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OPTIMIZATION OF INFLUENZA A VIRUS ISOLATION ON MDCK-SIAT1 CELLS: INFLUENCE OF CELL CONFLUENCE ON VIRAL PRODUCTION

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ABSTRACT

Introduction: Influenza A virus is a major cause of viral respiratory infections and a significant global public health concern. Isolation and propagation of the virus in cell culture are crucial steps for diagnosis, epidemiological surveillance, and research. Madin-Darby Canine Kidney (MDCK) cells are considered the gold standard for influenza virus culture. However, several experimental parameters, such as cell confluence at the time of inoculation, can affect the efficiency of viral replication.

Methods: This study aimed to optimize the inoculation of Influenza A/H1N1 virus onto MDCK cells by examining how different cell confluences influence viral production After amplification and maintenance of the MDCK-SIAT1 cells, four cell densities were prepared in T25 flasks: 6×10^5, 1.2×10^6, 2.5×10^6, and 3×10^6 cells per flask. For each condition, one non inoculated flask and one inoculated flask with the A/H1N1 virus were used. The cultures were monitored by microscopic observation of the cytopathic effect (CPE) and then analyzed by RT-qPCR to assess viral replication.

Results: Microscopic observations showed that CPE was absent at low confluences (6×10^5 and 1.2×10^6 cells/flask), while massive CPE, characterized by cell rounding, loss of adhesion, and detachment of the cell mat, was observed at high confluences (2.5×10^6 and 3×10^6 cells/flask). RT-qPCR results revealed a progressive decrease in Ct values with increasing cell density, reflecting a growing viral load. The condition corresponding to 3×10^6 cells per flask exhibited the lowest Ct values (Flu A1 = 17.4; Flu A2 = 19.1), suggesting maximum viral genome production. These results highlight that cell confluence is a determining factor in the efficiency of Influenza A/H1N1 virus isolation and propagation on MDCK-SIAT1 cells. Under the tested conditions, a density of 3×10^6 cells per T25 flask appears to be the most favorable for viral production.

Conclusion: This study contributes to optimizing viral culture protocols and can serve as a basis for future work aimed at improving the reproducibility and standardization of virological techniques in cell culture.

Keywords: MDCK-SIAT1, Influenza A/H1N1, Confluence, Optimization

1 INTRODUCTION

Viral respiratory infections represent a major public health challenge due to their frequency, transmissibility, and clinical impact, particularly among vulnerable individuals. Among the agents responsible for these infections, influenza viruses play a significant role. Influenza A viruses, in particular, are closely monitored because of their high genetic diversity, their ability to infect multiple species, and their potential to trigger epidemics or pandemics. The Influenza A/H1N1 subtype, for instance, serves as an important model for research, surveillance, and isolation via cell culture.

Cell culture remains a key tool in virology, enabling the isolation, propagation, and characterization of viruses within a controlled environment. For influenza viruses, MDCK cells are commonly used as a reference model, owing to their permissiveness to numerous strains and their ability to form an adherent cell monolayer suitable for viral inoculation. Nevertheless, the success of viral culture depends on several experimental parameters, such as the physiological state of the cells, the homogeneity of the cell monolayer, the composition of the inoculation medium, and the cell confluence at the time of infection.

Cell confluence is a key factor in viral inoculation assays. Low cell density can reduce the number of cells permissive to viral replication, while excessive density may alter the metabolic state of the cell monolayer and affect viral spread. It is therefore essential to determine a confluence condition that enables optimal viral production—one that can be detected through morphological, molecular, and functional methods.

This experimental in vitro optimization study examines how different densities of MDCK-SIAT1 cells influence the production of the Influenza A/H1N1 virus. The cells were cultured in T25 flasks at four initial densities: 6×10^5, 1.2×10^6, 2.5×10^6, and 3×10^6 cells per flask. For each condition, a non-inoculated negative control flask was compared with a flask inoculated with the Influenza A/H1N1 virus.

This study investigates how the confluence of MDCK-SIAT1 cells at the time of inoculation affects the in vitro production of the Influenza A/H1N1 virus.

