Characterization of cytopathogenicity of classical swine fever virus isolate induced by Newcastle disease virus

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Abstract Classical swine fever virus (CSFV), the causative agent of classical swine fever, belongs to the family *Flaviviridae* and genus *Pestivirus*. Some pestiviruses exhibit cytopathic effect in cell culture but exact phenomenon is unknown. Over expression of NS2-3 gene, presence of defective interfering particle and exaltation of Newcastle disease virus (END) phenomenon could be the reasons of cytopathogenicity. In the present study, a CSFV isolate exhibiting cytopathic effect (CPE) in Madin-Darby Canine Kidney (MDCK) cell line was characterized. To characterize cytopathogenicity of such isolate, END test was carried out. Interference of Newcastle disease virus (NDV) in MDCK adapted CSFV was confirmed by RT-PCR and virus neutralization test. Absence of CPE and NDV specific nucleic acid after neutralization confirmed the induction of CPE by NDV. Further, identity of the CSFV isolate in MDCK cell line by immunoperoxidase test, immunoblotting and RT-PCR post NDV neutralization established the virus replication without CPE (non-cytopathic isolate). Findings suggest that, there could be a chance of mixed infection of both CSFV and NDV in the piglet from which the sample was collected for virus isolation.

Keywords CSF · Virus · Cytopathogenicity · END phenomenon · VNT · MDCK

Introduction

Classical swine fever (CSF) is economically important contagious viral disease of swine, caused by the classical swine fever virus (CSFV) [18]. The causative agent is a small (40–60 nm) enveloped, single stranded ribonucleic acid (ssRNA) virus with positive polarity [17]. CSFV belongs to the *Pestivirus* genus of the *Flaviviridae* family [13, 27, 30] having one serotype [19]. Virus isolation is still a “gold standard” for identification of any virus. CSFV isolation/adaptation has been reported in both homologous [16, 22, 29] as well as in heterologous [21, 22, 26] cell lines. Based on replication characteristics in cell-culture, pestiviruses can be divided into two biotypes, noncytopathogenic and cytopathogenic. Majority of isolates do not exhibit cytopathogenicity in cell-culture [16] but, few isolates have been reported to produce CPE [5, 9, 11]. The mechanism responsible for the CPE is poorly understood. There are different schools of thought like over expression of NS2-3 [2], presence of defective interfering (DI) particle [14, 15] and exaltation of Newcastle disease virus (END) phenomenon [7, 10]. To elucidate the cytopathogenicity in heterologous cell-line, a “cytopathic” CSFV isolate adapted in MDCK cell line characterized in this study.

Materials and methods

Virus

The original sample (Spleen) was collected during an outbreak at Agartala, Tripura (2009) from a pig suspected for CSF infection. The sample was isolated in MDCK cells and kept in CSF repository. The adapted virus isolate at passage level twenty five (CSFV P-25) obtained from

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DBT-CSF repository, Division of Virology, IVRI campus, Mukteshwar was used in present study.

Cell line

The MDCK cell line at passage level sixty three maintained in the repository were used for further passaging and growth of CSFV isolate. The cells were propagated in Eagle's minimum essential medium (EMEM) containing 200 mM glutamine, 7.5 % (w/v) sodium bicarbonate and $1 \times$ antibiotic and antimycotic solution supplemented with 10 % horse serum. Pestivirus (CSFV and BVDV) free status of cell-culture components (cell lines, serum, media and trypsin) were checked using standard primers by reverse transcription-polymerase chain reaction (RT-PCR) (Data not shown).

Freshening of MDCK adapted CSFV isolate

The CSFV isolate was freshened-up in MDCK cells for further passage (up to 35th passage) by co-cultivation method. Following infection (one m.o.i-multiplicity of infection), first media change was done at 48 h post infection (hpi) with maintenance medium (MM; EMEM having 2 % horse serum) and harvested at 4th days post infection (dpi) after observing 60–80 % of CPE. After two rounds of freezing and thawing, the harvested virus was used for further studies.

Characterization of CSFV isolate

Virus neutralization test (VNT)

VNT was carried out in 96-well plates as per the technique given in European Union (EU) diagnostic manual for CSF (2007), http://www.tiho-hannover.de/uploads/media/technical_annex_draft_2007_02.pdf with few modifications. The reference positive CSFV antiserum available in the repository was heat-inactivated at 56 °C for 30 min. Both virus and antiserum were diluted two-fold (1:2–1:1024 dilutions) and mixture was incubated at 37 °C for 1 h (hour). Then in 96-well culture plate, 100 µl of mixture was added per well on to preformed monolayer of MDCK cells keeping 4 wells per series. Virus control (received only virus and no serum) and serum control (having only serum and no virus) wells were also kept. The plate was incubated at 37 °C with 5 % CO₂ tension. Medium was changed on 2nd day with MM and culture was observed for CPE till 4th day.

