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Comparative Evaluation of Different Cell lines for Adaptation of Orf virus vaccine strain Passaged in Primary lamb testes cells

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ABSTRACT: Orf virus (ORFV) is a poxvirus that causes non-systemic epitheliotropic disease in sheep and goats, resulting in economic losses to the welfare of farmed small ruminants and belongs to the genus *Parapox virus* and family *Poxviridae*. Vaccination is the only way to control orf infection in sheep and goats. In India, available vaccine for orf is produced from primary lamb testes cells (LT), which is not viable for scale up in large production and also virus yield varies from different batches. To overcome this, an attempt was undertaken to adapt and characterize ORFV vaccine strain in three different commonly available cell lines. In this study, LT adapted ORFV Mukteswar-59/05 vaccine strain was used for adaptation in Vero, Madin Darby Bovine Kidney (MDBK) and Baby Hamster Kidney (BHK-21) cell lines. In order to adapt vaccine virus in cell lines, it was blindly passaged up to 7 times and the presence of the virus and its replication was demonstrated by presence or appearance of characteristic cytopathic effect(s) and PCR. CPE was observed from initial passages (P1-P5) in all the infected cell lines and disappeared after P6 in Vero cell line whereas MDBK and BHK-21 cell lines shown the replication of virus till P7. It was observed that ORFV vaccine strain was successfully adapted and propagated in MDBK and BHK-21 cell lines evidenced from CPE and PCR. However, further studies are needed to determine the infectivity titre of virus and production of complete virus in cells by genome wide sequencing and analysis. This work is a step towards future development and evaluation of cell line based ORFV vaccine, which is cost effective and scalable for mass production.

Keywords: Primary lamb testes cells, Orf virus, BHK-21, MDBK, Vero, Cytopathic effects, Adaptation.

INTRODUCTION

Orf is an acute, zoonotic, contagious dermatitis virus infection that normally infects sheep and goats caused by orfvirus (ORFV) of the genus *Parapoxvirus*, family *Poxviridae.* Itconsists of linear double stranded DNA molecule of approximately 132-140 kbp in length (Chan *et al.,* 2009; Karki *et al.,* 2019). The disease is prevalent worldwide and affects all age groups although young/immune compromised animals are most susceptible. The lesions are usually found on the lips, around the muzzle, eyes, on the feet and occasionally on the udder (Abuelzein and Housawi, 2009).Typical orf lesions progress through clinical stages of erythematous macula, papules, vesicles, pustules and scab formation (Robinson *et al.,* 1982). ORFV can infect and grow on various primary cells of ovine origin such as embryonic sheep skin (Greig, 1957), lamb testes, lamb kidney (Plowright *et al*., 1959; Said *et al.,*2013), fetal lamb muscle cells (McInnes *et al*., 2001), ovine fetal turbinate cells (Delhon *et al*., 2004), bovine origin such as calf testes, calf kidney (Traykova, 1982; Rosenbusch and Reed, 1983), fetal bovine muscle cells, fetal bovine lung cells (Inoshima *et al*., 1999; 2002), fetal bovine spleen cells (Lard *et al*., 1991), goat testes (Kumar *et al.,* 2014) and rabbit kidney cells (Ivanov *et al.,* 2016). ORFV also replicate in cell lines, commonly used cell lines in different laboratories are Vero, Baby Hamster Kidney (BHK-21), Madin Darby Bovine Kidney (MDBK), Madin Darby Ovine Kidney (MDOK), OA3.Ts (Ivanov *et al.,* 2016; Nashirudddullah *et al.,* 2016; Karki *et al.,* 2019; Gulyaz *et al.,* 2020). Control against ORFV infection is done with vaccination using live virus preparations derived either from a fully virulent strain or live attenuated ORFV. In India, live attenuated ORFV vaccine strain (Mukteswar-59/05) has been developed using ORFV isolated from goats attenuated in primary lamb testes (LT) cells and found to be safe, potent and effective in sheep and goats (Bhanuprakash *et al.,* 2012). However, primary cells are laborious, time consuming, prone to contamination, expensive to produce, can only be propagated for limited passages, their characteristics may change with subsequent passages and difficult in massive vaccine production because it is not easily available, have to sacrifice animals for preparation of primary cells. There is an immense need to use cell lines, which are economic, easily available, and easy to handle/maintain for production of live vaccine. Therefore, we attempted to adapt and characterize the LT adapted ORFV Mukteswar-59/05 (vaccine strain, P50) in different three cell lines; Vero, MDBK and BHK-21 cells.