The general objective of this work is to optimize the inoculation process for the Influenza A/H1N1 virus in MDCK-SIAT1 cells by determining the cell density most favorable for in vitro viral production.

2 MATERIAL AND METHODS

2.1 The biological material for this study is based on a standardized host-pathogen system:

- Cell line: MDCK-SIAT1 cells, this genetically modified cell line has been optimized for the efficient isolation and culture of influenza viruses.
- Viral isolate: Clinical strain of Influenza A/H1N1 from a sample taken from patient MK, isolated, characterized, and stored at -80°C in the CVMIT cell culture unit laboratory.

2.2 Maintenance, Thawing, and Amplification of the MDCK-SIAT1 Cell Line:

Reculture and quantitative expansion of the cells were performed to obtain the cell load required for inoculations according to the WHO manual [1].

2.2.1 Thawing and Priming:

An MDCK-SIAT1 cell cryotube, initially stored long-term in liquid nitrogen at -196°C, was subjected to rapid thawing at 37°C.

2.2.2 Initial Culture:

The cell suspension was immediately transferred to an adherent culture in a T25 flask (25 cm^2). The culture medium used was DMEM supplemented with 10% FBS to ensure the supply of growth factors, promote rapid adhesion, and maintain optimal cell viability. Incubation was performed in a standardized incubator at 37°C, in a humid atmosphere enriched with 5% CO2.

2.2.3 Trypsinization and amplification:

Once a confluent cell mat of more than 90% was obtained, the culture medium was removed and the cells were washed with PBS. A trypsinization step (trypsin-EDTA) was performed to dissociate the intercellular junctions. To massively

amplify the cell biomass, the cells were progressively subcultured, moving from T25 to a T75 flask (75 cm^2), to ensure the quantity of cells necessary for the distribution of the different experimental densities.

2.3 Preparation of Experimental Cell Densities

To evaluate the impact of confluence on viral kinetics, the cell mat from the amplified T75 flask was trypsinized and subjected to precise cell counting on a counting slide to determine the exact concentration of the cell suspension. This suspension was then distributed in a standardized manner in a series of identical T25 flasks, suitable for microscopic observation, cell maintenance, and viral inoculation. Four target initial densities were established (Table 1).

Table 1: Organization of the experimental plan

Condition	Cell density per flask T25	Control flask (uninoculated)	Test flask (inoculated with IAV-H1N1)
C1	6 x 105 cel	Yes	Yes
C2	1,2 x 106 cell	Yes	Yes
C3	2,5 x 106 cell	Yes	Yes
C4	3 x 106 cell	Yes	Yes

For each condition, one flask was inoculated, while a second, rigorously identical and uninoculated flask was kept as a negative control.

3 VIRAL INFECTION PROTOCOL AND MONITORING OF CYTOPATHIC EFFECT

Before inoculation, the DMEM culture medium supplemented with 10% FBS was replaced with a specific serum-free inoculation medium. This step is essential because serum contains growth factors that can activate cell proliferation and thus mask the effect of the virus. The inoculation medium was therefore supplemented with TPCK trypsin to a final concentration of 2 µg/ml, as mentioned in the WHO manual [1]. TPCK trypsin plays an important role in the in vitro infectious cycle of influenza viruses, as it allows the proteolytic cleavage of the viral hemagglutinin HA0 into two functional subunits, HA1 and HA2. This cleavage is essential for the activation of fusion peptides, which allows for efficient viral entry into host cells. [1,2]

Inoculation was performed by introducing a standardized volume of 500 µL of Influenza A/H1N1 viral suspension into the inoculation medium of the test flasks after washing with PBS. Control flasks were treated under the same experimental conditions, but with the inoculation medium alone, without the viral suspension. This experimental setup allowed for the comparison, for each cell density studied, of an uninfected condition and an infected condition. [1,2]

After the addition of the viral inoculum, the flasks were kept in contact with the virus for 30 minutes, the adsorption period. During this period, gentle agitation was performed every 10 minutes to ensure homogeneous distribution of viral particles on the surface of the cell mat. At the end of this step, the infected cultures were incubated at 35 °C in an atmosphere containing 5% CO_2 [1]

