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Asma Latif, Aamer Bin Zahur, Geneviève Libeau, Rabaab Zahra, Aman Ullah, Afshan Ahmed, Muhammad Afzal, - Sajjad-Ur-Rahman

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RESEARCH ARTICLE

Comparative Analysis of BTS-34 and Vero-76 Cell lines for Isolation of Peste des Petits Ruminants (PPR) Virus

Asma Latif*[1,2], Aamer Bin Zahur[2], Genevieve Libeau[3], Rabaab Zahra[1], Aman Ullah[2], Afshan Ahmed[2], Muhammad Afzal[4] and Sajjad-Ur-Rahman[5]

[1]Department of Microbiology, Quaid-i-Azam University, Islamabad; [2]Animal Health Laboratories, Animal Sciences Institute, NARC, Islamabad, [3]CIRAD, UMR CMAEE, F-34398 Montpellier, France; INRA, UMR 1309 CMAEE, F-34398 Montpellier, France; [4]Progressive Control of PPR in Pakistan, FAO-UN, Islamabad 44000, Pakistan; [5]Institute of Microbiology, University of Agriculture, Faisalabad, Pakistan
*Corresponding author: asmachudary@yahoo.com

A B S T R A C T

Peste de petits ruminant (PPR) is a highly contagious viral disease affecting predominantly sheep and goats. The virus belongs to the genus Morbillivirus accounting for causing immunosuppression and severe lymphopenia in host lymphoid cells principally due to the presence of a glycoprotein known as Signaling Lymphocyte Activation Molecule (SLAM) acting as cellular receptor. Limitations regarding the efficiency of PPR virus isolation using a variety of cell lines paved the way to the use of a new cell line (BTS-34) expressing these receptors. In the present study, a total of 18 PPRV confirmed strains were inoculated onto low passage Vero-76 and BTS-34 cell lines simultaneously to compare the susceptibilities of Vero and BTS cells for PPR virus isolation. The recovered viruses were then re-confirmed by Ic-ELISA and RT-PCR. In order to investigate the differences in titers of virus strains, serial tenfold dilutions were made separately using both cells at a density of 0.01×10^6 and tissue culture infective dose fifty (TCID$_{50}$) was calculated. The results revealed that the characteristic pattern of the CPEs was more obvious in BTS-34 cell line marked by giant cells ultimately transforming into distinguished syncytia in 2 to 3 days. The average incubation time for virus isolation on Vero cells was about 10 days and syncytia formation were less marked. The growth curve indicated a one log increase in virus titer in case of BTS-34 cell line as compared to Vero-76. Statistically, there was significant difference between two types of cell lines in terms of number of days taken for PPR virus isolation as determined by single factor ANOVA (P<0.001). The study showed that BTS cells produced high virus titer with clearly distinct CPEs in short time due to the presence of SLAM receptors as compared to Vero cells suggesting the BTS cells to be more efficient and sensitive for PPRV isolation.

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INTRODUCTION

Peste de petits ruminant (PPR) also referred to as goat plague is an infectious viral disease of domesticated sheep and goats as well as wild small ruminants (Albina *et al.*, 2013) causing huge economic losses (Jones *et al.*, 2016). After the successful eradication of rinderpest in cattle, PPR has been declared as next eradicating target by Food and Agriculture Organization of United Nations (FAO) and the World Organization for Animal Health (OIE) by 2030 (Baazizi *et al.*, 2017). The disease is caused by a morbillivirus (PPRV) belonging to the family Paramyxoviridae (Chowdhury *et al.*, 2014) and is characterized by high fever, ocular and nasal discharges; oral erosion, bronchopneumonia, gastroenteritis, necrotic stomatitis, and can lead to death. The morbidity and mortality rates can reach upto 100% (Hailat *et al.*, 2018) and 50-90% respectively in susceptible populations (Rojas *et al.*, 2017).

