Chromosomes Stability and Growth Assessment of a Newly Established Lamb Heart Cell Line Sensitive to Lumpy Skin Disease Virus

Samah Rizk[2], Nour El-Din Hussein Saleh[1], Abdel-Hamid Bazid[2], and Mona E. Abd El-Gawad [3]

[1]Department of Zoology, Faculty of Science, Suez Canal University, Ismailia, Egypt.
[2]Middle East for Veterinary Vaccines (MEVAC), Sharkia, Egypt.
[3]National Gene Bank, Agricultural Research Centre, Cairo, Egypt.

Abstract:

Lumpy skin disease virus (LSDV) belongs to the *Poxvirus* family and is usually isolated, characterized and grown on embryonated eggs or primary cells. While embryonated chicken eggs and primary cells may not be available at all labs and could be risky at use due to potential endogenous or exogenous contamination, primary cell cultures usually show a limited number of passages. Nevertheless, both the Madin-Darby bovine kidney (MDBK) cell line and Vero cells were found to be susceptible to LSDV, but only MDBK showed cytopathic effect and the obtained virus titers were lower than those of primary fetal heart cells.

In this study, a stable lamb heart cell line sensitive to LSDV has been developed from the primary fetal heart cells. Studies on the characterization and stability of this cell line and optimal growth conditions were conducted. The obtained results revealed a successful passaging until the 60^{th} subculture. Chromosome analysis of heart cells at passage 60 revealed that >90% of cells were diploid cells. GTG-banding karyotyping confirmed that the chromosome diploid number was 54 (2n=54, XY). Viability of cells was 80 % after recovery from cryo-preservation. Population doubling time (PDT) was about 75 h. GMEM, and McCoy's media supplemented with 10% FBS supported optimum growth conditions compared to the other types of tested media.

The cells were highly susceptible to the *LSDV*, demonstrating the presence of severe cytopathic effects and an increase of two log10 titers in the virus harvest compared to MDBK cells. In conclusion, this study indicated that the developed heart cell line was stable and positively reflected on the quality and cost of vaccine production.

Keywords: Heart cell line, Characterization, GTG-banding, Karyotyping, chromosome counts Cryopreservation, *Lumpy Skin Virus (LSDV)*.

1. Introduction:

Cell culturing is one of the most used techniques in the life sciences. It was a term used for the establishment of an artificial environment suitable for the growth and multiplication of cells derived from human, animal, insect, and plant origin ***(Verma et al., 2020)***. Manufacturing of many biopharmaceuticals depends on using different cell culture systems for the development of multiple products. Animal cell cultures were used for a variety of purposes including; the establishment of model systems for research, the analysis of cellular structures or mechanisms, drug discovery, and stem cell research ***(Zahoor et al., 2016; Ajjarapu et al., 2023)***. Production of specific products like antibodies is a complex process. Therefore, animal cells have been recently gaining attention in this field due to the inability of some microorganisms to produce them efficiently. Furthermore, many cells were considered products because many types were used in the field of regenerative medicine ***(Horie et al., 2022)***.

The cell lines played a significant role in the developing process, optimization of techniques, and assessing quality variations for multiple viral strains and antigen production ***(Kiesslich and Kamen, 2020)***. The discovery of new vaccines against viral diseases or the development of existing ones is currently a worldwide high priority. Accordingly, the establishment of cell culture-based production gained attention owing to the trend of moving away from the classical approaches, like production in chicken eggs or primary cell lines with advantageous features like diminishing of cross-contamination or allergic reactions adding to processing in scalable bioreactors, need less space that allows for rapid manufacturing, especially during times of pandemics when vaccines are needed urgently ***(Aubrit et al., 2015)***. On the other side, the usage of live animals is more time-consuming, and expensive, and includes removing individuals from populations. Accordingly, scientists have created cell lines to be used in their research ***(Jordan and Sandig, 2014)***.

Identification of the cell line's origin, purity, and other significant features are important parameters for characterization. Therefore, many approaches, including; immunohistochemistry, western blot, flow cytometry, karyotyping, confocal microscopy, and next-generation sequencing, were commonly used ***(Richter et al., 2021)***.

Lumpy skin disease virus (LSDV) or the Neethling virus is classified in the genus *Capri pox virus* of the family *Poxviridae*. So, it is closely related to the *sheep pox virus (SPPV)* and *goat pox virus (GTPV)* ***(Sareyyüpoğlu et al., 2023)***. It is one of the highly emerging bovine threats due to its direct significance on the animal's productivity and

major impact on national output, as an outbreak occurrence might cause a restriction of live animal movement and trading of products globally ***(Farra et al., 2022).*** It is widely distributed and endemic in many countries, including Egypt, which implemented a mass vaccination strategy adding to other appropriate measures to control the disease (***Elhaig et al. 2021; Bazid et al., 2023)***.

As propagation of *LSDV* is crucial for diagnostics and vaccine production. It was found that the primary sheep testicular Sertoli cells (STSCs) were suitable for studying the molecular and pathogenic processes of *Capripoxviruses*. However, limitations, including their primary nature, the high cost of isolation, time-consuming operation, and short lifespan, greatly affected their practical application ***(Du et al., 2023)***. Another study tested the susceptibility of many cells, including cow skin fibroblasts cleared its ability to be a candidate cell line for basic and applied research, clinical application, and *LSDV* vaccine development ***(Ma et al., 2023)***. The fetal ovine heart (FOH-SA) cell line was developed by continuously passing fetal ovine heart cells, and it was discovered that this cell line is susceptible to the viruses that cause camelpox, peste des petits ruminants (PPR), lumpy skin disease (LSD), sheep pox, and RVF **(Khalid *et al.*, 2022).** Therefore, the presented study targeted the development of a stable lamb heart cell line and tested its sensitivity to *LSDV*. This is the first step and the cornerstone in the establishment of tools used for isolation of the *LSDV*, studying its pathogenesis, and developing of vaccines for controlling the disease.

2. Material and methods:

2.1. Ethical approval:

Before starting this study, the animal ethical committee of the Faculty of Medicine at Suez Canal University, Egypt, approved the proposed design, and experiments (no., 2018/156). This work was conducted in the MEVAC Factory for veterinary vaccines, New Salhia City, Egypt, following its guidelines for cell isolation, biosafety, and standards of good microbiological techniques (GMT).