4 KINETIC MONITORING AND QUANTITATIVE VALIDATION

Monitoring took place over a 5-day period (Day 1 to Day 5), using three complementary approaches:

4.1 Microscopic Photography

Cellular cytoplasmic encephalopathy (CCE) was investigated by observing characteristic morphological changes, such as cell rounding, loss of adhesion, progressive disorganization of the cell mat, or partial or total destruction of the monolayer. The morphological evolution of the cultures was documented by photographs taken using a camera integrated into the microscope. [1,3]

4.2 Molecular Analysis by RT-PCR

The presence of the Influenza A/H1N1 virus genome was confirmed and quantified in the culture supernatants using the GeneXpert molecular diagnostic analyzer. The samples were analyzed using the Xpress SARS-CoV-2_Flu_RSV plus multiparameter cartridge. The method is based on a reverse transcription step followed by real-time quantitative amplification. Positive infection was confirmed by specific amplification of the molecular targets Flu A1 and Flu A2. The generated Cycle Threshold (Ct) values served as indicators of viral RNA load (a low value indicating a high amount of

genetic material). RT-PCR allows for comparison of different cellular conditions and verification of the absence of a signal in control flasks [3,4]

5 INTERPRETATION CRITERIA AND DATA ANALYSIS

Interpretation is based on a combination of cellular observations, hemagglutination results, and the obtained Ct values. Confluence is considered favorable if associated with viral production confirmed by RT-PCR. Optimal conditions are defined by a high-quality cell monolayer, a negative control, and a Ct value consistent with significant production; Table 2 presents the interpretation for each result profile.

Table 2: Criteria for interpreting results

Result profile	Interpretation
Low Ct	High viral production
Moderate/high Ct	Limited viral production
Positive RT-PCR	Presence of viral genome with low production of hemagglutinating particles
RT-PCR négative	Absence de production virale détectable
Negative RT-PCR	No detectable viral production
Positive non-inoculated control	Suspected contamination or technical error
Negative non-inoculated control	Control validated

6 BIOSAFETY CONSIDERATIONS

The handling was done using the equipment typically used in cell culture (sterile consumables, micropipettes, equipment for maintaining controlled conditions). All operations involving MDCK-SIAT1 cells and the Influenza A/H1N1 virus were carried out in strict compliance with biosafety rules under a Microbiological Safety Cabinet (MSC), and according to the internal procedures of the Center for Virology and Tropical Infectious Diseases. Biological waste, contaminated consumables, and viral cultures were treated according to applicable standards.

7 RESULTS

7.1 Macroscopic Evaluation and Control Validation

Before characterizing the efficiency of the infection kinetics and viral production, the validity of the in vitro experimental system was assessed through careful monitoring of uninfected negative control flasks (TC). Daily microscopic observation from day one (D1) to day five (D5) of incubation showed that the MDCK-SIAT1 control cells, across all four cell densities tested (6 x 10^5, 1.2 x 10^6, 2.5 x 10^6, and 3 x 10^6 cells per T25 flask), maintained optimal viability, with no turbidity or changes in the culture medium. It is worth noting that throughout the post-inoculation monitoring phase, the cell monolayer in the control flasks remained stable, with no apparent proliferation or increase in cell biomass. This maintenance of a zero growth rate is the direct result of using an inoculation medium without FBS, in order to keep the cells viable in the short term while setting up the optimal conditions for viral replication only.

This cellular integrity and stability were confirmed at the molecular level by analyzing the culture supernatants collected on day 5. All control flasks tested negative in the RT-PCR test, with a complete absence of fluorescent signal for the Flu A1 and Flu A2 targets. Validating these negative controls confirms that there was no detectable influenza viral contamination during the handling, ensuring that the ECP and genomic load measured in the test flasks come solely from the inoculation with the Influenza A/H1N1 virus isolate.

7.2 Kinetic monitoring of infection and characterization of CPE

The assessment of cellular permissiveness was based on daily kinetic monitoring using inverted microscopy. Comparing the cellular profiles documented on Day1 and Day5 highlights major variability in the speed of appearance and intensity of CPE, directly correlated with the initial cell confluence.

