



Adaptability of a field isolate of Classical swine fever virus to PK-15 cells

R AKSHAYA, A RAMESH, K LOGESH, R SANGEETHA, K VIJAYARANI, K KUMANAN*,
P TENSINGH GNANARAJ and G DHINAKAR RAJ

Tamil Nadu Veterinary and Animal Sciences University, Chennai, Tamil Nadu 600 007 India

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Classical swine fever (CSF), an economically important disease of domestic and wild pigs is highly contagious and caused by Classical swine fever virus (CSFV) (Leifer *et al.* 2013). CSF is recognized by OIE as a Transboundary Animal Disease (TAD) and included under the Global Early Warning and Response System (GLEWS) framework. In India, CSF is endemic (Patil *et al.* 2010, Sarma *et al.* 2011, Rathnapraba *et al.* 2014, Baskar *et al.* 2015, Sangeetha *et al.* 2018) and clinical form of the disease varies based on the strain of the virus, age and susceptibility of the pigs (Moennig *et al.* 2003). The etiological agent belongs to *Pestivirus* genus of the family *Flaviviridae*. Because of its high mortality and morbidity, prevention of CSF is very important for the pig farmers and vaccination is the best way of preventing CSF. In India, live attenuated lapinized CSF vaccine is used to control CSF which is not freely available. Cell line based vaccines are considered to be always advantageous since these vaccines will be easy to scale up, devoid of the adventitious agents and comparatively cheaper, and attempts have been made to adapt CSFV to *in vitro* cultured cells (Dhar *et al.* 2008, Medhi *et al.* 2012, Badasara *et al.* 2017, Vadivoo *et al.* 2017). The present study describes the adaptability of a virulent, field isolate of CSFV in PK-15 cell line.

Porcine Kidney-15 (PK-15) cell line: Porcine Kidney-15 (PK-15) cell line obtained from ATCC, USA, and available at the Department of Animal Biotechnology, Madras Veterinary College, Chennai was used for the isolation and propagation of CSFV. PK-15 cells were cultured in Dulbecco's Minimum essential medium (DMEM, Sigma-Aldrich, USA) supplemented with 10% fetal bovine serum (Gibco, BRL, USA, pestiviruses free) at 37°C in 5% CO₂. The flasks containing confluent monolayer were used for virus adaptation. Sub-culturing of PK-15 cell line was done employing standard procedures.

Adaptation of CSFV in PK-15 cell line: Spleen and lymph node suspension available at Department of Animal Biotechnology, Madras Veterinary College collected from a diseased pig (Sample no. CSFV-Ind-TN/Veng-09-002

collected from a CSF outbreak from Vengaivasal village, Tamil Nadu) was prepared and treated with 200 IU of Penicillin and 200 µg of Streptomycin for 2 h at 37°C, centrifuged at 5,000 rpm at 4°C for 10 min. Supernatant was inoculated onto confluent monolayer of PK-15 cells and incubated in a CO₂ incubator for 5 days. At 5 days post infection (DPI), infected cells were subjected to 3 cycles of freezing and thawing for complete release of the virus from the infected cells. Cell lysate was clarified by centrifugation at 4,000 rpm at 4°C for 15 min and the virus containing supernatant was used for subsequent passages. Presence of CSFV was confirmed by Fluorescent antibody virus neutralization (FAVN) test and Reverse Transcription–Polymerase Chain Reaction (RT-PCR) with CSFV specific primers.

Fluorescent antibody virus neutralization (FAVN) test: FAVN test was conducted as described earlier (Rathnapraba *et al.* 2014) for the confirmation of the virus in PK-15 cells and also for quantification at different passage levels. Briefly, ten fold serial dilutions of the cell culture supernatant was prepared. Five replicates of 100 µl of each dilution was seeded into preformed PK-15 cells grown in 96 well plate. After 4 DPI, medium was discarded and the monolayers were washed gently in PBS. Cells were fixed in 4% ice cold acetone in PBS for 10 min. After fixing, the plates were washed with PBS and the monolayers were incubated with 1:50 dilution of polyclonal reference serum for CSFV diluted in PBST. Finally, the monolayers were stained with Anti-pig IgG conjugated with FITC and observed under fluorescent microscope for the presence of virus specific fluorescence. Virus titres were calculated as TCID₅₀ using standard procedures.

