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RESEARCH

Comparative growth kinetic of Egyptian LSDV isolate in Vero and MDBK cell line

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ABSTRACT

Background: Lumpy skin disease outbreaks were observed in different governorates of Egypt affecting cattle throughout the year and are still present. The progressive shift of the outbreak in Egypt during 2018 causing severe losses in cattle population despite vaccination, which may incur some debates on the efficacy of current live attenuated vaccines against CaPVs. The virus isolation, identification, and vaccine or other diagnostic preparations mainly depend on the proper culture used.
Objective: Our aim was to study the growth kinetic of recent LSDV isolate to determine the culture properties of recent LSDV isolated from skin nodule that could be useful in the attempts to develop more effective LSDV vaccine and diagnostic purposes.
Methods: In order to isolate the LSDV MDBK continuous cell line was used for six serial passages. The obtained LSDV isolate was molecularly identified and deposited to the GenBank accession number of MN271728. In addition, a comparative study of LSDV growth curves of two permissible continuous cell lines (MDBK and Vero). The growth curve of LSDV was determined by inoculating both cultures, harvesting at intervals up to 24 hours and titrating the virus culture. To confirm LSDV accumulated in cell cultures, molecular identification is required; we carried Real-time PCR.
Results: This study has revealed that the virus culture period until 95% CPE was 48hr for MDBK and 72hr for Vero culture. The significant difference in growth behavior could be found between its growth on MDBK and Vero cells that it reaches its highest peak at 48hr for MDBK and 72hr for Vero cells. The maximum destruction in cell monolayers was observed within 72hr to 96hr post-infection. With an optimal condition for both cultures, the LSDV titers were 5.2 in MDBK after 48hr and 5.67 in Vero after 72hr. This rise in virus titer was followed by a sharp decrease after 48hr P.I. on MDBK unlike Vero cells that continue its rise in virus titer until reaching its peak at 72hr P.I.
Conclusion: Comparison of growth kinetic of LSDV on MDBK and Vero cell line revealed that Vero cell was considered the best susceptible cell for the propagation of LSDV with best harvesting time 72hr P.I to obtain a maximum titer for subsequent vaccine production.

Keywords: LSDV; Isolation; MDBK; Vero; Growth kinetic; Cytopathic effect; Real-time PCR; Egypt.

BACKGROUND

Lumpy Skin Disease (LSD) is the pox disease of cattle, is of great economic importance in the cattle industry (Babiuk *et al.*, 2008; Kumar, 2011; OIE, 2017). LSDV is one of the largest viruses that belong to genus Capri-poxvirus [Family *Poxviridae*] which has a single linear ds-DNA genome (Moss, 1996; Tulman *et al.*, 2001; Maclachlan and Dubovi, 2011).

The disease is endemic in central, Southern Africa and Middle East Region occurs at regular intervals in these endemic areas (Tuppurainen *et al.*, 2017). The first confirmed outbreak in the Middle East was in Egypt since 1988-1989 and since 2012 it is widespread throughout the Middle East, including Saudi Arabia, Israel, Kuwait, Iraq, Lebanon, Jordon, Syria, Turkey and Egypt (Al-Salihi and Hassan, 2015; EFSA, 2015; Şevik and Doğan, 2017; Kasem *et al.*, 2018). Most recently, LSD has been aggressively spreading in the European Union with the first

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confirmed outbreak in Greece and Russia (Tuppurainen *et al.*, 2014, 2017; Agianniotaki *et al.*, 2017; OIE, 2017; Salnikov *et al.*, 2018).

In Egypt, Spreading of LSD remains locally during the summer of 1988 that introduced via cattle importation from Somalia (Ali *et al.*, 1990; Davies, 1991a,b). Over 17 years of apparent absence, the disease causes a severe outbreak in 2006 thought to be introduced via cattle importation from Ethiopia (El-Kholy *et al.*, 2008; OIE, 2017). It was reported among cattle and buffaloes in Qaliubiya in 2011 (El-Nahas *et al.*, 2011; Sharawi and Abd El-Rahim, 2011). While, OIE notifications and follow-up reports indicating LSD outbreak occurred in Egypt during 2014 and thought that uninterrupted animal movement across borders or arthropod vectors (flies and ticks) maybe its source raising the possibility of introduction of new strains (EFSA, 2015).

