



Phylogenetic analysis of recent classical swine fever virus isolates (CSFV) from Karnataka, India

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ABSTRACT

Classical swine fever (CSF) outbreaks are detected throughout the year. Tissue (spleen and lymph nodes) samples (6) collected from 5 classical swine fever outbreaks in different districts of Karnataka state were subjected to virus isolation in PK-15 cells and virus could be recovered from all the 6 samples. Virus isolation was confirmed by reverse transcription-PCR using primers specific to 5'UTR region and sequenced. Alignment of 150 nucleotides of 5'UTR region with that of 33 published sequences available in Genbank, including 22 Indian CSFV sequences was done to get better epidemiological information on classical swine fever virus (CSFV). Based on the phylogenetic analysis, the isolates from Karnataka could be grouped into subgroup 2.2. Phylogenetic tree confirmed that members of 2.2 subgroup show higher divergence among themselves when compared to other cluster within subgroup 1.1. The study also revealed involvement of 2 different isolates in an outbreak. Most of the outbreaks in our study are due to recent introduction of new stocks of pigs from other places. Even though group 1 viruses are predominant in India, the group 2 viruses are also gradually spreading as confirmed by frequent detection/ isolation of group 2 viruses in the recent years. It appears that subgroup 2.2 viruses are gradually replacing the subgroup 1.1 viruses, indicating the changing epidemiological scenario.

Key words: 5 UTR, CSF, Classical swine fever, Epidemiology, Karnataka, Phylogenetic analysis

Classical swine fever (CSF) causes serious economic losses (Edwards *et al.* 2000). Classical swine fever is one of the top 5 viral diseases of livestock in the country according to data available in PD ADMAS (Patil *et al.* 2012). CSF is caused by classical swine fever virus (CSFV), whose genome is a positive polarity RNA of about 12.3 kb in length which contains un-translated regions (UTR) at 5' and 3' ends and encodes a single polyprotein that is both co- and post-translationally processed to yield 4 structural (C, E0, E1 and E2) and 8 non structural (Npro, p7, NS2, NS3, NS4A, NS4B, NS5A and NS5B) viral proteins (Meyers and Thiel 1996). CSF viruses are classified into a single serotype, although minor antigenic variability is found among viral strains. A compilation of data from OIE indicated that there were 1,308 outbreaks of classical swine fever in India during 1996–2008 (Patil *et al.* 2010). Genetic typing is widely used to assist epidemiological studies (Pereda *et al.* 2005). Three regions of the genome, 5'UTR (150 nt), E2 envelope glycoprotein gene (190 nt) and NS5B polymerase gene (490 nt), are most commonly used for classifying CSFV strains/ isolates into groups and subgroups

(Greiser-Wilke *et al.* 1998, Lowings *et al.* 1996, Paton *et al.* 2000). The isolates analysis showed that they could be assigned into 3 main genetic groups (1, 2 and 3) and 11 subgroups including a new subgroup 1.4 from Cuba (Postel *et al.* 2013b).

CSF has not been studied systematically in Karnataka and therefore epidemiology of the disease is largely unknown. The study involving CSFV isolates from different regions/districts of Karnataka has not been carried out till date. The present study involving the nucleotide sequence analysis of 5'UTR region was carried out, to understand the movement of virus in Karnataka and also to create sequence database of CSFV isolates for future use.

