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SENSITIVITY OF SOME CELL LINES TO ADAPT PESTE DES PETITS RUMINANTS' VIRUS FOR VACCINE PRODUCTION

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ABSTRACT

Preparation of *the peste des petits ruminant*s virus (PPRV) vaccine is traditionally carried out by adapting the virus in Vero cells with typical cytopathic effects (CPEs). Alternative cell lines would be evaluated for the virus propagation as Baby Hamster Kidney cells (BHK) and Madin-Darby Bovine Kidney cells (MDBK). We aim to establish an alternative cell line for the production of live attenuated PPR vaccine in case of loss or unavailability of the required Vero cells for such purpose. The Nigerian PPR virus (N75/1) was passaged 10 successive times in Vero, BHK21, and MDBK cell lines, followed by virus titration and growth curve studies. Cell rounding, cytoplasmic vacuolation, and syncytia formation were the most noticeable characteristics of CPE for PPRV. The PPRV multicycle growth analysis in the supernatant of cells revealed a constant increase in the virus quantity that indicates PPRV adaptation to the used cell lines, having virus titer 7, 6, and 6.5 log10 Tissue Culture Infective Dose 50 (TCID50) within 120, 144, and 96 hours post cell infection for Vero, BHK21, and MDBK, respectively. The obtained findings suggest that Vero, BHK21, and MDBK cells can be reliable alternatives for propagation of PPRV for vaccine production.

Key words: *Peste des petits ruminants, cell lines, virus titration, growth kinetics.*

INTRODUCTION

The French acronym Peste des Petits Ruminants (PPR) is the most commonly used term worldwide, and it is classified as an OIE (Office International des Epizooties)-listed disease. Also, many common terms have been used, such as plague of small ruminants, goat plague, ovine rinderpest, or Kata. In Africa and South Asia, sheep and goats are of great importance in their livelihood. So, PPR has great importance as a food security concern. PPRV and rinderpest virus (RPV) are closely related Morbilliviruses (Kumar *et al.,* 2014).

It affects sheep, goats, and some wild types of small ruminants. In the most endemic countries, PPR morbidity may be up to 100%, while mortality rates can reach 90%. Lower mortality rates have been noticed in endemic areas; meanwhile, the disease shows serious influences on the

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productivity of the flock. High economic Losses, estimated at USD 1.2 to 1.7 billion per year, are caused by mortalities, reduced production, and disease control costs (WOAH, 2024).

The clinical signs appeared as oculo-nasal discharge, fever, dyspnea, diarrhoea, and leukopenia. Additionally, mucopurulent discharge with a fetid odour was recorded from the nostrils and eyes (Wohlsein and Saliki, 2006).

Death usually occurs 4–6 days after the onset of fever, as well as abortion in pregnant ewes (Singh *et al.,* 2009). PPRV infection is mainly due to close contact between animals. Goats are generally more susceptible than sheep, while sheep show a higher recovery rate. Notably, PPRV infection has been recorded in cattle, but no evidence was found to suggest transmission of the disease to other hosts (Khan *et al.,* 2008).

Primary cell cultures and various cell lines were utilized for the isolation and propagation of PPRV in vitro. African green monkey kidney (Vero), Madin-Darby Bovine Kidney (MDBK), Baby Hamster Kidney Clone 21 (BHK-21), and normal African green monkey kidney fibroblast cells (CV1) were found to be the most sensitive cell lines to PPRV (Silva *et al.,* 2008).

Isolation and propagation of PPRV for vaccine production are most commonly performed on the Vero cell line (Nanda *et al.,* 1996). Several blind passages on Vero cells are required for PPRV isolation to observe the characteristic cytopathic effects (CPE) (Kumar *et al.,* 2014; Sreenivasa *et al.,* 2006).

