Isolation, Morphological Characterization and Molecular Detection of Orf Virus from Infected Sheep.

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Abstract:
Parapoxvirus and pox-like disorders belong to a category of systemic diseases that, in their classical form are characterized by variously structured skin lesions, the diagnosis of these skin lesions is based on the suspicion of three viruses that might produce comparable lesions FMD, VS, and BT, CE has recently caused more deaths, the causative agent has just been genetically identified. The infected sheep isolate has been submitted to TEM morphological characterization and isolation of Orf virus on Vero cell line for 30 multiple passages. This study aims to better understand the sheep virus's biology in order to generate stronger vaccinations for Iraq.

Keywords: Orf virus, Vero cell, TEM, Parapoxvirus, and CE.

1. Introduction:

Contagious ecthyma (CE) is an emerging, infectious, zoonotic viral skin disease characterized by a large, benign, vascularized lesion that may be treated surgically or with antiretroviral drugs (**1**)(**2**). CE is classified by the ICTV as a large dsDNA virus with an envelope that replicates in the cytoplasm of infected host cells, exhibiting a well-defined ovoid shape that provides the foundation of a unique group of PPVs within the Poxviridae family (**2**). Sheep, goats, alpacas, camels, and reindeer are affected. CEV may cause 100 % morbidity and 15 % secondary mortality (**3**).

Diagnostic criteria for infectious ecthyma include clinical signs, lesion features, histology, and detection of poxvirus particles under an electron microscope (**4**). In contrast, serological tests such the agar gel precipitation test (AGPT), agglutination test, complement fixation test (CFT), enzyme linked immunosorbent assays (ELISAs), and serum neutralization test (SNT) might be used (**5**)(**6**). Abdullah et al (**7**) demonstrated that the molecular identification of Orf virus using specific primers targeting genes such as B2L and F1L might serve as diagnostic confirmation. Typically, the development of skin lesions implies active infections, which may be recognized and diagnosed most efficiently by regular viral isolation and characterization (**8**). Rapid and efficient virus isolation and culture are essential for the diagnosis and treatment of viral infectious illnesses, as well as the prevention and management of disease. Consequently, the sensitive nature of Vero cells (**9**).

2. Materials and methods

2.1. TEM microscopy

For TEM microscopy, sample was centrifuged 10000 rpm for particles removal and after that ultra-centrifuge 30000 rpm for 30 minutes (Sigma 3-30k) performed. Precipitated particles was eluted in 1 ml H2O and negatively stained with 2% uranyl acetate on carbon-coated copper grid with standard procedure. TEM images were captured in high resolution analytical TEM (FEI Tecnai G2 F20 SuperTwin TEM) located in University of Tehran (Iran).

2.2. Processing of Samples

The samples transported with medium was RPMI 1940 medium. Serum-free and duplicated antibiotic-folded, we homogenized 100 mL of skin crust in (PBS), centrifuged at 1000 rpm for 10 minutes to collect the supernatant, then filtered through a 0.45-M Millipore filter and utilized to inoculate cultures.

2.3. Virus isolation and cell line

80% confluent Vero cells were inoculated with 0.1 mL of diluted ECV and incubated for 1 hour at 37°C with flask rolling every 10 minutes, after incubation, cells were maintained in 7 ml of DMEM (5% fetal bovine serum) for 3-7 days at 37°C, with 5% Co_2. Daily cell inspections for cytopathic consequences (CPE).

2.4. Virus passageways

Vero cells were washed three times with PBS at 90% confluence (PBS). The cells were incubated at 37 C° in a humidified 5% CO2 incubator for 1 h with 1 mL of CEV diluted 1:3000 in viral growth medium DMEM with antibiotics (100 U/mL penicillin, 100 g/mL streptomycin, and 0.25% trypsin solution (Elabscience). Daily cytopathic effects were checked after introducing 2 mL of viral growing medium (CPEs). When CPEs were found in 80% of Vero cells, the flask was frozen and thawed three times, supernatants and cells were pipetted together, and kept at -80C°. Next passage employed seed cells. 30 times per requirement (**10**) (**11**).