2.2. Primary cell culture' preparation:

An ewe in the last week of pregnancy was brought to the animal experiment laboratory at the MEVAC factory for veterinary vaccines. After slaughtering, the uterus was immediately obtained and transported to the laboratory on a transport medium phosphate buffer with pH 7.2–7.6 in a pre-chilled box. The fetal heart tissues were processed according to the OIE guidelines ***(Janeiro 2018)***. After twice washing with PBS containing antibiotics, the heart sample was cut into pieces about 1 mm in diameter and seeded into a 25-cm^2 culture flask with 10 ml of Glasgow's MEM (GMEM, supplemented with 10%

FBS) and cultured at 37°C for 48 hr. Subsequently, 10 ml of fresh GMEM containing 10% FBS was added to the flask and exchanged every 2–3 days until the adherent cells migrated out and grew to monolayers. The cells were digested with trypsin and passaged in a ratio of 1:2.

2.3. Cell viability and growth curve determination (cell doubling time):

The cells were examined using an inverted microscope to assess the heart cell morphology and to determine its viability, before freezing and after recovery from the frozen status using the trypan blue (0.4% trypan blue in PBS). The number of nonviable cells was determined by cell counting ***(Verma 2014)***. Cell doubling time was determined using growth curves. Generally, the developed heart cells were seeded into a 25-cm^2 culture flask (2x10^^5 cells/ml, 2.5 mL/flask). The cell count was determined every day (3 flasks per time point) using a hemocytometer (MARIENFELDM, Germany) in conjunction with 0.4% trypan blue. From the log or exponential phase of the growth curve, the population doubling times (PDT) of the heart cell line was determined using a PDT calculating website (https://www.doubling-time.com/compute.php) calculated by the following equation:

$$\text{Doubling time} = \frac{duration * log(2)}{log(FinalConcentration) - log(InitalConcentration)}$$

Where "log" is the logarithm to base 10 or 2 or any other base ***(Singh et al. 2011)***.

2.4. Determination of optimal growth conditions:

Attachment capability and cell growth were studied using five different culture media. The cells were harvested after trypsnization then centrifuged at 1200 RPM for 5 min then seeded into five sets, each containing five cell culture vessels (n=25). Cells were seeded at (3 x 10^^5 cell/flask) in 25cm^2 cell vessels; each of the five vessels was incubated with a different culture media (GMEM, DMEM, EMEM, McCoy's, and RPMI 1640 media) separately and complemented with FBS (10%), penicillin (100 IU mL^{-1}), and streptomycin (100 µg mL^{-1}). The cell culture vessels were examined and counted then the optimum media was used as the basic culture media for all lasting tests ***(Verma et al. 2020)***.

Cells at a concentration of 5 x 10^5 were seeded in 25 cm^2 cell culture vessels supplemented with GMEM containing 10% FBS, 100 IU mL^{-1} penicillin, and 100 µg mL^{-1} streptomycin, and were cultured at 37°C for the duration of the experiment to determine the optimal serum per cent. The following day, the medium was removed, the cells were washed once with PBS, and fresh culture medium containing 5, 10, and 20% FBS was evaluated in triplicate flasks individually. The flasks were incubated at 37°C and observed for four days. At 24-h

intervals, the relative number of viable cells in triplicate flasks in each set was estimated microscopically using a hemocytometer ***(Verma 2014)***.

2.5. Cell cryopreservation and recovery:

The developed heart cells during the logarithmic growth phase of each passage were isolated, counted using a hemocytometer, and resuspended in freezing media containing 10% dimethyl sulfoxide (DMSO) (SIGMA, ALDRICH) and 90% FBS at a density of 2X $10^{\wedge 6}$ viable cells/ml. The cell suspension was aliquoted into sterile plastic cryo vials that were labeled with the passage number, freezing serial number, and date. The vials were capped and kept at −20◦ C for 60 min to equilibrate the DMSO and then transferred to −80◦ C for 24 h, and finally transferred into liquid nitrogen for long-term storage ***(Mehrabani et al. 2016b).*** For cell recovery was conducted at different time intervals till 4 years after cryopreservation, the cryo vials were removed from the liquid nitrogen and quickly thawed in a 37°C water bath. When the ice clump was almost thawed, frozen cells were transferred to a falcon tube containing 10 mL of complete cell culture medium (GMEM supplemented with10% FBS, 1% penicillin, and streptomycin), followed by centrifugation at 1200 rpm, and the cells were transferred into a 25 cm2 cell culture flask containing complete growth media and kept at 37°C with 5% CO2 for adhesion and further work ***(Mehrabani et al. 2016a).***

2.6. Characterization of heart cell line using chromosome counts and GTG-banding karyotype:

The chromosomes count of the heart cell line metaphases was performed at the Cytogenetics and Animal Cell Cutler Laboratory, National Gene Bank, Agriculture Research Institute. Briefly, this study was conducted with three groups, each group has a control and 12 passages. The adherent cells were treated with colchicine at a final concentration of 0.05 μg/ml for 1 hour to block cell division during metaphase by binding to microtubules. The cells were then treated with a hypotonic solution of 0.075 mol/L KCl for 30 minutes, followed by five fixation times using a 1:3 (v/v) ratio of acetic acid: methanol solution. After fixation, chromosomes were spread out on slides, followed by Giemsa staining. The chromosome counts were conducted in 28-33 metaphase for each passage ***(LIU et al. 2011)***. The GTG-banding technique was applied to the metaphase chromosomes of the developed heart cells as previously described ***(EAbdElGawad et al. 2019).***

2.7. Microbial sterility and mycoplasma detection:

The daily observation was done to check for any contamination and adherence of primary cells. Continuous monitoring for any microbial or fungal contamination was done under an inverted

microscope. A sample of cell culture supernatant from the first cell passage to the last one was added to a mycoplasma specific agar medium (solid PPLO agar). The sample of cell culture supernatant was cultivated on a mycoplasma agar medium and was incubated in an aerobic condition at 37ºC for two weeks, followed by examination of the agar plates under an optical light microscope ***(FDA 2010)***.

2.8. Sensitivity of the established heart cells to the *LSDV*:

Evaluation of cell sensitivity was carried out based on virus titration. The $TCID_{50}$ titration method was applied as described in the OIE Terrestrial Manual. The assay was done by serially diluting the *LSDV* and then infecting both the established heart cells and the MDBK cells as well. The plate wells were examined for evidence of CPE by light microscopy after five days, and the end points were determined. The virus titer was expressed by $TCID_{50}$ using Reed and Muench method ***(Rhazi et al. 2021)***.

2.9. Statistical analysis:

The data were edited in Microsoft Excel (Microsoft Corporation, Redmond, WA, USA). A Shapiro-Wilk test was conducted to check for normality as previously described (Wah and Razali, 2011). The significant differences between the different passage numbers as well as media types were examined using the One-way ANOVA (PROC ANOVA; SAS Institute Inc., 2012) with the level of significance set at $\alpha = 0.05$. Results were expressed as means ± standard error, SE. One sample t-test was used to perform pairwise comparisons between means and initial seeding count. Statistical significance between means was set at a p-value less than 0.05. Figures were created using GraphPad Prism software 9.0 (GraphPad, USA).