The infection caused by members of genus morbillivirus results in severe immunosuppression

including leucopenia in respective hosts, since lymphoid cells are a major target of morbilliviruses (Rajak *et al.*, 2005). The immunosuppression triggers secondary opportunistic bacterial and parasitic infections responsible for severity of disease and its symptoms (Seki *et al.*, 2003). This response is associated with the presence of a cell surface protein receptor CD150, the signaling lymphocyte activation molecule (SLAM) preferentially used by wild-type morbilliviruses for attachment to the host cell (Adombi *et al.*, 2011; Baazizi *et al.*, 2017). All morbilliviruses including PPR virus are capable of utilizing two receptors, CD150 (Tatsuo *et al.*, 2001) and CD46. CD150 is a glycoprotein belonging to the CD2 subset of the immunoglobulin super family and is expressed on the surface of a proportion of primary B cells, Epstein-Barr virus (EBV) transformed B cells (B95a cells), T cell clones, memory T cells, activated T cells, immature thymocytes, mature dendritic cells, and activated monocytes (Tatsuo *et al.*, 2001) whereas, CD46 is a complement regulatory protein expressed on all cells except red blood cells (Sannat *et al.*, 2014).

For PPR virus isolation, primary cultures of ovine and bovine kidney, African green monkey kidney (vero cells), and lung cells have been used (Adombi *et al.*, 2011). However, isolation using primary cultures is laborious, time consuming and needs technical expertise. To overcome this problem, continuous cell lines are used for isolation and cultivation of morbilliviruses. The most commonly used cell line is Vero cell line (Sannat *et al.*, 2014) but these cells do not favor efficient PPR virus isolation due to less probability of virus growth and require numerous blind passages for the development of visible cytopathic effects (CPEs) (Albayak and Alkan, 2009; Adombi *et al.*, 2011).

The identification of SLAM receptors used preferably by wild-type PPR virus strains and other members of morbillivirus as a mode of entry and attachment revealed the importance of these receptors for isolation of virus. The present study was designed to investigate the efficiency of BTS-34 cell line expressing the bovine SLAM receptors in comparison with Vero-76 cells (Sannat *et al.*, 2014).

MATERIALS AND METHODS

Cell lines: Two types of cell lines were used in this study for the isolation of PPR virus. Vero cells (Vero-76, ATCC Number: CRL-1587™) were maintained in tissue culture laboratory of Animal Health Program, Animal Sciences Institute, National Agriculture Research Center [taken from American type culture collections (ATCC)]. These cells were grown in Glasgow Modified Minimum Essential Medium (GMEM) supplemented with 10% fetal bovine serum (FBS) (Sigma-Aldrich), 1% mixed antibiotics/antimycotic solution. BTS-34 cell line was received with the courtesy of CIRAD (Center for International Cooperation in Agricultural Research for Development), France. These cells are CV1 cells constitutively expressing bovine SLAM (using the Flp-In system) maintained in the Dulbecco's Modified Eagle Medium (DMEM) 10% FBS 1% mixed antibiotic/antimycotic solution supplemented with 600μg/ml of hygromycin B (Sigma-Aldrich).

Sub culturing of cells: A pre-cultured flask of Vero and BTS cells each with 80-90% of confluency was trypsinized following split ratio method (Burleson *et al.*, 1992). Briefly, the cells were washed with phosphate buffered saline (PBS). After washing, the PBS was discarded and 1 ml working solution of versene 0.02% and trypsin 0.05% was added into the flask. After 1 minute, this solution was discarded and flask was incubated at 37ºC for 1 to 2 minutes. Once the cells became rounded and detached, 18 ml of growth media was added into the culture flask. This cell suspension in the medium was distributed equally into three 25 cm^2 tissue culture flasks and incubated at 37ºC with 5% CO2 necessary for the growth of cell. The cells were observed under the microscope after 24 and 48 hours of incubation for cell attachment and confluency.