MATERIALS AND METHODS

Cells. Secondary lamb testes (LT) cells, different cell lines (Vero, MDBK and BHK-21) maintained in the Poxvirus laboratory (PVL), Division of Virology, Indian Veterinary Research Institute (IVRI), Mukteswar, India, were used in the study. The secondary LT cells, Vero, and MDBK cell lines were revived and cultured in Eagle's Minimum Essential Medium (EMEM)

(Sigma, St. Louis, USA) with 10% fetal bovine serum (FBS) (Cell clone, Genetix Biotech, New Delhi, India) and antibiotics. For BHK-21, Glasgow minimal essential medium (GMEM) (Sigma, St. Louis, USA) was used.

Revival of ORFV-Mukteswar-59/05 (vaccine strain, P50). The freeze dried ORFV vaccine available at the repository of Poxvirus laboratory, Division of Virology, Indian Veterinary Research Institute (IVRI), Mukteswar was revived in the secondary LT cells. The inoculum was prepared from freeze dried vaccine of ORFV reconstituted in EMEM and theninoculated into secondary LT cells. After formation of 70-80% complete monolayer, growth media from the flask was removed and washed with EMEM for two times. The inoculum containing125 µL of virus was added into 25 cm^2 flask (TPP, Zollstrasse, Switzerland) to infect the cells by pre-adsorption method. The flask was incubated at 37°C and tilted after every 15 min to spread virus uniformly and keep cells moist. Following virus adsorption for 1 hr, the unadsorbed virus was removed and 5 ml maintenance mediacontaining 2% FBS was added, and then observed daily for appearance of characteristicCPE under inverted microscope. The infected flask was harvested after appearance of 70-80% CPE by repeated freezing-thawing for three times and stored at -80°C until further use.

Passage and adaptation of ORFV vaccine strain in different cell lines. The LT adapted ORFV vaccine strain was attempted to adapt in different cell lines (Vero, MDBK and BHK-21). Vero, MDBK and BHK-21 cells were seeded into 25 cm^2 cell culture flask at a concentration of $1x10^6$ cells/ flask. The cells were kept for attachment for 3 hr, then infected with 300 µL of ORFV Mukteswar-59/05 (vaccine strain, P50), kept for 1 hr adsorption to facilitate proper adsorption of virus onto the cells and tilted after every 15 min. The unadsorbed viruses were removed. Then, maintenance medium containing 2% FBS was added into the flask and incubated at 37°C for 3-7 days. The flasks were observed daily for characteristic CPE. Non-inoculated cell culture flasks were kept as control and evaluated in parallel with the inoculated flasks. The virus was then harvested by repeated freezing and thawing. The virus was cultivated on the same cell culture for five or more consecutive passages.

Detection of ORFV DNA in infected cells by PCR. Nucleo-pore DNASure® Tissue Mini Kit was used for extraction of total genomic DNA (gDNA) from the virus infected Vero, MDBK and BHK-21 cell cultures at different passage levels. The extracted DNAs were checked by using diagnostic PCR targeting DNA polymerase gene (Venkatesan *et al.,* 2014) or PCR based on other genes (Karki, 2017). The PCR reaction was performed in total volume of 25 µL containing 12.5 µL of GoTaq™ Green master mix, 1µL each of forward and reverse primers (10 pmol) and1µLgDNA template and then subjected to following PCR cycling conditions in a thermal cycler as per standard protocol (Venkatesan *et al.,* 2014). Thermal cycler conditions were set as initial denaturation at 95°C for 5 min, 35 cycles of denaturation at 94°C for 30 sec, annealing at 60°C for 30 sec, extension at 72°C for 30 sec and final extension at 72°C for 7 min. The amplified products thus generated were resolved on a 1% agarose gelelectrophoresis.