Reverse Transcription–Polymerase Chain Reaction: Total RNA was isolated from PK-15 cells infected with CSFV at every 5th or 10th passages using RNA Isoplus (Takara, Japan) as per manufacture's protocol. cDNA synthesis was carried out using random hexamers with total RNA from each sample using the High capacity cDNA synthesis kit (Applied Biosystems, USA) following manufacture's protocol to a final volume of 20 µl. RT-PCR was performed for three regions of the CSF viral genome,

*Corresponding author e-mail: kumananrani@gmail.com

Table 1. Details of primers used for the detection of CSFV

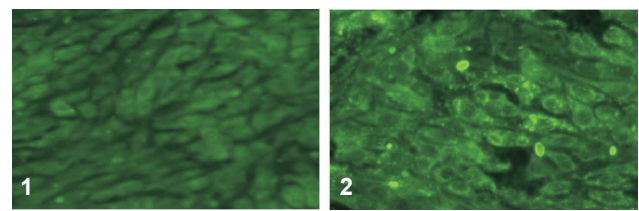
Primer	Primer Sequences and their regions	Annealing temperature	Expected product size
5'UTR Outer	FP: 5' CTA GCC ATG CCC WYA GTA GG 3' (94-113 nt) RP: 5' CAG CTT CAR YGT TGA TTG T 3' (514-496 nt)	50°C	420 bp
5'UTR Inner	FP: 5' AGC TCC CTG GGT GGT CTA 3' (146-163 nt) RP: 5' TGT TTG CTT GTG TTG TAT A 3' (417-399 nt)	50°C	271 bp
E2 Outer	FP: 5'AGR CCA GAC TGG TGG CCN TAY GA 3' (2228-2250 nt) RP: 5' TTY ACC ACT TCT GTT CTC A 3' (2898 - 2880 nt)	50°C	670 bp
E2 Inner	FP: 5' TCR WCA ACC AAY GAG ATA GGG 3' (2477 – 2497nt) RP: 5' CAC AGY CCR AAY CCR AAG TCA TC 3' (2748 – 2726nt)	55°C	271 bp
NS5B	FP: 5' GAC ACT AGY GCA GGC AAY AG 3' (11138- 11157 nt) RP: 5' AGT GGG TTC CAG GAR TAC AT 3' (11158 -11567 nt)	56°C	449 bp

viz. E2, NS5B and 5' UTR (Rathnaprabha *et al.* 2012) for confirmation of the virus presence. Primers used in the study are furnished in Table 1. PCR reaction mixture was prepared in a 25 µl volume with 12.5 µl of Taq DNA Polymerase 2x Master Mix RED (Ampliqon), 10 pmol each of forward and reverse primer, 3 µl of cDNA and the final volume was made up with nuclease free water. Amplified products were electrophoresed in 1.5% and 2% agarose gels.

Virus titration: Ten fold serial dilutions of culture supernatant were prepared and three replicates of 100 µl of each serial dilution (10^{-1} to 10^{-10}) were incubated for 5 days on PK-15 monolayers in 96 well plate. After 5 DPI, the spent medium was discarded and the monolayers were washed gently with PBS and fixed in 4% ice cold acetone in PBS for 10 min. After fixing, the plate was washed in PBS and the monolayers were incubated for 1 h with 1:50 dilution of polyclonal CSFV reference serum (EU reference laboratory, Germany) diluted in PBST. Finally, the monolayers were stained with anti pig FITC (Sigma) and observed under fluorescent microscope. Virus titres were calculated as TCID₅₀.

Animal inoculation: Safety of the adapted virus after 45 and 75 passages in PK-15 cells was studied by inoculating 2 ml each of the 45th and 75th passage virus in 3 Large White Yorkshire pigs of 12 weeks age procured from Post Graduate Research Institute of Animal Sciences, TANUVAS, Kattankolathur. Animals were observed for clinical signs of the disease. Conjunctival and nasal swabs were collected and tested for the presence of the virus.

