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Propagation and Growth Kinetics of Sheep Pox Vaccine Virus in MDBK Cells

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Abstract

Capripoxviruses include lumpy skin disease virus, sheep pox virus, and goat pox virus, which lead to economic impacts in ruminants. Live, attenuated commercial vaccines can be applied in cattle against lumpy skin disease virus because of cross-protection. Bakırkoy sheep pox vaccine is a live, attenuated vaccine used against lumpy skin disease virus in cattle in Turkey. Continuous cell lines are used in viral vaccine production. This study was performed to the propagation of vaccine virus in the Madin–Darby bovine kidney cells and determination of growth kinetics. At the seventh day post-inoculation, the cells became round and moved away from the surrounding cells and cytopathic effect formation started. After 80% cytopathic effect was formed on the tenth day post-inoculation, virus suspension was harvested and identified as sheep pox virus with real-time polymerase chain reaction analysis and serum neutralization test. The titre of the sheep pox–Madin–Darby bovine kidney vaccine virus was calculated as $10^{5.75}$/mL $TCID_{50}$. The highest value was observed on the seventh day but the titre decreased on the ninth day while virus titre of supernatant was detected the highest on the seventh day post-infection and it decreased on the eighth day post-inoculation. The highest titre was reached in the third freezing and thawing and decreased in the fifth thaw. In conclusion, the Madin–Darby bovine kidney cells can be used in sheep pox vaccine production.

Keywords: MDBK, propagation, sheep pox, virus, vaccine

Introduction

Capripoxviruses (*CaPV*) include *lumpy skin disease virus* (LSDV), *sheep pox virus*, and *goat pox virus*, which cause serious economic impacts in ruminants (Tuppurainen et al., 2017). They are not serologically distinguishable and can cause experimental cross-infection as well as stimulate cross-protection as heterologous (Diallo & Viljoen, 2007). Capripoxvirus isolates are highly conserved with at least 96% genome similarities (Tulman et al., 2002).

Live, attenuated commercial vaccines derived from *sheep pox* (SP), *goat pox* (GP), and *lumpy skin disease* (LSD) viruses can be used to vaccinate cattle against LSD because of cross-protection among them (Diallo & Viljoen, 2007; Kitching, 1983). Yugoslavian RM65, Romanian SP, and Bakirkoy SP vaccine virus strains were used to protect cattle against LSD (Brenner et al., 2009; Tuppurainen & Galon, 2016; Tuppurainen & Oura, 2012). Sheep pox vaccine prepared from Bakirkoy strain of SP virus is a live, attenuated vaccine that is applied heterologously against LSD in cattle in Turkey. Bakirkoy strain was attenuated at primary lamb kidney cell at 65 passages and was produced as a lyophilized vaccine to contain at least $10^{2.5}$/doses $TCID_{50}$ titer and has been successfully used for the prevention of SP, GP, and LSD (FAO, 2017). In a study on the genomic relationships between Bakırköy SP vaccine virus and other CaPV, it was reported that potential vaccine strains protecting not only against SP and GP but also against the LSD are significantly important in eradicating the disease (Hasoksuz et al., 2014). In recent years, LSD outbreaks significantly has reduced due to mass vaccination with SP Bakırkoy strain and only six outbreaks have been reported since 2020 in Turkey (Uzar et al., 2022).

Capripoxviruses can be generally replicated in many cells because of their tropism for epithelial cells. Sheep, goat, and bovine origin cells can be used in the isolation of SPV. Embryo, fetal organs, testes, primary and secondary cell cultures of kidney origin, kid testicle cell culture of goat origin, and kidney cells of bovine origin were utilized in many studies. In addition, SPV can be produced in pig kidney, testis and embryonic skin, and muscle cells. Vero, BHK-21, and KEM-La