Sample processing and screening: A total of eighty suspected tissue samples (spleen, lymph nodes and lungs) collected from 16 outbreaks in different areas of the country during 2008–2015 were processed. All the samples were tested for PPR virus antigen by indirect competitive enzyme-linked immunosorbent assay (Ic-ELISA) and reverse transcriptase polymerase chain reaction (RT-PCR). Briefly, the inoculum was prepared by grinding approximately 1g of infected tissue using sterilized pestle and mortar. After grinding, 500μl of sterile PBS with antibiotic solution was added and a 10% suspension (W/V) was made. The homogenate was then transferred to a sterile centrifuge tube and clarified by centrifuging at 1500 rpm for 15 minutes at 4°C and the supernatant was collected. A further fivefold dilution was made by adding 2 ml of supernatant to 8 ml of sterile GMEM and filtered through a 0.2 μm filter (Minisart NML). The resultant filtrate was used as inoculum in Ic-ELISA, RT-PCR and virus isolation.

Immunocapture enzyme-linked immunosorbent assay (Ic-ELISA): A total of 80 samples were tested by immunocapture-ELISA kit as described by Libeau *et al.* (1994). The Ic-ELISA kit produced jointly by Biological Diagnostic Supplies Limited (BDSL) with the collaboration of Flow Laboratories and CIRAD, EMVT, France was used for this purpose. The absorbance was measured at a wavelength of 492 nm. Percent positivity (PP) values were calculated by ELISA Data Interchange (EDI) software (FAO/IAEA, Vienna, Austria). The optical densities were converted to percentage positivity by using following formula:

$$PP = \frac{100 - (OD\ control/test\ sample)}{OD\ of\ PPR\ ref.\ antigen} \times 100$$

The samples with PP value greater than 18% were considered positive.

Identification of PPR virus by RT-PCR: Cell culture supernatant was tested for the confirmation of PPR virus by RT-PCR. A Macherey Nagel GmbH & Co. KG kit was used for extraction of RNA from the specimens according to manufacturer's instructions. PPRV N gene was amplified partially in a 25 μl reaction using Verso One-Step RT-PCR kit (Thermo Scientific) using PPRV-

specific primers NP3 (5′-TCTCGGAAATCGCCTCAC AGACTG-3′) and NP4 (5′-CCTCCTCCTGGTCCTCCA GAATCT-3′) as described by Couacy-Hymann *et al.* (2002). Briefly, a reverse transcription was carried out at 50°C for 30 min followed by initial denaturation of DNA at 95°C for 15 minutes. Then there were 35 cycles of denaturation at 95°C for 20 s, annealing at 58°C for 30 s and elongation at 72°C for 30 s, and a final extension at 72°C for 10 min. Amplification was carried out in a Veriti 96 well Thermal Cycler 9902 (Applied Biosystems, USA). The analysis of amplified DNA was carried out by gel electrophoresis in 1.5% agarose gel stained with syber safe @ 7 µl / 100 ml. A total of sixty four samples were found positive by PCR, out of which 24 samples were inoculated onto Vero and BTS cell lines simultaneously.

Virus isolation: A sub-confluent flask of Vero and BTS cells was washed with PBS. After thorough washing, 0.5 ml of prepared inoculum of suspected samples was added into the flasks after filtration with 0.2µ filter. The flasks were incubated at 37°C for 1 hour to adsorb the virus. The inoculum was discarded and 6 ml of growth media was added to the respective flasks. The control flask was inoculated with 500µl of sterile GMEM only. The flasks were labeled and incubated at 37°C. The media was changed at alternate days. Flasks were observed daily until the CPEs became visible. The flasks showing 80% of CPE's were harvested. However, for the cultures showing no CPE's, a blind passage was made using freeze thaw method. If no CPEs were observed till 3 successive blind passages, the sample was considered negative.

Growth curve: Tenfold serial dilutions of seven PPR virus local isolates were made and added in micro-titration plates seeded with Vero and BTS cells at a density of 0.01×10^6. The tissue culture infective dose fifty ($TCID_{50}$) was calculated as described by Reed and Muench (1938). The plate was incubated at 37°C for 24 hours. The CPE's were observed and recorded.

