Adaptation of Classical Swine Fever Virus in Cell Culture System and Confirmation by Electron Microscopy

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Abstract

Adaptation studies of field isolates of Classical Swine Fever (CSF) virus was carried out in Porcine Kidney (PK 15) cell line. Bulk culture of the virus at 25th passage was subjected to purification and concentration by ultracentrifugation and also confirmation by RT-PCR using NS5B primers. The Transmission Electron Microscopy (TEM) studies of CSF virus revealed 40-50 nm virus like particles which were surrounded by an asymmetrically arranged poorly defined sac like membrane. This was taken as confirmation for the adaptation of the CSF virus in PK 15 cell line.

Key words: classical swine fever virus; PK 15 cell line; Ultracentrifugation; TEM analysis

Classical swine fever virus (CSFV) is the etiological agent of a highly contagious disease of pigs. Together with bovine viral diarrhoea virus and border disease virus CSFV belongs to the genus Pestivirus within the family Flaviviridae. CSF is a serious, economically damaging disease of swine which can spread in an epizootic form as well as establish enzootic infections in domestic and wild pig populations. The RNA genome is approximately 12.5 kb and contains a single large open reading frame (ORF), which is flanked by 5' and 3' nontranslated regions (NTRs). The virus can be adapted in porcine cell lines and the “EU Diagnostic Manual for CSF Diagnosis”, recommends the use of the permanent cell line PK15 derived from porcine kidney.

(Grummer et al., 2006). The present study describes the adaptation of two field CSFV isolates in PK15 cell and its purification and concentration for confirmation by electron microscopy.

Materials and Methods

Tissue samples received from CSFV suspected outbreaks that were confirmed by multiplex panpest RT-PCR which differentiates CSFV and other pestivirus infections of swine (de Arce et al., 2009) was used for isolation and adaptation of the virus in PK 15 cell lines. For infection of the Pk15 cell line ATCC(CCL33) one in ten dilution of 10 per cent w/v tissue suspension prepared was smeared over the PK15 cell monolayer and incubated at 37°C for one hour for viral adsorption purpose. Then the inoculum was removed and the cell sheet was again washed with MEM and replaced with sterile maintenance medium (MEM with 2% FBS) and incubated at 37°C for 5 to 10 days. Since the CSF virus is a non-cytopathic virus, no cytopathic changes could be observed except for elution of the infected PK15 cells. Five blind passages were carried out before confirming the virus by RT-PCR by amplifying with NS5B primers (Ruggli et al., 1996). The two CSFV field isolates were further passaged in PK15 cells for 25 times and confirmed for the presence of CSF virus by RT-PCR using the 5'UTR nested primer at every 5th passage.

Purification of the virus was done at 25th passage as per the method described by Gould and Clegg (1985) with modifications. The cell associated CSF virus was extracted from PK 15 cell culture after four days of post infection by repeated freeze thawing for 5 times. The tissue culture fluid (TCF) was clarified at 6000 g for 10 min. Seven percent (wt/vol) of polyethylene glycol 8000 in 0.5 M NaCl was added to the TCF very slowly and the suspension was stirred in a magnetic stirrer for 2 hrs at 4°C and kept at

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4°C overnight. The viral suspension was centrifuged at 10,000 g for 30 mins and the pellet was dissolved in 1/10th the original volume of Glycine-Tris-Sodium chloride-EDTA (GTNE) buffer (50 mM Tris HCl, 200 mM glycine, 100 mM NaCl and 1mM EDTA). Further the suspension was clarified by centrifugation at 15,000 rpm for 15 min, and the supernatant was applied to a rate zonal centrifugation in 10 per cent and 50 per cent (wt/vol) sucrose in GTNE. Centrifugation was performed at 20,000 g for 1 ½ hrs at 4°C in swing out rotor in a Beckman ultracentrifuge. The pellet was suspended in a minimal volume of PBS and dialyzed twice against PBS, pH 7.2.

The presence of CSF virus at the 5th, 10th, 15th, 20th and 25th passage were confirmed by RT-PCR amplification of CSFV specific genes using NS5B primers.

The purified virus was processed for negative staining by phosphotungnistic acid by the method described by Gelderblom et al., (1991). A 400-mesh copper collodion coated grid was used and the samples were subjected to negative staining. A drop of sample was placed over the grid and allowed 45 minutes for adsorption. Then a drop of 1 per cent phosphotungstic acid was placed over the sample for 30 seconds and the excess stain was removed by using filter paper, air-dried and the grid was examined under 80KVA Technai-10-Philips Transmission Electron Microscope (TEM).

Results and Discussion

Tissue samples received from CSFV suspected outbreaks that were confirmed by multiplex panpest RT-PCR were used for isolation and adaptation of the virus in PK 15 cell lines. The multiplex PCR amplified a 174 bp for CSFV and 119 bp for pestivirus. The PK 15 cell lines infected with CSFV was checked at every 5th passage for the presence of virus using NS5B gene primers. The presence of the virus was confirmed by the 409bp amplification product of the NS5B gene of the CSF virus (Fig 1). Bulk quantities of CSF virus at passage 25th were prepared using PK15 cells grown in Roux flasks. The CSF virus purified and concentrated by ultra centrifugation was subjected to TEM analysis. The TEM studies of CSF virus revealed 40-50 nm virus like particles which were surrounded by an asymmetrically arranged poorly defined sac like membrane (Fig 2). The findings of the present study are in concordance with Cunliffe and Rebers 1968. Virus particles of members of the Flaviviridae consist of an inner complex of viral RNA genome and core protein that together form the nucleocapsid,
and an outer lipid layer containing the viral glycoproteins. The size of the CSFV shows an inner core structure of about 30 nm, surrounded by a spherical envelope with diameters ranging between 40 and 60 nm. The virus particles are hexagonally shaped with an electron-dense inner core structure of about 30 nm, surrounded by a spherical envelope with diameters ranging between 40 and 60 nm (Moennig et al., 2003).

The morphological structure of different strains (T strain, C strain and F strain) of CSFV was studied by ultra-thin section electron microscopy by Wang et al., (2000). The studies showed that the virions of CSFV were roughly round and approximately 70 nm in diameter with a 40 nm core, and were wrapped by membrane. The TEM studies by Nunez et al., (2005) in the CSFV infected animals, the cytoplasm of some kuffer cells and neutrophils showed intracytoplasmic vesicles containing electron-dense material, or membranous structures, or both. The membranous structures, which were usually close to the vesicle wall, were spherical (45–50 nm in diameter) and, electron-dense nucleoids (virus-like particles). Since the CSF virus does produce any cytopathological changes in the PK 15 cells and isolation of virus in cell cultures is a more sensitive but slower method for diagnosis of CSF, a confirmatory method like TEM would help in establishing the presence of the CSF virus in the cell culture system for the preparation of an effective vaccine.

**Summary**

Tissue samples received from CSFV suspected outbreaks were adapted in PK 15 cell lines. For virus isolation and confirmation bulk cultures of the virus at the 25th passage were subjected to gradient ultracentrifugation. The TEM studies of the purified and concentrated CSF virus revealed 40-50 nm virus like particles which were surrounded by an asymmetrically arranged poorly defined sac like membrane confirming the presence and adaptation of the virus in PK15 cell lines.

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**References**