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are also accepted for the isolation and propagation of SP virus (Babiuk et al., 2007; Bhanuprakash et al., 2006). The Madin–Darby bovine kidney (MDBK) cells were obtained in 1957 from renal tissue of an adult calf (Madin & Darby, 1958). It has high sensitivity to many viruses, especially vesicular stomatitis (Indiana strain), infectious bovine rhinotracheitis virus (IBR), bovine parvovirus, bovine adenovirus I–III, and parainfluenza virus 3. The most significant utilization area of MDBK cells is industrial vaccine production and propagation of attenuated vaccine viruses. Currently, BVD, parainfluenza-3, IBR, and bovine respiratory synctialvirus vaccines are produced in MDBK cell culture (Hu, 2020), and contagious ecthyma virus (Orf) which belongs to the *Poxviridae* family *Parapoxvirus* genus can also be produced in MDBK cells (Gülyaz et al., 2020; Oem et al., 2013).

It is suggested that while continuous cell lines are easy, safe, and economic in viral vaccine production, the risk of contamination and costly and troublesome preparation limit the use of primary cell cultures. Up to date, there are no data on SP vaccine produced in cells of bovine origin. Therefore, this study was designed for the propagation of the SP vaccine virus in MDBK cells and the determination of growth kinetics.

Methods

Cell Culture

The Madin–Darby bovine kidney cell line (ECACC, 90050801) was used to propagate SP vaccine viruses. *Pestiviruses*-free status of cells was confirmed in the manufacturer's certificate statement and also laboratory pre-process controls. Cell lines were grown in Dulbecco's modified Eagle's medium (DMEM, Biochrom, Cat. no: F0445) containing $NaHCO_3$, supplemented with 10% fetal bovine serum (FBS, Biochrom S0115), 1% penicillin–streptomycin (Sigma-Aldrich, Cat. no: P0781), and 1% L-glutamine (Wisent, Cat. no: 609-065EL).

Propagation of SP Vaccine Virus

Sheep pox Bakirkoy strain, passaged 60 times in primary lamb kidney cells, was obtained from Pendik Veterinary Control Institute, Viral Vaccine Production Laboratory. Monolayer MDBK cells prepared in 75 cm^2 flasks by using DMEM containing 10% FBS, 1% penicillin–streptomycin, 1% L-glutamine, and SP virus (Bakirkoy strain at 60th passage) were inoculated with titers of $10^{4.0}$/mL $TCID_{50}$ and allowed to adsorb for 1 hour. After the incubation at 37°C, the inoculum was removed and 10 mL DMEM containing 2% FBS was added to each flask. Cells were checked daily for vitality and cytopathic effect (CPE). When the 80%–90% CPE formation was seen, the flasks (within 4–8 days) were taken to the freezer at −20°C in a sterile condition, and five serial passages were made in MDBK cells, and bulk vaccine virus (SP–MDBK vaccine virus) was stored at −20°C.

Identification of Sheep Pox Vaccine Virus Adapted to MDBK Cells

To identify the SP vaccine virus in the cell culture supernatant (bulk vaccine), virus titration test, neutralization test with SP hyperimmune serum, and SP-DNA detection by real-time polymerase chain reaction (PCR) (Bowden et al., 2008) were done.

Virus titration test was used to determine the both value of $TCID_{50}$/L and CPE formation. Ten-fold dilutions of the SP vaccine virus were made with phosphate-buffered saline (PBS); 100 µL of each dilution was placed in 4 wells of the 96-well plate; 100 µL MDBK cells ($3–5 \times 10^5$ cells/mL) was added on all dilutions. For cell control, 100 µL of medium and 100 µL of cell suspension were added to the last four cells of the plate. The plate was incubated at 37°C in an environment with 5% CO_2 for 10 days, and CPE formations were observed due to the propagation of the virus. $TCID_{50}$ values of the produced SP virus strains were calculated according to the Spearman–Karber method (Hierholzer & Killington, 1996).

The SP hyperimmune serum (collected from the rabbit) was obtained from the Viral Vaccines Production Laboratory of the Pendik Veterinary Control Institute. About 1 mL SP–MDBK vaccine virus and 1 mL SP hyperimmune serum were equally mixed and incubated at 37°C for 1 hour. At the end of the incubation period, 0.1 mL virus and hyperimmune serum mixture were inoculated into MDBK cells in a 25 cm^2 flask and followed for 10 days. One flask was kept as negative control and inoculated with only PBS.

