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Molecular Characterization of Orf Virus Isolated from Sheep and Goats in Egypt

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Abstract: Twenty five (25) samples from affected sheep and goats from four governorates in Egypt showed skin lesions were collected for isolation, identification and molecular characterization of the Orf virus (contagious ecthyma). The prepared samples were inoculated in SPF embryonated chicken eggs (ECE) for three blind passages by the CAM route. The positive pathological changes on CAM were characterized by odema, thickening, hemorrhages, small grayish white foci and pock lesions. Eight samples from each of both positive and negative results on ECE were inoculated on confluent sheet of MDBK cell culture. All the eight samples which gave positive results and only six out of eight that gave negative results with inoculation on ECE were gave positive results with inoculation on MDBK cell culture after the third passage. The developed CPE on MDBK cells 5-7 days post inoculation appeared in the form of cell rounding, multinucleated cells, then progressing of the CPE till distortion of the monolayer and cell detachment. The MDBK cell line was more sensitive than ECE for isolation and propagation of Orf virus. Identification and confirmation of isolated CPD virus was done on the molecular and biological levels. The inoculated MDBK cells showed clear specific yellowish green fluorescence by indirect fluorescent antibody technique (IFAT). The physico – chemical characterization of the obtained isolates were carried out. The examined harvested CAM by electron microscopy revealed ovoid shape virus particles with ball of wool appearance. The viral isolates showed reduction in virus titre due to treatment with (IUDR) ranged from 3.3 Log 10 to 4.5 Log 10 $TCID_{50}$/ 0.1 ml. The results of exposing the Orf viral isolate to the effect of temperature at 37°C and 56°C were showed reduction in the viral isolate titre with variable Degrees. Finally the isolated virus was sensitive to the action of diethyl ether reflecting the presence of an essential lipids in the envelop. PCR test was done using known primer on tissue samples. Amplification and running of characteristic fragments of Orf viral DNA on ethidium bromide stained agarose gel gave typical results in many segments on DNA fragment as reference virus which indicate that the given samples contain Orf viral antigen. The PCR technique proved to be more rapid and efficient for diagnosis of CPD virus infection than other tests. Phylogenic analysis of P55 gene and matching the field isolates with reference strains in the gene bank was carried out.

Key words: Orf virus · CPD · PCR · Electron microscopy and Phylogenic analysis

INTRODUCTION

Contagious pustular dermatitis (Orf or contagious ecthyma) is a common viral skin disease of sheep and goats. The disease has also been described in a broad range of wild ruminants [1] as well as human where it represent an occupational hazard among people who handle with infected animals [2]. The causative agent is Orf virus, a type species or prototype within the genus parapoxvirus, family Poxviridae. The epithliotropic Orf virus infects damaged skin and replicates in epidermal keratinocytes [3]. Orf viral particles are ovoid, 260 x 160 nm containing linear dsDNA with closed hairpin loop ends and have an outer membrane that consists of a single, long spiral tubule. In Egypt CPD was firstly observed among an imported flock of foreign breed sheep imported for the Department of Breeding of Ministry of Agriculture [4], then several outbreaks of variable severity were recorded [5-10].

Laboratory diagnosis of the disease is achieved by negative stain electron microscopy from scabs of affected animals [11] and serological tests include indirect

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flourcent antibody technique [12], virus neutralization test, agar gel immunodiffusion, complement fixation test and ELISA [14].

The development of PCR methods for the molecular detection of parapox DNA has met the demands for specific and sensitive laboratory diagnosis of the disease [15-16].

The present work aimed to study the molecular characters of the recent isolates from sheep and goats, phylogenic analysis and identity for matching the field isolates with reference strains in gene bank.

MATERIALS AND METHODS

Virus Isolation and Propagation: Twenty five samples were collected on 50% glycerin buffer saline from affected sheep and goats with skin lesions (15 from sheep and 10 from goats). These lesions were obtained from lips, commisures and gums.

These animals located in different localities in Ismailia, Menoufia, Sharkia and Giza Governorates.

The samples were crushed and prepared in maintenance media to make 10% suspension. The suspension was rapidly frozen and thawed for three successive cycles, then centrifuged at 3000 rpm for 10 minutes at 4°C, the supernatant fluid was collected and inoculated on (CAM) of 11 day old ECE and (MDBK) cell line. They were propagated for three blind passages on (CAM) of ECE and for further three passages in MDBK cells for virus isolation and propagation according toTantawi *et al.* and Kahrs [17, 18].

