



Replacement of Animal Model for Propagation of Classical Swine Fever Challenge Virus by Adaption in the PK-15 Cell Line

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ABSTRACT

Classical swine fever (CSF) challenge virus has been adapted in PK-15 cell line from infected splenic suspension of the challenge virus maintained hitherto by pig to pig passages. Confirmation of viral presence was done by reverse transcription-polymerase chain reaction (RT-PCR) and Fluorescent Antibody Technique (FAT). A reasonably good titre of $10^{6.5}$ TCID₅₀/ml was obtained at 6th passage level. The cell culture adapted challenge virus at a dose of $10^{5.0}$ TCID₅₀ produced CSF symptoms in pigs from 2nd days post infection (dpi) onwards and succumbed to the infection between 11-12 dpi. Cell culture adapted CSF challenge virus offers advantage to inoculate exact virus particles over the traditional tissue suspension (20% w/v) in potency testing. Adapted challenge virus will replace the use of pigs for propagation of challenge virus; hence follows 4 R's (replacement, reduction, refinement and rehabilitation) principle. This challenge virus can be attenuated by further serial passages and can be used to develop indigenous live attenuated cell culture based vaccine.

Keywords: CSF challenge virus, adaptation, PK-15 cells, potency testing

Classical swine fever (CSF) is a highly contagious viral disease of pigs and wild boars causing significant economic losses in swine industry worldwide (Leifer *et al.*, 2013; Lindenbach *et al.*, 2013; Rossi *et al.*, 2015; Gong *et al.*, 2016). In India, disease is endemic and several outbreaks have been reported from most of the states (Patil *et al.*, 2010; Nandi *et al.*, 2011; Sarma *et al.*, 2011; Chander *et al.*, 2014). A Lapinized swine fever vaccine is being used in the country since 1963-64 for control of the disease. The vaccine is efficacious and strict QC test, particularly potency test, is essential for every batch of the vaccine produced by any manufacturer.

The efficacy of swine fever vaccine is tested by the challenge of vaccinated and control pigs by swine fever challenge virus to calculate the PD₅₀ content of the vaccine (Indian Pharmacopoeia, 2014). A CSF challenge virus is currently being used in the Biological Standardization

Division, IVRI and in other State Biological Units of the country for potency tests of CSF vaccines and the virus has been characterized biologically as well as at molecular level (Kumar *et al.*, 2014). The challenge virus is presently maintained by regular passages in pigs and a 20% splenic suspension derived from experimentally infected animals is used for potency testing. The disadvantages of this procedure are need of additional pigs every year for propagation of challenge virus and splenic tissue suspension cannot be specified exact number of viral particles in potency test. Therefore, replacement of pig model by *in vitro* method (PK-15 cell line) for propagation of CSF challenge is an essential requirement as per the guidelines of 4R's (replacement, reduction, refinement and rehabilitation) principles and strict regulations for use of animals in experimental purposes. Another advantage of this works is the use of exact number of virus in term

of TCID₅₀ will enable consistent efficacy testing of the vaccine batches.

In India, there are many reports on adaptation or passaging of CSF virus field isolates in PK-15 cells and confirmation by RT-PCR and/or FAT (Sarma *et al.*, 2007; Medhi *et al.*, 2012; Rathnapraba *et al.*, 2012). However, those cell culture adapted CSF isolates have never been checked in pigs for virulence. In the present work, identity of the challenge virus during adaptation in PK-15 cells were confirmed by 5'UTR, E2, NS5B genes based RT-PCR, Fluorescent Antibody Technique (FAT) using CSF specific monoclonal antibody and by re-passaging in pigs to check for virulence.

MATERIALS AND METHODS

Cell line

Mycoplasma and pestiviruses free PK-15 cells available in Division of Biological Standardization were used to adaptation and titration of CSFV challenge virus. The PK-15 cells were cultured in Eagle's Minimum Essential Medium (EMEM, Sigma-Aldrich, USA) supplemented with 10% foetal bovine serum (HyClone, USA, pestiviruses free) at 37°C in 5% CO₂.