Two PPR vaccine strains showed different CPE in Vero cells. The Sungri vaccine virus strain showed visible CPE from the 4th day post-infection, whereas the CPE of the Arasur virus strain appeared 36-48 hours post-infection. CPE in Vero cells for both virus strains initially induced cell rounding, aggregation, and syncytial formation, with generalized CPE by the 6th day for the Sungri strain and by 96 hours post-infection for the Arasur strain, where complete detachment of the cells was seen by 120 hours post-infection with the Arasur strain. Stained cultures with H&E revealed cell vacuolation, cytoplasmic extension, and syncytia, in addition to intranuclear and acidophilic intracytoplasmic inclusion bodies (Raveendra *et al.,* 2009). Regarding another observation, the CPE began to be visible 24 hours post-infection of Vero cells, with initial aggregation, syncytial formation, and cell rounding. Cell degradation and the development of inclusion bodies were observed by 72 hours post-infection, followed by full detachment of the cells by 84 hours post-infection (Msi *et al.,* 2021)."

In BHK-SLAM cells, the CPE after virus infection appeared earlier and was more obvious, with large syncytia, compared to Vero cells. BHK-SLAM and Vero cells were infected with PPRV/GFP at an MOI of 0.01. Both cell types were evaluated for growth curves and viral titers. Vero cells showed higher titers than BHK-SLAM cells. Both cell lines showed the peak titer simultaneously (4 days post-infection) (Jialin *et al.,* 2016).

Vero cell culture has been established for the production of the live attenuated PPR vaccine since 1998 (Khodeir and Mouaz, 1998), but sometimes these cells are not viable, which may decrease or delay vaccine production.

A host system with high virus yield is crucial for large-scale virus propagation in vaccine production attempts. Therefore, it was essential to search for an alternative cell line to continue the required vaccine production. Accordingly, we chose BHK and MDBK cell lines for this purpose, as these lines are the most available and commonly used in VSVRI.

MATERIALS AND METHODS

Virus:
The PPR virus (Nigerian 75/1) was attenuated by 6 passages on lamb kidney cell culture, followed by 77 passages on Vero cells (AU-PANVAC), which represents the master seed of the PPR virus. It was kindly obtained from the African Union 'Pan African Veterinary Vaccine Centre (PANVAC),' Debrazit, Ethiopia, and maintained in the Department of Rinderpest Vaccine Research (DRVR), Veterinary Serum and Vaccine Research Institute (VSVRI), Abasia, Cairo, Egypt. This virus is used for experimental propagation in three cell lines: Vero, BHK, and MDBK.

Cell lines:
African green monkey kidney (Vero), Baby hamster kidney (BHK21), and Madin-Darby bovine kidney (MDBK) cells were supplied by VSVRI, passaged, and maintained in DRPVR with Minimum Essential Medium (MEM; Gibco, USA), supplemented with 10% newborn calf serum and 50 µg/ml Gentamycin, with an adjusted pH of 7.2–7.4. The cells were used at a density of 4.8 × 10^4 cells/ml in 25 cm^3 tissue culture flasks, incubated at 37°C in a 5% CO2 atmosphere.

Virus propagation:
The PPR virus (Nigeria 75/1) with an MOI of 0.01 was used to infect Vero, BHK, and MDBK cell flasks, which were incubated at 37°C. Cell viability was evaluated daily by microscopic examination. Whenever the viability dropped below 15%, the cell flasks were frozen at -20°C. Freeze-thawing of the suspension fluid was then performed, followed by clarification through centrifugation (10 minutes at 2000 rpm). The virus harvests were titrated (OIE, 2017) and finally frozen at -70°C. Bacterial, fungal, and mycoplasma contamination were assessed after each manipulation (FAO, 1994). The virus was passaged 10 successive times in each cell line, with the observed CPE and virus titer determined for each cell line.

Virus titration:
Each passage of the PPR virus in the three cell lines was titrated in the corresponding cell culture using the microtiter technique, according to Burleson *et al.* (1997). The virus titer was expressed as $\log_{10}$ $TCID_{50}$/ml, following the method of Reed and Muench (1938).

Confirmation of the incidence of the PPR virus in cell cultures
A virus neutralization test was applied to samples from each virus passage in each of the used cell cultures to confirm the incidence of PPR, using specific PPR immune serum supplied by DRPVR, according to OIE (2018). In addition, the direct fluorescent antibody technique (FAT) was performed on the tenth virus passage in each of Vero, BHK, and MDBK cells, using specific anti-PPR serum conjugated with fluorescein isothiocyanate, according to Ehizibolo *et al.* (2013).