DNA of SP–MDBK vaccine virus was extracted according to the kits (Qiagen, DNeasy Blood & Tissue Kit, cat.no:69506) protocol using reference Bakırköy SP vaccine virus DNA as a positive control. Real-time PCR analysis was performed with BioRad CFX Connect 96 Devices to amplify the 89 bp P32 gene of CaPV (Bowden et al., 2008).

Growth Kinetics of Adapted Vaccine Virus

The growth kinetics of the SP vaccine virus in MDBK cells were examined in terms of determining the applications to be carried out during the vaccine production process.

Determination of Virus Titre in Supernatant and Vaccine Bulk

Monolayer MDBK cells were grown in 16 flasks. Each flask was inoculated with 0.1 mL of SP (BK) $LK_{60}MDBK_5$ vaccine virus and incubated at 37°C in a 5% CO_2 incubator for 9 days and daily observed with an inverted microscope. From the second to the ninth day post-inoculation (dpi), two flasks were periodically taken out from the incubator. The supernatant of one of these flasks was collected, the other one was frozen 3 times at −20°C and thawed at 37°C, and all virus suspensions were centrifuged at 3000 rpm for 15 minutes and the supernatants were stored at −20°C. These procedures were carried out by taking two flasks for 9 days. The titer of virus suspensions was calculated according to the Spearman–Kaerber method by applying a virus titration test.

The Effect of Repeated Freezing and Thawing on Vaccine Virus Titre

Monolayer MDBK cells were grown in four flasks. Sheep pox–MDBK vaccine virus was inoculated into three of them. One flask was kept as a negative control and inoculated with PBS. All flasks were incubated for 7 days in an incubator with 5% CO_2 at 37°C and daily observed with an inverted microscope. At the end of the incubation, the flasks were moved to −20°C and frozen and thawed 5 times. After each thawing process, a 0.5 mL sample of vaccine virus suspension was taken. Virus titers were calculated by taking the average of three flasks with the Spearman–Kaerber method by applying the virus titration test.

Results

Propagation of Vaccine Virus in Confluent MDBK Cells

On the 7 dpi, CPE formation started with the cells in some foci became round and moved away from the surrounding cells. After 80% CPE was formed on the tenth day (Figure 1A), the flasks were

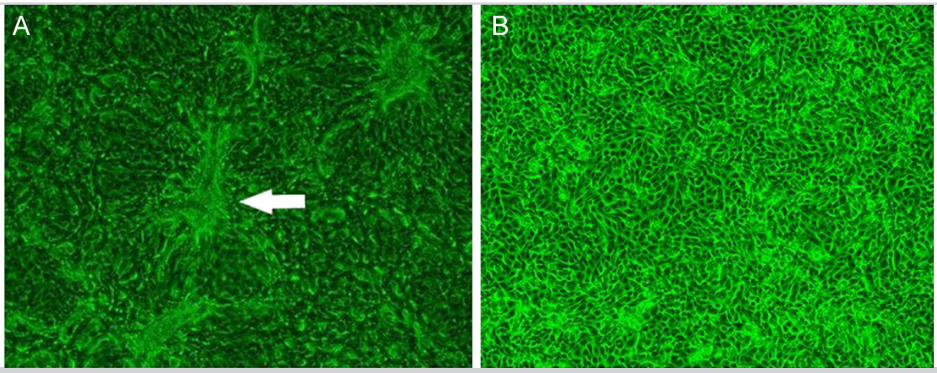

Figure 1.
Appearance of Monolayer MDBK Cells. (A) MDBK Cells Inoculated with Sheep Pox Bakırköy Strain at the 10 dpi. (B) Negative Control. MDBK = Madin–Darby bovine kidney; dpi = day post-inoculation.

picked up to the freezer at −20°C. No CPE formation was observed in control cells (Figure 1B).

Identification of Vaccine Virus

Cytopathic effect formation of SP–MDBK vaccine virus was observed down to 10^{-5} dilution steps (Figure 2), and titer was calculated as $10^{5.75}$/mL $TCID_{50}$.