- Low confluence (6 × 10^5 and 1.2 × 10^6 cells/flask): On D1, the appearance of cells in the inoculated group remains strictly comparable to that of the control flasks. On D5, the absence of visible CPE could be linked to the low initial cell density, which does not allow clear visualization of lysis foci. Although viral replication is

metabolically active within the host cells, the low initial cell density does not allow the expansion of visible lysis foci under the microscope. The cell layer maintains a continuous structure and retains a normal appearance phenotype.

- High confluences (2.5 × 10^6 and 3 × 10^6 cells/plate): Under these high cell density conditions, the infection kinetics speed up a lot. On day 1, we can see a confluent cell layer, which makes the comparison with day 5 visible. The progress to day 5 shows major structural and macroscopic disorganization of the cell system. The CPE is massive (~90% CPE) and shows as a general flattening of the MDCK-SIAT1 cells, loss of adhesion, and complete peeling off of the cell layer. The plates that reached this stage of total destruction were immediately frozen at -80 °C to lyse the remaining structures and release all virions.

All these morphological observations are illustrated by the comparative photographic plates shown in Figures 1 and 2.

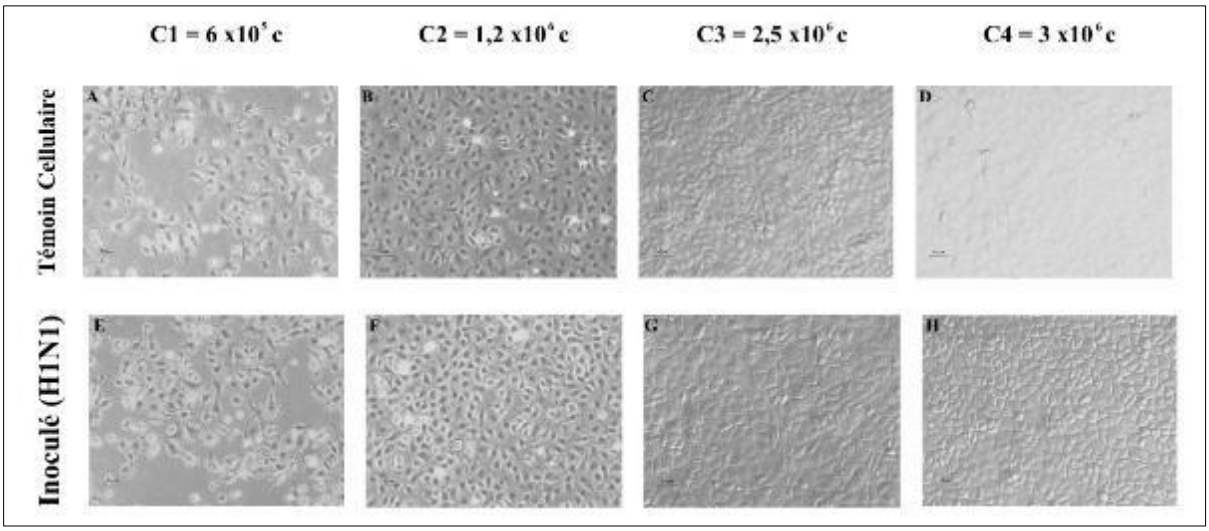


Figure 1: Morphological monitoring of MDCK-SIAT1 cell cultures one day post-inoculation (D1)