3. Results:

3.1. Examination of the developed cells:

It was discovered that fibroblast-like cells gradually started to proliferate from the cardiac tissue after the cells had been connected in culture for around a week. After that, the characteristic long spindle form of the cells suggested that they were in good health and that the culture conditions were perfect. The microscopic examination of the prepared cells revealed viable proliferation of spindle-shaped cells that were adherent along the flask wall. It was found that the developed cells could grow up to the 60^{th} passage. Fig. (1)

3.2. Cell viability and doubling time:

The viability percentage of cells before cryopreservation and after recovery was 90 % and 80 %, respectively Fig. (2). Interestingly, the growth kinetics of the developed heart cells represented by the growth curve (Fig.3) exhibited an "S"-shaped appearance. The cells had the ability to grow for a long period with a very low potentiality of cell apoptosis, especially at the

beginning of the third day of the culture initiation. At the same time, a plateau phase could be observed by the end of the same day. The heart culture flasks had more than 2-fold cell number, suggesting the superior proliferative ability of the developed cells. Furthermore, the population doubling time (PDT) of 24 h and 75 h interval was 19 and 19.01 h, respectively.

3.3 The best growth conditions:

The effect of different growth media was examined. The best growth and surviving rates represented by the higher percentage of attached cells were observed using McCoy's medium complemented with 20% FBS which gradually minimized to 10%. On the same side, the use of GMEM medium showed good results under the same conditions. However, in contrast to the GMEM medium and McCoy's medium, the cultures did not produce satisfactory results when DMEM, RPMI 1640, and EMEM were utilized. Because GMEM combined with 10% FBS can maintain cell culture in optimal condition while saving and decreasing costs in our research, it was the preferred method for culture propagation in subsequent trials Fig. (4, 5), Table (1).

For detection of the best serum concentration, the results showed that the cells grew well using the GMEM media supplemented with 20% serum during the initial passages, and decreasing the serum percentage obviously affected the cell proliferation and the total cell number per flask. The low serum levels caused cell vacuolation and gradual detachment of the cell sheet. The cultures reached confluency in 2 days only when complemented the GMEM medium with 20% serum for all passages. While after the 50th passage, the cells could propagate in 10% serum, by lowering the percentage of FBS, the cells have been noticed to take more time in cell proliferation and growth Fig (6). The cells were observed to proliferate effectively in GMEM medium complemented with 10% FBS with a split ratio of 1:3, even after the 60th passage, and they reached confluency three days after subculturing.

3.4. Cryopreservation and recovery of the cell lines:

At each passage, the cells were cryopreserved with 10% DMSO used as a cryo-protectant. The recovered cells even after 4 years of cryopreservation in liquid nitrogen, showed 80% viability and grew to confluency within 3-5 days. Upon retrieval the preserved cells, an examination revealed no discernible changes to the morphology or growth pattern.

3.5. Characterization of the heart cell line by chromosome counts and GTG-banding karyotype:

Chromosomes count was used to detect the numerical chromosomal abnormality frequency of polyploidy and aneuploidy. These numerical chromosomal abnormalities related to the genetic stability among all passages were examined, for every

five passages, the metaphase of all passages was counted to determine the number of chromosomes in each metaphase. From the 5th to the 45th passage, the frequency of 2n=54 was 100%, while in the three other passages from the 50th to the 60th passage, the proportion of normal metaphases started to decline. With many passages, Numerical chromosomal abnormality tended to increase, which suggests that cells' ancestry was marginally impacted by in vitro culture. Also, these cells displayed a normal karyotype over the whole successive passages. No aneuploidy metaphases were recorded at all (Table 2).

GTG-banding karyotype were used in an attempt to provide fine karyotype details and characterize the chromosomes of heart cell line. All the passages revealed that, the number of chromosomes was 54 (2n=54, XY), and the karyotype formula was 2n=54 = Lm6 + Ma22 + Sa24 + sex chromosomes Fig (7).

3.6. Microbial sterility and mycoplasma detection:

Microscopic examination of the cultured cells revealed the absence of microbial contamination, including bacteria, fungi, and yeasts. In the heart cultures, there was no evidence of vacuolation, granulation, or clumping of cells. There was no evidence of a sudden decrease in pH, a sign of bacterial contamination, nor were any mycelium-containing hyphae seen in the cultures. However, routinely maintained cultures were promptly rejected if any signs of bacterial, yeast, or fungal contamination were found. Cultivation of different samples of different passages on PPLO culture media plates for 14 days showed no mycoplasma contamination.

3.7. Sensitivity of the established heart cells to LSDV:

To determine the sensitivity of heart cells for LSDV, a wild-type strain isolated from clinically infected cases was inoculated to the established heart cells, and the cells were examined microscopically. A typical poxvirus cytopathic effect (CPE) was noted, characterized by cell rounding and then developing irregular shapes with long, thin projections (Fig. 8). A star-like image was seen as the cells seemed to have contracted or collapsed. It was noted that the integrity of the monolayers was compromised by an increase in granulated and fragmented dying cells.

Comparing the growth criteria of the LSDV on the developed heart cell line to the MDBK cells was conducted. The growth titer of the LSDV harvested from the heart cells was approximately 7 log10 which was more than 2 logs higher than those obtained from the MDBK cells (4.5 log10), which reflects the high efficiency and sensitivity of the developed cells to LSDV growth and multiplication.

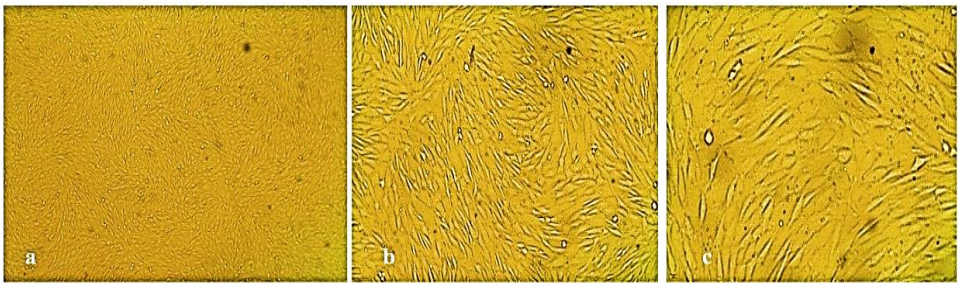

Figure 1: *Microscopic examination and determination of cell morphology. The figure shows identical fibroblastic cell shape visualized by an Inverted light microscope: Leica, Germany, this morphology remained constant in the successive subcultures till passage 60, ((a) fig shows established heart cells at the power of 4X, (b) Heart cells at the power of 10 X, and (c) Heart cells at the power of 20 X.*