Sheep pox–MDBK vaccine virus and SP hyperimmune serum were incubated for 1 hour at 37°C and then inoculated into MDBK cells. No CPE formation was observed in the MDBK cells during the 10-day incubation period (Figure 3), while the CPE was observed at the 4 dpi in MDBK cells infected by non-neutralized SP–MDBK virus control cells (Figure 4).

In the molecular analysis of the SP–MDBK vaccine virus DNA, the CT value was determined as 24.32 with real-time PCR (Figure 5).

Growth Kinetics of Adapted Vaccine Virus

Both daily virus titres of vaccine bulk and its supernatant were calculated from the 2 to the 9 dpi. Vaccine bulk virus titre reached the highest value on the seventh day, but it decreased on the ninth day, while virus titre of supernatant was found to be the highest on the 7 dpi and it decreased on the 8 dpi (Figure 6).

Average virus titres were calculated after five freezing and thawing processes (Table 1). While the highest titre was reached in the third freezing and thawing, it decreased in the fifth thawing.

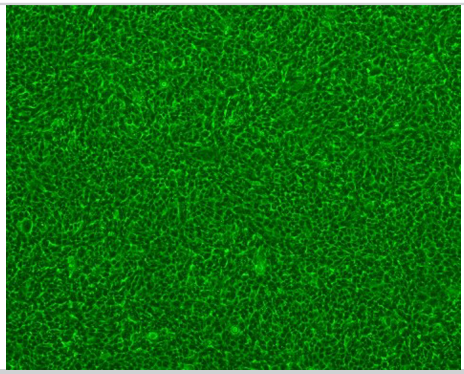

Figure 3.
No CPE Formation in MDBK Cells after Inoculation of Neutralized SP–MDBK Vaccine Virus at the 10 dpi. CPE = cytopathic effect; SP–MDBK = sheep pox–Madin–Darby bovine kidney; dpi = day post-inoculation.

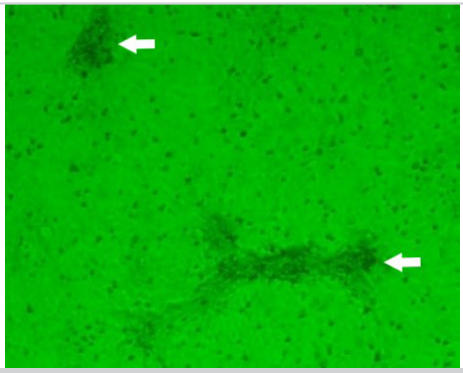

Figure 2.
CPE Formation of SP–MDBK Vaccine Virus at 10^{-5} Dilution. SP–MDBK = sheep pox–Madin–Darby bovine kidney; CPE = cytopathic effect.

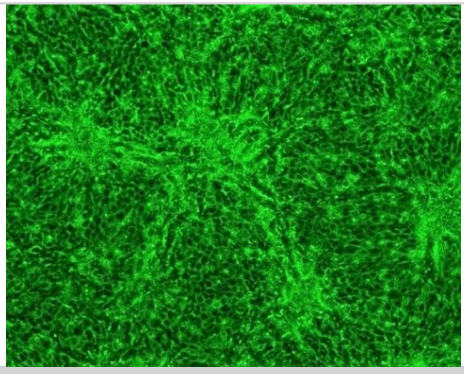

Figure 4.
CPE Formation in MDBK Cells Infected by Non-neutralized SP–MDBK Virus at the 4 dpi. CPE = cytopathic effect; SP–MDBK = sheep pox–Madin–Darby bovine kidney; dpi = day post-inoculation.