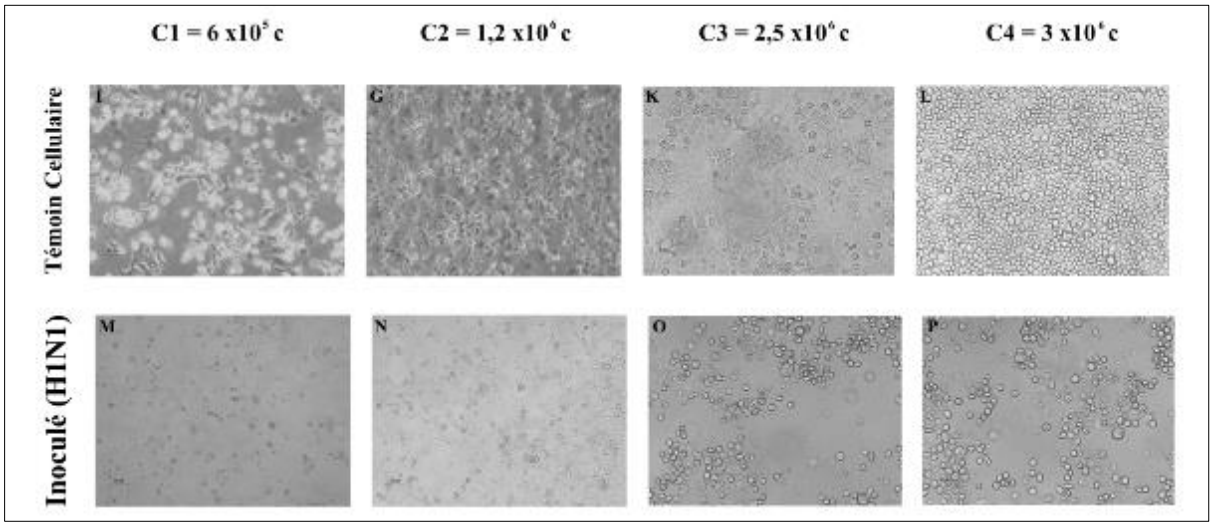


Figure 2: Morphological monitoring of MDCK-SIAT1 cell cultures five days post-inoculation (D5)

Observations made with an inverted microscope. (A to D) Uninfected control flasks (Tc): Respective densities of 6 x 10^5, 1.2 x 10^6, 2.5 x 10^6, and 3 x 10^6 cells/flask. The cell layer shows healthy epithelial morphology and normal adhesion. (E to H) Flasks inoculated with Influenza A/H1N1 virus: Corresponding densities. At this early stage, the morphological appearance of the infected cells remains similar to that of the control cells, with no visible CPE. The (I to L) Control flasks (Tc): Maintained an intact and healthy cell layer at all tested densities. (M to P) Flasks inoculated with IAV/H1N1: The intensity of the CPE is proportional to the initial confluence. Low confluences (M, N) show a layer that looks continuous with no visible lysis. In contrast, high confluences (O, P) show massive and nearly total destruction of the cell layer (~90% CPE), characterized by widespread rounding and complete detachment.

7.3 Detection of viral genome by RT-PCR (GeneXpert)

Molecular analysis of the clarified culture supernatants on day 5 was carried out using the GeneXpert system with the Xpress SARS-CoV-2_Flu_RSV plus multiparameter cartridge. This technique made it possible to confirm the presence of viral nucleic acids and indirectly quantify their load by assessing the Ct values for the two specific genomic targets (Flu A1 and Flu A2); the results are shown in Table 3.

Table 3: Molecular quantification of the Influenza A/H1N1 genome by RT-qPCR

Initial cell confluence	Target analyte	Ct value	Analyte results	RNA load interpretation
C1: 6 x 10^5 cells	Flu A 1 Flu A 2	22,2 23,5	Positive Positive	Moderate viral RNA load
C2 : 1,2 x 106 cells	Flu A 1 Flu A 2	21,6 23,1	Positive Positive	Moderate viral RNA load
C3 : 2,5 x 106 cells	Flu A 1 Flu A 2	18,1 19,8	Positive Positive	Very high viral RNA load
C4 : 3 x 106 cells	Flu A 1 Flu A 2	17,4 19,1	Positive Positive	Maximum viral RNA load

RT-PCR data reveal a direct and inversely proportional correlation between the initial cell density of the inoculation layer and the Ct values obtained. A significant drop in Ct is observed when moving to high confluences, indicating an exponential increase in viral nucleic acid load. The maximum confluence condition (3 × 10^6 cells) provides the highest genomic replication rate, with a minimum Ct of 17.4 for the Flu A1 target and 19.1 for the Flu A2 target.