RESULTS

Virus confirmation: The pathological samples were collected from infected sheep and goats in 16 suspected PPR outbreaks. A total of 80 samples including tissues (Lymph node, Spleen, lungs and liver) and swabs (Ocular and nasal) were used to check the presence of PPR virus. Out of these, 52 samples (65%) were found positive by Ic-ELISA and 64 samples (80%) were found positive by RT-PCR (Table 1).

Virus recovery: Eighteen out of 24 samples attempted for virus isolation were recovered (Table 2) by propagating in two different cell lines (Vero-76 and BTS-34). Both cell lines revealed different patterns of CPEs in terms of morphology as well as time taken for their development (Table 3). The Vero cell line showed a delayed onset of CPE's with 1 to 2 blind passages from Vero to Vero cell line (Table 4). The CPE's in this cell line developed as elongated granular cells followed by cell shrinkage and rounding. At the third day post infection, a significant number of hollow round and refractive cells (approx. 90-100%) were prominent with very few intact cells (Fig. 2).

In BTS-34 cell line, the PPR virus revealed visible CPEs from 72 hours post infection. About 40-50% of CPEs were seen as round giant cells followed by formation of syncytia and layer detachment. On fourth day post inoculation, 80-90% CPEs were observed with an increased number of round cells, large cluster of cells and with very few intact cells (Fig. 3). The complete process of PPRV infection on BTS-34 cell line took approximately 4 to 6 days (Table 4).

Confirmation of virus recovery by Ic-ELISA and RT-PCR: Out of 24 tissue and swab samples declared positive by both Ic-ELISA and RT-PCR were attempted for virus isolation onto Vero-76 abd BTS-34 cell lines. A total of 18 PPR virus strains recovered on both cell lines showed characteristic CPE's of PPR virus. The recovered viruses were then re-confirmed by both techniques (Ic-ELISA and RT-PCR). It was found that 13 viruses were confirmed by RT-PCR (72.22%) (Fig. 1) while Ic-ELISA confirmed 6 PPR virus isolates (33.33%) as shown in the Table 2.
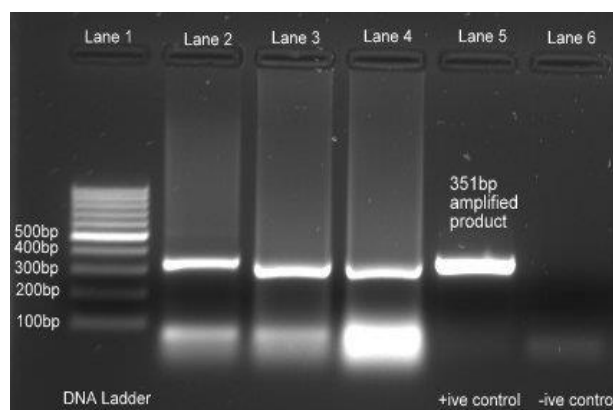

Fig. 1: A representative gel image showing band size of 351bp of viral N gene (from left – right). First lane, DNA ladder of 100bp. Lanes 2-4: study samples, Lane 5: Positive control, Lane 6: Negative control.

Table 1: Initial screening of PPR suspected samples from different outbreaks by Ic-ELISA and RT-PCR

Area	No. of samples	Ic-ELISA		RT-PCR	
		Positive	% Positive	Positive	% Positive
Karachi	13	8	61.54	10	76.92
Islamabad	20	14	70	18	90
Taxila	4	4	100	4	100
Rawalpindi	8	7	87.5	8	100
Muzzafarabad	6	4	66.67	6	100
Bhimber	10	4	40	6	60
Faisalabad	4	4	100	4	100
Gilgit Baltistan	2	1	50	1	50
Murree	2	1	50	1	50
Bhakkar	6	3	50	3	50
Thar	5	2	40	3	60
Total	80	52	65	64	80