Reverse transcription-polymerase chain reaction (RT-PCR)

Total RNA was isolated from cell culture lysate using Tri reagent LS. In this method, modified Guanidium-phenol–

chloroform procedure [4] was followed. Quantity and purity of extracted RNA was checked by spectrophotometer based on the UV-light absorbance at 260/280 nm wavelengths. Out of total RNA isolated from cell culture lysate, approximately 1000 ng was reverse transcribed using RevertAid TM First Strand cDNA Synthesis Kit (M/s MBI Fermentas Life Sciences, Maryland, USA).

The primers and PCR conditions applied for amplification of 5'UTR gene; 150 bp (sense: 5'GGACAGTCGTCA GTAGTTCG3', antisense: 5'CTGCAGCACCCTATCAGG TC3') and E2 gene; 171 bp (sense: 5'CTGTGGCTAATA GTGACCTAC3', antisense: 5'CATTCTTTATGGGCTC ATC3') of CSFV isolate were as per the published Ref. [8]. The stepwise cyclic conditions were: step I, 1 cycle, 94 °C for 3 min; step II, 35 cycles, 94 °C for 30 s, 55 °C for 30 s, 72 °C for 30 s; step III, 72 °C for 10 min. The PCR products were analyzed by electrophoresis using 1.5 % agarose gel along with molecular weight marker.

Immunoperoxidase test (IPT)

This test was carried out using both CSFV antiserum and specific Monoclonal antibody (mAb, directed against E2 protein, Bio-X diagnostics, Jemelle-Belgium). The test was performed as per the manufacture's protocol with little modifications. Briefly, 25 cm² flask of MDCK cell was sub-cultured and co-infected with virus keeping one m.o.i. 500 µl/well of infected cell-suspension was seeded to 24 well-plates (one each for antiserum and mAb) and incubated for 4 days at 37 °C under 5 % CO₂ tension. The wells for healthy cell control were also kept. Medium was changed using MM on third day and plate was harvested for staining on fourth day. The Culture medium was removed thoroughly from the wells intended for staining. The cells were fixed with 2 % paraformaldehyde (one ml per well) at 25 °C for 15 min followed by washing thrice with 1X PBS. 200 µl of mAb (1:40 dilution with PBS) was added to each well, incubated at 25 °C for 1 h and washed thrice with 1X PBS. Similarly, antiserum (1:20 dilution with PBS) plate was also treated. Thereafter, 200 µl of rabbit anti-mouse IgG HRPO conjugate (Sigma-Aldrich, St. Louis, USA) at 1:200 dilution with PBS was added to each well in both the plates and incubated at 25 °C for 1 h. Once again, wells were washed thrice with 1X PBS. Finally, color was developed using 200 µl of 3-Amino-9-Ethyl Carbazole (AEC) at 25 °C. Plate was observed under microscope for color development.

Immunoblotting

The assay was carried out as per Sambrook et al. (2001) [25]. Both the supernatant and cell lysate of CSFV infected MDCK cell was used for the assay. After treatment with

sample buffer, samples were run into gel along with standard pre-stained protein marker. Electrophoresis was carried out at a constant voltage at 130 V. After completion of electrophoresis, gel was transferred to Hybond nitrocellulose membrane (NCM) (M/s Pharmacia Biotech, USA) using semi-dry blot system (M/s ATTO corporation, Japan). A constant current of 15 V was applied for 45 min to transfer the gel image on NCM. After transfer, the gel was removed and membrane was blocked overnight at 4 °C with blocking buffer (5 % skim milk powder in PBS). The membrane was washed thrice with washing buffer (Phosphate buffer saline, pH 7.4 with tween-20, PBS-T). Immunodetection of the blotted proteins was carried out using CSFV specific MAb against E2 protein (Bio-X diagnostics, Jemelle-Belgium) (1:20 dilution in blocking buffer). After 1 h of incubation, the membrane was washed and incubated at 37 °C with anti-pig IgG HRPO conjugate (M/s Sigma-Aldrich, St. Louis, USA) (1:2000 dilution) for 1 h. The unbound conjugate was removed after washing. Colour reaction was carried out with DAB (M/s Sigma-Aldrich, St. Louis, USA) using 1 mg/ml dilution in PBS. The color reaction was stopped with excess distilled water and finally documented.