In an attempt to evolve a vaccine candidate virus for CSF, a local field isolate of CSFV was subjected to continuous passages in PK-15 cell line. Since the virus do not produce characteristic cytopathic effects (CPE), the virus was subjected to five blind passages in PK-15 cells. FAVN test conducted after 5 passages revealed a bright green cytoplasmic fluorescence in infected PK-15 cells as compared to uninfected cells (Figs 1 and 2). To further confirm the presence of the virus in infected cells, RT-PCR was conducted for three genes of CSFV. Specific amplification was observed with NS5B, E2 inner and 5' UTR inner genomic regions of CSFV with 449 bp, 271 bp and 271 bp products respectively as expected (Greiser-



Figs 1–2. 1. Uninfected PK-15 cells stained with FITC (100×). 2. CSFV infected PK-15 cells at 75th passage stained with FITC showing cytoplasmic fluorescence (100×).

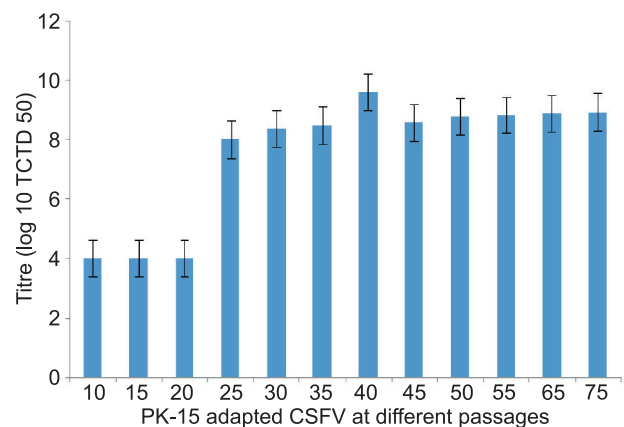


Fig. 3. TCID₅₀ titres of PK-15 adapted CSFV at different passages indicating only a marginal increase after 45 passages.

Wilke *et al.* 1998; Paton *et al.* 2000). After confirming the presence of the virus after 5 passages, presence of virus was confirmed by FAVN and RT-PCR after every 5th or 10th passages as well.

Virus titration studies indicated that, the titre stood around 104.0 TCID₅₀ up to 20 passages (Fig. 3) which increased substantially at 25th passage indicating the adaptability of the virus to PK-15 cells. This was followed by a gradual increase in titre until 40th passage ($10^{9.58}$ TCID₅₀). There after the titre decreased at 45th passage followed by a gradual increase from 50 passage until 75th passage conducted during 2017–2018. It is likely that minor variations could occur in virus titres during stabilization of the virus to the host system which could be the reason for the variations in virus titres between 40–50 passages. The results further indicated that the virus got adapted and

stabilized to PK-15 cells at around 40–45th passage.

Further to assess the attenuation status of the field virus, susceptible pigs were inoculated with the infected PK-15 cell lysate at 45th and 75th passage. In both instances, inoculated pigs were found to be healthy and no clinical signs were recorded such as rise in body temperature, petechial markings on the skin, huddling, weakness, drowsiness, anorexia and conjunctivitis characteristic of CSF (Sangeetha *et al.* 2018). However, presence of the virus in these animals was confirmed by demonstrating the presence of CSFV in nasal and conjunctival swabs collected 5 days post inoculation. This clearly indicated that, though the adapted virus was circulating in the pigs, it was not causing any clinical disease confirming that the virulent virus got attenuated at 45th passage. This attenuation persisted after 75 passages also.

It is concluded that, though the virus did not cause any disease after 45th and 75th passage, the virus titre was found to be high and got fixed around 40–45th passages. Hence the attenuated virus around this passage level can be used as a candidate virus for future vaccine production.