2.5. Cell-culture harvesting

Harvesting detaches adherent cells from a cell culture flask using proteolytic enzymes. First, the growing medium was discarded. Twice, cells were washed with PBS. Subsequently, enzymatic harvesting solution was added. After 15 minutes, serum-containing culture media neutralized the proteolysis, collected tissue culture flask cells using different enzymatic solutions of trypsin and EDTA.

2.6. Molecular detection

By homogenizing scabs suspended in a 1.5ml tube, a 10% (PBS) viral solution was generated. DNA was extracted using a G-spin kit/ Korea. Two set of primers table (3-6) were designated according to published GeneBank accession number (KX951407.1, KX951408.1) by online program (**Primer3Plus**) https://www.bioinformatics.nl/cgi-bin/primer3plus/primer3plus.cgi to generate particular PCR primers for both B2L, F1L genes for detection orf virus genetically and optimize the pcr conditions for both primers (**Optimase ProtocolWriter™**).

Genes	Sequences		Product sizes
B2L	**F**	GTGGCCGTTCTCCTCCATC	967 bp
	R	GAGCAGCTTGGTGTTGTTCG	
F1L	**F**	GCCTACATAATCGGGGTTGC	854 bp
	R	GTGAGGAAGTAGATGGCGCC	

PCR Conditions detected with Optimase ProtocolWriter™ program.

Table (3-7) PCR conditions for both genes.

Temperature/Times	*Initial Denaturation*	*Denaturation*	*Annealing*	*Extension*	*Final extension*
	95Cº/ 2 min	95Cº/30 sec	55Cº/30 sec	72Cº/90.0 sec.	72Cº/5min.
Cycles		35			

Gel Electrophoresis 1% agarose gel, prepared by 1.5 g of agarose powder added to 100 ml of 1X TBE buffer and microwaved until the gel particles dissolved. After cooling to 50-60Cº, 2 µl of ethidium bromide was added and mixed well.

TEM microscopy

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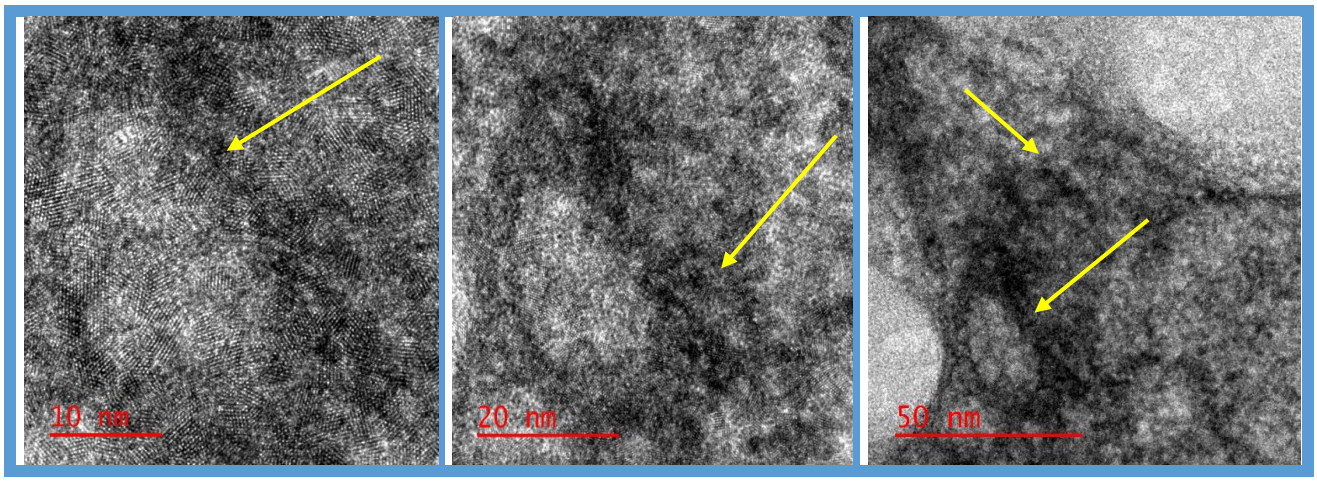