Virus growth kinetics:
Growth curves of the PPR virus in the tested cell cultures (Vero, BHK, and MDBK) were conducted in tissue culture tubes, where each cell culture was infected with its propagated virus at the 10th passage, at an MOI of 0.01. One hour was allowed for adsorption, after which the inoculum was discarded, and the cells were washed twice with MEM. Each tube was then supplied with 1 ml of MEM containing 2% newborn calf serum and incubated at 37°C. Infected tissue culture tubes were collected daily up to 6 days post-cell infection and stored at -80°C until subjected to virus titration.

The 50% tissue culture infectious dose ($TCID_{50}$) of the virus was estimated at different time points using the previously mentioned method, after three freeze-thaw cycles (Reed and Muench, 1938).

RESULTS

The successive passage of the PPR virus in Vero, BHK, and MDBK cell lines indicated that the virus can be propagated in each of them, showing the same features of specific CPE, including cell rounding and aggregation, which led to the fusion of adjacent cells, forming a syncytium. CPE began at 72–96 hours post-infection, with harvesting taking place at 144–168 hours post-infection, as shown in Figures 1-b, 2-b, and 3-b. The virus recorded its highest titers (7, 6, and 6.5 $\log_{10}$ $TCID_{50}$/ml) for Vero, BHK, and MDBK, respectively, by the 10th passage, with a gradual increase from the 1st to the 10th passage (Table 1).