MATERIALS AND METHODS

Tissue samples such as spleen and lymph nodes collected from suspected field cases of CSF in different districts of Karnataka were used in the study (Table 1). PK-15 (ATCC) cells maintained using Eagle's minimal essential medium supplemented with 5% horse serum were used for virus isolation. Briefly, tissue materials were cut into small pieces using sterile scissors and ground in a mortar and pestle with a small amount of phosphate buffered saline (PBS) and sterile sand. The suspension was centrifuged at 1000 × g for 15 min and the supernatant was used for inoculation

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onto preformed monolayer of PK-15 cells. At each passage, positive and negative (mock infected) controls were kept along with test samples. The infected PK-15 cells were observed daily for any changes up to 5 days. After 5 days of infection, total viral RNA was extracted from cell culture supernatant using viral RNA mini kit according to manufacturer and stored at -80°C . The concentration and quality of RNA extracted was measured at a dual wavelength of 260 and 280 nm using spectrophotometer. Reverse transcription-PCR (RT-PCR) was performed targeting the 5' UTR region using RNA template and previously published primer pairs (Paton *et al.* 2000) by using one-step RT-PCR kit. Reverse transcription was performed at 50°C for 30 min, followed by initial enzyme activation step at 95°C for 15 min. Subsequent PCR amplification was carried out with 35 cycles of denaturation at 94°C for 1 min, annealing at 50°C for 1 min, extension at 72°C for 1 min followed by final extension for 10 min at 72°C .

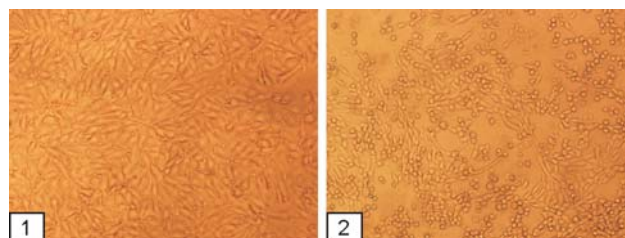
The PCR products were analyzed by electrophoresis on 1% agarose alongside 250 bp DNA ladder. The amplified products were purified from the gel using gel extraction kit. After gel purification, all the amplicons were sent for nucleotide sequencing of both strands at a commercial service. Phylogenetic trees were constructed as described previously (Greiser- Wilke *et al.* 2000). Briefly, 150 nucleotides of the 5'UTR region were aligned with other reference strain sequences available in Genbank using the Clustal X, Ver. 1.8.3 programme and edited manually using GeneDoc (Thompson *et al.* 1997). Nucleotide divergence/ similarity was calculated using Megalign module of Lasergene package. The phylogenetic tree constructed by using the neighbor-joining method. The nomenclature of the groups and subgroups was as per Paton *et al.* (2000).

RESULTS AND DISCUSSION

Classical swine fever is endemic in Karnataka and outbreaks of the disease are reported every year. Regular monitoring of CSF outbreaks and subtypes involved, are valuable in tracking movement of the virus and spread of disease. Among several genomic regions like E2, NS5B and 5' UTR of CSFV, 5' UTR region is targeted for

epidemiological study, as it is highly specific for CSFV. In the present study, 6 tissue (spleen and lymph node) samples collected from 5 outbreaks in different districts of Karnataka (Table 1) were subjected to virus isolation in PK-15 cells. Since, CSFV does not generally induce cytopathic effect, presence of virus after each blind passage was determined using RT-PCR. Upon 3 blind passages, virus could be recovered from all the 6 samples; 5 of the 6 isolates did not induce cytopathic effect (Fig. 1; CPE), however the isolate (KAR-1698/12) induced CPE from second passage onwards (Fig. 2). Specificity of the 6 RT-PCR amplicons of 421 bp size (Fig. 3) obtained using primers specific to 5'UTR were confirmed by using restriction enzyme *Pst* I digestion which yielded 2 fragments of 188 bp and 233bp size. Subsequently the products were gel purified and sequenced on both strands. The nucleotide sequences were aligned with the other relevant UTR sequences of the virus and analyzed. Briefly, for nucleotide sequence alignment and analyses, 150 nucleotide sequences from position 200–349 of Riems (GenBank Accession AY 259122) were used.