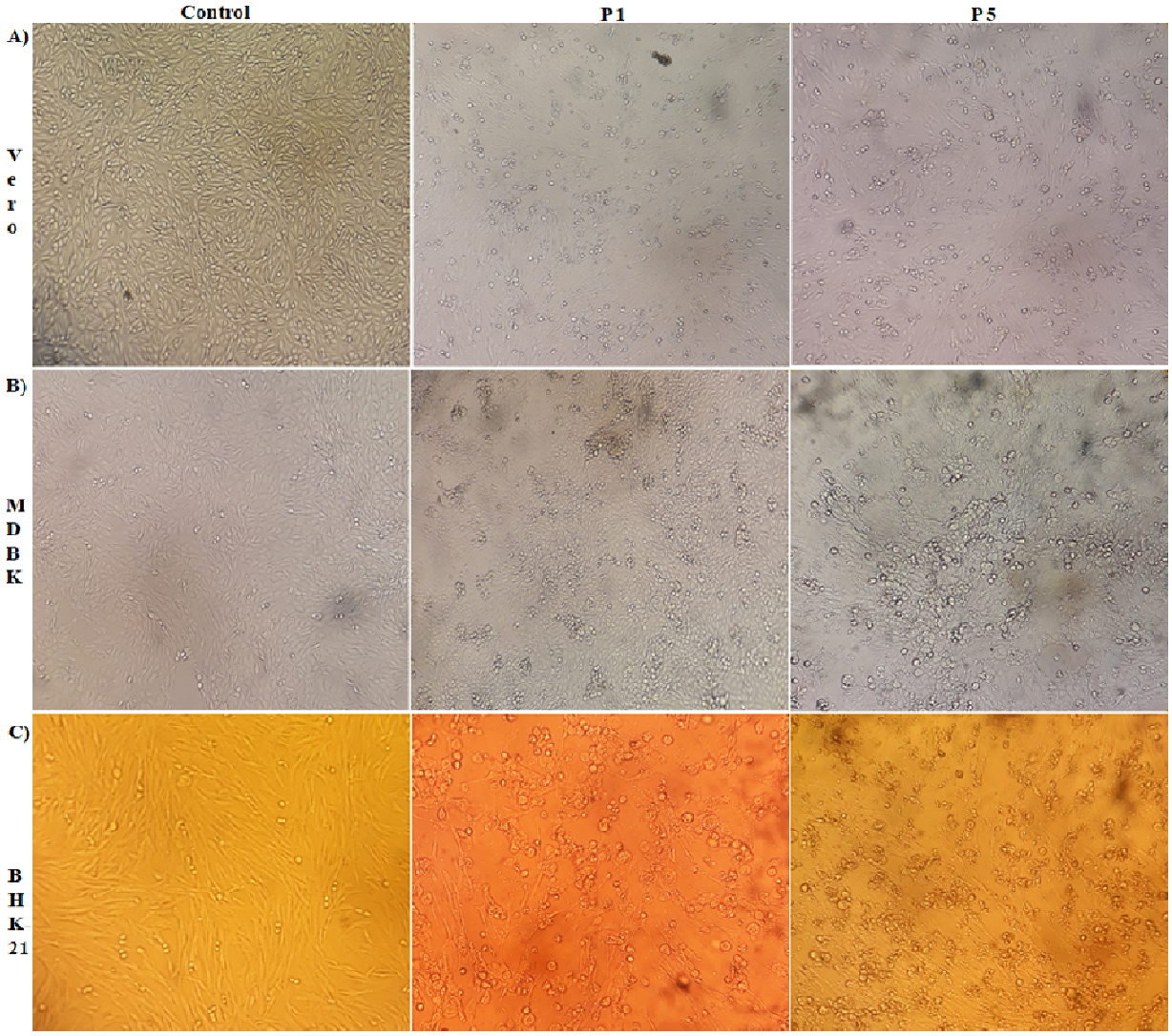
RESULTS AND DISCUSSION

Vaccination is the eventual way to control and eliminate any viral pathogen including orf in any endemic country like India. Ideal vaccine should have cardinal features namely safety, immunogenicity, protective efficacy and stability. Nevertheless, viability to produce of the vaccine at industrial scale is equally important. Available vaccine for orf in India using indigenous virus strain (Mukteswar 59/05) to use in sheep and goats is found to be safe, efficacious and potent (Bhanuprakash *et al.,* 2012). However, production of this vaccine involves preparation of primary cells from lamb testes, which is labor and cost intensive being not viable economically, subject to batch variation and presence of potential contaminating elements such as endogenous viruses. Therefore, orf vaccine virus strain that has been adapted in continuous cell lines can be useful for large scale production and mass vaccination in sheep and goats during control program.

The ORFV vaccine available in the Poxvirus laboratory, Division of Virology was revived in secondary LT cells. After 2nd day post-infection, the characteristic CPE were observed as cell rounding, ballooning, increased refractivity, clumping and degeneration of cell monolayer. Virus infected cells were harvested after appearance of 80% CPE by repeated freezing-thawing for three times. Total gDNA was extracted, confirmed by PCR showing an expected size of 214 bp, used as a positive control for amplication of ORFV and stored at -20ºC until further use. The different cell lines (Vero, MDBK and BHK-21) revived from the repository of author's laboratory was used for adaptation of ORFV vaccine strain. The LT adapted ORFV