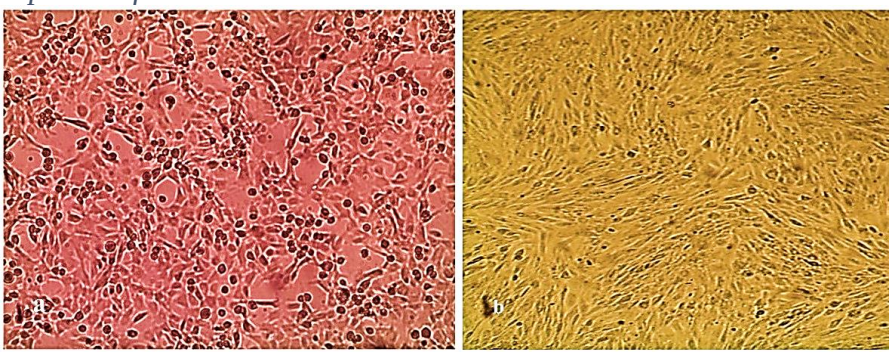

Figure 2: *shows the viability of cells before and after cryopreservation. (a)Cells before cryopreservation were healthy and in the mitotic phase, (b) heart cells after recovery; the cells were cultured for 48 h after thawing.*

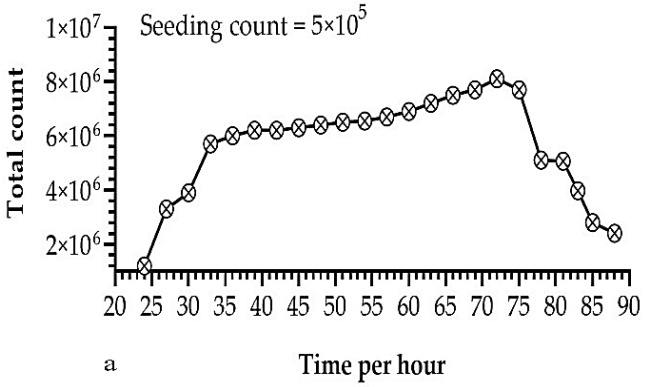

Figure 3: *Shows the growth curve of the developed heart cells. The highest peak of the count was on the third day of the cultural initiation, the curve showed a typical 'S' shape. The growth curve included the lag phase, exponential growth phase and stationary phase.*

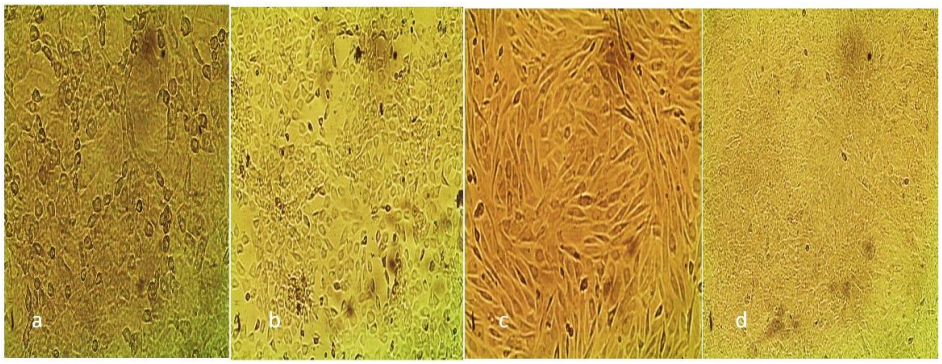

Figure 4: *Effect of different media in the propagation of heart cells. (a) DMEM media, (b) EMEM media, (c) RPMI1640 media, and (d) McCoy's media.*

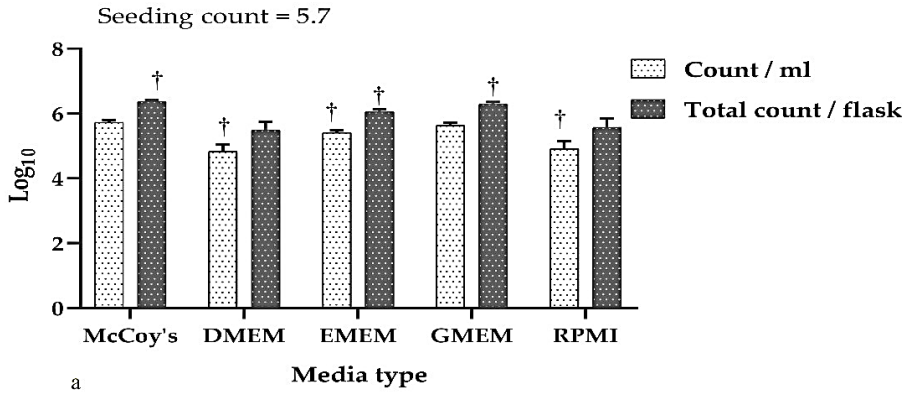

Figure 5: *The Histogram shows the effects of different growth media types on the growth of the heart cells. The different media types showed good results in the propagation of established heart cells; the best media were McCoy's and GMEM media.*

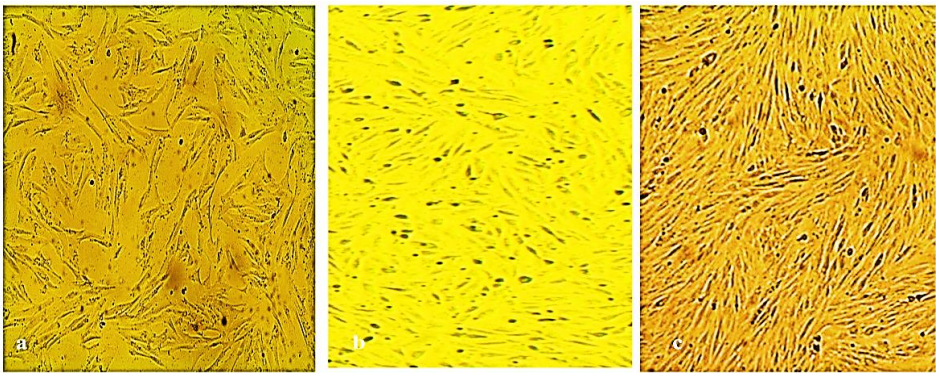

Figure 6: *The effect of different fetal serum concentrations on the growth of heart cells. (a)growth of heart cells on GMEM media supplemented with 5 % FBS, (b) growth of heart cells on GMEM media supplemented with 10 % FBS,(c) growth of heart cells on GMEM media supplemented with 20 % FBS.*