Under the field condition in Egypt, it has not been possible to implement control strategies, and thus, vaccination with live attenuated sheep pox vaccine has been adopted to control LSD for many years (Davies, 1991a, b; Michael *et al.*, 1994). Over three last years, LSD has been aggressively distributed in Egypt causing severe losses in animal wealth, the situation raising concerns that the disease continues to spread despite excessive vaccination campaigns.

Virus isolation and virus neutralization test still gold standard assays used to investigate the viability of the virus in the samples. The development of vaccine and diagnostic assays largely depends on the virus isolate. LSDV can be propagated in a variety of primary cells or cell lines of bovine, ovine or caprine origin. Owing to it grows slowly on cell cultures and may require several passages; polymerase chain reaction (PCR) assays have replaced virus isolation as a primary reliable diagnostic assay (Binepal *et al.*, 2001; Gari *et al.*, 2008, 2012; Awad *et al.*, 2010; Zhivodeorova *et al.*, 2017).

Due to unavailability of primary and secondary cell cultures and lack of facilities to maintain and handling of these cultures for vaccine production and development of LSD diagnostics which depend mainly on the proper choice of cell culture (Menasherow *et al.*, 2014; Tuppurainen *et al.*, 2014), the continuous cell lines are promising for LSDV isolation and propagation as they support growth and production of a large amount of virus. For choosing the proper cell culture, we make trails on the species-specific cells of bovine, ovine or caprine origin (Kara *et al.*, 2003). The use of heterologous continuous cell lines like Vero and BHK-21 excludes the presence of antibodies to homologous cells which need further purification of antigen to facilitate the use of sera in diagnostics. Our aim was to study the growth behavior of recent LSDV isolate to determine the most suitable time for harvesting of LSDV could be useful in the attempts to develop safer and more effective LSDV vaccine and diagnostic purposes.

MATERIALS AND METHODS

Virus isolation

LSDV isolation and propagation were carried out on Madin Darby Bovine Kidney (MBDK) [NBL-1] [ATCC-CCL-22], cells were grown and maintained in Eagle's minimum essential medium with Earle's salts (MEME) (SIGMA-Aldrich, USA) supplemented with 10% fetal calf serum (FCS) (SIGMA-Aldrich, USA), procedure was performed according to OIE guidelines (OIE, 2017).

Virus adaptation and infectivity titration

For virus adaptation to used cell lines, serial passages were carried out. Cell culture of Vero was grown in Eagle's medium supplemented with 10% fetal calf serum [FCS]. 70% confluent monolayer was inoculated. The virus is allowed to adsorb by incubating the inoculated monolayers at 37 ºC for 1-1.5 hours. The inoculum was removed and the culture is then washed twice with warm PBS and covered with supplemented maintenance medium containing 2% fetal calf serum [FCS], the prescriptions were incubated at 37.0±0.5 °C at a 5% CO_2 incubator (Shel-lab, USA for 5-7 days. The culture freeze-thawed three times at -80 °C, thoroughly mixed, clarified supernatant subsequently inoculated on to fresh culture for another two passages till the development of characteristic CPE observed under an inverted microscope. The presence of CPE and change of viral titer during the passage indicate the permissively of cell culture to the virus. Infectivity titration of the virus was determined by titration in 96 well tissue culture plates according (Payment and Trudel, 1993). Virus titer was calculated by Reed and Muench methods expressed in log $TCID_{50}$/100µL (Reed and Muench, 1938).

Growth behavior of LSDV in MDBK and Vero cell line

The growth curve LSDV isolate was conducted cell line. This was done to compare and contrast the growth of LSDV local isolate. Recently prepared cell monolayers in 25 cm^2 tissue culture examined under an inverted microscope for 60-70% confluency. T25 cm^2 tissue culture flasks were inoculated in duplicate with LSDV. Two flasks were removed at various time intervals 8, 16, 24, 36, 48, 72, 96 hours post-infection. Flasks placed at -80 °C until titrated. Flasks for each time point were freeze/thawed three times in total and the resulting crude virus stock was titrated using the $TCID_{50}$ titration method. An average titer was taken for each time point.