8 DISCUSSION

The results show that the initial confluence of MDCK cells strongly influences the efficiency of inoculation and the production of Influenza A/H1N1 virus in vitro. Combining microscopic observations with RT-qPCR results highlights a clear difference between low and high cell densities. Cultures inoculated at low density show detectable viral replication but limited CPE, whereas high-density cultures display significant disruption of the cell layer, suggesting more intense viral replication. These observations are consistent with the work of Eisfeld et al, who emphasize that the efficiency of influenza virus isolation strongly depends on the quality and physiological state of the cell layer used for inoculation [3].The absence of visible CPE at 6×10^5 and 1.2×10^6 cells/flask does not necessarily mean there is no viral infection. RT-qPCR results actually show the presence of the viral genome under these conditions. This phenomenon has also been reported by Dai et al., who point out that molecular methods like RT-PCR are much more sensitive than microscopic observation and can detect viral replication before any visible morphological changes appear [4].So, the absence of CPE can't be considered a reliable indicator of inoculation failure. The conditions with the highest cell densities (2.5×10^6 and 3×10^6 cells/plate) showed the lowest Ct values, indicating an increase in viral load. This observation is biologically plausible since having more permissive cells available at the time of inoculation promotes viral replication. According to Dou et al, the replication cycle of Influenza A virus closely depends on the availability of host cells that can be infected, with each cell serving as a potential site for producing new viral particles [5]. Similarly, Carter and Iqbal point out that the efficiency of viral culture is directly tied to the interactions between the virus and target cells in the culture [6]. The condition with 3×10^6 cells/flask showed the lowest Ct values, suggesting it corresponds to the highest viral genome production among the conditions tested. This trend matches the observations of Karakus et al., who highlight that optimizing cell culture parameters is a key factor in increasing viral yields in Influenza A production systems [10]. The authors specifically show that cell density directly affects the amount of virus recovered after inoculation. The massive CPE seen in high-density conditions confirms the impact of viral replication on the integrity of the cell layer. The observed phenomena, including cell rounding, loss of adhesion, and progressive cell detachment, correspond to the classic signs of Influenza A virus infection described in the literature [5,6]. These changes result from the effects of intracellular viral replication and the release of new viral particles, which gradually affect the viability of the cell layer. The use of TPCK trypsin in the inoculation medium likely contributed to the effectiveness of the observed viral replication. Indeed, trypsin is necessary for the cleavage of viral hemagglutinin (HA0) into HA1 and HA2, a crucial step for the virus to efficiently enter cells and spread the infection. Seitz and colleagues showed that the presence of trypsin significantly boosts Influenza A virus production in MDCK cells by not only activating hemagglutinin but also promoting viral spread within the culture [2].Moreover, several studies have shown the excellent ability of MDCK cells to support the growth of influenza viruses. The historical work of Tobita established MDCK cells as a reference model for isolating influenza viruses [7]. More recently, Hatakeyama et al. and Takada et al. confirmed that MDCK cells are highly permissive to human influenza viruses due to the expression of sialic acid

receptors that facilitate viral entry [8.9]. However, the interpretation of results should remain cautious. RT-qPCR detects viral genetic material but does not distinguish between infectious and non-infectious particles. As pointed out by the WHO and Eisfeld et al [1, 3], a complete assessment of viral production ideally requires complementary methods to measure the infectivity or biological activity of the particles produced [1,3]. In this perspective, the hemagglutination test not carried out in this study can serve as a useful complementary tool to estimate the presence of functional viral particles. Finally, the stability of the negative control flasks is an important aspect of experimental validation. The absence of morphological changes and the lack of detectable signal in RT-qPCR in the controls confirm that the changes observed in the inoculated cultures are specifically linked to infection by the Influenza A/H1N1 virus. This stability also reflects adherence to cell culture conditions and biosafety rules implemented during the study. Overall, the results suggest that an increased cell density promotes the production of Influenza A/H1N1 virus in MDCK-SIAT1 cells. Among the conditions tested, a density of 3×10^6 cells per flask appears to be the most favorable for viral replication.