SUMMARY

Classical swine fever (CSF) is an economically important pig disease affecting rural pig farming and vaccines are not freely available for control. In the present study an Indian isolate of CSF virus was passaged 75 times in PK-15 cells. At different passages, presence of virus was confirmed by Fluorescent Antibody Virus Neutralization (FAVN) test and Reverse Transcription-Polymerase Chain Reaction (RT-PCR) for NS5B, E2 and 5'UTR genes. TCID₅₀ titers were found to range between 4.00 and 8.97 at 10th and 75th passages respectively. Back passage and pathogenicity studies in susceptible pigs, the natural host, indicated that the virus was found to be attenuated following PK-15 passages and did not produce any clinical signs after 45th and 75th passages.

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REFERENCES

Badasara S K, Mohan M, Sah V, Kumari P, Upmanyu V, Dhar P and Gupta V K. 2017. Replacement of animal model for

propagation of classical swine fever challenge virus by adaptation in the PK-15 cell line. *Journal of Animal Research* 7: 581–86.

Bhaskar N, Ravishankar C, Rajasekhar R, Sumod K, Sumithra T G, John K and Aishwarya J. 2015. Molecular typing and phylogenetic analysis of classical swine fever virus isolates from Kerala, India. *Virus disease* 26: 260–66.

Dhar P, Rai A, and Verma R. 2008. Adaptation of classical swine fever virus (lapinized strain) in PK-15 cells and confirmed by reverse transcription-polymerase chain reaction (RT-PCR) and fluorescent antibody technique (FAT). *Indian Journal of Animal Sciences* 78: 135–37

Greiser-Wilke I, Depner K, Fritzemeier J, Haas L and Moennig V. 1998. Application of a computer program for genetic typing of classical swine fever virus isolates from Germany. *Journal of Virological Methods* 75: 141–50.

Leifer I, Ruggli N and Blome S. 2013. Approaches to define the viral genetic basis of classical swine fever virus virulence. *Virology* 438: 51–55

Medhi P, Sarma D K and Sarmah R. 2012. Adaptation and propagation of classical swine fever virus in different cell lines. *Indian Journal of Animal Sciences* 82: 1281–84.

Moennig V, Floegel-Niesmann G and Greiser-Wilke I. 2003. Clinical signs and epidemiology of classical swine fever: a review of new knowledge. *Veterinary Journal* 165: 11–20.

Patil S S, Hemadri D, Shankar B P, Raghavendra A G, Veeresh H, Sindhoora B, Chandan S, Gagendragad M R, Sreekala K and Prabhudas K. 2010. Genetic typing of recent classical swine fever isolates from India. *Veterinary Microbiology* 141: 367–73.

Paton D J, McGoldrick A, Greiser-Wilke I, Parchariyanon S, Song J Y, Liou P P, Stadejek T, Lowings J P, Björklund H and Belak S. 2000. Genetic typing of classical swine fever virus. *Veterinary Microbiology* 73: 137–57.

Rathnapraba S, Kumanan K, Vijayarani K, Palanisammi M and Gunaseelan L. 2014. Molecular typing of Classical swine fever viruses from field outbreaks in Southern India. *Indian Journal of Animal Sciences* 84: 723–27.

Rathnapraba S, Vadivoo V S, Manoharan S, Logesh K and Kumanan K. 2012. Molecular detection of classical swine fever from a field outbreak in Tamil Nadu. *Indian Journal of Veterinary Pathology* 36: 22–27.

Sangeetha R, Hemalatha S, Ramesh A, Vijayarani K and Kumanan K. 2018. A Classical swine fever outbreak-A recent postulation of laboratory pathogenicity. *Indian Veterinary Journal* 95: 9–13.

Sarma, D K, Mishra N, Vilcek S, Rajukumar K, Behera S P, Nema R K, Dubey P, and Dubey S C. 2011. Phylogenetic analysis of recent classical swine fever virus (CSFV) isolates from Assam, India. *Comparative Immunology, Microbiology and Infectious Disease* 34: 11–15.

Vadivoo V S, Rathnapraba S, Ramesh A, Logesh K, Manoharan S, Raja A and Kumanan K. 2017. Adaptation of Classical swine fever virus in cell culture system and confirmation by electron microscopy. *Indian Veterinary Journal* 94: 38–40.