Molecular identification of LSDV

QIAamp® DNA Mini Kit (QIAGEN GmbH, Germany) was used for LSDV DNA purification following the manufacturer instructions with minor modification by snap-frozen in liquid nitrogen and overnight incubation at 56 °C after re-suspends in AL buffer and Proteinase K Solution. It was performed according to the manufacturer instructions using LSDV dtec-qPCR kit, supplied by Genetic PCR solutions GPS®, Spain that contains a mixture of specific forward/reverse primers and probe specific for detection of LSDV and 5x MixStable qPCR mix.

RESULTS

LSDV isolation and adaptation

In the third passage, the characteristic cytopathic effect (CPE) was observed in infected MDBK culture with nodular suspension. The CPE was characterized by cell rounding with retraction of the cell membrane from surrounding cells and clustering to form rosette shape aggregations scattered all over the monolayer sheet starting from the 3rd-day post-inoculation which gradually increased leading to complete detachment of the cell monolayer at 6th-day post-inoculation [Fig.1]. the virus titer in MDBK cell culture was $10^{5.52}$ $TCID_{50}$/100 µL. The isolated strain (identified and deposited to Genbank under accession no. MN271728) was further used in this study.

The Vero and BHK-21 cell culture was used to adapt the strain and to identify its cytopathogenic behavior. The time required for the development of 95-100% CPE for Vero and MDBK cell cultures took 48-72 hr. P.I. While the BHK-21 showed CPE as soon as 24 hr. P.I.

with rounding cells which followed by a detachment of sheet accompanied with a sharp change in medium pH to be acidic.

Growth kinetics of LSDV on MDBK and Vero cell culture

First, we try to optimize FCS concentration, the infected cells cultured in a maintenance medium without serum and with the addition of 3; 5 and 10% serum. The highest virus titer was observed at 3-5% FCS compared to 10%.

The virus infectivity obtained from $TCID_{50}$ titration (Table 1), A slight lag phase can be observed over the first 16 hr. P.I followed by a rapid increase that can be observed for both cell types with similar growth rates throughout the first 36 hr. P.I., thereafter, the virus titer on MDBK found slightly higher than that of Vero through the first 48 hr. P.I., This rise in virus titer was followed by a sharp decrease after 48 hr. P.I. on MDBK unlike Vero cells that continue its rise in virus titer until reaching its peak at 72 hr. P.I., as shown in table 1.

The characteristic cytopathic effect (CPE) in both cell cultures was not the same. So, in MDBK The CPE was characterized by cell rounding with retraction of the cell membrane from surrounding cells and clustering to form rosette shape aggregations scattered all over the monolayer sheet after 24 hr. P.I. which gradually increased leading to detachment of the cell monolayer after 72 hr. P.I. After 96 hr. P.I. most of the sheet showed vacuoles due to cell detachment and the remaining cells were either aggregated or elongated.