END test

This test was conducted to see the presence of NDV, which could enhance the multiplication/replication of CSFV to produce CPE in cell-culture. To do so, the identity of virus was checked by amplifying fusion (F) gene fragment and VNT.

RT-PCR of NDV

RNA isolation and cDNA synthesis was done similar to CSFV as described earlier. To check the viral nucleotide, PCR was conducted as per the published Ref. [28]. In brief, PCR reaction was set alike CSFV in 25 µl volume of 0.2 ml PCR tube. The thermal profile was kept as: initial denaturation (94 °C for 5 min) and cyclic denaturation (94 °C for 1 min), annealing (58 °C for 1 min), extension (72 °C for 1 min) for 30 cycles with final extension (72 °C for 5 min). The amplified product was resolved by 1.5 % agarose gel electrophoresis.

VNT of NDV

Neutralization test was carried out in 96-well plates as per the technique given in European Union (EU) diagnostic manual with few modifications. The heat-inactivated (56 °C for 30 min) reference positive serum against NDV was used. Procedure for neutralization was similar to VNT

as mentioned for CSFV. Plate was observed for presence/absence of cytopathic effects and documented.

Results

Freshening and characterization of CSFV isolate

Virus freshened in MDCK cell line exhibited CPE characterized by rounding, clumping and detachment of cells on 2nd, 3rd and 4th dpi, respectively (Fig. 1a–h). No virus specific changes were observed in mock infected cells. Identity of the CSFV isolate was confirmed by amplifying the 5'UTR (150 nts) and E2 (171 nts) gene specific fragments (Fig. 2a–b).

In neutralization test, antiserum against CSFV neutralized the virus at 1:32 dilutions and no viral gene could be detected by RT-PCR up to this dilution. However, CPE was very well appreciable even post neutralization. To check the stability and reversion of neutralized virus, five back passages were given and virus was found stable (result not shown).

Characterization of NDV and assessment of END phenomenon

Amplification of F-gene specific to NDV was observed post RT-PCR (Fig. 3) in CSFV isolate. Identity of virus was established by neutralization with NDV reference serum. CPE could not be observed up to the 1:16 dilutions. This was further confirmed by post-neutralization RT-PCR (Fig. 4a). Similar to CSFV, stability and reversion of neutralized virus was also checked for NDV. It was found stable without showing any CPE after five back passages. Replication of CSFV alone post NDV neutralization was established by E2 gene based PCR (Fig. 4b), western blot (Fig. 5) and IPT. Positive IPT signal (both in CSFV antiserum and mAb) evidenced by development of dark brown colour at infected foci was noticed (Fig. 6a–b). Presence of non-cytopathic population was achieved after neutralizing the cytopathic CSFV isolate with NDV antiserum.

Discussion

CSFV usually does not produce CPE in cell-culture but, cytopathic isolates are also reported. The exact reason for cytopathogenicity is largely unknown; however role of NS2-3, presence of DI particles and END phenomenon have been described. In this study, a CSFV isolate adapted in MDCK cell lines was found to be cytopathic in nature. The reason for cytopathogenicity was explored both at virological and molecular level. After infection of virus to

Fig. 1 **a–h** CPE in Madin-Darby Canine Kidney (MDCK) cell-line infected with cytopathic CSFV (P25): **a, c, e** and **g**: Healthy cells (1st to 4th dpi); **b** look alike healthy cell (1st dpi); **d** rounding (2nd dpi); **f** clumping (3rd dpi) and **h** detachment (4th dpi) of cells

MDCK cell, CPE appeared which was characterized by rounding, clumping and detachment of infected cells in focal area. Similar finding has been reported by Kosmidou (1998) [9] but in homologous cell lines (Swine testis epitheloid cells). The idea behind adapting the CSFV in heterologous cell-line is to avoid the chance of adventitious viruses of porcine origin. Use of heterologous cell for CSFV has been reported by Petkova et al. (1986) [20], who attempted to grow bovine *Pestivirus* in heterologous swine

kidney cell line and passaged up to 15 passages. Rivero et al. (1988) [22] used RK-13 (rabbit kidney) for adapting the lapinized CSFV. The C-strain virus was adapted to Bovine faetal muscle (BFM) with good virus yield [21]. Recently, CSFV Agartala isolate was adapted both in MDCK and RK-13 cell lines. CSFV isolate adapted to MDCK cells was found to be cytopathic in nature [26]. Identity of this isolate has been done by RT-PCR and sequencing (Accession No.: GU969035, JX556415 and

