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GENETIC RELATEDNESS OF LUMPY SKIN DISEASE VIRUS FIELD ISOLATE WITH VACCINES USED IN THE FIELD

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ABSTRACT

In the present study, thirty four skin nodules from clinically diseased cattle that showed clinical signs suspected to be lumpy skin disease (LSD) were collected during the period from summer, 2009 until summer 2011 in Dakahlia Governorate, Egypt. Four samples were collected from cattle vaccinated with Kenyan strain of sheep poxvirus (SPV) vaccine and 12 were collected from cattle vaccinated with RM65 strains of SPV vaccine. Polymerase chain reaction (PCR) and dot blot hybridization (DBH) were used to identify the viral DNA in the collected samples. Thirty two out of 34 samples (94.1%) were found positive by PCR while 29 (85.3%) were found positive by DBH. P32 gene (~1027bp) that contains the major immunogenic determinants was sequenced to elucidate the genetic relationship between LSDV field isolate, tissue culture adapted strain of LSDV-Ismailyia88, and currently used SPV vaccines (Kenyan, Roumanian and RM65 strains). The nucleotide sequence analysis showed that LSDV field isolate was more closely related to LSDV-Ismailyia88 strain than to the currently used SPV vaccines. Also, antigenic analysis of inferred amino acids sequences was done by protean software (DNASTAR, Lasergene ®) to study the influence of changes of nucleotide sequence on the antigenicity of the p32 protein. The result showed that antigenicity of LSDV isolate and LSDV-Ismailyia88 strain was highly similar than to SPV vaccines. In conclusion, this study revealed that LSDV isolate was more genetically and antigenically related to tissue culture adapted LSDV-Ismailyia88 strain than to the currently used SPV vaccines.

Key Words: Lumpy skin disease virus, sheep poxvirus, polymerase chain reaction (PCR), dot blot hybridization (DBH), vaccine, antigenicity, sequencing.

INTRODUCTION

Lumpy skin disease (LSD) is an acute, subacute or inapparent viral disease characterized by pyrexia, localized or generalized skin pox lesions, and generalized lymphadenopathy. The disease caused by a virus (Neethling strain) in the genus Capripoxvirus of the family Poxviridae **(Davies, 1991 and Mercer et al., 2007).**

LSDV genome is a linear dsDNA molecule. The 151 kbp LSDV genome 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).**

P32 protein, one of the the structural proteins present in all capripoxviruses, contains major immunologic determinants **(Heine et al., 1999).**

Diagnosis of LSD is depending initially on clinical signs and laboratory diagnosis is based on virus isolation, histopathology and serological methods **(House et al., 1990, Tuppurainen, 2005 and Awad et al., 2010).** These conventional diagnostic methods are time consuming, laborious and most of them of low specificity. Polymerase chain reaction (PCR) has been described for detection of LSDV and proved to be simple, rapid and specific diagnostic method **(Ireland and Binepal, 1998).**

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 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**). In Israel has used the RM65 strain (Ramyar strain) to control all its LSD outbreaks since 1989 **(Yeruham et al., 1995 and Brenner et al., 2006).**

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)**.

The aim of the present work lies in sequence analysis of P32 gene of LSDV isolated from cattle and the currently used sheep pox virus vaccines (Kenyan, Roumanian and RM65 strains) to elucidate the genetic relatedness among these viruses.

MATERIALS AND METHODS

Samples collection:

A total of 34 skin nodules from clinically diseased cattle (one lesion per cow) that showed clinical signs suspected to be lumpy skin disease (LSD) were collected during the period from summer, 2009 until summer 2011 in Dakahlia Governorate, Egypt. Diseased cattle exhibited multiple skin nodules either localized or generalized with or without systemic signs. Among collected cattle samples, there were 4 from cattle vaccinated with Kenyan strain of SPV vaccine or RM65 strains of SPV vaccine. Each sample was taken in sterile dry bottles containing phosphate buffer saline (PBS) with antibiotics then transported in an icebox to the virology laboratory for virus identification by polymerase chain reaction (PCR) and dot blot hybridization (DBH). Skin biopsies from three normal cows were included as negative controls.