Indirect Fluorescent Antibody Technique: It was carried out on infected MDBK cells according to Majewska *et al.* [19].

The infected cells were covered with reference rabbit anti- CPD positive serum (CLEVB, Abbassia, Cairo) and incubated for 30 minutes in humid chamber, then washed and recovered with anti-rabbit IgG conjugated with fluorescien-isothiocyanate developed in rabbits (CLEVB, Abbassia, Cairo).

Physico-Chemical Characterization

Electron Microscopy: It was applied in Electron Microscopical Centre of Veterinary Hospital – Egypt.

Effect of 5-Iodo-2-Desoxyuridine (IUDR): The type of nucleic acid associated with the viral isolates were determined by means of 5-Iodo-2-Desoxyuridine (IUDR) in tissue culture tubes according to the method described by Lillie and Mohanty [20].

Effect of Heat "Thermostability": Sensitivity to heat at 37°Cand 56°C was carried out according to Snowdon [21]. Two sets of viral isolate suspensions were distributed into 1 ml amount small tubes and stoppered well. One set of tubes were dipped in a water path and heated at 56°C for 5, 10, 15, 20 and 25 minutes. Second set of tubes were incubated at 37°C for 1, 2, 3, 4, 5, 6, 7 and 10 days.

Effect of Diethyl Ether: It was carried out according to Griffin *et al.* [22]. It was obtained from BDH Chemicals Ltd, Poole, England and prepared in a final concentration 10% and 20% of total volume of viral isolate.

Conventional PCR: PCR of Orf virus was done in the tissue samples for DNA virus detection. DNA extraction was done by using QIAquik PCR purification kits and thermal technique using Thermo-mix PCR kit (thermo, UK). The oligo-nucleotide primers for DNA amplification by PCR was done according to Min Zheng *et al.* [23].

The primer sequence for the amplified gene was: OVS: AGGCGGTGGAATGGAAAGA and OVA: CCAGCAGGTATGCCAGGATG.

Sequencing and Phylogenetic Analysis of P55 Gene: Determination of nucleotide sequences for the p55 gene of the Egyptian isolates were carried out for the amplified gene using a Bigdye Terminator Kit (version 3.1; Applied Biosystems, Foster City, CA) on a 3130 Genetic Analyzer (Applied Biosystems, Foster City, CA), of gene analysis unit, Animal Health Research Institute, Dokki, Egypt, using specific internal primers for partial gene sequencing.

Comparative analysis of the amplified three p55 gene sequences for the orf viruses was carried out and compared with the available sequences on the Genbank using the National Center for Biotechnology Information (NCBI) database. The data of 3 partial p55 gene sequences were submitted to the GenBank database under the following accession numbers: KC511809, KC511810 and KC511811.

For the sequence analysis, the sequences were compared with other international strains from the Genbank. The genetic data were assembled using BioEdit software, V.7 [24].

The alignment and phylogenetic analysis was done using the Clustal V method and the Neighbor-Joining method, the Phylogenetic tree was constructed on the partial p55 gene, using DNA star software (DNASTAR Inc., Madison,WI).

RESULTS

Inoculation of Embryonated Chicken Eggs (SPF) and MDBK Cell Line: The results of inoculation of prepared clinical samples in ECE for three successive passages were illustrated in Table (1). The number of positive suspected cases and the specific changes were clearly detected in (10) samples (66%) and (6) samples (60%) respectively for sheep and goats clinical specimens. The pathological changes were observed in the form of odema, thickening, hemorrhages, small grayish white foci and pock lesions.

All the eight inoculated suspected samples which gave positive results and only six out of eight that gave negative results with inoculation on ECE were gave positive results with inoculation on MDBK cell culture after the third passage as shown in Table (1). The developed CPE on MDBK 5-7 days post inoculation appeared in the form of cell rounding, multinucleated cells, then progressing of the CPE till distortion of the monolayer and cell detachment (Photo 1 & 2).

Identification of the Cytopathic Viral Agents by IFAT: The stained MDBK cells inoculated with the positive isolates showed clear specific yellowish green fluorescence, Photo (3). While the non infected one was free from any fluorescence.

Physico-Chemical Characterization

Electron Microscopy: The electron microscopic examination of the harvested CAM revealed that the isolated virus was ovoid in shape and characteristic ball of wool appearance as shown in Photo (4).