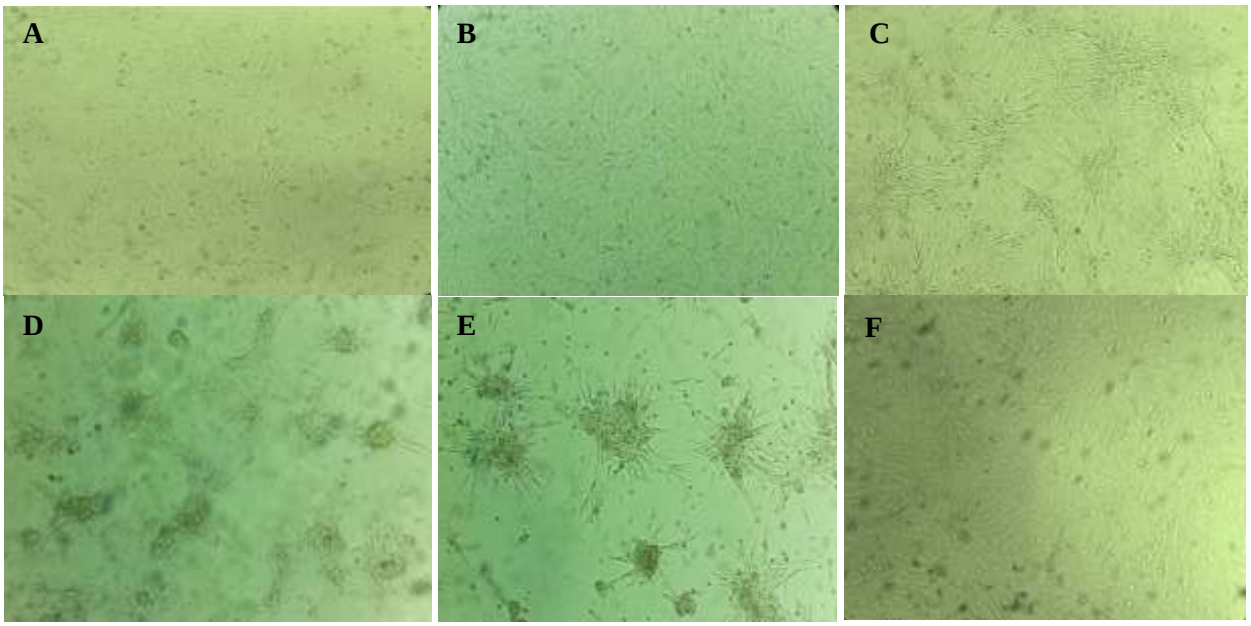
In Vero culture, there were spindle-shaped cells, rounding and aggregation formed 48 hr. after inoculation and detaching of infected cells were observed after 72 hr post-inoculation. There was an increased cell rounding in the case of an infected Vero cell. After 72 hr. P.I. the cells were rounded and formed singly and in aggregation with a detachment of sheet. After 96 hr. P.I. most of the sheet showed detachment and the remaining cells were aggregated with giant cell formation.

No noticeable difference in cell morphology could be observed in both cultures during the first 24 hr. CPE developed only in infected cells and not in uninfected control cells.

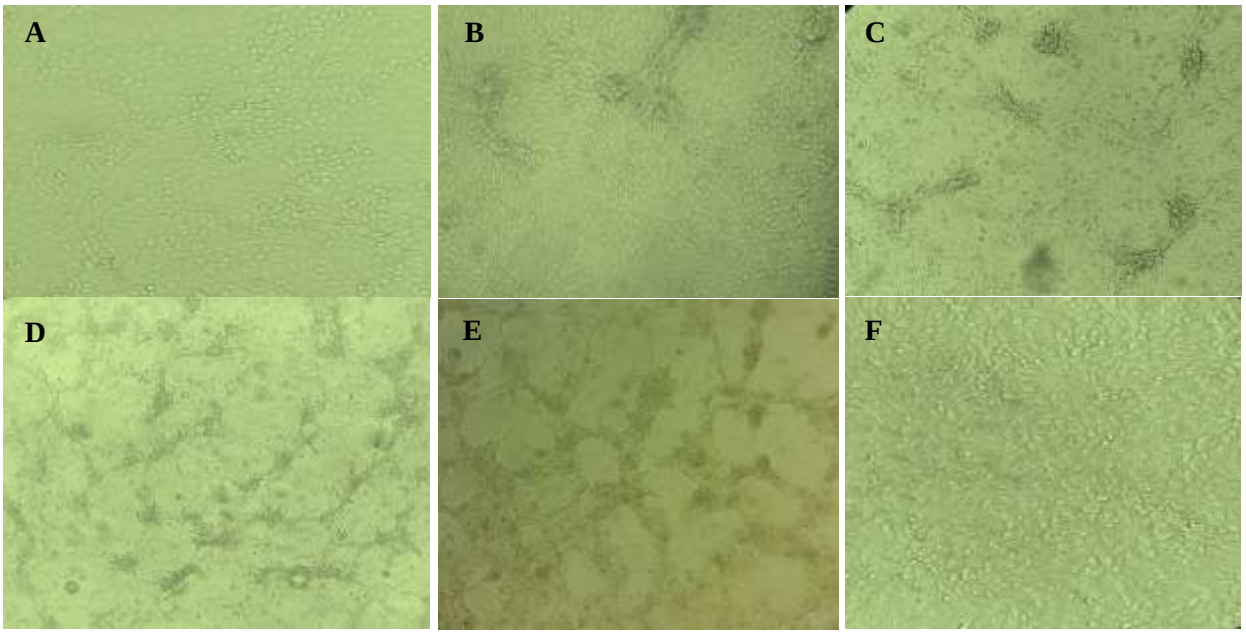
Table 1: Growth kinetic of LSDV in MDBK and Vero cell cultures. (log values obtained from $TCID_{50}$ titration).