Virus strains:

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 Madian Darby Bovine Kidney (MDBK) and had a titre of $10^{4.5}$ $TCID_{50}$/mL. SPV/ Kenyan vaccinal strain was also obtained from the VSVRI. SPV/Roumanian strain was produced by Biopharma, in Morocco, had a titre of $10^{3.5}$ $TCID_{50}$/ml. SPV/RM65 was produced by Jordan Bio- Industries Center (JOVAC) in Jordan, had a titre of $10^{3.5}$ $TCID_{50}$/ml. Both Roumanian and RM65 strains were purchased from El-Ghanam pharmaceutical company.

Preparation of skin biopsies for virus identification:

Sample preparation was performed as described by **(OIE, 2008).**

Polymerase chain reaction (PCR):

Oligonucleotide primers

Oligonucleotide primers were designed according to **Ireland and Binepal, (1998)** for amplification of the attachment gene of capripoxvirus. Oligonucleotide primers used in the PCR reactions were synthesized by Metabion International AG Company, Germany.

DNA extraction

DNA extraction was done as described by **Viljoen et al., (2005**).

PCR amplification

This was carried out as described to **Ireland and Binepal, (1998)**.

Amplified product analysis

This was carried out according to **Viljoen et al., (2005**).

Dot blot hybridization (DBH):

DBH was applied to detect LSDV. Labelled DNA probe was prepared from the product obtained by amplification of attachment gene of capripoxvirus using primers designed by **Ireland and Binepal, 1998** according to **Sambrook and Russell (2001)** and labeled with digoxigenin using nonradioactive labeling kit (Roche, Germany). Hybridization technique was applied according to **Khandijan, (1987).**

PCR amplification of P32 gene of capripoxviruses:

Sequencing of P32 gene that contains the major immunogenic determinants of lumpy skin disease virus (LSDV) isolate, tissue culture adapted strain of LSDV Ismailyia88 strain and a current used sheep poxvirus vaccines (Kenyan, Roumanian and RM65 strains) was done using Oligonucleotide primers designed according to **Heine et al, (1999)** for amplification of P32 gene of capripoxvirus (1024 bp). The primers sequences were as follows: forward primer (A95), 5´- CAC GGA TCC ATG GCA GAT ATC CCA TT-3´ and reverse primer (B7), 5´- AAC AAG CTT ACT CTC ATT GGT GTT CGG-3´.

Nucleotide sequencing and analysis of sequencing data:

Sequencing of the PCR amplicons were performed by JenaGen GmbH Biotechnologie-Gentechnik-Diagnostik (Jena, Germany) using the BigDye® Terminator v1.1 Cycle Sequencing Kit on a 3130 sequencer (Applied Biosystems). The obtained 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 with 1000 repeats of bootstrap tests analysis by MegAlign (DNASTAR, Lasergene ®). Sequence divergence and identity percents were calculated by MegAlign (DNASTAR, Lasergene ®). Molecular analysis of amino acids sequence was done by protean(DNASTAR, Lasergene ®).

RESULTS

Identification of LSDV nucleic acid in skin nodules after DNA extraction:

Thirty two out of 34 samples (94.1%) were found positive by PCR while 29 (85.3%) were found positive by DBH.

Sequencing of P32 gene of lumpy skin disease viruse and sheep poxvirus vaccines:

The PCR products obtained from amplification reaction of the DNA extracted from tissue culture adapted strain of LSDV Ismailyia 88 strain and currently used sheep poxvirus vaccines (Kenyan, Roumanian and RM65 strains) beside the previously extracted DNA from our lumpy skin disease isolate using specific primers that amplify P32 gene of capripoxviruses were analyzed by gel electrophoresis which revealed the positive amplification of P32 gene with correct size (1027 bp) and **figure (1)** represents the obtained bands. After PCR products identification on the gel, sequencing of the P32 gene of the viruses [lumpy skin disease isolate, LSDV Ismailia 88strain and sheep poxvirus vaccines (Kenyan, Roumanian and RM65 strains)] was done.

Analysis of sequencing data

The obtained nucleotide sequence of 1027 bp PCR fragments representing the whole P32 gene of LSD viruses and SP viruses revealed open reading frame of 969 bp**.** The multiple alignment of the obtained sequences along with P32 sequences available in Gene bank **(table 1)** was created using ClustalW (http://www.ebi.ac.uk/clustalw/). The multiple alignments revealed that cattle LSDV isolate was found to be differ from tissue culture adapted LSDV (Ismailyia88 strain) at 13 positions: A72T, A140T, T142C, T143C, T144C, A145T, A164T, A334T, T801G, T881G, G907T, G908T and A936T. It was found that Cattle LSDV isolate differ from Kenyan, RM65 and Roumanian sheep poxvirus vaccines at 449, 467 and 347, respectively.