Effect of IUDR: The usage of 5- iodo-2-deoxyuridine (IUDR) inhibited the production of infectious viral particles of Orf viral isolates in inoculated MDBK cells, the viral isolates showed reduction in virus titre ranged from 3.3 Log 10 $TCID_{50}$/ 0.1 ml to 4.5 Log 10 $TCID_{50}$/ 0.1 ml Table (2).

The Effect of Heat Treatment on the Infectivity of Orf Viral Isolates at 37°C: The rate of reduction in the virus isolate titre started in the first day followed by gradual decreasing in titre at the second day till complete inactivation by the tenth day Table (2).

The Effect of Heat Treatment on the Infectivity of Orf Viral Isolates at 56°C: The rate of reduction in viral activity was much accelerated, with bulk decreasing in titre (2.7 Log units) in the first five minutes, then rapid decreasing in virus isolate titre till complete inactivation in less than 25 minutes Table (2).

Treatment of Orf Viral Isolate with 10% & 20% Diethyl Ether at 4°C: The treated virus was completely inactivated while the control virus titre was not changed. This indicated the destruction of essential lipids in the envelope Table (2).

Detection of CPD Viral Nucleic Acid Using PCR: The amplification and running of characteristic 708 (bp) fragments of CPD viral DNA were shown in Photo (5). Four tested samples gave positive results, while two samples gave negative results.

DISCUSSION

Sheep and goats are considered one of the most important economic sources in Egypt. They are of great value due to high quality meat and wool so, any disease affect these animals has an economical importance. Orf or CPD is a highly contagious viral disease of sheep, goats and occasionally humans with worldwide distribution. It is caused by filterable virus which is the type species of the para-poxviruse genus in the family Poxviridae [25, 26]. The disease is a potential problem of live sheep exports due to the close confinement of animals and the feeding of sheep with pellets and hay that cause minor abrasions to the mouth and lips [27-29].

Repeatedly reinfection of sheep and goats with Orf virus is related to several reasons; firstly, the infection is acute and apparently restricted to epidermal keratinocytes in vivo, this may allow the virus time to replicate prior to recruitment of a critical level of antiviral immunity. Secondary, virus infection may not stimulate an appropriate protective response. Finally, the virus may have evolved mechanisms to subvert or Interfere with components of a protective immune response as has been demonstrated with other poxviruses [30-31].

Because of its contagious nature, public health aspects and economic importance of the CPD, This study focused on isolation and identification of locally isolated Orf virus in sheep and goats in some localities of Egypt Governorates. Trials for isolation of Orf virus on ECE (SPF) from prepared skin lesions (nodules, pustules and scabs) of clinically suspected sheep and goats at different localities were demonstrated in Table (1). The obtained

Table 1: Results of inoculation of ECE and MDBK cell line

	No. of Samples	No. of Passages					
		1st passage		2nd passage		3rd passage	
		+ve	%	+ve	%	+ve	%
Result of Inoculation on CAM of ECE	25	3	12	9	36	16	64
Result Of Inoculation on MDBK cell line	8 (+ve)*	2	25	6	75	8	100
	8 (-ve)*	0	0	2	25	6	75

*Result of inoculation on CAM OF ECE.

Table 2: Showing Physico- Chemical Properties of Viral Isolates.

Physico- Chemical Properties												
Effect Of IUDR			Effect of Temperature						Effect of Diethyl ether			
*Virus titre			At 37°C			At 56°C			10%		20%	
Untreated isolates	Treated isolates	Amount of reduction * in titre	Time interval in days	*Titer of treated virus	Reduction in titre	Time interval in days. min.	*Titre of treated virus	Reduction in titre	Treated *viral isolate	Untreated *viral isolate	Treated *viral isolate	Untreated * viral isolate
5.4	1.4	4.0	0	5.9	0	0	5.9	0	Zero	5.9	Zero	5.9
5.1	1.2	3.9	1	5.4	0.5	5	3.2	2.7				
4.3	1.0	3.3	2	5.1	0.8	10	2.2	3.7				
5.9	1.4	4.5	3	3.5	2.4	15	1.6	4.3				
3.6	0	3.6	4	3.1	2.8	20	1.2	4.7				
4.9	1.0	3.9	5	2.5	3.4	25	0	5.9				
3.4	0	3.4	6	1.8	4.1							
4.6	1.2	3.4	7	1.2	4.7							
			10	0	5.9							

*Expressed in Log 10 $TCID_{50}$ /0.1 ml.