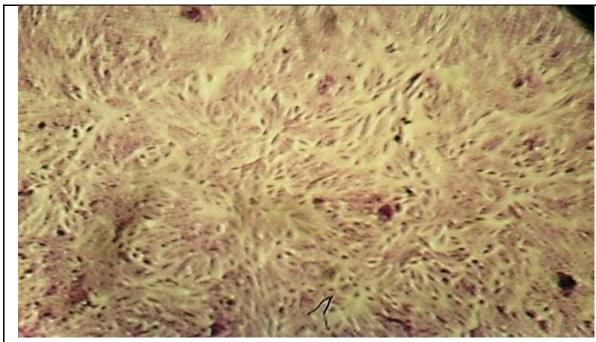

Fig. (1-a): Normal Vero cells

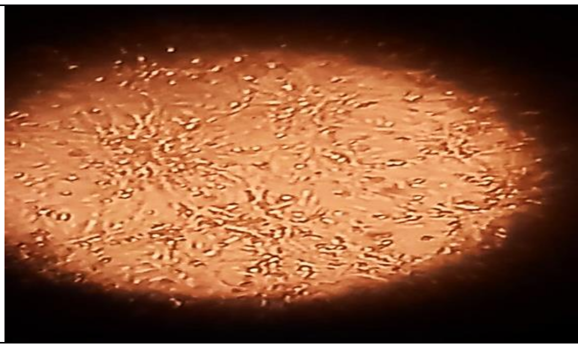

Fig. (1-b): PPR virus infected Vero cells

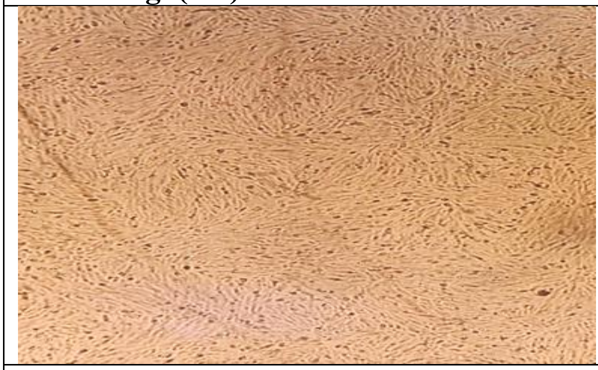

Fig. (2-a): Normal BHK

Fig. (2-b): PPR virus infected BHK

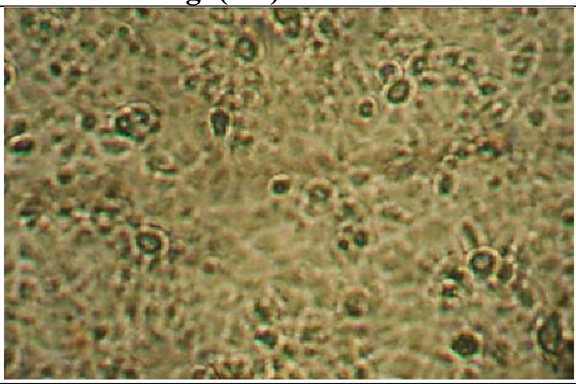

Fig. (3-a): Normal MDBK

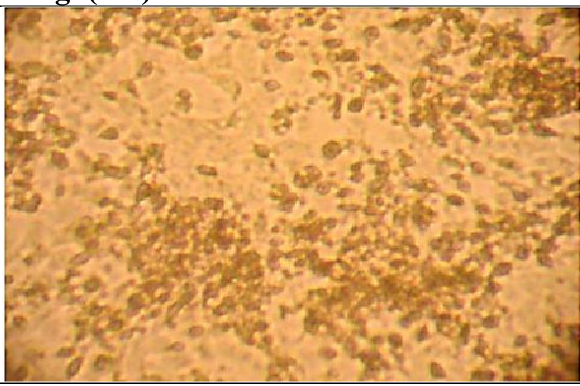

Fig. (3-b): PPR virus infected MDBK

Infected cell lines showed CPE, characterized by cell rounding and aggregation, which ultimately led to the fusion of adjacent cells, forming a syncytium.

The results of the virus neutralization test confirmed that the observed CPE was specific to PPR virus, and the direct FAT results, showing an intracytoplasmic apple-green reaction, further supported the successful propagation of PPR virus in Vero, BHK, and MDBK cell lines (Fig. 4a, b, & c).

Fig. 4 (a, b, and c) shows a positive FAT revealing an intracytoplasmic apple-green reaction in both of the cell lines used, with 3+ to 4+ CPE, while Fig. 4d shows a negative reaction.

Table 1: Propagation of PPR virus (Nigerian strain 75/1) in different cell lines.

Passage number	PPR virus propagation in								
	Vero			BHK			MDBK		
	CPE Onset	HT*	VT**	CPE Onset	HT	VT	CPE Onset	HT	VT
	HPCI***			HPCI			HPCI		
1	The virus is already adapted to Vero cell line showing CPE started by the 72-96 hours post cell infection and harvested by 120-144 hours with a titer ranged from 6log10 to 7log10 TCID50/ml.			96	144	4	96	144	3.5
2				96	168	4.5	72	144	4
3				72	168	4	48	120	4
4				72	168	4.5	48	120	5
5				72	168	5	48	120	5
6				72	168	5.5	48	120	5.5
7				72	144	5.6	48	120	5
8				72	144	5.5	48	96	6
9				72	144	6	48	96	6.5
10				72	144	6.0	48	96	6.5

*HT= harvest time **VT= virus titer (log10TCID$_{50}$/ml) ***HPCI= hours post cell infection