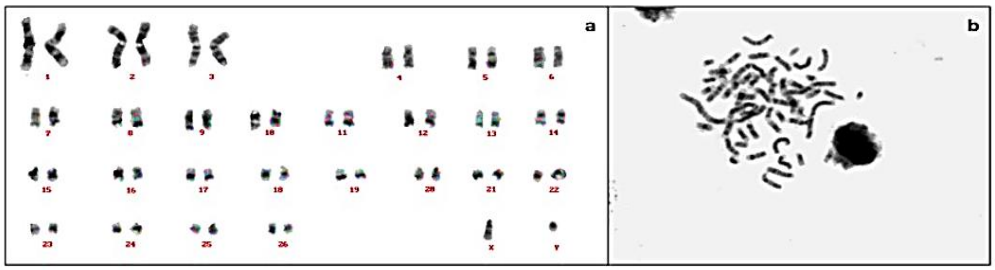

Figure 7: *showed GTG-banding karyotype, a: normal male karyotype of the established heart cells and (b) Chromosomes at normal male metaphase. The karyotype of the heart fibroblast cells consisted of 54 pairs of chromosomes. The sex chromosome type was XY (♂).*

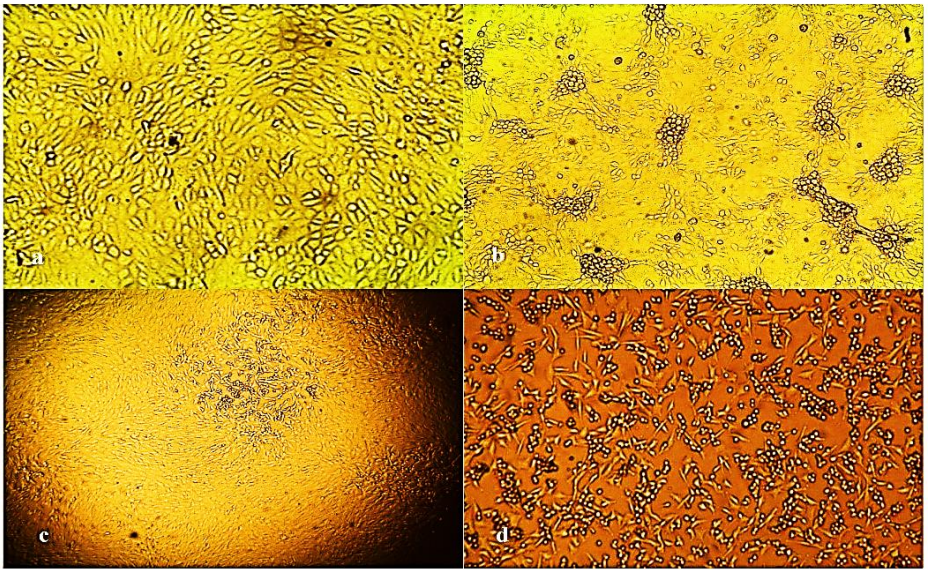

Figure 8: *Sensitivity of Heart cells and MDBK cells to LSD virus CPE characterized by cell rounding, foci formation (cell -clustering), also degenerative changes was observed in both heart cells and MDBK cells ((a) control MDBK cells, (b) MDBK Cells infected with LSDV on the 5th-day post-inoculation, (c) Heart Cells infected with LSDV on the 3rd-day post-inoculation, and (d) Heart Cells infected with LSDV on the 5th -day post-inoculation.*

Table 1: *Effect of culture medium on growth of heart cells, statistical analysis shows the difference between media types, differ significantly with seeding count ($p<0.05$); ¶: p-value was calculated by the one-way analysis of variance to assess the significant differences among different media types.*

Media type	**Seeding (log_{10})**	**Count/ml (log_{10})**	**Total count/flask (log_{10})**
McCoy's	5.70	5.73 ± 0.07	6.37 ± 0.05†
DMEM		4.83 ± 0.21†	5.49 ± 0.26
EMEM		5.41 ± 0.08†	6.05 ± 0.09†
GMEM		5.64 ± 0.08	6.30 ± 0.06†
RPMI		4.91 ± 0.23†	5.57 ± 0.28
p-value¶		0.0015	0.0081

Table 2: *Showed the mean of total metaphase counts, mean of normal metaphase counts and mean of polyploidy metaphase counts of all heart cells passage.*

Passage No	Mean of total metaphase counts	Mean of Normal metaphase counts	Mean of polyploidy metaphase counts
Control	28.00±0.00	28.00±0.00	0.00±0.00
P5	28.00±0.00	28.00±0.00	0.00±0.00
P10	28.00±0.00	28.00±0.00	0.00±0.00
P15	28.00±0.00	28.00±0.00	0.00±0.00
P20	28.00±0.00	28.00±0.00	0.00±0.00
P25	30.33±2.33	30.33±2.33	0.00±0.00
P30	28.33±0.33	28.33±0.33	0.00±0.00
P35	29.33±1.33	29.33±1.33	0.00±0.00
P40	28.00±0.00	28.00±0.00	0.00±0.00
P45	27.33±0.67	27.33±0.67	0.00±0.00
P50	30.84±3.33	30.67±3.18	0.17±0.23†
P55	27.52±0.33	27.33±0.33	0.19±0.10†
P60	31.93±3.21	31.67±2.91	0.26±0.23†
p-value¶	0.3242	0.5385	0.1206

it shows non significant differences with the control ($p>0.05$): p-value was calculated by the one-way analysis of variance to assess the significant differences among different passage no.

4. Discussion

Most of the recently developed biological sciences depend on cell culturing as a tool for studying the mechanisms of infection for many pathogens, developing, and evaluating many products. This technology involved the isolation of cells from their original host organ and multiplication in favorable artificial conditions ***(Swain 2014)***. Since using of animal cell lines more than four decades ago, their importance has increased dramatically, and the technology for processing was greatly improved depending on serum-based media to serum-free, then protein-free chemically defined media ***(Ho et al. 2021)***. In this study, the establishment of the ovine heart fibroblast cell line was particularly challenging work. The ovine heart cells had developed successfully and proliferated under optimized growth conditions.

The microscopic examination of the obtained heart cells revealed fibroblastic cell characteristics; the cell shape was almost identical to typical elongated fibroblast-like. Also, after subculturing, the morphology of the cells had not undergone any changes to their shape until the sixty passages. The same spindle shape was recorded for many different tissue types including skin ***(Singh et al. 2011)***, and ear marginal tissue fibroblast cell line ***(Li et al. 2009).***

The proportion of viable cells in a culture accurately reflects the

culture's health status. The developed cells were examined for viability after reviving from preservation in liquid nitrogen; it has been demonstrated that cells have a viability percentage of 80% even after four years of conservation. The same percentage was previously reported in a different cell type as the *Puntius sophor*e caudal-fin (PSCF) cell line of the pool *barb Puntius sophore* showed a 75% viability percentage with no noticeable changes in morphology or growth pattern ***(Lakra and Goswami 2011).*** On the other side, the Guinea pig fetal fibroblast cells showed a viability percentage of 95% in the second passage and 74.5% in the eighth passage respectively ***(Mehrabani et al. 2014)***.