Alignment of 150 nucleotides of 5'UTR region with that of 33 other sequences available in Genbank, including 22 Indian CSFV isolates was done. Phylogenetic tree constructed from the above aligned sequences is shown in Fig. 4, and it was observed that most of Karnataka isolates have clustered into subgroup 2.2 within the group 2. These 5' UTR sequences from Karnataka isolates are moderately divergent from the members of the subgroups 1.1 and 2.2. It can also be observed that members of this cluster showed higher divergence among themselves compared to other cluster within subgroup 1.1. The tree is out grouped to the



Figs 1–2. 1. Uninfected PK–15 cells (10 $\times$). 2. Cytopathic changes (rounding and detachment of cells) induced by CSFV isolate from H. D. Kote, Mysore (KAR-1698/12) (10 $\times$).

Table 1. Details of collection of different samples suspected for CSF from various parts of Karnataka, India

Sample No.	Place of origin	District	Month and year of outbreak	No. of pigs died/ total no. of pigs in farm	Procurement of pigs
KAR-1698/12	H.D. Kote	Mysore	September 2012	60/230	Border areas of Kerala
KAR-1701/12	Kempapura	Bengaluru	October 2012	120/900	HosakoteBangalore
KAR-1702/12	Hesaraghattahobli				
	Silvepura	Bengaluru	October 2012	40/150	HosakoteBangalore
	Hesaraghattahobli				
KAR-1875/12	Koppa	Chikkamagalure	November 2012	08/24	Tumkur district
KAR-1884/12	Hagari	Bellary	December 2012	14/32	Border areas of Andra Pradesh
KAR-1885/12	Hagari	Bellary	December 2012	14/32	Border areas of Andra Pradesh

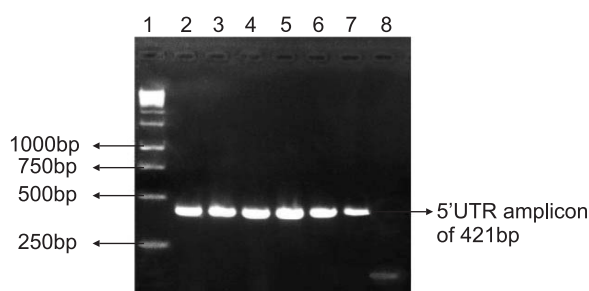


Fig. 3. Amplification of 5' UTR region of CSFV in different samples from 4 districts of Karnataka, India.

corresponding sequence of the Kanagawa/74 (Genbank Acc. No. AB252185) isolate (Fig.4).

In the present study, all the isolates (KAR-1698/12, KAR-1702/12, KAR-1702/12, KAR-1875/12, KAR-1884/12 and KAR-1885/12) recovered from 5 different outbreaks have grouped within subgroup 2.2 along with other Bengaluru isolates Genbank Acc. No HM138667, HM13868, HM13869 (Chakroborthy *et al.* 2011) and IND-294/08 isolate which was from an outbreak in Harohalli, Karnataka (Patil *et al.* 2010). The sequences of KAR-1701/12 showed 100% homology with KAR-1702/12 indicating the possible single source of virus responsible for both the outbreaks. This is not surprising, considering the fact that both outbreak locations are just 2 km apart from each other and frequent exchanges of pig and materials occur between these farms. Interestingly, isolates KAR-1884/12 and KAR-1885/12 which were from the same outbreak in Bellary district were not identical. The reason for this could be attributed to buying behavior of farmer. The said farmer who had small stock of his own was involved in buying and selling of pigs from various other sources and incidentally in the process had CSF outbreak at the same time due to viruses from different sources. The isolates KAR-1698/12 and KAR-1875/12 which were from 2 different districts, Mysore and Chikkamagalur are distinctly placed in the phylogenetic tree (Fig.4). The study also indicated that, lots of genetic divergence exists among viruses isolated from Karnataka and are distinctly different from the earlier isolates reported by Chakroborthy *et al.* (2011) and Patil *et al.* (2010). Most of the outbreaks in our study were due to recent introductions of new stocks of pigs from other places (Table 1).