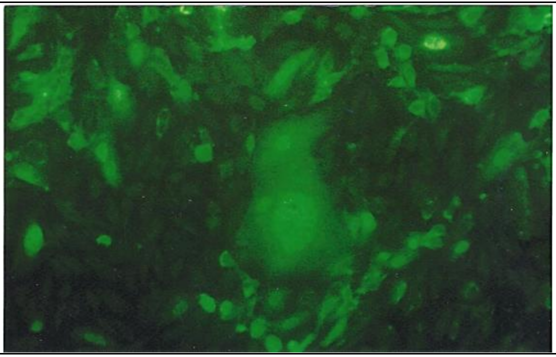

Fig. (4-a): Positive FAT in Vero

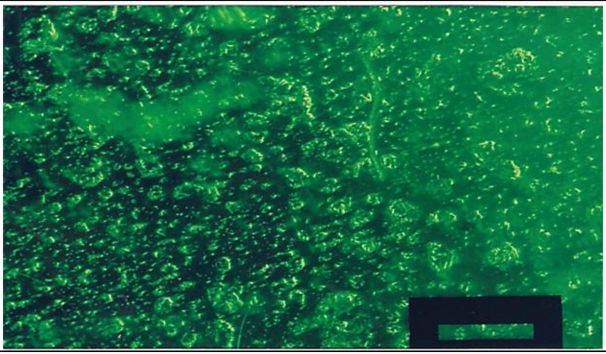

Fig. (4-b): Positive FAT in BHK

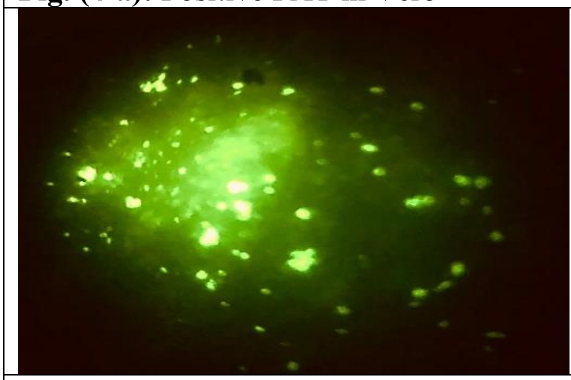

Fig. (4-c): Positive FAT in MDBK

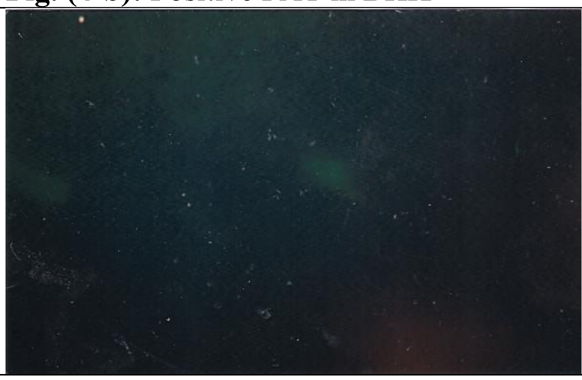

Fig. (4-d): Negative FAT in cell lines

Tracking the induced titer of PPR virus over a period of 168 hours post-cell infection, a gradual increase was recorded at 24-hour intervals for all Vero, BHK, and MDBK cell lines, ranging between 0.5 to 1 log. The highest titers (7, 6, and 6.5 log_{10} TCID$_{50}$/ml) were reached at 168 hours post-infection for Vero, BHK, and MDBK, respectively, as demonstrated in Table 2 and Fig. 5.

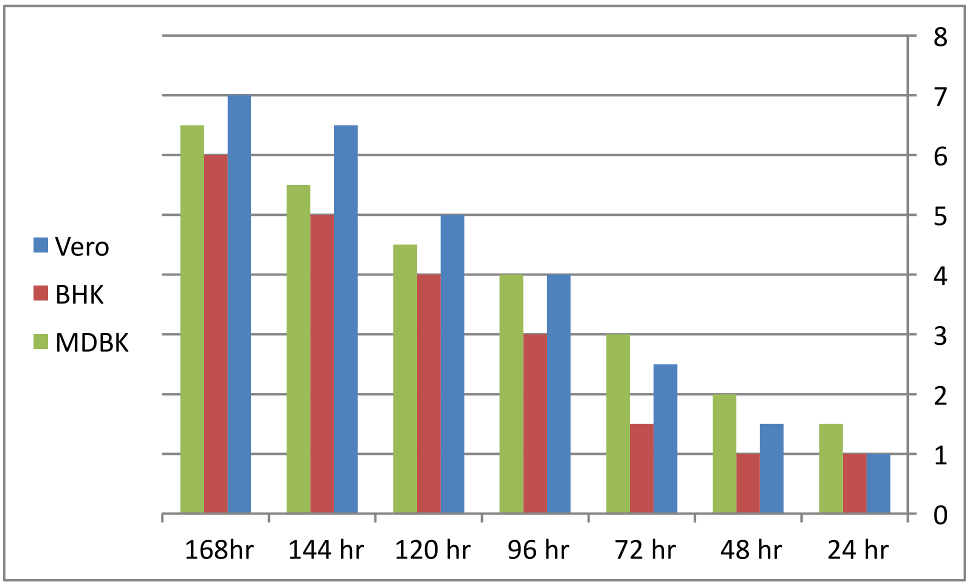

Fig. (5): Growth curves of PPR virus in Vero, BHK and MDBK cell lines at the 10th passage