The population growth rate is an important characteristic of any cell culture ***(Lindström and Friedman 2020)***. Accordingly, growth kinetics including determination of doubling time was carried out. It was found that cells had a 19 h cell doubling time and that the monolayer only became confluent after 75 h. Cells could be handled twice a week, which is advantageous in terms of production consideration and the concept of time. The PDT of the developed Guinea pig fetal fibroblast cells of the second passage was about 23h and of the eighth passage was about 30 h ***(Mehrabani et al. 2014)***. While that of ear marginal tissue fibroblast cell line was 24.9 h ***(Li et al. 2009)***.

Evaluation of cell growth rates for the newly developed cell lines is so important. Multiple approaches could be applied to determine it including the fluorescence-based method ***(Pereira et al. 2020)***. While the most commonly used method was the cell growth curves. The obtained heart cells growth curve was typically S shaped representing the basically known lag, log, and stationary phases. Similar results were previously recorded in different cell types including the cancer cells ***(Assanga 2013; Toloudi et al. 2014)***.

The cell lines' excellent plating efficiency and cell proliferation are suggestive indicators of genotypic alterations or transformation characteristics ***(Verma 2014)***. The split ratio was calculated to assess the cells at their optimum growth and viability. High split ratios are indicative of immortalized cells ***(Verma et al. 2020)***.

Optimization of growth condition for the newly developed cell lines is crucial ***(Verma et al. 2020)***. Different types of media were tested for cell growth and multiplication. The obtained results revealed that the McCoy's and GMEM media were the most suitable for optimum growth conditions with no significant difference. The relative applicability of various media demonstrated that GMEM was appropriate for promoting the proliferation of cardiac cell lines. As of right now, McCoy's has been employed in about 80% of the cell

lines created since 1994 ***(Yang and Xiong 2012).*** The study's separated cells appeared to grow best in GMEM containing 20% FBS for primary cultures, whereas 10% FBS was the ideal concentration for cell lines. The OCF cell line, which was derived from the Amphiprion ocellaris (ocellaris clownfish), grew at its fastest rate in a medium that contained 1% of 0.2 M NaCl and 20% FBS at 28 °C. However, all passages demonstrated satisfactory results with a 15% FBS concentration ***(Yashwanth et al. 2020)***.

Cryopreservation of heart cell line in liquid nitrogen using 90% FBS and 10% DMSO at different passage levels has been successfully done. The revived cells showed an 80% survival rate even after four years of storage. It was found that, the viability of the revived Guinea pig fetal fibroblast cell cultures was 74.5% ***(Mehrabani et al. 2014)***. While, cryopreservation of the Fars native goat fetal skin fibroblast cell lines culture using the freezing solution containing the same constituent ratio revealed a viability percentage of (88.32 ± 5.17)% after thawing ***(Mehrabani et al. 2016b)***. Though serum is frequently added in high concentrations to the medium, serum functions as a complex protective agent that may shield cells from injury during freezing and thawing.

The freezing medium constituents differ according to the type of cell preserved. The Mongolian sheep ear marginal tissue fibroblast cell line (MSF32) was preserved in medium (10% dimethyl sulfoxide + 50% fetal bovine serum + 40% DMEM) (LIU et al. 2011). While for preservation of a liver cell line, derived from yellowfin sea bream, a cryopreservation medium (containing 30% FBS and 10% dimethyl sulphoxide) was used ***(Li et al. 2022)***.

The analysis of chromosomes is a crucial aspect for identifying and characterizing the cell lines, particularly those derived from tumors. Furthermore, karyotyping can be utilized to differentiate between subclones and to monitor their stability over time ***(Rosenberg and Rosenberg 2012)***. Based on chromosomal research, the generated cardiac cells were shown to be a stable diploid cell line. Similar results on many cell types were previously documented. ***(Li et al. 2019; Pan et al. 2022)***. The produced cell lines, like the fibroblast cell line derived from red-rumped agouti skin, surprisingly kept their original properties following retrieval, according to the majority of previously reported karyotyping investigations. Nevertheless, even after cryopreservation, most of the parameters analyzed were unchanged ***(Praxedes et al. 2021)***.

It was established that the generated cells were sterile against mycoplasma, fungus, and bacteria. The creation of cell cultures is a crucial procedure since

contamination can happen at any stage of the production process, either from contaminated raw materials or from infected primary cultures ***(Geraghty et al. 2014)***. Accordingly, for the production of the presented heart cells, high precautionary measures were taken into consideration to prevent the source of infection ***(Rhazi et al. 2021)***.

The developed heart cells were highly susceptible to the LSDV, demonstrating by the presence of severe cytopathic effects and an increase of two log10 titers in the virus harvest compared to MDBK cells. Usually, the LSDV could be isolated in primary cells of ovine origin with a disadvantages of a limited number of passages ***(Salnikov et al. 2018)***. Although different cell lines including MDBK and Vero cells could be used for isolation but mostly no CPE could be detected specially in the first passages and the produced virus titer could be relatively low comparing to the primary cells ***(Kumar et al. 2021)***. On the other side, while a lot of scientists used cell cultures yielded their harvests by the 5th day post infection, heart cells yielded the highest virus titer (7 Log10 TCID50/ml) followed by MDBK cells (4.5 Log10 TCID50/ml). It is worth to note that, the develop heart cell line was successfully used for Commercial production of a live attenuated *LSDV* vaccine ***(Bazid et al. 2023)***. To our knowledge, the presented study is the first successive trial for establishment of a novel lamb heart cell line as a candidate for production of *LSDV* vaccine.

Conclusion:

A stable lamb heart cell line was developed. Continuously dividing cell lines were derived from the primary fetal tissues extracted from embryonic lamb under sterile conditions. Primary cell growth was routinely observed by determination of growth curve analysis. Isolated heart cells were passaged till reached 60 continued passages. The cell line was successfully cryopreserved by a slow freezing procedure at − 80 °C with a revival efficiency of 70-80 %. Aliquots of cell lines were routinely archived in liquid nitrogen for further analysis. The origin of this cell line was confirmed using Karyotype analysis. The chromosome analysis of Heart cells at passage 60 revealed that >90% of cells were diploid karyotypes. The cells were highly susceptible to lumpy skin disease virus (LSDV), which was demonstrated by the presence of a severe cytopathic effect with an increase of two logs in the obtained harvest's titer in comparison to those from the MDBK cells. In conclusion, this study indicated that the developed heart cell line was stable. The ability of cell growth up to the 60^{th} passage and increase in the obtained virus titer will positively reflect on the quality of vaccine production and its cost reduction. Furthermore, the

newly established Heart cell line would be an excellent in vitro platform for the study and isolation of the pox viruses.