Time in hours	Titer $TCID_{50}$ (log 10 /100 µL)	
	MDBK cell culture	Vero cell culture
8	2.39	1.83
16	3.42	3.14
24	4.25	3.71
36	4.66	4.2
48	5.2	4.83
72	4.32	5.67
96	3.9	4.21

The virus accumulated in cell cultures was confirmed by the detection of LSDV nucleic acid by real-time PCR.



Vero cells infected with LSDV, cells as seen A) 8 hr. P.I B) 24 hr. P.I C) 48 hr. P.I D) 72 hr. P.I E) 96 hr. P.I F) Vero cells left uninfected [5 days post seeding]



MDBK cells infected with LSDV, cells as seen A) 8 hr. P.I B) 24 hr. P.I C) 48 hr. P.I D) 72 hr. P.I E) 96 hr. P.I F) MDBK cells left uninfected [5 days post seeding]

Fig. 1: Cytopathic effect of LSDV on continuous cell lines of African green monkey kidney Vero [Upper] and Madin derby bovine kidney MDBK [Lower] [magnification power x100, inverted microscope, Olympus Co., Japan).

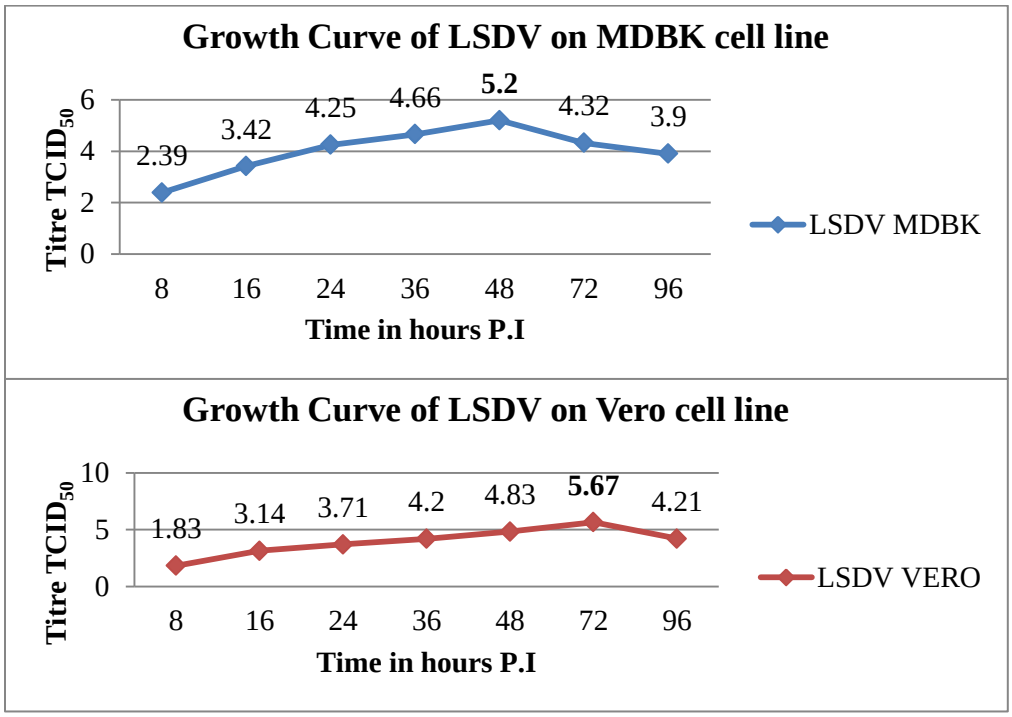


Fig. 2: Growth curves assay of LSDV on MDBK [Upper] and Vero [Lower] cell lines**.**