Table 2: Growth curves of PPR virus in Vero, BHK and MDBK cell lines

HPCI*	PPR virus titer (log10TCID$_{50}$/ml) in		
	Vero	BHK	MDBK
24	1	1≤	1.5
48	1.5	1	2.0
72	2.5	1.5	3.0
96	4	3.0	4.0
120	5.0	4.0	4.5
144	6.5	5.0	5.5
168	7	6.0	6.5

*HPCI: hours post cell infection

DISCUSSION

Peste des petits ruminants (PPR) is an economically significant disease of small ruminants, with an increasing global incidence (Dhar, 2002; Ozkul *et al.,* 2002). The PPRV strain Nigeria 75/1 was attenuated by multiple passages in the Vero cell line (Ozkul *et al.,* 2009). Given the urgency to control PPR outbreaks and the limitations of scalable Vero cell culture, our present work aims to evaluate alternative cell lines for the production of the PPR attenuated vaccine in case of a shortage of Vero cell supplements.

The cytopathic effect (CPE) in the current study refers to the death or morphological modification of host cells during virus multiplication (cytopathogenic virus). Common examples include cell rounding, fusion of cells to form a syncytium (polykaryocytes), and the occurrence of nuclear or cytoplasmic inclusion bodies. These effects result from the lysis of host cells by the infecting virus or due to cell death when the cells are unable to reproduce. The appearance of cytopathic effects (CPE) due to virus infection is a preliminary protocol for determining the presence of viruses in clinical samples (Suchman and Blair, 2007).

Through ten passages of the PPR virus in Vero, BHK, and MDBK cell lines, it was found that the virus can be propagated in each of them, showing the same forms of specific CPE, including cell rounding and aggregation, which ultimately led to the fusion of adjacent cells, forming a syncytium. This process began at 72–96 hours and ended at 144–168 hours post-cell infection, as shown in Fig. 1-b, 2-b, and 3-b. Peak titers of the virus (7, 6, and 6.5 log$_{10}$ TCID$_{50}$/ml) were recorded for Vero, BHK, and MDBK, respectively, by the 10th passage, showing a gradual increase from the 1st to the 10th passage (Table 1). These characteristics were described and confirmed by Raveendra *et al.* (2009) in Vero cells, Jialin *et al.* (2016) in BHK cells, and Silva *et al.* (2008) in MDBK cells. Control cells appeared with nearly the same morphology upon microscopic examination. Mao *et al.* (2022) successfully propagated the PPR virus in Vero, BHK, and MDBK cell lines, describing the same features of its CPE.

The use of virus neutralization and direct FAT using specific antisera to confirm the presence of PPR virus in Vero, BHK, and MDBK infected cells is in agreement with Hu et al. (2012) and OIE (2019).