Experimental animals

Three seronegative, 8-12 weeks old, Landrace cross pigs were procured from Swine Production Farm, Indian Veterinary Research Institute (IVRI), Izatnagar. The animal experiment was approved by the Committee for the Purpose of Control and Supervision of Experiments on Animals (CPCSEA), Ministry of Environment and Forests, New Delhi.

Virus isolation

The CSF challenge virus is being maintained in the Division of Biological Standardization through regular pig to pig passages. Tissue suspension of CSF challenge virus (20% w/v, which is equivalent to 10^{5.0} PID₅₀/4 ml) was used for experimental reproduction of the disease in a pig. Leukocytes (approx 2 millilitre) were isolated from 10ml blood of infected pig at peak temperature (108°F). One ml of the leukocytes were used as virus inoculums for

infection of PK-15 cells (3 × 10⁶ cells in 25 cm² flasks) by co-cultivation method. The cells were incubated at 37°C in a CO₂ incubator for 66 hrs and subsequently stored at -80°C. The cells (5 ml) were subjected to two cycles of freezing-thawing and the cell debris was removed by centrifugation at 15000 rpm for 10 min at 4°C. One ml of the supernatant was used for subsequent serial passages up to third passage of the virus in 25 cm² flasks. At third passage level, the virus was confirmed by RT-PCR and its titre was determined by FAT. From 4th passage onwards, the virus was inoculated in 75 cm² flasks at 0.006 multiplicity of infection (MOI). Virus was produced in bulk at 6th passage level and was again confirmed by RT-PCR and its titre was determined by FAT.

Reverse transcription-polymerase chain reaction (RT-PCR)

Total RNA was isolated from cell culture flasks at 3rd and 6th passages of infected PK-15 cell monolayer using TRI Reagent (Sigma-Aldrich, UK) and cDNA were synthesized using QuantiTect Reverse Transcription Kit (QIAGEN, USA). RT-PCR was carried out using published primers specific for three regions of the CSF viral genome viz., 5'UTR, E2, NS5B to detect viral RNA (Table 1).

Table 1: Primers used for amplification of 5'UTR, NS5B and E2 regions of CSFV

Region	Sequence	Product size (bp)	References
5'UTR	F CTAGCCATGCCCWYA GTAGG	421	Greiser- Wilke <i>et al.</i> , 1998
	R CAGCTTCARYGTTG ATTGT		
E2	F AGRCCAGACTGGTGGC CNTAYGA	671	Paton <i>et al.</i> , 2000
	R TTYACCACTTCTG TTCTC		
NS5B	F GACACTAGYGCAGG CAAYAG	449	Paton <i>et al.</i> , 2000
	R AGTGGGTTCCAGGA RTACA		

Abbreviations: 5'UTR=Un-translated region

Reactions were set in 25µl volume with 12.5µl of 2x Dream Taq PCR master mix (Thermo scientific), 10 pmol each of forward and reverse primer, cDNA (100ng) and final volume was adjusted with nuclease free distilled water. RT-PCR was performed using a initial denaturation temperature of 94°C for 5 minutes followed by 35 cycles of denaturation at 94°C for 30 sec, annealing temperature for 30 sec and extension of 72°C for 45 sec. Final extension was carried out at 72°C for 10 minutes. Annealing temperature (Ta) was standardized at 56°C for NS5B and 50°C for 5'UTR and E2 gene amplification. The amplified products were electrophoresed in 1.5 % agarose gel in 1X TAE buffer with ethidium bromide (0.5 µg/ml) and visualized by a gel documentation system (Bio Rad, USA).

Fluorescent Antibody Technique (FAT)

Titration of cell culture adapted CSF challenge virus was done at 3rd passage level in PK-15 cells in cover slip cultures in 12 well tissue culture plates. Cells ($5 \times 10^5/900 \mu\text{l}$) were simultaneously infected with 100 µl of each virus dilutions (10^{-1} to 10^{-6}) in duplicates and incubated at 37°C for 66 hrs. Two uninfected controls were also kept. FAT was performed after 66 hrs post infection for detection of viral particles in infected cells using a protocol developed by Dhar *et al.* (2008) with modifications. Triton X -100 (0.1%) was used in place of Tween-80 for permealization of the cells. Secondly, rabbit hyper immune serum was replaced by FITC labelled CSFV MAb (BIO 272, Bio-X Diagnostics) and manufactures protocol was followed. Virus titres were calculated according to the method of Reed and Muench (Reed and Muench, 1938) and expressed as TCID₅₀/ml. Similarly, FAT was conducted at 6th passage level virus as mentioned above.