Percent identity of the nucleotide sequence of the P32 gene of LSDV (Ismailyia88 vaccinal strain), LSDV isolate, the currently used sheep poxvirus vaccines (Kenyan, RM65 and Roumanian strains) and reference capripoxviruses retrieved from the Gen Bank database revealed that LSDV isolate showed 98.7%, 94.6%, 94.0 and 93.1% identity with LSDV-Ismailyia88, LSDV-59, LSDV-99 and LSDV-58, respectively. Also this LSDV isolate revealed 53.6%, 51.8 and 64.2% identity with Kenyan, RM65 and Roumanian strains of sheep

poxvirus vaccine, respectively. GPV-MU, GPV-UT, SPV-RF and SPV-Shanxi showed identity of 92.9%, 92.6%, 84.2% and 77.5%, respectively with LSDV isolate.

The Phylogenetic tree pattern for the alignment of the sequenced viruses and references capripoxviruses is shown in **figure (2).** The Phylogenetic tree pattern for the alignment revealed that sequences of LSD viruses were grouped together. SPV-RF, SPV-Shanxi, SPV-Roumanian strain and GPV/Gans/2009 were grouped together but GPV/Gans/2009was in separate branch. SPV-Kenyan strain and SPV-RM65 strain were in a separate cluster. The phylogenetic tree showed that LSDV isolate was more related to LSDV Ismailia 88 strain than to other capripoxviruses. Also, the LSDV cattle isolate was more related to SPPV (Roumanian strain) than its relation to the Kenyan and RM65 strains of SPPVvaccines.

Antigenic analysis of deduced amino acids sequences of P32 protein:

Antigenic analysis of inferred amino acids sequences was done by protean (DNASTAR, Lasergene ®) to study the effect of changes of nucleotide sequence on the antigenicity of the p32 protein. The analysis showed that the surface probability, antigenicity, flexibility and hydrophilicity of LSDV isolate and tissue culture adapted LSDV (Ismailyia88 strain) were highly similar; on the other hand, the cattle LSDV showed highly antigenic differences when compared with the currently used sheep poxvirus vaccine (Kenyan, RM65 and Roumanian strains). However, the LSDV isolate showed the highest similarity to the Roumanian strain compared to the Kenyan and RM65 strains. **Figure (3)** showed antigenic analysis of deduced amino acids of P32 protein by protean (DNASTAR, Lasergene ®): LSDV isolate in comparison to LSDV (Ismailyia88 strain) and the currently used sheep poxvirus vaccines (Roumanian & kenyan strains).

Discussion:

In the present study, thirty-four skin nodules from clinically diseased cattle were collected as aseptically as possible, among these collected cattle samples; there were 4 from cattle vaccinated with Kenyan strain of SPV vaccine and 12 from cattle vaccinated with RM65 strains of SPV vaccine**.** These samples were prepared then subjected to virus identification by PCR & DBH. Out of 34 skin biopsies collected from diseased cattle, viral DNA was detected in 32 (94.1%) samples by PCR and in 29 (85.3%) samples by DBH. The result indicate sensitivity of PCR over DBH in virus detection in field samples and this may be due to low concentration of viral nucleic acid in some field samples which amplified by

PCR but in case of DBH the viral DNA was blotted on nylon membrane without amplification (**Wiedbrauk and Farkas,1995**).

Molecular identification of the virus are in concurrence with previous reports who recorded that PCR could be used in detection of LSDV in biopsy samples, tissue culture and semen (**Tuppurainen, 2005)**, skin and blood samples (**Awad et al., 2010**) and in CAMs of ECEs (**El-Kenawy and El-Tholoth, 2010)**. Also, the results are in agreement with **Awad et al., 2010** who succeeded in identification of LSDV in skin samples by DBH.

The sequence analysis of P32 gene illustrated that LSDV isolate was more closely related to tissue culture adapted LSDV-Ismailyia 88 strain than to the currently used sheep poxvirus vaccines. Also, the sequence revealed that the highest similar vaccine with cattle LSDV isolate was Roumanian sheep poxvirus vaccine (which is not commonly used in vaccination program against LSD) and the lowest was RM65 sheep poxvirus vaccine (which is routinely used in vaccination program). So, further study should be applied on using of Romanian sheep poxvirus vaccine instead of the commonly used RM65 and Kenyan strains of sheep poxvirus vaccine and also on using of a LSDV vaccine prepared from LSDV in protection of cattle against LSD.