References:

Ajjarapu SM, Tiwari A, Kumar S (2023) Applications and Utility of Three-Dimensional In Vitro Cell Culture for Therapeutics. Futur Pharmacol 3:213–228. https://doi.org/10.3390/futurepharmacol3010015

Assanga I (2013) Cell growth curves for different cell lines and their relationship with biological activities. Int J Biotechnol Mol Biol Res 4:60–70. https://doi.org/10.5897/IJBMBR2013.0154

Aubrit F, Perugi F, Léon A, et al (2015) Cell substrates for the production of viral vaccines. Vaccine 33:5905–5912. https://doi.org/10.1016/j.vaccine.2015.06.110

Bazid A-H, Wasfy M, Fawzy M, et al (2023) Emergency vaccination of cattle against lumpy skin disease: Evaluation of safety, efficacy, and potency of MEVAC® LSD vaccine containing Neethling strain. Vet Res Commun 47:767–777. https://doi.org/10.1007/s11259-022-10037-2

Du G, Wu J, Zhang C, et al (2023) Generation and application of immortalized sertoli cell line from sheep testis. J Virol Methods 316:114727. https://doi.org/10.1016/j.jviromet.2023.114727

EAbdElGawad M, AEllItriby H, MSobhy H, HAHussein E (2019) Fish- Mapping and Standard Gtg-Banding Karyotype of Three Egyptian Sheep Breeds. Int J Adv Res 7:374–387. https://doi.org/10.21474/ijar01/8827

Elhaig MM, Almeer R, Abdel-Daim MM (2021) Lumpy skin disease in cattle in Sharkia, Egypt: epidemiological and genetic characterization of the virus. Trop Anim Health Prod 53:. https://doi.org/10.1007/s11250-021-02711-5

Farra D, De Nardi M, Lets V, et al (2022) Qualitative assessment of the probability of introduction and onward transmission of lumpy skin disease in Ukraine. Microb Risk Anal 20:100200. https://doi.org/10.1016/j.mran.2021.100200

FDA (2010) Guidance for Industry Cell Characterization and Qualification of Cell Substrates and Other Biological Materials Used in the Production of Viral Vaccines for Infectious Disease Indications. FDA Guid Ind 1–50

Geraghty RJ, Capes-Davis A, Davis JM, et al (2014) Guidelines for the use of cell lines in biomedical research. Br J Cancer 111:1021–1046. https://doi.org/10.1038/bjc.2014.166

Ho YY, Lu HK, Lim ZFS, et al (2021) Applications and analysis of hydrolysates in animal cell culture. Bioresour Bioprocess 8:93. https://doi.org/10.1186/s40643-021-

00443-w
Horie M, Yamano-Adachi N, Kawabe Y, et al (2022) Recent advances in animal cell technologies for industrial and medical applications. J Biosci Bioeng 133:509–514. https://doi.org/10.1016/j.jbiosc.2022.03.005
Janeiro DORIODE (2018) No 主観的健康感を中心とした在宅高齢者における 健康関連指標に関する共分散構造分析Title. 1–9
Jordan I, Sandig V (2014) Matrix and Backstage: Cellular Substrates for Viral Vaccines. Viruses 6:1672–1700. https://doi.org/10.3390/v6041672
KHALID,M., SULEIMAN, M., ALJULIDAN, G.,HUSSEIN, and HABIB,N., 2022. Establishment of a novel fetal ovine heart cell line by spontaneous cell fusion bioRxiv Cell Biology.https://doi.org/10.1101/2022.07.14.500071
Kiesslich S, Kamen AA (2020) Vero cell upstream bioprocess development for the production of viral vectors and vaccines. Biotechnol Adv 44:107608. https://doi.org/10.1016/j.biotechadv.2020.107608
Kumar N, Chander Y, Kumar R, et al (2021) Isolation and characterization of lumpy skin disease virus from cattle in India. PLoS One 16:. https://doi.org/10.1371/journal.pone.0241022
Lakra WS, Goswami M (2011) Development and characterization of a continuous cell line PSCF from Puntius sophore. J Fish Biol 78:987–1001. https://doi.org/10.1111/j.1095-8649.2010.02891.x
Li J, Jia P, Chen X, et al (2019) Establishment and characterization of a fin tissue cell line derived from silver pomfret, Pampus argenteus. J Fish Dis 42:1391–1399. https://doi.org/10.1111/jfd.13059
Li LF, Guan WJ, Li H, et al (2009) Establishment and characterization of a fibroblast cell line derived from Texel sheep. Biochem Cell Biol 87:485–492. https://doi.org/10.1139/O09-005
Li Y, jia P, Yu F, et al (2022) Establishment and characterization of a liver cell line, ALL, derived from yellowfin sea bream, Acanthopagrus latus, and its application to fish virology. J Fish Dis 45:141–151. https://doi.org/ 10.1111/jfd.13543
Lindström HJG, Friedman R (2020) Inferring time-dependent population growth rates in cell cultures undergoing adaptation. BMC Bioinformatics 21:583. https://doi.org/10.1186/s12859-020-03887-7
LIU C-Q, GUO Y, GUAN W-J, MA Y-H (2011) Establishment and characterization of a fibroblast cell line derived from Mongolian sheep. Anim Sci J 82:215–222. https://doi.org/10.1111/j.1740-0929.2010.00824.x
Ma C, Ren S, Afera TB, et al (2023) The comparative study revealed that the hTERT-CSF cell line was the most susceptible cell to

the Lumpy skin disease virus infection among eleven cells. J Virol Methods 317:114745. https://doi.org/10.1016/j.jviromet.2023.114745

Mehrabani D, Booyash N, Aqababa H, et al (2016a) Growth kinetics, plasticity and characterization of hamster embryonic fibroblast cells. Ital J Zool 83:306–311. https://doi.org/10.1080/11250003.2016.1194487

Mehrabani D, Mahboobi R, Dianatpour M, et al (2014) Establishment, culture, and characterization of guinea pig fetal fibroblast cell. Vet Med Int 2014:. https://doi.org/10.1155/2014/510328

Mehrabani D, Tajedini M, Tamadon A, et al (2016b) Establishment, characterization and cryopreservation of Fars native goat fetal fibroblast cell lines. Asian Pacific J Reprod 5:247–251. https://doi.org/10.1016/j.apjr.2016.04.013