Animal inoculation and biological characterization of cell culture adapted CSF challenge virus

To assess the virulence of the cell culture adapted challenge virus at 6th passage level, two 8-12 weeks old susceptible pigs were inoculated with $10^{5.0}$ TCID₅₀ viruses by subcutaneous and intramuscular routes keeping one naive animal in the group to examine contact-mediated transmission. Animals were observed daily for rectal temperature, appearance of systemic and local clinical signs. Blood, nasal, and rectal swabs were collected every

2-3 dpi for isolation of viral RNA for RT-PCR by 5'UTR, E2, and NS5B regions primers as described earlier. Total blood leukocytes were enumerated every 2-3 dpi interval by haemocytometer using the standard method. The pigs were observed for CSF symptoms and the course of infection. Tissue samples (spleen, kidney, tonsils, and mesenteric lymph nodes) were tested by RT-PCR in case of death or sacrifice of the animal following infection.

RESULTS AND DISCUSSION

The virus identity of adapted virus at 3rd and 6th passage level was confirmed by RT-PCR targeting 5' UTR, E2 and NS5B gene fragments of CSFV. Expected amplification products of 421, 671 and 449 bp were obtained by 5' UTR, E2 and NS5B PCRs respectively (Fig. 1) as reported earlier (Greiser-Wilke *et al.*, 1998; Paton *et al.*, 2000).

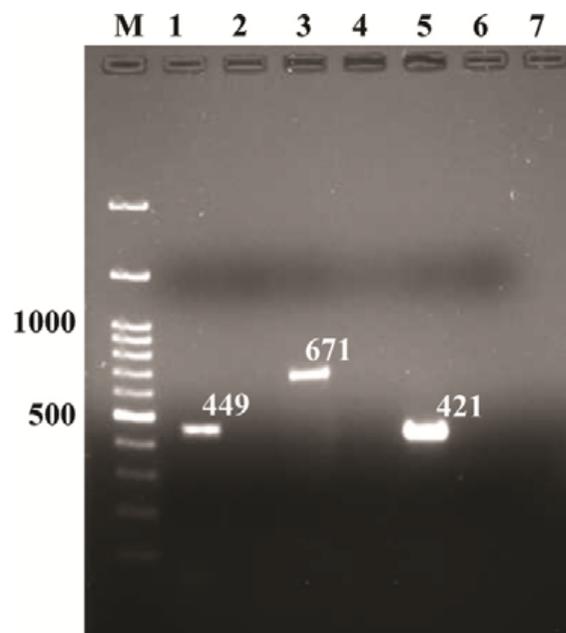


Fig. 1: Amplified products of 6th passage CSFV by RT-PCR. NS5B (Lane 1), NTC (Lane 2), E2 (Lane 3), NTC (Lane 4), 5' UTR (Lane 5), NTC (Lane 6) and Lane M: 100 bp DNA Ladder (Br Biochem Life Sciences Pvt. Ltd.)

The titre of the cell culture adapted CSF challenge virus in PK-15 cell line at third passage was $1 \times 10^{5.5}$ TCID₅₀/ml and the titre increased to $1 \times 10^{6.5}$ TCID₅₀/ml at 6th passage, as detected by direct fluorescent antibody technique (Fig. 2). The increase in the titre from lower to higher passage level indicated that challenge virus was adapted in PK-15 cells.