Mukteswar-59/05 (vaccine strain, P50) was attempted to adapt in Vero, MDBK and BHK-21 cell lines along with mock infected cell control was passaged up to 7 times in cell lines and characterized by PCR. Comparative assessment of three different cell lines with respect to appearance of CPE, change of media, time of harvest and virus detection are displayed in Table 1. Mock infected cell controls have shown intact cell monolayer throughout the passage levels. Vero cells have started showing typical CPE from passage 1 (P1) at 2nd day post-infection namely rounding of few cells, progressed gradually to aggregation of rounding cells at 4-5th day and there was detachment of cell monolayer at 6-7thday post-infection. During initial three passages, the cells were just started to change its shape from 2-3rd day and from P4 showing its changes earlier on 2nd day post-infection though the clear CPE was not observed after passage 6. Replication of ORFV in Vero cells has been reviewed and reported elsewhere (Karki *et al.,* 2019) and Vero cells are commonly used to isolate, propagate and produce capripox viruses or vaccine antigens (Bhanuprakash *et al.,* 2012). Similar to Vero cells, MDBK cells infected by ORFV have also shown CPE and its progression from P1-P6 on 2nd day onwards and the onset of CPE was earlier in subsequent passages till P7. In BHK-21 cells, CPE was initially observed from P1 on 1st day, P2 on 2nd day post-infection as rounding and ballooning, up to P7 showed CPE.

Table 1: Comparative evaluation of ORFV in different cell lines.

Cell lines	Vero	MDBK	BHK-21
First detection of CPE	2-3rd day	2-4th day	1-2nd day
Change of media	Alternate days	Alternate days	Daily
Harvested	6-7th day	6-7th day	4th day
PCR	P1-P6	P1-P7	P1-P7

