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Molecular typing of classical swine fever viruses from field outbreaks in Southern India

S RATHNAPRABA[1], K KUMANAN[2], K VIJAYARANI[3], M PALANISAMMI[4] and L GUNASEELAN[5]

Tamil Nadu Veterinary and Animal Sciences University, Chennai, Tamil Nadu 600 007 India

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ABSTRACT

Classical swine fever (CSF) has serious economic implications and laboratory confirmation is important in early diagnosis. The present study evaluated the ability of the RT-PCR for the detection of CSFV in Southern India. So far the available information on molecular characterization of CSFV isolates from Southern states of India is limited. Hence the outbreaks occurred during 2009–2011 were studied. The outbreaks were confirmed by virus isolation and molecular detection methods employing reverse transcriptase nested PCR (RT-nPCR) targeting CSFV specific 5'UTR. Molecular detection of swine fever suspected samples (84) targeting 5'UTR resulted in 63% positivity. Virus isolation was done in PK15 cell line from 4 positive tissue samples, after 5 blind passages and the virus multiplication in cell culture system, it was checked on fifth day post infection by indirect immnunofluoroscent antibody technique (FAT). After confirming the isolates by PCR targeting CSFV specific 5'UTR the isolates were genotyped based on the 150 nt of 5'UTR inner nested region, which revealed that the 3 isolates belonged to group 1 including 1.1 and 1.2 subgroup and 1 isolate in 2.2 subgroup suggesting the circulation of group 1and 2 viruses in this region.

Key words: Classical swine fever, RT-nPCR, 5'UTR, Phylogenetic analysis

Classical swine fever (CSF), a highly contagious viral disease of domestic pigs and wild boars, is one of the most devastating porcine diseases worldwide (Moennig *et al.* 2003). Despite close relation with bovine viral diarrhoea types 1 and 2 (BVDV 1 and BVDV2) and border disease virus (BDV), CSFV forms a distinct group that can be differentiated based on genotyping (Paton *et al.* 2000). Current diagnostic methods include detection of viral antigen in fluorescent antibody test (FAT), or antigen capture enzyme linked immunosorbant assay (ELISA) or detection of genomic RNA by RT-PCR. In India outbreaks of the disease have been reported from Uttar Pradesh, Maharashtra, Punjab and the North East Indian states of Arunachal Pradesh, Manipur, Mizoram, Nagaland, and West Bengal (Barman *et al.* 2003). CSF is endemic in Tamil Nadu, Southern India and earlier in 2003 the disease outbreak in Tamil Nadu was reported (Govindarajan *et al.* 2003). Southern states of India has only 1.2 million of the total 13.519 million Indian pig population, and because of the lower preference for the Indian pig husbandry in Southern India, this disease has not been studied extensively. Genotyping and phylogenetic analysis of CSFV isolates of Southern states of India have not been done so far. The present study was aimed at molecular detection and genetic characterization of the swine fever isolates involved in the outbreaks during the year 2009–2011 in the Southern states of India. Sequence data generated have also been used to subdivide CSFV isolates into types and subtypes, which can assist in epidemiological tracing of the routes of virus spread.

Present address: [1]Assistant Professor (prabakanraj @gmail.com), [3]Professor, [4]Professor and Head, Department of Veterinary Biotechnology; [2]Director of Research; [5]Professor and Head, Department of Veterinary Epidemiology and Public Health, Madras Veterinary College, Chennai.

MATERIALS AND METHODS

Samples for RNA extraction: Samples (84) comprising spleen (18), tonsil (9), liver (14), kidney (22), lymphnodes (14) and nasal swabs (7) collected from 9 suspected outbreaks in Southern India during 2009–2011 were used in this study. Total RNA was extracted from 250 µl of homogenized samples using trizol as per manufacturer's instructions. RNA was suspended in 10 µl of nuclease free water.

cDNA synthesis and RT-PCR: cDNA was synthesized using superscript III RT kit; 5µg of RNA, 1 µl of random primers (50µg/µl) and 8 µl of NFW in a 20 µl reaction volume, heated for 65°C for 5 min and snap cooled on ice for 1 min followed by addition of 2x reaction buffer and 200 U enzyme mix. The reaction mix was held at 25°C for 10 min and 85°C for 5 min for enzyme inactivation. The CSFV specific 5'UTR nested primers and PCR thermoprofile were used (Lowings *et al.* 1996). The PCR amplicons were analyzed by agarose gel electrophoresis and the validity of the results were confirmed with positive (vaccine virus) and negative controls.