The deduced amino acids of the nucleotide sequences were analyzed by protean (DNASTAR, Lasergene ®) to study effect of changes of nucleotide sequence on the antigenicity of the p32 protein. The protean analysis confirmed the results concluded from the nucleotide sequence analysis as it showed that the surface probability, antigenicity, flexibility and hydrophilicity of cattle LSDV isolate and tissue culture adapted LSDV (Ismailyia88 strain) were highly similar; on the other hand, the cattle LSDV showed highly antigenic differences when compared with the currently used sheep poxvirus vaccine (Kenyan, RM65 and Roumanian strains). However, the LSDV isolate showed the highest similarity to the Roumanian strain compared to the Kenyan and RM65 strains. These findings are in concurrence with **El-Kenawy and El-Tholoth, (2010)** who cited that field cattle LSDV isolate is genetically related to LSDVIsmailyia88 strain than to Kenyan strain of SPPV vaccine but they depend on sequence of attachment gene (192 bp) which is not sufficient to give a sharp conclusion and other sheep poxvirus vaccine strains (RM65 and Roumanian) were not sequenced. The results are in harmony also with **Aboul-Soud, (1995) and Daoud et al., (1998)** who reported that LSD vaccine prepared from LSDV able to protect cattle against LSD.

The potency of sheep poxvirus vaccines under field using for protection of cattle against LSD not gives complete protection **(Bahgat and Khalil, 2007, Brenner et al., 2009 and Kumar, 2011).** This may be due to genetic variation as we concluded from sequence analysis of P32 gene. On the other hand **Capstick and Coackely, 1962, Davies, 1991 and Carn, 1993** mentioned that vaccine and vaccination failure to LSDV may occur due to lower dose of the virus in sheep poxvirus vaccine or bad storage and transportation of vaccine. All these points concerning vaccine and vaccination program need further investigation.

Table (1): List of Capripoxviruses sequences used for the phylogenetic analysis of P32 gene

Isolation country	Virus isolate	Isolation year	Genbank accession no.	Reference
Egypt	Cattle LSDV 011 isolate	2011	In process	**This Paper**
	Buffaloe LSDV 011 isolate	2011	In process	**This Paper**
	Ismailia 88 LSDV	1990	In process	**This Paper**
Kenya	LSDV-Neethling 2490(LSDV-58)	1998	AF325528	**Tulman et al., (2001)**
	SPPV kenyan strain	1976	In process	**This thesis**
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)**
Turkey	Sheep pox virus strain TU-V02I27(SPV-TU)	1970	AY077832	**Tulman et al., (2002)**
India	Goat Pox Virus-Mukteswar (GPV-MU)	1946	AY 159333	**Hosamani et al., (2004)**
	GPV-Uttarkashi (GPV-UT)	1978	AY382869	**Hosamani et al., (2004)**
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	
Rumania	SPV-Rumanian Fanar strain (SPV-RF)	Unknown	AY368684	**Hosamani et al., (2004)**
China	Sheep poxvirus AV40	2009	HQ607368	**Yan et al., (2011)**
	Sheep poxvirus Shanxi	2009	HM770955	**Yan et al., (2011)**
	GPV/GanS/2009	2009	HM57233	**Yan et al., (2011)**
Jordon	Sheep pox virus RM-65 vaccine strain	1967	In process	**This Paper**
Morocco	Sheeppox virus Roumanian vaccine strain	Unknown	In process	**This Paper**

Figures

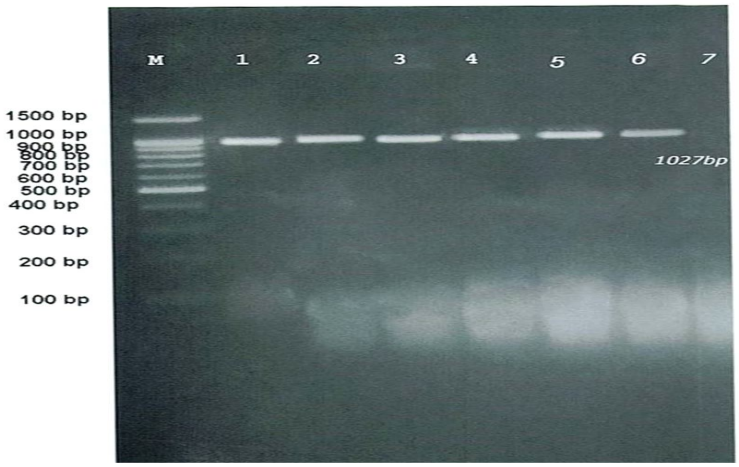

Figure (1): Gel electrophoresis of PCR products of P32 gene (1027 bp) of different capripoxviruses strains in agarose gel.