9 CONCLUSION

This study highlights the importance of cell confluence for optimizing the inoculation of Influenza A/H1N1 virus in MDCK-SIAT1 cells. The results show that high cell densities promote higher viral production, as indicated by the appearance of a marked CPE and low Ct values in RT-PCR. Under the experimental conditions studied, a density of 3×10^6 cells per T25 flask seems to be the most effective for producing new viruses. This work helps to better understand the factors influencing in vitro culture of Influenza A/H1N1 virus. It serves as a basis for improving the reproducibility of viral cultures in the cell culture unit and makes it easier to carry out further optimizations, including replicates, a more precise study of viral kinetics, and a functional analysis of the particles produced.

STATEMENTS ON COMPLIANCE WITH ETHICAL STANDARDS

Disclosure of conflict of interest

The authors declare no conflicts of interest regarding this manuscript.

REFERENCES

[1] World Health Organization (Éd.). (2011). Manual for the laboratory diagnosis and virological surveillance of influenza. World Health Organization.

[2] Seitz, C., Isken, B., Heynisch, B., Rettkowski, M., Frensing, T., & Reichl, U. (2012). Trypsin promotes efficient influenza vaccine production in MDCK cells by interfering with the antiviral host response. Applied Microbiology and Biotechnology, 93(2), 601-611. https://doi.org/10.1007/s00253-011-3569-8

[3] Eisfeld, A. J., Neumann, G., & Kawaoka, Y. (2014). Influenza A virus isolation, culture and identification. Nature Protocols, 9(11), 2663-2681. https://doi.org/10.1038/nprot.2014.180

[4] Dai, J., Li, H., & Li, L. (2016). Laboratory Test for Diagnosis of Influenza. In H. Li (Éd.), Radiology of Influenza (p. 17-20). Springer Netherlands. https://doi.org/10.1007/978-94-024-0908-6_7

[5] Dou, D., Revol, R., Östbye, H., Wang, H., & Daniels, R. (2018). Influenza A Virus Cell Entry, Replication, Virion Assembly and Movement. Frontiers in Immunology, 9, 1581. https://doi.org/10.3389/fimmu.2018.01581

[6] Carter, T., & Iqbal, M. (2024). The Influenza A Virus Replication Cycle: A Comprehensive Review. Viruses, 16(2), 316. https://doi.org/10.3390/v16020316

[7] Tobita, K. (1975). Permanent canine kidney (MDCK) cells for isolation and plaque assay of influenza B viruses. Medical Microbiology and Immunology, 162(1), 23-27. https://doi.org/10.1007/BF02123574

[8] Hatakeyama, S., Sakai-Tagawa, Y., Kiso, M., Goto, H., Kawakami, C., Mitamura, K., Sugaya, N., Suzuki, Y., & Kawaoka, Y. (2005). Enhanced Expression of an α2,6-Linked Sialic Acid on MDCK Cells Improves Isolation of Human Influenza Viruses and Evaluation of Their Sensitivity to a Neuraminidase Inhibitor. Journal of Clinical Microbiology, 43(8), 4139-4146. https://doi.org/10.1128/JCM.43.8.4139-4146.2005

[9] Takada, K., Kawakami, C., Fan, S., Chiba, S., Zhong, G., Gu, C., Shimizu, K., Takasaki, S., Sakai-Tagawa, Y., Lopes, T. J. S., Dutta, J., Khan, Z., Kriti, D., Van Bakel, H., Yamada, S., Watanabe, T., Imai, M., & Kawaoka, Y. (2019). A humanized MDCK cell line for the efficient isolation and propagation of human influenza viruses. Nature Microbiology, 4(8), 1268-1273. https://doi.org/10.1038/s41564-019-0433-6

[10] Karakus, U., Crameri, M., Lanz, C., &Yángüez, E. (2018). Propagation and Titration of Influenza Viruses. In Y. Yamauchi (Éd.), Influenza Virus (Vol. 1836, p. 59-88). Springer New York. https://doi.org/10.1007/978-1-4939-8678-1_4