Photo 1: Normal MDBK Cells

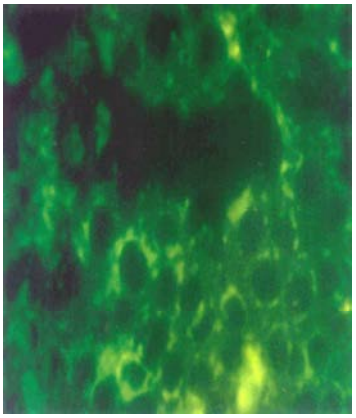

Photo 3:

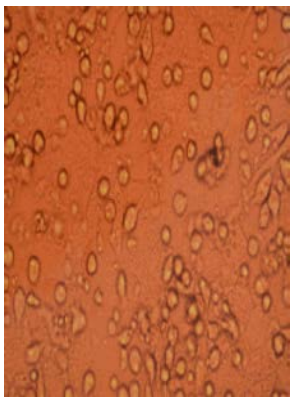

Photo 2:Inoculated MDBK after 5-7days

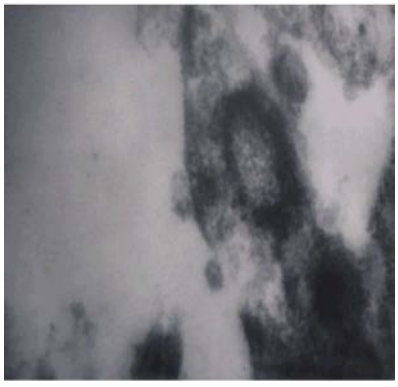

Photo 4:

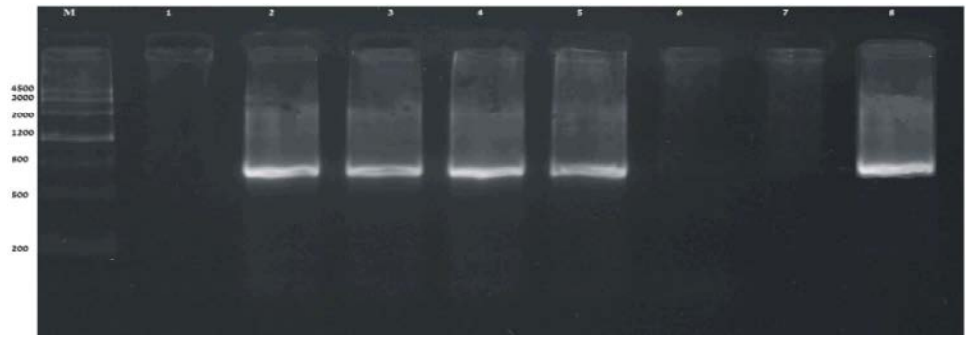

Photo 5:M: 100bp marker. Lane 1: Negative control Lane 2: Positive control-
Lanes 3, 4, 5, 8: positive samples.
Lanes 6, 7: Negative samples. Phylogenetic analysis of p55 gene nucleotide sequence of Orf virus

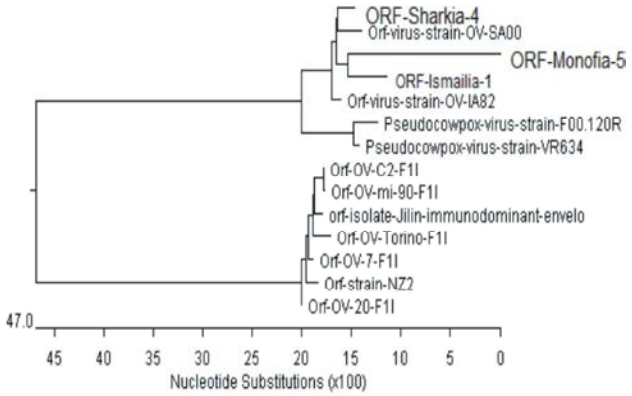

Fig. 1: Phylogenetic analysis of P55 gene nucleotide sequence of Orf virus

Table 3: Similarity percent orf p55 gene nucleotide sequence of Egyptian Orf viruses in relation to other reference viruses