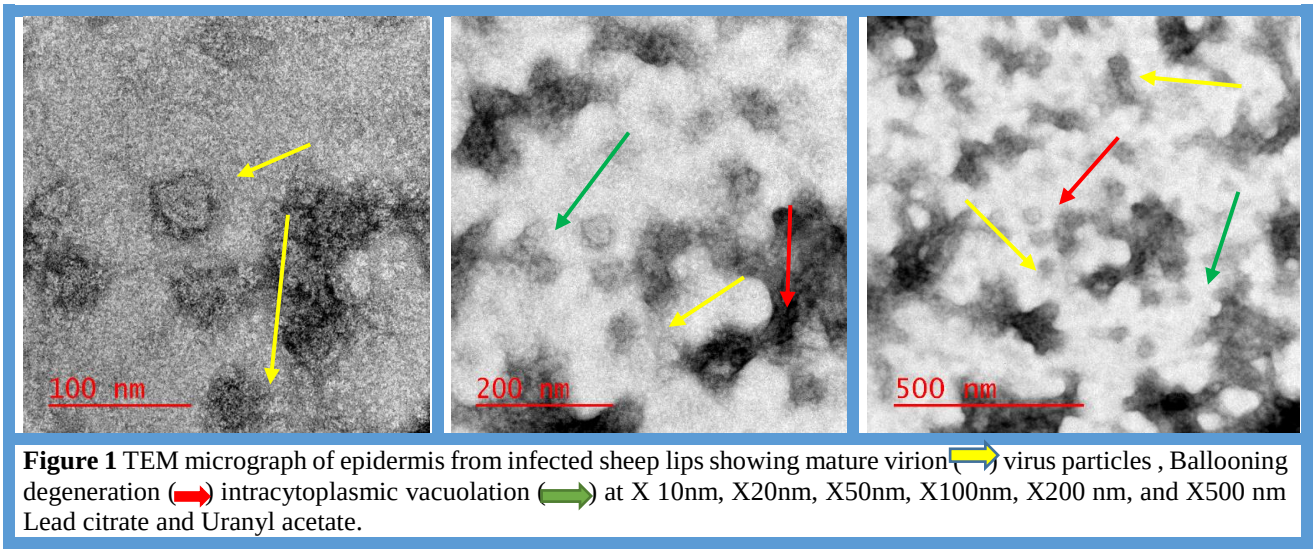

Figure 1 TEM micrograph of epidermis from infected sheep lips showing mature virion ( ) virus particles , Ballooning degeneration ( ) intracytoplasmic vacuolation ( ) at X 10nm, X20nm, X50nm, X100nm, X200 nm, and X500 nm Lead citrate and Uranyl acetate.

Tecnai G2 F20 SuperTwin TEM) located in University of Tehran (IRAN)
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3. Results

