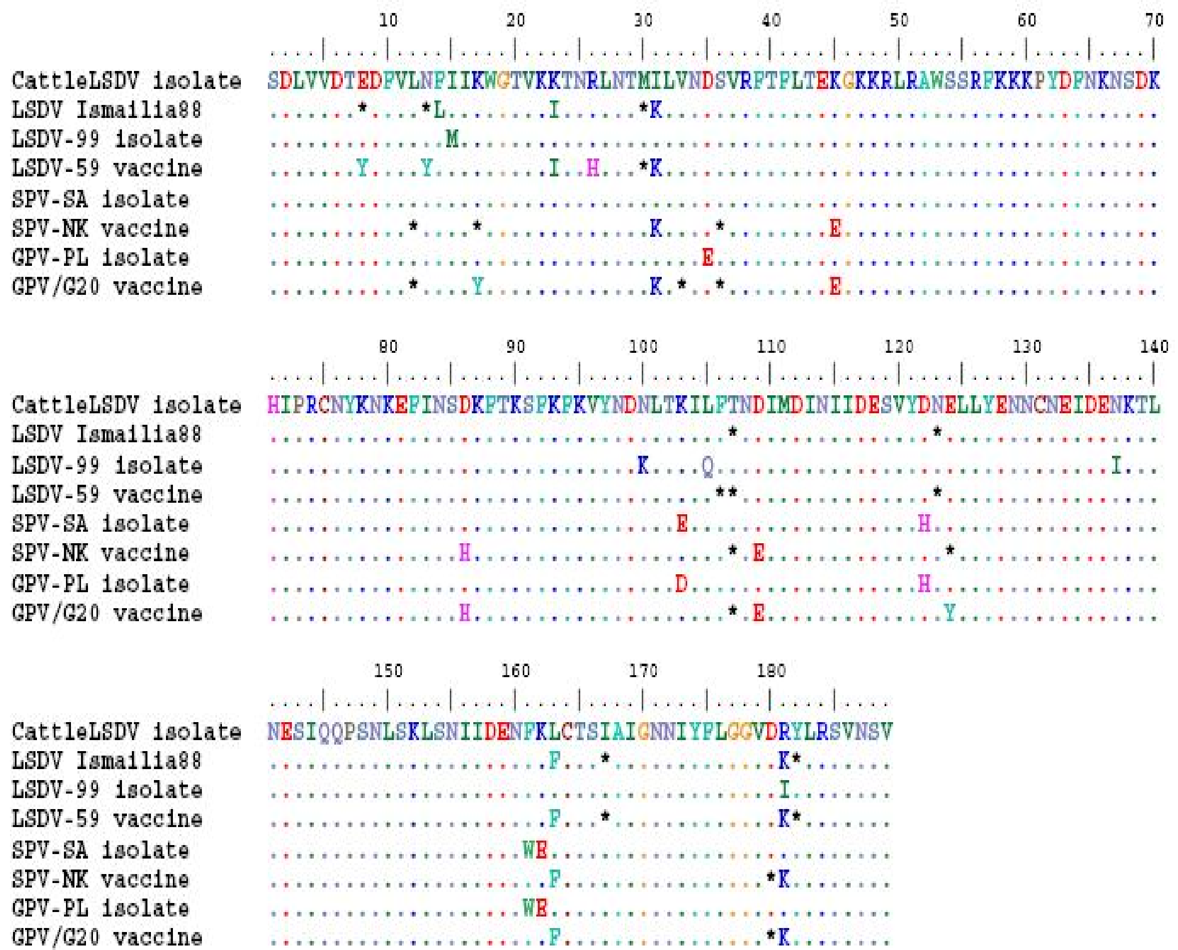

Figure (3): The multiple alignment of the deduced amino acids of Kelch-like gene of cattle LSDV isolate and tissue culture adapted strain of LSDV Ismailyia88 strain along with sequences of reference sheep poxviruses (isolate and vaccine strains), goat poxviruses (isolate and vaccine strains) and LSDV (isolate and vaccine strains) retrieved from gene bank.

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الملخص العربى

الجين شبة الفنجانى للتفرقة بين المعزول الحقلى لفيروس مرض الجلد العقدى و الفيروس المؤقلم على خلايا الزرع النسيجى

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** قسم الفيروسات – كلية الطب البيطرى – جامعة القاهرة

مرض الجلد العقدى هو احد الامراض الفيروسية العابرة للحدود و التى تصيب الابقار ويسببة فيروس فى جنس Capripoxvirus و الذى ينتمى الى عائلة الجدرى يعرف بفيرس نيثلنج .و في هذه الدراسة تم إجراء محاولة للتفرقة بين المعزول الحقلي لفيروس مرض الجلد العقدى و الفيروس المؤقلم على خلايا الزرع النسيجى (عترة الاسماعلية٨٨). و قد تم استخدام تحليل تتابع النيوكليوتيدات للجين لمحاولة التفرقة بين المعزول الحقلى و الفيروس المؤقلم على خلايا الزرع النسيجى. و قد أظهرت نتائج تحليل تتابع النيوكليوتيدات و الاحماض الامنية المستنتجة للجين شبة الفنجانى (Kelch-like gene) وجود رموز توقف جينية (stop codons) في حالة العترات المؤقلمة على خلايا الزرع النسيجي و ليس فى حالة المعزولات الحقلية. و خلصت الدراسة الى إمكانية استخدام تحليل تتابع النيوكليوتيدات و الاحماض الامنية المستنتجة للجين للتفرقة بين المعزول الحقلي لفيروس مرض الجلد العقدى و الفيروس المؤقلم على خلايا الزرع النسييجى. و نوصى بعمل دراسة مستقبلية على تحضير لقاح مستضعف معدل وراثيا يتم فية ازالة هذا الجين للاستخدام فى تحصين الابقار ضد مرض الجلد العقدى.

الكلمات الدالة: فيروس مرض الجلد العقدى ، تفاعل البلمرة المتسلسل ، التهجين بعد التلطيخ التنقيطى، الجين شبة الفنجانى، تحليل تتابع النيوكليوتيدات.