

5'-UTR-based phylogenetic analysis of Classical swine fever virus isolates from India

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Summary. – Classical swine fever (CSF) caused by Classical swine fever virus (CSFV) is a globally significant disease of pigs. Genetic typing of CSFV isolates can help in understanding the epidemiology of disease and trace down the source of outbreak. 5'-UTR sequence analysis and subsequent genetic classification of nine CSFV field isolates from India indicated that 3 isolates belonged to genotype 2.1 and were closely related to European CSFV strains. The remaining 6 isolates belonged to genotype 1 that contained old and new strains. However, the genotype 2.1 group consisted of recent field isolates only. The study showed circulation of both genotypes 1 and 2.1 in north-eastern part of India.

Keywords: Classical swine fever virus; 5'-UTR; genotype; India

Introduction

CSF is an OIE (Office International for Epizooties) notifiable, economically important, highly contagious viral disease of domestic pigs and wild boars caused by CSFV. Depending on various host and viral factors, the CSF may run an acute, chronic or inapparent course. It is an important trans-boundary disease endemic in Asia, Central and South America, some parts of Europe, and Africa. In India, indigenous pig breeds, crossbred and exotic breeds constitute the total pig population (13.5 million). Pig farming is an important agricultural activity in eight north-eastern states of India and CSF is an important disease affecting livestock farming in this region. However, sporadic episodes of CSF outbreaks have also been reported from other parts of India.

CSFV belongs to the genus *Pestivirus* of the family *Flaviviridae*. Bovine viral diarrhoea virus (BVDV), Border

disease virus (BDV) and tentative Pestivirus of giraffe are other members of the genus *Pestivirus* (Heinz *et al.*, 2004) that show close antigenic and genetic relationships among themselves and with CSFV. CSFV is an enveloped virus with 12.5 kb long single-stranded, positive sense RNA genome flanked by highly conserved 5'-UTR and 3'-UTR. The genome consists of a single ORF encoding about 4,000 amino acid long polyprotein that is processed to yield different viral proteins (Meyers *et al.*, 1996).

Gel based RT-PCR has been widely used for a highly specific detection and differentiation of CSFV and other pestiviruses (Lowings *et al.*, 1994; Vilcek *et al.*, 1996; Sandvik *et al.*, 1997). This method allows a rapid detection of CSFV especially in laboratories that cannot afford a real-time PCR machine (Liu *et al.*, 2007). 5'-UTR of the viral genome is frequently used as a target for detection of CSFV, since 5'-UTR is highly conserved among all pestiviruses. RT-PCR followed by a nested PCR can detect CSFV in blood samples of the infected pigs on average 2.8 days earlier than "Gold Standard" virus isolation method (Dewulf *et al.*, 2004). The nucleotide sequencing data generated by the amplified RT-PCR products of CSFV genome have been used for the comparative sequence analysis and interpretation of genetic relatedness

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Abbreviations: CSF = classical swine fever; CSFV = CSF virus

among CSFV isolates. Different CSFV isolates are classified in to three major genetic groups (Paton *et al.*, 2000). The groups 1 and 2 are composed of three subgroups each (1.1–1.3 and 2.1–2.3) and the group 3 contains four subgroups (3.1–3.4). Members of the group 1 are mostly old European isolates, old and recent American isolates, and some vaccine strains. Isolates in the group 2 are currently found worldwide and the isolates in group 3 are derived from different parts of Asia (Lowings *et al.*, 1996; Vilcek *et al.*, 1996; Paton *et al.*, 2000). New genetic variants are rapidly identified and the routes of virus spread are promptly traced down and recorded in order to combat and eradicate CSF (Belak, 2007).

The present paper describes the characterization of RT-PCR amplicons of genomic 5'-UTR region of 9 Indian CSFV isolates and their genotypic classification.

Materials and Methods

CSFV isolates. Nine CSFV field isolates obtained in years 1980s (from union territory of Andaman and Nicobar Islands) and between years 2000–2004 (from the states of Assam, West Bengal, and Uttaranchal) were included in the present study (Table 1).

RNA isolation. Homogenates from the infected tissue samples, infected cell culture (PK-15) or reconstituted CSFV vaccine were prepared in PBS. Total RNA was extracted from 100 µl each of the homogenates by TRIzol (Invitrogen) solution and was used directly for RT-PCR amplification.

RT-PCR. Total RNA was reverse transcribed using random hexamer primers with MMLV reverse transcriptase at 37°C for 1 hr. Primary PCR and nested PCR of 5'-UTR region was carried out according Greiser-Wilke *et al.* (1998). About 10 µl of nested PCR products (150 bp) were electrophoresed in 2% agarose gel and examined for specific amplification.

Cloning and sequencing. Nested PCR products were gel purified and cloned in pGEM-T Easy vector (Promega) and competent

Escherichia coli DH5α cells were transformed with these plasmids. The purified recombinant plasmid clones were subjected to the nucleotide sequencing from both ends on ABI Prism 377 automatic sequencer (Applied Biosystems).

Phylogenetic analysis. For phylogenetic analysis and comparison, 5'-UTR sequences of CSFV isolates from different parts of the world were obtained from the CSFV database, Institute of Virology, Hannover, Germany (Greiser-Wilke *et al.*, 2000) and aligned by ClustalW method. Phylogenetic tree was generated using the Neighbor-joining method in MEGA 4 (Tamura *et al.*, 2007) and the reliability of the tree was tested by 1000 bootstrap replicates. Sequence distances were calculated using Lasergene Software (DNASTAR). The CSFV sequences generated in this study were submitted to the GenBank and are available under Acc. Nos. presented in Table 1.