Pan H, Chen H, Chen L, et al (2022) Establishment and characterization of a liver cell line from black porgy, Acanthopagrus schlegelii. Aquac Reports 25:101213. https://doi.org/10.1016/j.aqrep.2022.101213

Pereira TF, Levin G, DeOcesano-Pereira C, et al (2020) Fluorescence-based method is more accurate than counting-based methods for plotting growth curves of adherent cells. BMC Res Notes 13:57. https://doi.org/10.1186/s13104-020-4914-8

Praxedes ÉA, Silva MB, Oliveira LRM de, et al (2021) Establishment, characterization, and cryopreservation of cell lines derived from red-rumped agouti (Dasyprocta leporina Linnaeus, 1758) – A study in a wild rodent. Cryobiology 98:63–72. https://doi.org/10.1016/j.cryobiol.2020.12.006

Rhazi H, Safini N, Mikou K, et al (2021) Comparative sensitivity study of primary cells, vero, OA3.Ts and ESH-L cell lines to lumpy skin disease, sheeppox, and goatpox viruses detection and growth. J Virol Methods 293:114164. https://doi.org/10.1016/j.jviromet.2021.114164

Richter M, Piwocka O, Musielak M, et al (2021) From Donor to the Lab: A Fascinating Journey of Primary Cell Lines. Front Cell Dev Biol 9:. https://doi.org/10.3389/fcell.2021.711381

Rosenberg LE, Rosenberg DD (2012) Chromosome Abnormalities. In: Human Genes and Genomes. Elsevier, pp 141–167

Salnikov N, Usadov T, Kolcov A, et al (2018) Identification and characterization of lumpy skin disease virus isolated from cattle in the Republic of North Ossetia-Alania in 2015. Transbound Emerg Dis 65:916–920. https://doi.org/10.1111/tbed.12818

Sareyyüpoğlu B, Uzar S, Saraç F, et al (2023) Immune response

against lumpy skin disease after simultaneous vaccination of cattle with sheep pox and goat pox and foot and mouth disease vaccines. Vet Microbiol 281:109726. https://doi.org/10.1016/j.vetmic.2023.109726

Singh M, Ma X, Amoah E, Kannan G (2011) In vitro culture of fibroblast-like cells from postmortem skin of Katahdin sheep stored at 4°C for different time intervals. Vitr Cell Dev Biol - Anim 47:290–293. https://doi.org/10.1007/s11626-011-9395-6

Swain P (2014) Basic Techniques and Limitations in Establishing Cell Culture: a Mini Review. Adv Anim Vet Sci 2:1–10. https://doi.org/10.14737/journal.aavs/2014/2.4s.1.10

Toloudi M, Ioannou E, Chatziioannou M, et al (2014) Comparison of the Growth Curves of Cancer Cells and Cancer Stem Cells. Curr Stem Cell Res Ther 9:112–116. https://doi.org/10.2174/1574888x0902140121163539

Verma A (2014) Animal Tissue Culture. In: Animal Biotechnology. Elsevier, pp 211–231

Verma A, Verma M, Singh A (2020) Animal tissue culture principles and applications. INC

Yang Z, Xiong H-R (2012) Culture Conditions and Types of Growth Media for Mammalian Cells. Biomed Tissue Cult 3–18. https://doi.org/ 10.5772/52301

Yashwanth BS, Goswami M, Kooloth Valappil R, et al (2020) Characterization of a new cell line from ornamental fish Amphiprion ocellaris (Cuvier, 1830) and its susceptibility to nervous necrosis virus. Sci Rep 10:20051. https://doi.org/10.1038/s41598-020-76807-7

Zahoor MA, Khurshid M, Qureshi R, et al (2016) Cell culture-based viral vaccines: Current status and future prospects. Future Virol 11:549–562. https://doi.org/10.2217/fvl-2016-0006

تقييم استقرار الكروموسومات ونموها لخط خلايا قلب الحمل المنشأ حديثًا والحساس لفيروس مرض الجلد العقدي

سماح رزق أحمد الريس .نورالدين حسين صالح شريف.عبد الحميد بازيد .منى إبراهيم حسين عبد الجواد

الملخص

ينتمي فيروس مرض الجلد العقدي (LSDV) إلى عائلة Poxvirus وعادة ما يتم عزله وتمييزه ونموه على البيض أو الخلايا الأولية. في حين أن بيض الدجاج والخلايا الأولية قد لا تكون متاحة في جميع المختبرات ويمكن أن تكون محفوفة بالمخاطر في الاستخدام بسبب التلوث الداخلي أو الخارجي المحتمل، فإن مزارع الخلايا الأولية عادة ما تظهر عددا محدودا من المقاطع. ومع ذلك، تم العثور على كل من خط خلايا الكلى البقري (Madin-Darby (MDBK وخلايا Vero عرضة لـLSDV ، لكن MDBKفقط أظهر تأثير الاعتلال الخلوي وكانت عيارات الفيروس التي تم الحصول عليها أقل من تلك الموجودة في خلايا قلب الجنين الأولية.

في هذه الدراسة، تم تطوير خط خلايا قلب خروف مستقر حساس لـ لمرض الجلد العقدى من خلايا قلب الجنين الأولية. أجريت دراسات حول توصيف واستقرار هذا الخط الخلوي وظروف النمو المثلى. كشفت النتائج التي تم الحصول عليها عن مرور ناجح حتى الثقافة الفرعية الستين. كشف تحليل كروموسوم خلايا القلب عند المرور 60 أن أكثر من 90% من الخلايا كانت خلايا ثنائية الصيغة الصبغية. أكد النمط النووي لنطاق GTG أن عدد الكروموسوم الثنائي كان 54(2 ن = 54، XY) بلغت صلاحية الخلايا 80% بعد التعافي من الحفظ بالتبريد. كان وقت تضاعف السكان (PDT) حوالي 75 ساعة. دعمت وسائط GMEM و McCoyالمكملة بـ 10 FBS %ظروف النمو المثالية مقارنة بالأنواع الأخرى من الوسائط التي تم اختبارها.

كانت الخلايا شديدة التأثر بفيروس مرض الجلد العقدى، مما يدل على وجود تأثيرات اعتلال خلوي حادة وزيادة قدرها 2 عيار log10 في حصاد الفيروس مقارنة بخلايا .MDBK وفي الختام أشارت هذه الدراسة إلى أن خط خلايا القلب المتطور كان مستقراً وانعكس إيجاباً على جودة وتكلفة إنتاج اللقاح.

الكلمات المفتاحية : خط خلايا القلب، التوصيف، نطاقاتGTG ، التنميط النووي، تعداد الكروموسومات، الحفظ بالتبريد، فيروس الجلد العقدي(LSDV) .