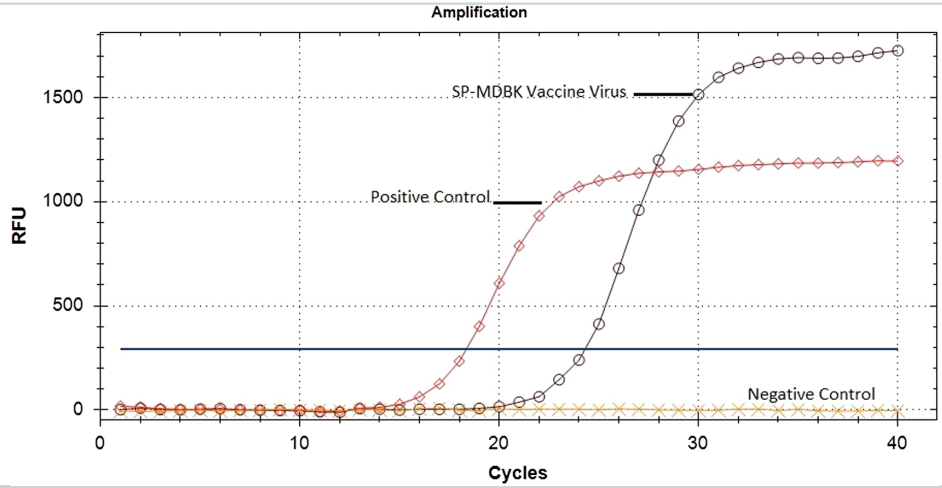

Figure 5.
Molecular Identification of SP Vaccine Virus by Real-Time PCR. SP = sheep pox; PCR = polymerase chain reaction.

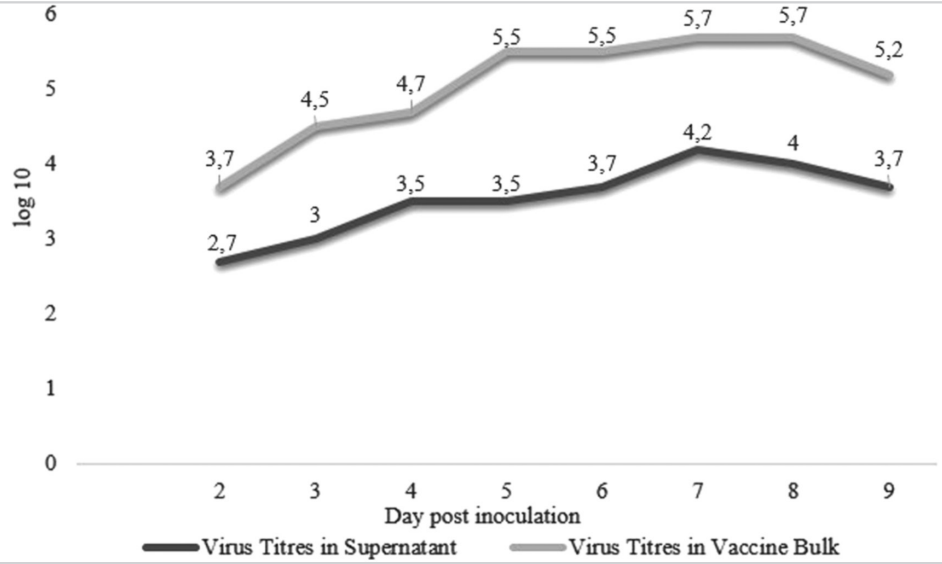

Figure 6.
Daily Virus Titres in SP–MDBK Vaccine Bulk and Its Supernatant. SP–MDBK = sheep pox–Madin–Darby bovine kidney.

Table 1.
Virus Titres of SP–MDBK Vaccine Bulk After Repeated Freezing and Thawing Process

Repetition Number	Virus Titres (log 10)
1	5.2
2	5.5
3	5.7
4	5.7
5	5.5

Note: SP–MDBK = sheep pox–Madin–Darby bovine kidney.

Discussion

At present, live, attenuated commercial vaccines derived from SP, GP, and LSD viruses can be used in cattles against LSD because of cross-protection among them (Kitching, 1983, 2003). Live, attenuated LSD vaccines are effective, but the major obstacle observed after the Neethling vaccination was the development of mild to severe clinical signs similar to LSD (Ben-Gera et al., 2015; Tasioudi et al., 2016), so a safer homologous LSD vaccine needs to be developed as well as a heterologous SP vaccine inducing better immunity. However, there is a need to improve this vaccine in terms of cell propagation and immunity.