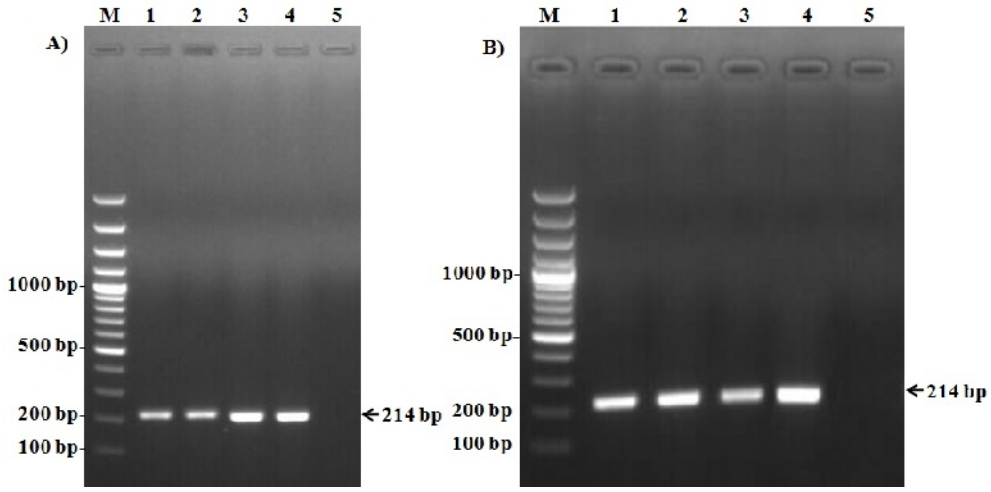


Panel A: Vero cells (Control), Vero cells infected with ORFV at P1 (6thday post-infection), at P5 (5th day post-infection).
Panel B: MDBK cells (Control), MDBK cells infected with ORFV at P1 (2ndday post- infection), at P5 (4th day post-infection).
Panel C: BHK-21 cells (Control), BHK-21 cells infected with ORFV at P1 (3rd day post-infection), at P5 (2nd day post-infection).
Fig. 1. Cytopathic effects observed in different cell lines infected with ORFV at different passage levels.

In general, it was observed that the time taken for appearance of CPE decreased with increased passage levels of the virus. Fig. 1 showed the characteristic CPE of different cell lines infected by ORFV along with mock infected cell control. Total gDNA of the virus infected Vero, MDBK and BHK-21 cells at different passage levels were processed for screening of target DNA polymerase gene (214 bp) by PCR method (Venkatesan *et al.*, 2014). PCR was amplified till P6 in Vero cell line and all the passages in both MDBK and BHK-21 cell lines (Fig. 2). In addition to this conserved gene located at central part of genome, genes responsible for virus pathogenesis and virulence were also targeted to amplify from cell line adapted viruses (Fig. 3 & 4). The result of adaptation of ORFV vaccine strain in different cell lines were confirmed by PCR is shown in Table 2. Cell culture is reported as one of the methods for isolation and propagation of ORFV (Karki *et al.*, 2019). Traditionally, researchers used primary cell cultures like LT and lamb kidney to support the growth of ORFV (Bhanuprakash *et al.*, 2012). The major drawbacks for primary cultures are limited life span, contamination and cells characteristic may change at each passage.

Table 2: Amplification of ORFV with diagnostic PCR and other virulence genes.

	Gene(s) targeted	Vero					MDBK					BHK-21				
		P1	P2	P3	P4	P5	P1	P2	P3	P4	P5	P1	P2	P3	P4	P5
Diagnostic PCR	DNA polymerase 214 bp	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
PCR based on virulence genes	VEGF 450 bp	+	+	+	-	-	+	+	+	+	+	+	+	+	+	+
	VIR 560 bp	+	+	-	-	-	+	+	+	-	-	+	+	+	+	+



Panel A: Lane M: 100 bp plus ladder; Lane 1: Vero infected cells at P1; Lane 2:MDBK infected at P1; Lane 3: BHK-21 infected at P1; Lane 4: Positive control; Lane5: No template control.
Panel B: Lane M: 100 bp plus ladder; Lane 1: Vero infected cells at P5; Lane 2:MDBK infected at P5; Lane 3: BHK-21 infected at P5; Lane 4: Positive control;Lane 5: No template control.

Fig. 2. PCR amplification of ORFV at P1 and P5 targeting DNA polymerase gene.