Growth curve: Titers of seven local isolates of PPR virus were compared separately using both cell lines (Vero-76 and BTS-34) by making tenfold serial dilutions and calculation of tissue culture infective dose fifty ($TCID_{50}$). It was found that there was approximately one log increase in viral titer in case of BTS cells as depicted in Fig. 4 which suggested that BTS cells produce high titer of virus as compared to Vero cells.
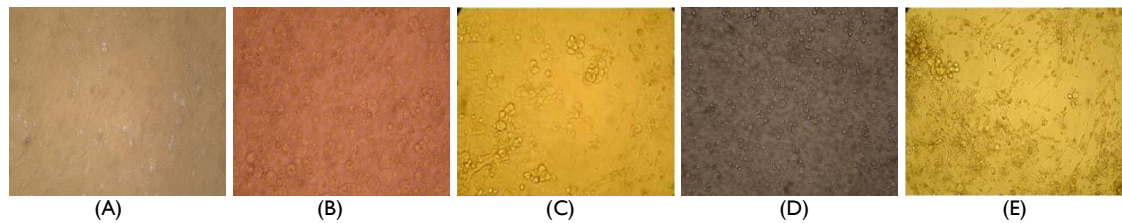
Fig. 2: Micrograph of Vero cell infection: (A) Cells at the day of virus inoculation (B) CPEs elongation (C) Clumping of cells (D) Rounding of cells (40 – 50%) (E) Rounding of cells (90 – 100 %).
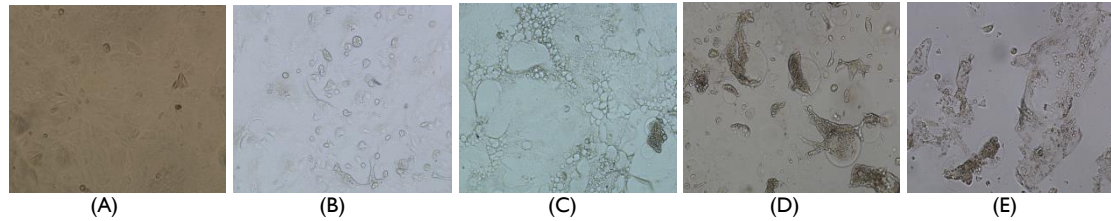

Fig. 3: Micrographs of BTS cells post infection (A) Normal cells (B) Cell rounding (C) Initiation of syncytia formation (2 days post infection) (D) Enlargement of syncytia (3 days post infection) (E) Detachment of cell monolayer (4 days post infection).

Table 2: Tissue and swab samples collected from suspected outbreaks inoculated onto Vero76 and BTS-34 cell lines and confirmation of virus recovery by Ic-ELISA and RT-PCR using N gene primers for PPR virus

| Area | Total samples | Samples attempted for Virus isolation | Virus recovery | Confirmation of virus recovery by | | | |
| | | | | Ic-ELISA | | RT-PCR | |
				Positive	% Positive	Positive	% Positive
Karachi	13	3	2	1	50	2	100
Islamabad	20	8	6	2	33.33	4	66.67
Taxila	4	1	1	1	100	1	100
Rawalpindi	8	2	2	-	0	2	100
Muzzafarabad	6	1	1	1	100	1	100
Bhimber	10	2	1	-	0	1	100
Faisalabad	4	2	1	1	100	1	100
Gilgit Baltistan	2	2	1	-	0	-	0
Murree	2	2	1	-	0	-	0
Bhakkar	6	2	1	-	0	-	0
Thar	5	2	1	-	0	1	100
Total	80	24	18	6	33.33	13	72.22