Percent Identity

	1	2	3	4	5	6	7		
1		95.3	92.8	96.3	95.7	86.8	88.1	1	ORF-Sharkia-4
2	4.0		94.2	94.7	94.4	86.5	88.2	2	ORF-Monofia-5
3	5.7	5.3		92.9	91.9	84.3	85.3	3	ORF-Ismailia-1
4	3.2	4.9	6.3		97.4	89.3	90.4	4	Orf-virus-strain-OV-IA82
5	3.6	5.3	7.3	2.6		89.0	90.1	5	Orf-virus-strain-OV-SA00
6	12.5	13.4	15.6	10.4	11.0		98.4	6	Pseudocowpox-virus-strain-F00.120R
7	11.1	11.6	14.5	9.1	9.6	1.5		7	Pseudocowpox-virus-strain-VR634
	1	2	3	4	5	6	7		

Divergence

results revealed that the number of the clinical samples that develop positive pathological changes on CAM of ECE after the third passage were 10 samples (66%) and 6 samples (60%) respectively for sheep and goats clinical specimens. These positive pathological changes were characterized by odema, thickening, hemorrhages, small grayish white foci and pock lesions. Those obtained results came in complete agreement with those of [32, 33]. Concerning with attempts for virus isolation onto MDBK and examined daily for cytopathic effect (CPE). The methods for isolation of CPD virus on MDBK cell line were planned in two patterns: firstly, inoculation of eight suspected lesions (five samples from sheep and three from goats) that gave positive results with (ECE), Secondly, inoculation of eight suspected lesions (four samples from sheep or goats) that gave negative results with ECE. The results of the first pattern were showed in (Table 1). It was obvious from the table that all the eight inoculated suspected samples were gave positive results as similar as the results obtained with inoculation on ECE. Giving the fact that CPD virus developed CPE 5-7 post inoculation in the form of cell rounding, multinucleated cells, then progressing of the CPE till distortion of the monolayer and cell detachment (Photo 2). This CPE was considered as a confirmatory for The presence of Orf virus. Similar findings were reported by [34]. In contrast, the results of second pattern revealed that six suspected samples out of eight with the ratio of 75% were gave cytopathic effect on MDBK cell line after the third passage (Table 1).

Related to the results evoked with the second pattern, we concluded that the MDBK cell line was more sensitive than ECE for isolation and propagation of Orf virus.

Our results on MDBK cell line culture came in accordance with those reported by Kottaridi *et al*. [35] who said that CPD virus can be isolated in various animal cell cultures on which it produces characteristic cytopathic changes. These obtained results also were came in compliance with those recorded by Dulbecco and Harold [36] who succeeded in isolation of Orf virus strain (ORFV-cc) from scab materials of lambs with clinical sore mouth symptom by passaging on MDBK cells.

Identification of isolated CPD virus was done either by serological and non serological tools. Indeed the serological investigation, identification and confirmation of viral isolates, indirect fluorescent antibody technique (IFAT) was done by using standard CPD antisera. The CPD viral antigens were detected in positive lesions with CPE post MDBK inoculation. The (IFAT) commonly used for identification of unknown viral isolates and in serodiagnosis due to its higher sensitivity than the direct fluorescent antibody technique [19]. The stained MDBK cells inoculated with the positive isolates showed clear specific yellowish green fluorescence, Photo (3). Those above mentioned results were coincided with that recorded by Koptopoulos, Reid and Pow [12], who suggested that for investigating field outbreaks of Orf virus, the indirect fluorescent antibody technique would appear to be the test of choice. Any way the IFAT is rapid, reliable and sensitive for CPD virus like other viruses among Poxviridae. Also, this result agrees with Dulbecco and Harold [36] who performed The IFA test on CPE-positive MDBK cells in order to detect the existence of a virus antigen. The virus-specific green fluorescence was found in the cytoplasm of affected MDBK cells. The second confirmatory tool applied for identification of viral isolates was done on the harvested CAM by using the electron microscope. The examined samples revealed that the isolated virus was ovoid in shape and characteristic ball of wool appearance as shown in Photo (4). The same results were recorded by Guo *et al*. and Kui Zhao *et al.* [11, 37], where, they proved that the laboratory diagnosis of orf virus was achieved by negative stain electron microscopy from scabs of infected animals where the characteristic ovoid shape of the virion is demonstrated with a ball of wool appearance. Treatment of CPD viral isolates with structural analogue of thymidine, 5-iodo-2-deoxyuridine (IUDR) as a potent inhibitor for replication of CPD virus at a concentration 10^{-4} M, inhibited the production of infectious virus particles of CPD virus in inoculated MDBK cells. The reduction in the titre of CPD isolates due to treatment with IUDR ranged from 3.3 log 10 $TCID_{50}$/0.1 ml to 4.5 log 10 $TCID_{50}$/0.1 ml (Table 2). These results agreed with that obtained by Lillie and Mohanty, [20], McElroy and Bassett and Berrios *et al*. [38, 39]. The results of exposing CPD viral isolate to different temperatures for varying times are shown in (Table 2). When the CPD viral isolate was held at 37°C, a 1.5 Log units decrease in the titre was evidenced by the first day, then, gradual decreasing till inactivation was complete by the tenth day. When the viral isolate was held at 56°C, the decrease in viral activity was much accelerated, with a bulk of the virus 2.7 Log units being inactivated within the first five minutes and the complete inactivation was achieved within 25 minutes.