- **M**: DNA marker.
- **Lane 1 & 2**: The amplified products prepared from lumpy skin disease virus isolate
- **Lane 3, 4 & 5**: The amplified products prepared from Kenyan, Roumanian and RM65 strain of sheep poxvirus vaccines**,** respectively.
- **Lane 6**: The amplified products prepared from LSDV Ismailia 88 strain.
- **Lane 7:** Control negative skin sample

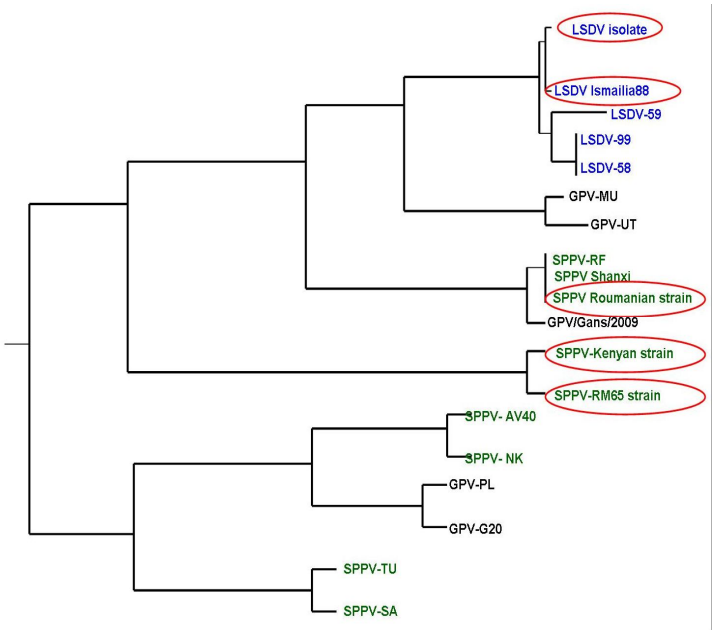

Figure (2): Phylogenetic tree of LSDV (Ismailyia88 strain), LSDV isolate and the currently used sheep poxvirus vaccines (Kenyan, RM65 and Roumanian strains) with others that were taken from the GenBank database based on P32 gene nucleotide sequences.