Results and Discussion

Different RT-PCR protocols generally focusing on the 5'-UTR region of CSFV genome were described and the generated nucleotide sequences were analyzed for a genetic typing (Hofmann *et al.*, 1994; Vilcek *et al.*, 1996; McGoldrick *et al.*, 1998) of the isolates. It has been found that for RT-PCR-based CSFV genome detection in non-degraded samples, 5'-UTR specific primers were considered as more sensitive than E2 region primers (Blacksell *et al.*, 2004). Therefore, in the present study, RT-PCR and nested PCR specific to 5'-UTR of CSFV genome were employed for amplification of 150 bp products from RNA samples and the amplification products were used for nucleotide sequencing.

The phylogenetic analysis (Fig. 1) of the generated 150 bp 5'-UTR nucleotide sequences revealed that along with vaccine strain the nine CSFV field isolates belonged to two different genotypes. CSFV isolates CSF40-02, Nbe2/44 and NG1/102 (all from the state of Assam), belonged to the genotype 2.1 and

Table 1. Details of Indian CSFV isolates

CSFV isolate	Source	Year of isolation	Origin	Genotype	Acc. No.
CSF6-2K	Infected spleen	2000	Assam	1	AJ605588
CSF8-2K	Infected spleen	2000	Assam	1	AJ605589
CSF11-89	Infected spleen	1980's	Andaman and Nicobar Islands	1	AJ605584
CSF23-02	Infected spleen	2002	Uttaranchal	1	AJ605585
CSF30-03	Infected spleen	2003	West Bengal	1	AJ605586
CSF40-02	Infected spleen	2002	Assam	2.1	AJ605587
Nbe2/44	Infected spleen	2004	Assam	2.1	EU606030
NG1/102	Infected spleen	2004	Assam	2.1	EU606029
HCVCAD22/14	Infected spleen passaged in PK-15 cells	2004	Assam	1	EU606028
VAC-IVRI	Lapinized vaccine	NA	China (C strain)	1	AJ605591

grouped with CSFV isolates from Europe. Isolates CSF6-2K, CSF8-2K, CSF11-89, CSF23-02, CSF30-03 and HCVCAD22/14 belonged to the genotype 1. The genotype 1 isolates were obtained from both recent (years 2000–2004) and old (years 1980s) CSF outbreaks and the genotype 2.1 CSFV isolates were obtained recently (years 2002 and 2004). The CSFV isolates originating from Assam belonged to both genotypes. The lapinized vaccine virus VAC-IVRI presently used for the control of CSF in India, was also grouped as genotype 1.

The nucleotide sequence analysis revealed maximum sequence variation of 9.3% between isolates CSFV 11-89 and NG1/102, wherein they belonged to the different genotypes and originated from distant geographical locations and in different years. We found a sequence identity of about 91.3% between Indian CSFV genotypes 1 and 2.1 with the inter-genotypic variation of about 9.3%. The cell culture-adapted strain HCVCAD22/14 was much closer (98.7%) to the 5'-UTR sequence of lapinized VAC-IVRI strain. The overall sequence identity between Indian 1 and 2.1 CSFV genotypes with the corresponding genotypes of the world considered in this study was about 94.0 to 98.0% indicating the conserved nature of the 5'-UTR sequence of CSFVs.

Though many CSF outbreaks occur periodically in India, satisfactory information about the molecular epidemiology and genotypes of prevalent CSFVs is not available. So far, only one study has been published about the phylogenetic characterization of a virulent CSFV/MP isolate obtained in 1983 in India, which has been classified as genotype 1.1 (Singh *et al.*, 2004). It is obvious from the present study that CSFV isolates in India belong to two major genotypes, namely genotype 1 and 2. However, the genotypic classification based on 5'-UTR did not allow precise differentiation of the subgroups 1.1 and 1.2 (Paton *et al.*, 2000). Except the isolate HCVCAD22/14, all Indian CSFV isolates were closer to the isolates that were classified as genotype 1.2. The closer relationship of three Indian CSFV isolates of genotype 2.1 to the European isolates implied the possibility that these CSFVs might have been introduced to India during import of the exotic breeds like Landrace and Hampshire from Europe for a piggery development. On the other hand, the closer relationship of HCVCAD22/14 isolate with VAC-IVRI indicated the possibility of its genotype 1.1. The VAC-IVRI isolate originated from Chinese C strain CSFV that has earlier been grouped as subgroup 1.1 along with other strains of CSFV (Pereda *et al.*, 2005).

It was shown that the lapinized Chinese strain CSFV vaccine offered a longer immunity in vaccinated animals lasting for years up to lifelong and conferred wide range of immunity against different strains and genotypes (Aynaud, 1988; Ferrari *et al.*, 1992; Tu *et al.*, 2001; Vandeputte *et al.*, 2001). For the effective control of CSF in India, there is an urgent need for sufficient quantity of lapinized CSFV vaccine, intensive and mandatory vaccination programs along with animal movement control and rapid elimination of the infected pig herds. The top priority is the availability of



Fig. 1

Phylogenetic grouping of Indian CSFV isolates

The genotypes are indicated on the right. (■) isolates of genotype 1; (◆) isolates of genotype 2.1.

diagnostic reagents and facilities for timely diagnosis, molecular study of the circulating CSFV isolates and a formation of epidemiological database. Though the present study is a preliminary epidemiological investigation involving only a few CSFV isolates, it provides a piece of information about the possible genotypes of CSFV circulating in Indian pig population. Though RT-PCR is used for the confirmation

of CSFV presence in diseased pigs, there is an urgent need for the intense molecular epidemiological investigations of CSFV isolates and development of comprehensive nationwide control program for CSF to sustain the livelihood of farmers and growth of piggy industry in India.

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