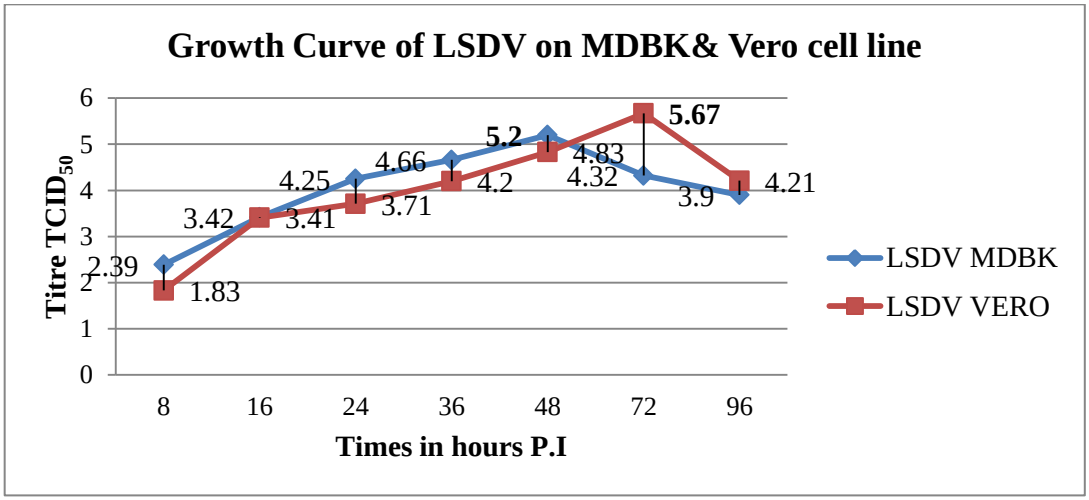


Fig. 3: Growth curves assay of LSDV on MDBK and Vero cell lines.

DISCUSSION

CaPVs are known to grow slowly in mammalian cell culture of animal origin and require several passages to propagate these viruses (Weiss, 1968) and CPE of LSDV cannot be detected before day 4 post-inoculation up to 14 days (Tuppurainen *et al.*, 2005). LSDV can be propagated in a variety of primary cells of bovine, ovine or caprine origin such as Lamb testis (LT) or kidney primary cells are most commonly used for primary isolation of LSDV (Binepal *et al.*, 2001; Babiuk *et al.*, 2007; OIE, 2017). There are many problem in availability of primary and secondary cell cultures and lack of source and facilities to maintain and handling of these cultures for vaccine production and development of LSD diagnostics which depend mainly on

the proper choice of cell culture (Menasherow *et al.*, 2014; Tuppurainen *et al.*, 2014), the continuous cell lines are promising for LSDV isolation and propagation. They provide replication and give high virus titer in the virological and molecular study of the virus. Therefore, it should determine the sensitivity of cell cultures and their permissivity to support the growth of the virus. We have adapted well-established cell lines like Madin-darby bovine kidney (MDBK) and African green monkey kidney (Vero) for LSDV isolation and propagation (Zhivodeorova *et al.*, 2017).

The Egyptian isolate of LSDV was originally isolated on MDBK infected initially with a nodular suspension showing the typical cytopathic effect (CPE) (El-Bagoury *et al.*, 1995; El-Nahas *et al.*, 2011; Abou Elyazeed *et al.*, 2012; Elhaig *et al.*, 2017). Initially, BHK-21, Vero cell lines were chosen for LSDV adaptation trials depend on the presumption that they are easily available for subculture in the lab and their mammalian origin (Binepal *et al.*, 2001; Menasherow *et al.*, 2014), the later one was successfully chosen due to high stability and slower growth rate that maintain slow-growth nature of LSDV to produce a detectable clear CPE (Babiuk *et al.*, 2007). MDBK and Vero cell line were chosen to study the growth behavior of our recent isolate by assessing the infectivity titer due to it provide high virus titer which was satisfying for studying growth kinetics of our isolate (Zhivodeorova *et al.*, 2017). The time required for the development of 95% of clear CPE for MDBK and Vero cell line, it took 48-72 hours for subsequent passages which are explained due to virus was adapted to cell culture that confirm the susceptibility of our isolates to this cell line as in consistence with (House *et al.*, 1990; El-Nahas *et al.*, 2011; Haftu, 2012; Ayelet *et al.*, 2014; Zhivodeorova *et al.*, 2017) who isolated LSDV from skin biopsies on MDBK and Vero cell cultures. Clear prominent CPE was observed when the culture maintained at 2-5% FCS compared to serum-free medium due to serum may support survival and replication of the virus. These findings agree with those mentioned by Zhivodeorova *et al.*, (2017).