To ensure the potency of the produced vaccine, the proper time for harvesting the virus should be determined to confirm the presence of the required amount of intact virus. Therefore, maximum virus titer, virus growth kinetics, and the onset and pattern of CPE induction are still required (Jadi *et al.,* 2010). The results obtained in the present study (Table 2 & Fig. 6) showed that Vero cells yielded the highest titer (7 log_{10} $TCID_{50}$/ml), followed by MDBK (6.5 log_{10} $TCID_{50}$/ml), and the lowest titer was obtained from BHK (6 log_{10} $TCID_{50}$/ml) by 168 hours post-infection. These titers are similar to those obtained by Saiful *et al.* (2021), who found that a PPR virus titer (Nigerian 75/1) of 6.2 log_{10} $TCID_{50}$/ml was obtained from Vero cells. However, their findings differ in that this titer was achieved within 72–96 hours, a discrepancy that could be attributed to the MOI used, cell age, and density. On the other hand, it could be suggested that the difference in the timing of CPE onset and harvesting from the different cell lines could be attributed to variations in the receptors for attachment and replication, as well as species differences in the origin of the three cell lines (Benjamin *et al.,* 2010). Furthermore, it was found that the standard CPE of the PPR virus appeared at different times for two different PPR vaccines. The Sungri vaccine strain showed CPE at day 4 post-infection, while CPE appeared after only 36–48 hours for the Arasur vaccine strain. Consequently, for the Sungri vaccine, CPE was generalized by day 6, whereas for the Arasur strain, it was observed by 96 hours post-infection, with complete detachment of the cell monolayer occurring by 120 hours post-infection (Raveendra *et al.,* 2009). Additionally, it was concluded that the development of CPE and the replication kinetics of viruses depend on various factors such as the cell line used, the passage or adaptation level, and the virus selection method (Jadi *et al.,* 2010; Chandrahas *et al.,* 2014).

Regarding the use of BHK for the adaptation of the PPR virus, it was concluded that the BHK-21 cell line would facilitate large-scale vaccine production due to its rapid growth and ease of maintenance (Mohan *et al.,* 2007). In the BHK-21 cell line, the peak titer of the PPR vaccine virus on the 5th, 10th, and 15th days at 72 hours post-infection was 8.23 log_{10}, 8.41 log_{10}, and 8.54 log_{10} $TCID_{50}$ / 0.1 ml, respectively (John and Koshy, 2006). These titers appear to be higher than those of other results, including ours, and could be attributed to the cell culture system used, either roller or suspension, whereas we used the stationary system.

Spotlighting the MDBK cell line, Olivier *et al.* (2011) successfully isolated the PPR virus in this cell line, and Yang *et al.* (2021) demonstrated that the MDBK cell line is sensitive and suitable for the propagation of the PPR virus, which could explain why it yielded a high titer similar to that obtained with Vero cells.

CONCLUSION

The development of CPE is a crucial indicator of full PPR virus adaptation in Vero, BHK, and MDBK cells. Therefore, any of these cell lines that enable extensive virus multiplication will be essential for a potent vaccine production strategy.

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حساسية بعض خطوط المزارع النسيجية المستمرة لأقلمة فيروس طاعون المجترات الصغيرة من أجل إنتاج اللقاح

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إن تحضير لقاح طاعون المجترات الصغيرة يتم عادة بتمرير الفيروس على خلايا Vero محدثة التأثير المرضي المميز(CPE) ، وتم تقييم خطوط خلايا بديلة لأقلمة الفيروس مثل BHK وMDBK. كان الهدف من ذلك هو إنشاء خطوط بديلة لإنتاج لقاح طاعون المجترات الصغيرة الحي المستضعف في حالة فقدان أو عدم توافر خلاياVero المستخدمة لهذا الغرض. قمنا بتمرير فيروس طاعون المجترات الصغيرة N75/1عشرة مرات متتالية على كل من خلايا Vero وBHK وMDBK، متبوعة بتقييم التراكيز الفيروسية ودراسة منحنيات النمو

(growth curve) . أظهر الفيروس تأثيرًا مرضيًا واضحًا على هيئة خلايا دائرية وظهور حويصلات في السيتوبلازم بالإضافة إلى تكوّن التكتلات الخلوية (syncytia formation) . من خلال دراسة وتحليل منحنيات النمو(growth curve) ، أظهرت تزايدًا ثابتًا في كمية الفيروس، مما يشير إلى حدوث تأقلم للفيروس على خطوط الخلايا المستخدمة في هذه الدراسة، وذلك بتترات ٧ و٦ و٦,٥ $TCID_{50}$ في خلال ١٢٠ و ١٤٤ و٩٦ ساعة بعد حقن الفيروس على خلايا Vero وBHK وMDBK على التوالي.

تستخلص هذه الدراسة أن جميع الخلايا المستخدمة في الدراسة يمكن أن تكون بدائل فعالة لزرع وتمرير فيروس طاعون المجترات الصغيرة أثناء عملية إنتاج اللقاح".