Fig. 2 **a, b** RT-PCR targeting 5'UTR and E2 genes of representative passages (P25, 30 and 35) of CSFV isolate adapted to MDCK cells: 5'UTR (**a**) and E2 (**b**) based amplification of representative passages

of CSFV isolate; *LVV* Lapinised vaccine virus (positive control), *M* 100 bp DNA ladder plus Marker (MBI, Fermentas), *NTC* Non template control

Fig. 3 Presence of NDV (356 bp) in CSFV isolate (P 25–35): *M* 100 bp ladder, *L1* P-25, *L2* P-30, *L3* P-35, *L4* Positive control, *NTC* Non-template control

JX556416). This isolate at passage level 25th is characterized for cytopathogenicity in current study.

The identity of CSFV was re-confirmed by RT-PCR targeting 5'UTR and E2 gene and found specific. CPE produced by the virus in cell culture have been reported to

be due to DI particles [14]. DI particles largely produced by deletion mutation which lack structural proteins C, E^{ms}, E1 and E2 [1, 9, 14], important for the immune response. In this study, we could successfully amplify the E2 region of the said virus, which was indicative of absence of such defective virus. However, presence of both defective and full length viral population in same culture has also been reported [1, 14]. Such study has not been done and would have added advantage in this investigation, though presence of structural gene had given indication of absence of DI-particles. Investigation was further undertaken for other plausible reason of CPE. According to the END phenomenon, a conspicuous CPE and large quantities of NDV is produced irrespective of the age of porcine cell culture, when inoculated in porcine cells already infected with virulent strain of swine fever virus. Hence, attempts were made to conduct END test to understand the cytopathogenicity of the isolates. In this direction, successful amplification of NDV F-gene in CSFV infected MDCK cell was achieved. Our finding of NDV interference is in agreement with Kumagai et al. (1960) [10], who reported

Fig. 4 **a, b** Post neutralization RT-PCR of NDV (**a**) and CSFV (**b**). NDV couldn't be detected up to 1:16 dilution, whereas CSFV gene amplified in the same dilution. 1:8 to 1:64: virus-serum dilutions used

for neutralization, *M* 100 bp DNA ladder, *VC* Virus control, *+Ve C* positive control, *NTC* non-template control

Fig. 5 Post-neutralization Western blot analysis of CSFV immunogenic protein (E2) using MAb.: *M* Prestained protein marker, *Lane 1* CSFV infected MDCK cell supernatant, *Lane 2* mock infected MDCK cell supernatant, *Lane 3* mock infected MDCK cell-lysate, *Lane 4* CSFV infected MDCK cell-lysate

that interference with the multiplication and CPE production in hog cholera infected tissue culture was due to presence of NDV (END phenomenon). Later on many studies in this direction were carried out to employ this CPE in titration and pathogenicity of the virus [2, 6, 23, 24]. It has also been reported that, END⁺ (Exaltation of NDV phenomenon) virus which did not produce CPE in cell-culture had strong signal of NS2-3 gene in END⁺ virus infected cells [2]. Later, involvement of NS3 gene in cytopathogenicity induced by apoptosis has also been reported [31]. However, study on non-structural genes in current study has not been undertaken.

Interference of NDV to CSFV was confirmed by RT-PCR and VNT. To get freedom of NDV, neutralization test was performed by using NDV antiserum. Neutralization of NDV and identity of CSFV was confirmed in post-neutralization study by RT-PCR (NDV & CSFV gene), immunohistochemistry and immunoblotting. Post-neutralization study by RT-PCR revealed the absence of NDV gene, whereas CSFV specific E2 gene showed amplification. Further, detection of

Fig. 6 Post-neutralization IPT staining of MDCK cells infected with CSFV: IPT showing dark brown colour in infected and no stain in mock infected cells by MAb (**ai**, **ii**) directed against CSFV E2 (1:40)

and CSFV antiserum (1:20) (**bi**, **ii**), antimouse conjugate (1:200), 3-Amino-9-Ethyl Carbazole (AEC) as chromogen and 3 % H₂O₂ as substrate

CSFV by immunohistochemistry and analysis of immunodominant protein (E2-protein) in immunoblotting established the CSFV replication post neutralization in MDCK cell. Based on these findings, it was suggested that CSFV isolate showing CPE in MDCK was primarily due to NDV and further, replication of CSFV in MDCK cell line without showing CPE, establish non-cytopathic nature of this isolate. Since, there are reports of isolation of NDV from swine [3, 12]. There is chance of mixed infection of piglet from which the sample was obtained.

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