3.1 Morphology of Orf virus

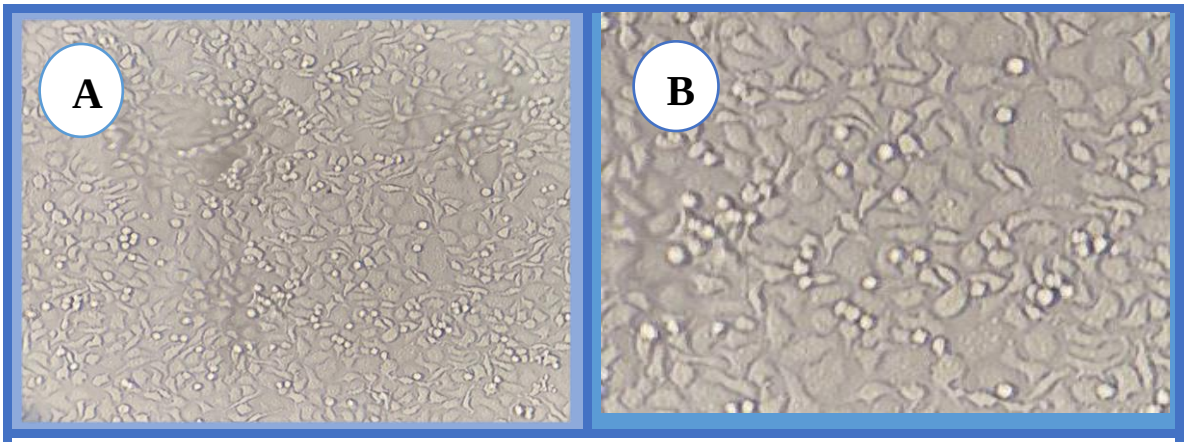
Transmission electron microscopy reveals the morphological properties of Orf virus.

3.2. Isolation on Vero cell

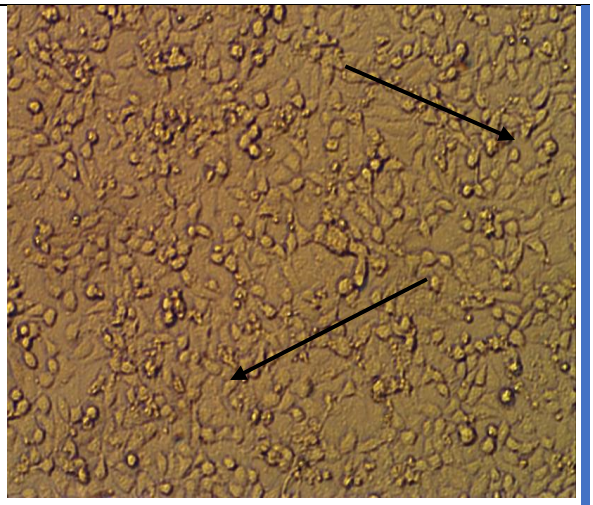
After 3–5 days in culture for either the first or second blind passage, infected Vero cells exhibited the characteristic pox virus-induced cytopathic effects (CPE) of rounded cells detaching from the monolayer (data not shown). 80% confluence Vero cells were employed for the replication of Orf virus, within the first 24 hours of infection, the inverted light microscope did not disclose any noticeable changes. However, 48-72 hours after infection, isolated virus produced rounded, sloughing cells that separated from the monolayer (Figure 2C). After 96h, CPE was characterized by the formation of syncytia containing multiple nuclei of fused cells (Figure 2D), which became apparent with increasing viral titer, and 80% of cells cultivated after 5days exhibited CPE and numerous floating cells in culture medium (Figure 2E). This result, along with the PCR screening results, verified the presence of infectious ORFV in infected sheep.