It can be noted that Patil *et al.* (2010) reported the involvement of subgroup 2.2 virus in one of the CSF outbreaks in Karnataka. Later Chakroborthy *et al.* (2011) confirmed the finding. Patil *et al.* (2012) showed the plausible Chinese origin of the Indian subgroup 2.2 viruses. Even though group 1 viruses are predominant in India, the group 2 viruses are also gradually spreading as confirmed by frequent detection/ isolation of group 2 viruses in the recent years, particularly from the North-Eastern states (Patil *et al.* 2010, 2012), Uttar Pradesh, Karnataka and from neighboring country like Nepal (Postel *et al.* 2013a). Thus it appears that sub group 2.2 viruses are gradually replacing the subgroup 1.1 viruses, which were hitherto

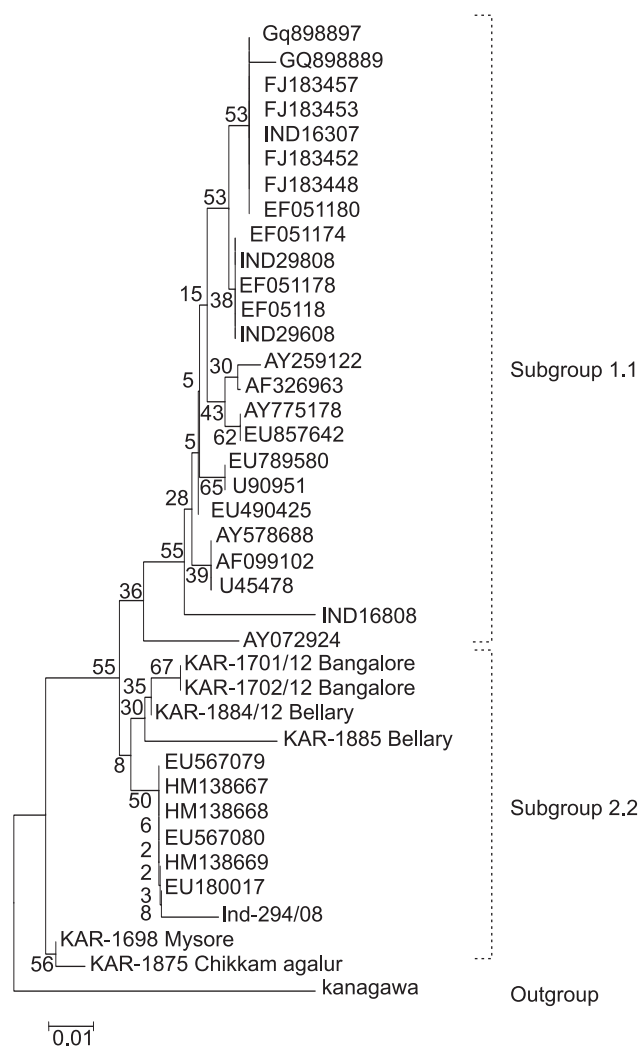


Fig. 4. Phylogenetic analysis of 5'UTR sequences of classical swine fever virus. The tree is constructed using the neighbour-joining method. Numbers at the node represent the bootstrap values after 100 replicates.

predominantly involved in CSF outbreaks. At present, it is not clear whether subgroup 2.2 viruses have any selective advantages over subgroup 1.1 viruses in infecting the hosts but nevertheless their presence in recent outbreaks is indicating the changing epidemiological scenario. It is worth mentioning that group 2 and 3 viruses are now predominantly involved in CSF outbreaks worldwide (Leifer *et al.* 2010). In this context, further studies involving other regions of viral genome such as NS5B and E2 may be useful for resolving the groups, as well as the movement and spread of the disease in Karnataka. Phylogenetic analysis conducted in this study proves to be useful for epidemiological investigation of the CSF outbreaks in Karnataka, India.

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