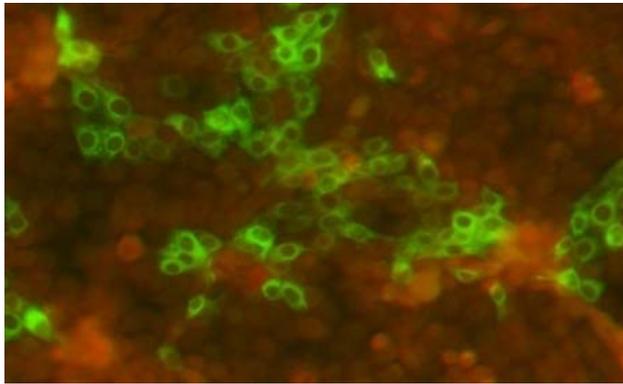


Fig. 2: Cell culture adapted CSF challenge virus (6th passage) infected PK-15 cells showing bright green cytoplasmic fluorescence foci by direct fluorescent antibody technique

Pigs showed typical clinical signs of CSF and thermal reaction after inoculation of cell culture adapted challenge virus. Fever was observed from 2 dpi and peak temperature was observed at 6 dpi (Fig. 3).

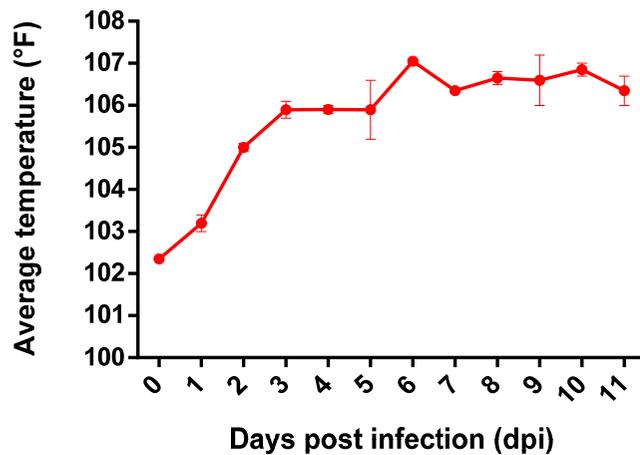


Fig. 3: Average daily body temperatures (°F) of infected pigs with cell culture adapted CSF challenge virus (6th passage) at different dpi

From 3rd dpi, animals had reduced feed intake, depression, reluctant to walk and on 5th dpi, the animals were completely off fed followed by diarrhoea, ataxia and swaying movement of the hindquarter. On 7th dpi, animals became recumbent with posterior paresis and vigorous belly movement. After 8-10 dpi, the animals had respiratory distress/dyspnoea, shivering, convulsions, purulent conjunctivitis and purple cyanotic discoloration especially at ear tips, muzzle, ventral abdomen, medial side

of legs and testes as reported earlier (Moennig *et al.*, 2003; Barman *et al.*, 2010). The severity of the above clinical signs increased gradually and consequently animals died at 11-12 dpi. The naive animal housed with challenged pigs was also started showing CSF symptoms from 10 dpi with rise in temp, off fed, recumbent etc and was sacrificed on 14 dpi when symptoms became severe.

Total blood leukocyte count (TLC) decreased from 21700 cells/ μ l on the day of infection to as low as 8400 cells/ μ l at 5 dpi and 4750 cells/ μ l at 11-12 dpi indicating marked leucopenia, which is most obvious in CSF infections. Leucopenia and immunosuppression are characteristics to virulent challenge virus as indicated earlier (Moennig *et al.*, 2003; Donahue *et al.*, 2012; OIE, 2014). The naive animal also showed reduced TLC count from 10 dpi (9650 cells/ μ l) which coincided with rise in temp and other CSF symptoms and has marked leucopenia on 14 dpi, when the TLC was only around 3800 cells/ μ l.