Table 3: Appearance of visible CPE's by different isolates onto Vero and BTS cells

| Isolates used | Isolation on cell type | | Characteristics of PPR CPE's on Vero and BTS cells | | | | | | | | | | | |
| | | | Cell rounding | | Detachment from surface | | Vacuolation | | Syncitia formation | | Clumping | | Cells with elongated processes | |
			Vero	BTS	Vero	BTS	Vero	BTS	Vero	BTS	Vero	BTS	Vero	BTS
NARC-1	GKC	BTS	+	+	+	+	+	+	+	+	-	-	-	-
NARC-2	GKC	BTS	+	+	+	+	+	+	+	+	-	-	-	-
NARC-3	Vero	BTS	+	+	+	+	-	-	-	+	+	+	+	+
NARC-4	Vero	BTS	+	+	+	+	-	-	-	+	+	+	+	-
NARC-5	Vero	BTS	+	+	+	+	-	-	-	+	+	+	+	-
NARC-6	Vero	BTS	+	+	+	+	-	-	-	+	+	+	+	-
NARC-7	Vero	BTS	+	+	+	+	-	-	-	+	+	+	+	-

DISCUSSION

Peste des petits ruminants (PPR) is considered as an important trans-boundary disease of small ruminants causing huge economic losses. Recently, Food and Agriculture Organization of the United Nations (FAO) and the World Organization for Animal Health (OIE) have started an eradication campaign to control the disease by 2030. Improvement in current vaccine formulations and development of effective, inexpensive, and sensitive diagnostic techniques can facilitate this control strategy (Fakri *et al.*, 2016). PPR virus isolation employing cell culture techniques is a gold standard test for better understanding of pathogen attributes and to investigate the disease pattern globally (Hematian *et al.*, 2016).

Primary cultures of mammalian kidney and lungs have been used for many years for isolation of morbilliviruses (Lefevre and Diallo, 1989; Taylor *et al.*, 1990) but the difficulty in maintaining these cultures and batch to batch variation makes their use less frequent. Therefore, continuous cell lines like Vero, BHK-21, CV1, and CHS-20 are preferred for cultivation of PPR virus and recently BTS cells are commonly used (Adombi *et al.*, 2011).

The present study reveals that PPR virus like other morbilliviruses also uses SLAM protein of their host species as a cellular receptor. A new cell line referred as BTS-34 was used in this study in comparison with the Vero cells for PPR virus isolation. This cell line constitutively expresses bovine SLAM receptors which

aids in attachment and entry of virus into the host cells. Alternatively, Vero cells are also used for propagation of PPR virus as the cells are accessible and can easily be maintained and grown for long time in-vitro. However, as the origin of Vero cells is not bovine, PPR virus isolation in these cells is inefficient due to the absence of virus specific receptors on their surface.
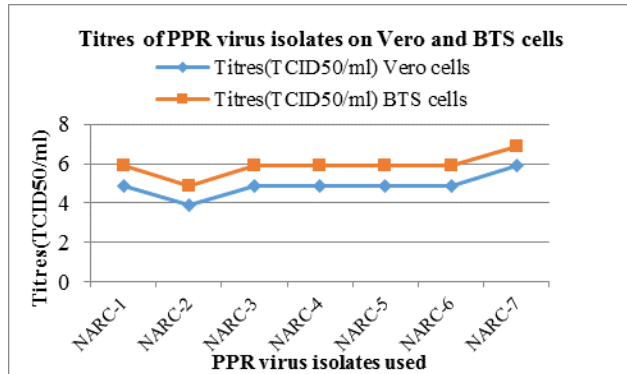

Fig. 4: Comparison of PPRV titres propagated onto Vero and BTS cells

Table 4: The IDs of isolates and the time taken for both cell lines (BTS & Vero) to develop characteristic CPE's

Isolate IDs	Days taken for appearance of CPE's	
	BTS cells	Vero cells
NARC-1	5	16
NARC-2	5	8
NARC-3	5	16
NARC-4	7	8
NARC-5	6	8
NARC-6	8	21
NARC-7	5	16
NARC-8	8	21
NARC-9	5	8
NARC-10	6	8
NARC-11	8	21
NARC-12	6	21
NARC-13	5	16
NARC-14	8	21
NARC-15	8	21
NARC-16	5	21
NARC-17	5	21
NARC-18	8	21
Mean	6.277778	16.27778
p-value	1.9E-08	