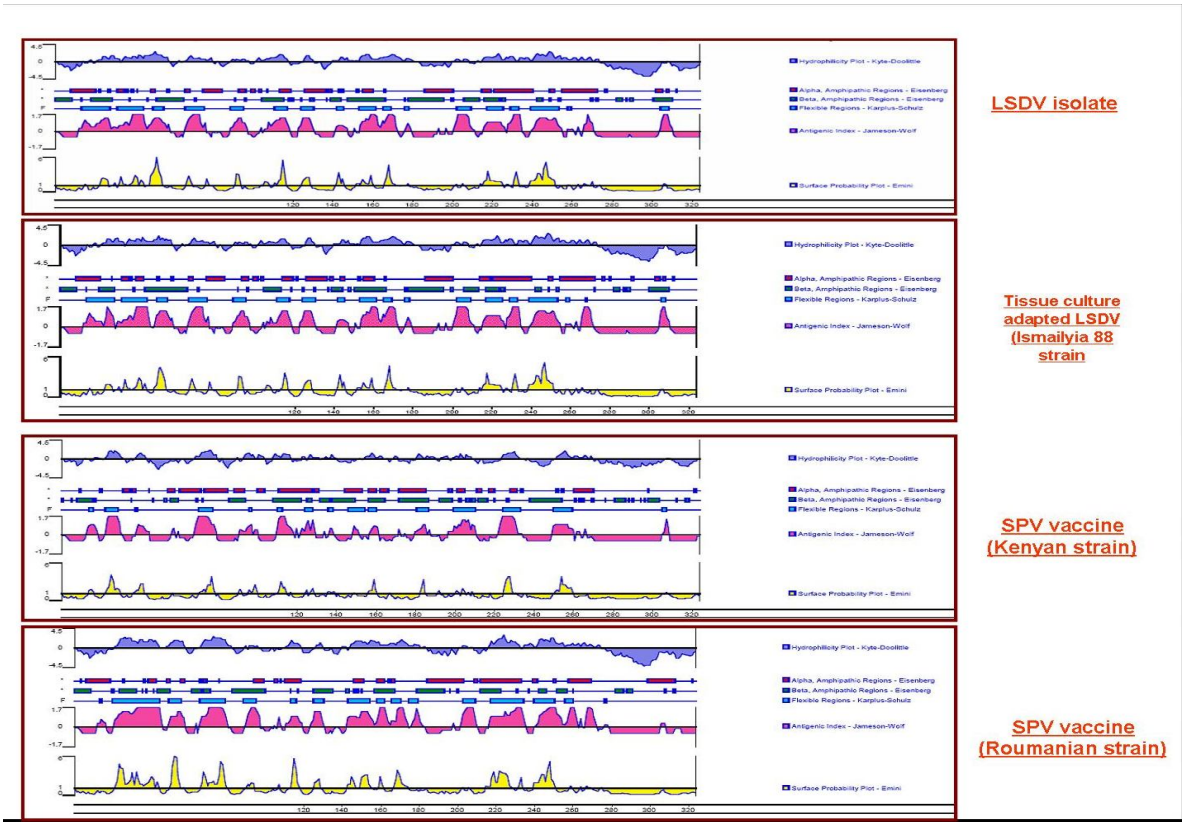

Figure (3): Protean analysis of P32 protein**:** LSDV isolate in comparison to tissue culture adapted LSDV (Ismailyia88 strain) and currently used sheep poxvirus vaccines (Kenyan, RM65 and Roumanian strains).

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الملخص العربى

العلاقة الجينية بين المعزول الحقلى لفيروس مرض الجلد العقدى و اللقاحات المستخدمة فى الحقل

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في هذه الدراسة تم تجميع عدد ٣٤ عينة من عقيدات جلد الابقار المصابة فى الفترة من صيف ٢٠٠٩ الى صيف ٢٠١١ من محافظة الدقهلية بمصر. من بين هذة العينات المجمعة هناك ٤ عينات من ابقار محصنة بالقاح جدرى الاغنام (العترة الكينية) و ١٢ عينة من ابقار محصنة بالقاح جدرى الاغنام (عترة راميرRM٦٥) . استخدام تفاعل البلمرة المتسلسل و التهجين بعد التلطيخ التنقيطي لتشخيص الفيروس .و قد تم تعريف الحامض النووى للفيروس فى عدد ٣٢ من اصل ٣٤ عينة (٩٤,١٪) باستخدام تفاعل البلمرة المتسلسل و فى عدد ٢٩ عينة (٨٥,٣٪) باستخدام التهجين بعد التلطيخ التنقيطى. كذلك تم دراسة التصنيف الجينى و الانتيجينى للمعزول الحقلى لفيروس مرض الجلد العقدى و مقارنتها بعترات لقاحات جدرى الاغنام المستضعفة و المستخدمة حاليا فى مصر و هى العترة الكينية و العترة الرومانية و عترة راميرRM٦٥ و ذلك عن طريق تحليل تتابع النيوكليوتيدات و الاحماض الامنية لجين بى ٣٢ (P32 gene). بتحليل تتابع النيوكليوتيدات للجين بى ٣٢ تبين ان فيروس مرض الجلد العقدى المعزول مرتبطا جينيا بفيروس مرض الجلد العقدى المؤقلم على خلايا الزرع النسيجى (الاسماعليةRM٨٨) اكثر من ارتباطه بعترات لقاح جدرى الاغنام المستخدمة فى مصر. و قد أكد التحليل الانتيجينى لتتابع الاحماض الامنية المستنتجة نتائج تحليل تتابع النيوكليوتيدات. من النتائج السابقة على جين بى ٣٢ نوصى بعمل دراسة مستقبلية على مميزات استخدام استخدام لقاح محضر من المعزول الحقلى المحلى لفيروس مرض الجلد العقدى لحماية الابقار من المرض بدلا من استخدام لقاح جدرى الاغنام.

الكلمات الدالة: فيروس مرض الجلد العقدى ، فيروس جدرى الاغنام ، تفاعل البلمرة المتسلسل ، التهجين بعد التلطيخ التنقيطى ، اللقاح، الانتيجنينية ، تتابع النيوكليوتيدات.