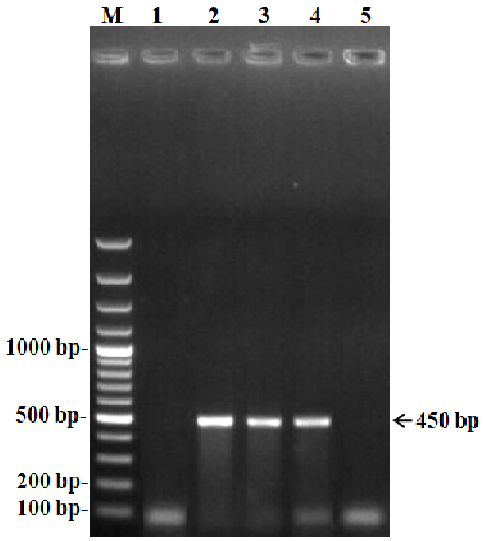


Fig. 3. PCR amplification of ORFV VEGF gene from infected cell lines at P5. Lane M: 100 bp plus ladder; Lane 1: Vero infected cells; Lane 2: MDBK infected cells; Lane 3: BHK-21 infected cells; Lane 4: Positive control (450 bp); Lane 5: No template control.

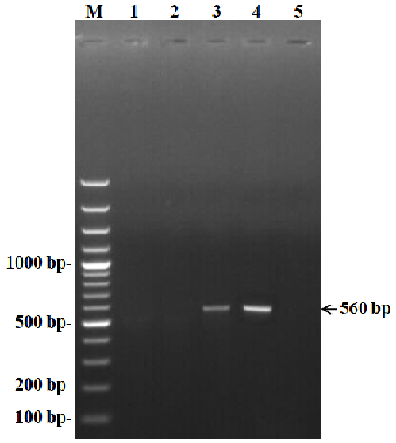


Fig. 4. PCR amplification of ORFV VIR gene from infected cell lines at P5. Lane M: 100 bp plus ladder; Lane 1: Vero infected cells; Lane 2: MDBK infected cells; Lane 3: BHK-21 infected cells; Lane 4: Positive control (560 bp); Lane 5: No template control.

By contrast, cell lines have the ability to proliferate indefinitely, have rapid large-scale preparations, more robust and easier to work with than primary cells. In Vero cells, CPE was observed till P6 and disappeared after this passage. This may be due to production of incomplete or defective viral particles that may interfere with the infectious virus particles. The present observation was found consistent with the findings of Traykova (1982) who reported CPE observed after P2-P3 and disappeared after P5 in Vero cells. Ivanov *et al.* (2016) reported CPE appeared from 3rd day post-infection in MDBK cells and 4th day post-infection in BHK-21 cells, covered a larger area of cell monolayer after 5-7th day, dropping off a great part of monolayer was observed by 10-12th day.

Gulyaz *et al*. (2020) reported CPE of vaccine virus was observed within 4-8th day post-infection and could be adapted in MDBK cells. Similar findings were also found in our study, which revealed observation of CPE at 7th passage level with ballooning, clumping or aggregation of rounding cells. The replication of ORFV was confirmed by targeting DNA polymerase gene (a highly conserved gene) at each passage level (data shown till P5). The used PCR successfully amplified DNA from vaccine virus adapted in MDBK and BHK-21 but in Vero cells it can be amplified till P6. To check whether the virus lost some virulence genes after adaptation in cell lines, we amplified virulence genes (VEGF and VIR) located at terminals of ORFV genome. It is observed that there was a variable degree of amplification or even not amplified of virulent genes at different passages in three cells (Fig. 3&4).The inability of adapted ORFV to amplify the virulence genes might be due to some degree of attenuation during continuous passage in primary or cell lines.

CONCLUSION

From the present study, ORFV vaccine strain was successfully adapted and passaged in MDBK and BHK-21 cell lines. However, Vero cells did not adequately support the virus replication and subsequent growth. Further study is essential to determine the infectivity titre and replication dynamics of virus in order to develop cost effective cell line based vaccine against orf. Also, other cell lines that may be suitable to support ORFV replication need to be screened.

FUTURE SCOPE

Such type of research and their findings have high potential in development of ORFV vaccine using cell lines, which could avoid use of animals for preparation of primary cells and minimize the batch to batch variation in virus production.

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Conflict of Interest. The authors declare that they have no conflict of interest.

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