3.3. Developmental kinetics

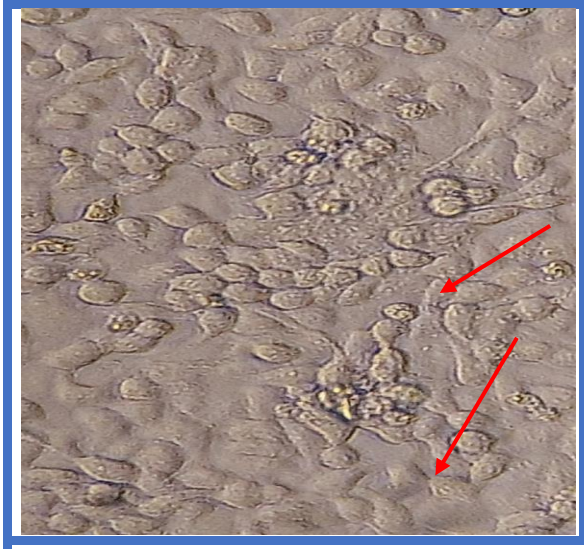
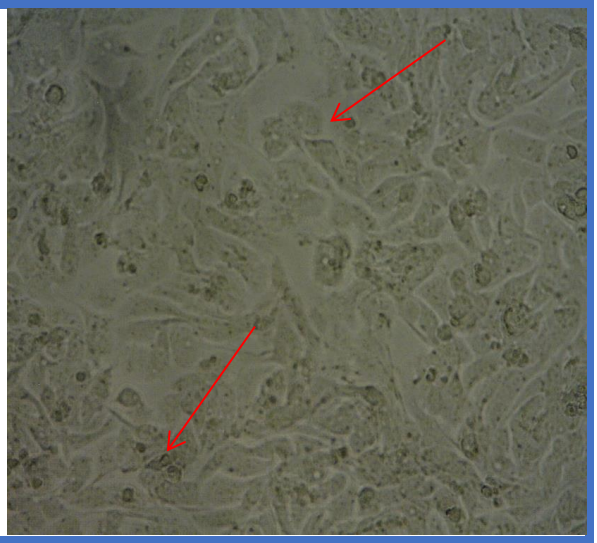
In 6-well plates, Vero cell monolayers were injected with Orf virus P8, P25, P30 infected at a MOI of 0.01 supernatants and cell lysates were obtained at 6, 48h post-inoculation. Cell culture samples were evaluated in 96-well plates to determine titration for 50% tissue culture infectious dose (TCID50/mL) following one round of freeze-thaw according to the Reed–Muench method (**12**).



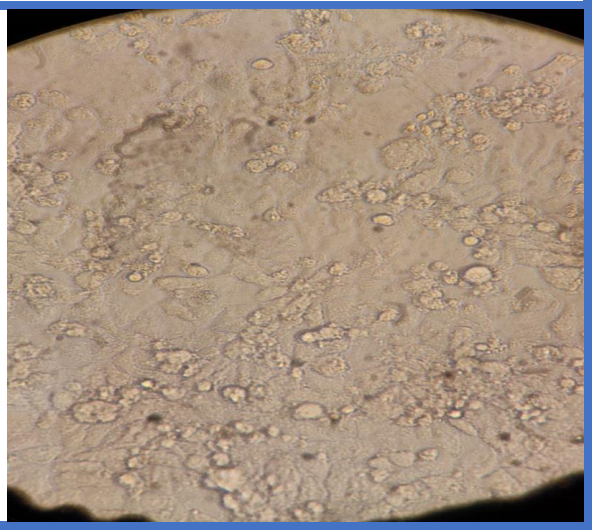
A.Vero cell 80% confluence line 20x appearance **B.** Vero cell 80% confluence line 40x Showing normal

C. Cell fusion observed at 48 -72h post infection rounding, shrinkage, and aggregation of the cells.

D. After 96h the cells marked by the production of syncytia containing several nuclei of merged cells which appeared to be more distinct as virus titer increased.

(Figure 2) E. 80% of cultured cells had CPE after 120h and many cells floated in the culture medium.

3.4 Molecular results of orf virus targeted B2L, F1L genes

The gel electrophoresis results figure (3) showed positive orf virus DNA in both targeted genes B2L gene in left at product size 967bp and F1L gene on the right in third lane positive control sample at product size 854bp.