Characteristic cytopathic effect (CPE) in both cell cultures was not similar started to appear after 24 hr. P.I and reached the maximal level after 72 hr. P.I after which complete destruction of the sheet after 96 hr. P.I was observed, these support those observed in earlier studies (Plowright and Witcomb, 1959; Nawathe *et al.*, 1978; Woods, 1988; Abraham and Zissman, 1991). So, in MDBK culture, the CPE was characterized by cell rounding with retraction of the cell membrane from surrounding cells and clustering to form rosette shape aggregations scattered all over the monolayer sheet after 24 hr. P.I. which gradually increased leading to detachment of the cell monolayer after 72 hr. P.I., After 96 hr. P.I. most of the sheet showed vacuoles due to cell detachment and the remaining cells were either aggregated or elongated (Pandey *et al.*, 1985; Davies, 1991a; Tuppurainen *et al.*, 2005; Omyma, 2008; OIE, 2017; Zhivodeorova *et al.*, 2017). *In Vero* culture, there were spindle-shaped cells, rounding and aggregation formed 48 hr after inoculation and detaching of infected cells was observed after 72 hr. post-inoculation. There was an increase in cell rounding in the case of infected Vero cells. After 72 hr. P.I., the cells were rounded and formed singly and in aggregation with a detachment of sheet. After 96 hr. P.I., most of the sheet showed detachment and the remaining cells were aggregated with giant cell formation (Haftu, 2012; Ayelet *et al.*, 2014; OIE, 2017). To confirm LSDV accumulated in cell cultures, molecular identification is required; we carried Real-time PCR (Bowden *et al.*, 2008; Stram *et al.*, 2008; Zeynalova *et al.*, 2016).

The virus infectivity obtained from virus titration, A slight lag phase can be observed over the first 16 hr. P.I followed by a rapid increase that can be observed for both cell types with a similar growth rate throughout the first 36 hr. P.I., thereafter, the virus titer on MDBK found slightly higher than that of Vero through the first 48 hr. P.I., This rise in virus titer was followed by a sharp decrease after 48 hr. P.I. on MDBK unlike Vero cells that continue its rise in virus

titer until reaching its peak at 72 hr. P.I., as shown in table 1. The comparative growth curves of LSDV in MDBK and Vero cells, the significant difference in growth behavior could be found between its growth on MDBK and Vero cells that it reaches its highest peak at 48 hr. for MDBK and 72 hr. for Vero cell, the faster growth rate also correlated with absence of immune cells and lack of immune regulation pathways in the cell-line used, where the sharp drop in virus titer might be related to the destructive action of LSDV on cell culture and the detachment of infected cells from the surface of the prescription, This result is in general agreement with (OIE, 2017) and many other researches findings that recommend the use of MDBK cell for primary isolation of LSDV (Pandey *et al.*, 1985; Tuppurainen *et al.*, 2005; El-Nahas *et al.*, 2011; Abdelwahab *et al.*, 2016), and in contrast with previous studies that not recommend the use of Vero cell in LSDV propagation (Davies *et al.*, 1971; House *et al.*, 1990; El-Bagoury *et al.*, 1995; Omyma, 2008; Kumar, 2011; Tuppurainen *et al.*, 2014; OIE, 2017).

CONCLUSION

We have shown a comparison of growth kinetic of LSDV on MDBK and Vero cell line. Thus, Vero cell was considered the best susceptible cell for propagation of LSDV with best harvesting time 72hr P.I to obtain a maximum titer for subsequent vaccine production.

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The authors declare that they have no competing interests.

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