Fig. 4: Dead carcass with cyanotic discoloration of skin throughout body

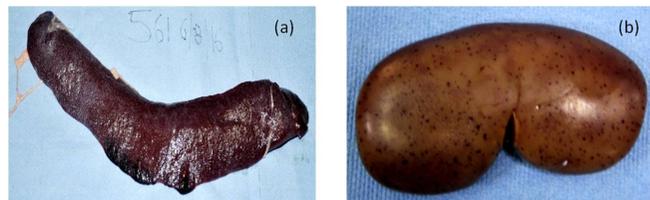


Fig. 5: (a) Enlarged and congested spleen with multiple splenic infarcts (b) Pinpoint haemorrhages in the sub capsular region of the kidneys (turkey egg kidney appearance)

Post Mortem examination of dead pigs showed purple cyanotic discoloration of the skin (Fig. 4), enlarged and haemorrhagic lymph nodes, haemorrhagic tonsils, turkey

egg kidney, multifocal splenic infarcts (Fig. 5), congested GI tract and mesenteric blood vessels, which suggests the acute nature of the disease (Moennig *et al.*, 2003; Everett *et al.*, 2010; Malswamkima *et al.*, 2015). Adapted CSF challenge virus is a virulent strain based on severe pyrexia and acute clinical symptoms in the infected pigs (Mittelholzer *et al.*, 2000; Asala *et al.*, 2010; Kumar *et al.*, 2014).

Viral RNA was detected by RT-PCR amplified products of all the three genes in blood of the inoculated pigs at 5 dpi and persisted till death. Viral RNA was detected in blood at 6-9 dpi by RT-PCR based on 5'UTR, as reported by Raut *et al.* (2015). Nasal and rectal swabs from inoculated pigs were positive for viral RNA at 2 days post viraemia (7 dpi). Blood of in-contact animal was positive by RT-PCR at 10 dpi but nasal and rectal swabs were negative by RT-PCR till 14 dpi and then animal was euthanized at 14 dpi. Tissues such as spleen, kidney, tonsil and mesenteric lymph node of inoculated as well as in-contact animal were found positive by RT-PCR (Fig. 6).

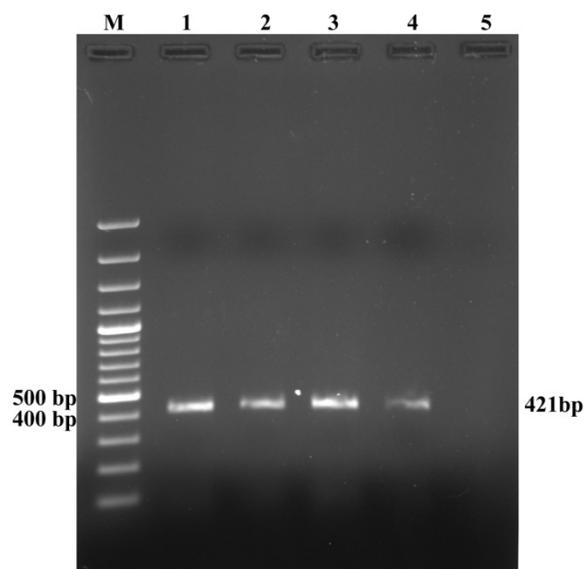


Fig. 6: Amplified products of CSFV by RT-PCR of 5' UTR in different tissues. Lane 1: spleen; Lane 2: kidney; Lane 3: mesenteric lymph node; Lane 4: tonsil; Lane 5: NTC and Lane M: 100 bp plus DNA Ladder (Thermo Fisher Scientific)

These results indicate that cell culture adapted virus was easily transmitted from infected animals to in-contact animal and in-contact animal showed similar disease pattern.

CONCLUSION

The present study showed that the cell culture adapted CSF virus at 6th passage retained its virulence showing characteristic clinical and pathological signs of CSF on experimental infection in pigs. The identity of adapted challenge virus was confirmed by 5'UTR, E2, and NS5B genes based RT-PCRs and fluorescent FAT using CSF specific monoclonal antibody. This cell culture adapted challenge virus can be used in potency testing of swine fever vaccines and there would be no need to maintain the challenge virus by regular pig passages, thereby reducing the use of animals and their sufferings. Also, use of the cell culture challenge virus would offer a great advantage to inoculate the exact amount of the virus (10^5 TCID₅₀) for potency test of CSF vaccines. This challenge virus can be further attenuated by serial passages in PK-15 cells and thus development of an indigenous live attenuated cell culture based vaccine seems plausible. Last but not the least, the present study has opened a vast scope to study host-pathogen interactions in-vitro for CSF virus.

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