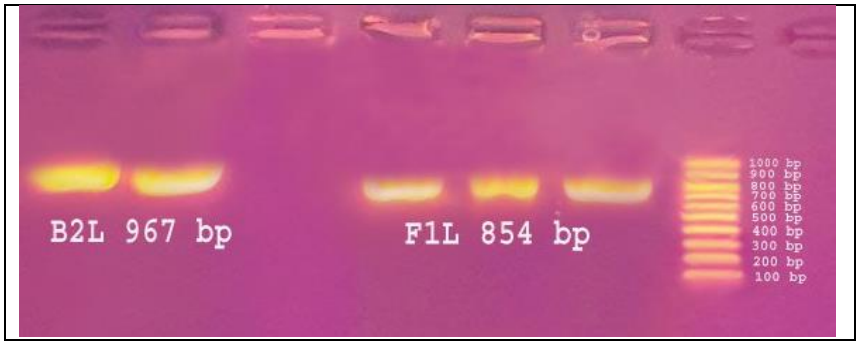


Figure (3) PCR findings for both B2L, F1L genes of Orf virus, agarose gel electrophoresis was used and DNA marker ladder lane (1000-100) bp. The gel showed 967bp, 854bp bands, respectively.

4. Discussion

There have been more reports of ORFV infections in sheep, goats, wild animals, and humans globally in recent years. Electron microscopy, histopathology, and serological tests such as fluorescent antibody method, virus neutralization test, agar gel immunodiffusion, and ELISA can be used to diagnose CE in the laboratory. Electron microscopy distinguishes CaPV from parapoxvirus (**13**). CaPV can be diagnosed by testing other poxviruses with similar clinical signs in animals, genetic sequencing can differentiate CaPV strains (**14**) (**15**). Currently, CE is confirmed by polymerase chain reaction (PCR), which has been demonstrated to be extremely sensitive and specific (**16**) (**17**). Manometer-scale TEM shows viruses, TEM helps identify viruses directly in biological materials, such as tissue slices or in vitro mammalian cells in clinical samples. In the 1990s, ELISA and PCR replaced TEM due to its "catch-all" character, this technique permits virus diagnosis without prior assumptions. TEM detected the pathogen when molecular methods failed. TEM can detect infections molecular testing missed, microbiological safety is checked, animal-cell biopharmaceuticals may contain viruses TEM's "catch-all" feature can find viruses (**18**) (**19**). Poxviridae are large, brick-shaped, double-stranded DNA viruses that can infect humans and animals. Parapoxviruses are 260 x 160 nm and contain a spiral coat that distinguishes them from other poxviruses (**20**), the main structure of the virion contains the DNA and numerous viral proteins, while the coat and two lateral bodies surround the core (**21**). The clinical symptoms and characteristics of the lesions, histology, and visualization of poxvirus particles under an electron microscope may be used to diagnose infectious ecthyma.

Vero cells are genetically stable and cancer-resistant, high viral titers make cells vulnerable to infection and robust to different culture conditions and can grow on microcarriers, making them useful in vaccine development (**22**). Due to genetic anomalies, Vero cells are incapable of producing interferon and them wide susceptibility of cells has led to their usage as a vaccination substrate. Vero cells are more efficient than primary and diploid cell lines at producing biological products. First, the cell bank is simple to establish and maintain, and its growth is rapid (**23**). Cytopathic effect (CPE), defined by progressive degeneration in VERO cells due to virus multiplication, was utilized as a first phase screening of materials by assessing cell morphology after virus transfection. Syncytia development was detected. Different passages of cells were studied to detect the virus's cytopathic effect, the results showed all viral suspensions were toxic to the cells, which was a pre-confirmatory test for the virus (**24**). Fig 2 (A, B, C, D, E) depicts VERO cell morphology before and after virus transfection. For genetic detection in this study we used both B2L, F1L genes figure (3) for many reasons they were highly immunogenic and possessing a multitude of functional properties and none of different Iraqi studies genetically defined B2L, F1L a major and immunogenic membrane protein in sheep The results agreed with (**25, 26**). They have been extensively used B2L, FIL genes for quick clinical diagnosis of ORFV.

Conclusions:

In conclusion, the standard approach for diagnosing Orf is not accurate for determining the amount of infection, and as a result, it is not possible to determine the severity of the infection.

Aknowlgment:

This search applied in Rawafed Al Uloom Company for the development and training of human resources and educational and research services, unfunded.

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