Pathological samples were grown on two different cell lines for PPR virus recovery. BTS-34 cell line developed visible cytopathic effects within 4 days post infection (Fig. 3) while in Vero cells, 2 or 3 blind passages (Vero to Vero) were needed to isolate viruses successfully (Fig. 2). Both cell lines exhibited different forms of changes *in-vitro*. In a similar experiment Emikpe and coworkers (2009) compared the susceptibility of PPR virus towards BHK and Vero cell line revealing that the cytopathic effects and their pattern was earlier and more obvious in BHK cells than in Vero cells. The infected cells experienced variations in their morphology as the infection progressed. They became well defined, rounded, swollen giant cells with increased refractivity. In Vero cells typical rounding was observed on 21 days post infection. In the late stages of infection, the cells became refractile. Large cluster of cells were seen which later formed clumps, eventually leading to cell disintegration and detachment whereas, BTS cells presented a drastic

change in morphology prior to the development of visible CPEs. Small polykaryons with many intercellular spaces were observed that became more prominent and larger the next day ultimately transforming into big syncitia. Finally, the cells got detached from glass surface and were seen floating in the culture media.

The SLAM protein present on the cell surface aids in the attachment of virus with the host cell facilitating efficient PPRV isolation. This is supported by a similar work in which goat SLAM receptors were introduced in monkey CV1 cells referred to as CHS-20 cells, facilitating PPR virus growth and isolation from clinical samples. The study revealed that the CPEs appeared after 11 to 12 weeks post infection in Vero cells while in infected CHS-20 cells syncytia produced within 1 to 2 days following infection (Adombi *et al*., 2011). It was also proposed that Vero cells expressing canine SLAMs were extremely susceptible to infection with canine distemper virus (CDV) in pathological specimens (Seki *et al*., 2003). In another study scientists compared PPR virus infection in Vero cells expressing canine SLAM (Vero Dog SLAM) with Vero cells expressing Nectin-4 (VeroNectin-4) depicting VeroNectin-4 cells as more component than Vero Dog SLAM cells (Fakri *et al*., 2016). Recently a new suspension cell line (F9 lymphoid cells) has been used for PPRV isolation and it was suggested that this cell line is more preferable over Vero cells in terms of high titre and can be used to propagate PPR virus for vaccine production (Mofrad, 2016).

The study shows that Vero cell line infected with PPRV developed CPEs after 2 to 3 blind passages while the same PPRV strains showed visible infection after 3 to 4 days post infection in susceptible BTS cells suggesting that PPR virus adapted to the BTS-34 cell line to a greater extent as compared to the Vero cells. This high affinity of PPRV towards BTS cells could be due to the presence of SLAM protein used as cellular receptor. Nevertheless, un-attenuated PPRV strains may possibly use other receptors for entry into the host cells but less competently (Takeda *et al*., 2007; Tahara *et al*., 2008).

Conclusions: BTS-34 cell line demonstrated to be more sensitive and efficient *in-vitro* system for PPR virus isolation possibly because of the presence of SLAM receptors. This protein is specifically used as attachment site for PPR virus. Although PPR virus has ability to infect cells which do not possess SLAM receptors on their surface but the pace of CPEs formation in these cells is very slow. These finding provide an insight to enhance the host cell potential facilitating efficient PPR virus isolation from clinical samples.

Authors contribution: AL sample processing, virus isolation on Vero-76 and BTS-34 cell lines, experimental design, all experimental work in cell culture laboratory, manuscript write-up, ABZ funding for the experiment, experimental design and CPE's observation, GL supplied BTS cell line and helped in CPE's observation, RZ proof reading of manuscript, AA maintenance of Vero cell line, helped in manuscript write up. AU statistical analysis, MA guidance throughout experimentation, SUR guidance in write-up of manuscript.

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