The above results agreed with Tomoko Suzuki *et al*. [40] who reported that that temperatures of 50°C and 56°C showed slight drop in the titre of the Orf virus after 30 min. However, heating for 30 min. at 60°C completely inactivated. The treated virus with 10% and 20% was completely inactivated while the control virus titre was not changed. This indicated the destruction of essential lipids in the envelope [39] and Tomoko Suzuki *et al*.[40].

Virus isolation is thought to be a gold standard but it's time-consuming [41]. With the development of molecular biology, the PCR technique has become widely used to amplify the desired genomic fragments from tissue specimens and it has become a powerful tool in molecular diagnosis. The PCR method is able to diagnose Orf viral infection in field specimens from affected animals [34]. To confirm whether the causative agent was present in affected skin samples, PCR of p55 gene (H3L genomic region) was used in this study [23]. The expected PCR fragments, approximately (708bp) in length, were obtained from DNA which had been extracted from affected skin samples; no fragments were obtained from the negative control.

In this study, six original samples (three that gave positive results and three that gave negative results on isolation) were examined by PCR technique where the results were strongly confirmed through presence of CPD virus fragments (708bp) and were observed on gel electrophoresis. As DNA amplification was not observed when negative CPD virus samples were used as a control negative template, the PCR was therefore verified as specific for CPD virus (Photo 5).

All the three tested samples that gave positive results with inoculation on ECE & MDBK gave positive results with PCR, while only one sample from the three tested samples that gave negative results with inoculation on ECE & MDBK gave positive result with PCR.

Related to the results describe the use of sequence data to characterize the Orf virus recovered from the different outbreaks in Egypt, nucleotide sequence data are widely used for phylogenetic analysis to track the movements of Orf virus and can also be used to define antigenic determinants of the virus.

Furthermore, sequence data can also be used to design sensitive and specific molecular tests to detect Orf virus from affected animals.

Nucleotide sequence analysis of the 680 nucleotide of p55 gene of the Egyptian isolates (Sharkia-4-sheep, Monfia-5-goat and Ismailia-1-sheep) in comparison with the field isolate OV-IA82, which obtained from nasal secretions of a lamb from Iowa Ram Test Station, USA during an orf outbreak in 1982 and Orf virus OV-SAOO which is isolated from skin lesions of a goat kid in Texas, USA, 2000 confirm high homology percent of the three Egyptian isolates with both reference strains were (96.3%, 94.7%, 92.9%) with Orf virus strain OV-IA82 respectively and also high homology percent with strain OV-IA00 (95.7%, 94,4%,91,9%) respectively. The isolate Ismailia-1 showed the higher variability among other isolates where it recorded about (92,9-91,9) with other two Egyptian isolates and the two strains OV-IA82 and OV-IA00. On the other side low homology percent with pseudo cow pox virus strains as indicated in (Table 3).

Interspecies sequence variability is observed in all functional classes of genes but is highest in putative virulence/host range genes, including genes unique to PPV. At the amino acid level, OV-SA00 is 94% identical to OV-IA82 and71% identical to BV-AR02 show an unusual degree of intraspecies variability [42].

In conclusion, Orf virus is distributed among sheep and goats in some Egyptian Governorates. Orf virus is preferably isolated in MDBK cell line than CAM of ECE. Egyptian isolates have high homology % with some Orf isolate in gene bank and the most diversion isolate was Ismailia 1 where it had (92,9-91,9) identity with reference strains.

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