Capripoxvirus can easily propagate in tissue culture of bovine, ovine, or caprine origin. Primary or secondary cell cultures of

kidney and testis cells are the most susceptible to both virus isolation and vaccine production (OIE Terrestrial Manual, 2021). Live viral vaccines against LSD are attenuated by performing multiple passages in the cell culture or embryo egg (Davies & Mbugwa, 1985; Van Rooyen et al., 1969). The fact that primary cells can maintain their resistance up to a certain number of passages and the propagation of new primary cells frequently limits and complicates the industrial vaccine production process (Gulyaz, 2003). In addition, primary cell cultures are more likely to be contaminated with viral diseases such as *Pestiviruses* (Giangaspero, 2013). In a study, bluetongue virus contamination was reported in SP vaccines used to fight against LSD (Bumbarov et al., 2016). Therefore, the propagation of vaccine viruses to continuous cell lines will provide convenience and safety in vaccine production processes. In this study, the Bakırköy SP vaccine virus was easily adapted to MDBK cells. The titre of the vaccine virus was calculated as $10^{5.75}$/mL $TCID_{50}$, and it was found appropriate according to the World Organisation for Animal Health Manual of Diagnostic Tests and Vaccines for Terrestrial Animals (OIE Terrestrial Manual, 2021). Similar studies have been performed in the propagation of SP and GP virus in MDBK cells (Joshi et al., 1995; Pandey et al., 1985). In a propagation study of Bakırköy SP vaccine virus to Vero cells, it was reported that the first CPE formations were observed in the fifth passage and the propagation process was completed in the twelfth passage in Vero cells (Gulyaz, 2003). In a study conducted on the growth kinetics of the Bakırköy SP vaccine virus in primary calf kidney cells, the virus titre reached the highest level on the 6 dpi (Chifney et al., 1973). In this study, the SP–MDBK vaccine virus reached the highest titre on the 7 dpi while the titre decreased in the 9 dpi. Also, the highest titre was reached in the third time freeze–thaw process to reveal the virus remaining in the cells by exploding the cells, and a decrease in titre occurred after the fourth thaw. The virus titre in the supernatant was found to be the highest on the 8 dpi, and the same results were got as in a study (Gulyaz, 2003). They can move CaPVs out of the cells in two ways either by budding from the cell through the Golgi membrane to form extracellular enveloped virus or through the rupture of the host cell, where intracellular mature virion is released and can infect surrounding cells (Babiuk, 2018). Considering the titre of supernatant and vaccine virus, mature virion particles within host cells increase the total virus titre from 1 to 2 log10. As a result of the growth characteristic study of the SP–MDBK vaccine virus, it was concluded that the vaccine virus would reach the highest titre by harvesting on the 7 dpi, followed by three freeze–thawing processes. Also, viruses in the supernatant can be harvested as an intermediate product in the vaccine bulk production processes, where medium change is predicted. MDBK cell culture has been widely used in vaccine production processes. In order to avoid the disadvantages of using primary cell culture, it is important to use stable continuous cell lines with high growth kinetics. Many studies have been conducted on the use of existing vaccine strains in continuous cell lines instead of primary cell cultures (Gülyaz, 2003; Gülyaz et al., 2020; Uzar et al., 2022).

Conclusion and Recommendations

In conclusion, SP vaccine production in MDBK cells did not cause any unwanted effects on the virus titre and, considering the efficacy and safety studies, MDBK cells can be conveniently used to avoid the disadvantages of primary cells.

Ethics Committee Approval: All procedures were conducted in accordance with EU Directive for animal experiments and national regulation.

Peer-review: Externally peer-reviewed.

Author Contributions: Design – S.U., N.T.; Analysis and/or Interpretation – S.U., N.T.; Literature Review – S.U., N.T.; Writing – S.U.; Critical Review – N.T.

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Declaration of Interests: The authors declare that they have no conflict of interest.

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