Sequencing and genetic typing: 5' UTR amplicons were purified using commercial kit and sequenced in an automated sequencer using a terminator kit. The sequences obtained were assembled in Seqman of DNASTAR and the aligned sequences were subjected to BLAST analysis. Additional sequences for comparison were obtained from CSFV database held at European reference laboratory for classical swine fever virus (Gresier –Wilke *et al.* 2000). All the nucleotide sequences were aligned using the Clustal W method. The percentage of nucleotide sequence similarity was determined using MEGALIGN programme of DNA STAR. Computer assisted phylogenetic tree was constructed and bootstrap analysis was performed for 150 nucleotide fragment of 5'UTR by Neighbour-joining method.

Virus isolation in PK15 cell line and titration by FAVN: Out of the 9 suspected outbreaks tissue samples screened, 4 PCR positive tissues (CSF-TRP2, CSF-KAR, CSF-ERD and CSF-CHN) were selected and pooled tissue samples were subjected for virus isolation in PK15 cell line. Antibiotic treated 10% w/v suspensions of the pooled tissue sample (spleen, liver and lymphnode) were used as inoculum to infect monolayers of PK15 cells grown in minimum essential medium supplemented with 10% fetal bovine serum free of BVDV. The infected PK15 cells were frozen on fifth day post infection (DPI). After 5 blind passages, the virus multiplication in cell culture system was checked on 5 DPI by indirect immnunofluorescent antibody technique (FAT) using 1: 50 diluted positive CSF poloyclonal serum and 1: 100 diluted anti - pig IgG FITC conjugate. The 5^{th} and 10^{th} passage infected PK15 cell culture fluids were confirmed for the presence of CSFV by reverse transcriptase PCR targeting 5'UTR gene, with passaged lapinized vaccine virus as positive control.

Virus titration was performed to quantify the virus passaged in PK15 cells. Ten-fold serial dilutions of culture supernatant were prepared. Three replicates of 100 µl of each serial dilution (10^{-1} to 10^{-8}) were incubated for 4 days on a monolayer of PK15 cells in a 24 well plate. After 4 dpi growth medium was discarded and the monolayers were washed gently in PBS and fixed in 4% ice cold acetone in PBS for 10 min. The plate was washed in PBS and the monolayers were incubated for 1 h with 1: 50 dilution of polyclonal reference serum (EU reference laboratory) diluted in PBST and were stained with anti - pig IgG FITC followed by observation under fluorescent microscope. Virus titres were calculated as $TCID_{50}$ using Reed and Muench method.

Comparative sensitivity of the RT- nPCR targeting 5'UTR was assessed by extracting total RNA from CSFV/TRP2 infected PK15 cell line at 10^{th} passage which had a $TCID_{50}$ of log $10^{3.7}$. Serial ten-fold dilutions of the infected genomic RNA in MEM were prepared for RNA extraction by TRIzol method, followed by cDNA synthesis by a kit. RT – n PCR amplification was done for the genes targeting 5'UTR regions. The amplicons were checked in 2% agarose gel.

RESULTS AND DISCUSSION

PCR amplification and sequencing: CSF suspected samples (84), processed for detection of CSFV viral RNA using 5'UTR nested RT-PCR, revealed that the 5'UTR outer primer sets produced a specific 420 bp amplicon (Fig. 1) and inner primer set amplifies a 270 bp product (Fig. 1a). Out of 84 samples resulted in 63% showed positivity by 5' UTR. A comparative study by Dewulf *et al.* (2000) testing the analytical performance of the laboratory diagnostic techniques, virus isolation, antigen ELISA and RT-nPCR, reported that RT-nPCR performed significantly better than virus isolation, and represents the best diagnostic tool available for early detection of CSFV infection. Here in this report, the viral genome was confirmed by RT-PCR amplification targeting CSF specific 5'UTR gene. The primers used are specific to swine fever viruses and have no cross reactivity to other pestiviruses. The RT nested PCR is more sensitive for routine diagnosis particularly to detect the low virulent strain of the virus (Handel *et al.* 2004). RT-PCR could detect the viral genome from the clinical samples with higher positive percent in lymphnodes (78.5%), spleen (78%), liver (78%), tonsil (55%), kidney (45%) and intestine (43%) for 5'UTR. Van Oirschot *et al.* (1992) reported that almost all the lymphoid organs like tonsil, liver, spleen had highest concentration of virus in early phase of infection. Here in this report the comparatively lower percentage positivity in tonsil and intestine might be due to a lesser number of samples (9) screened.

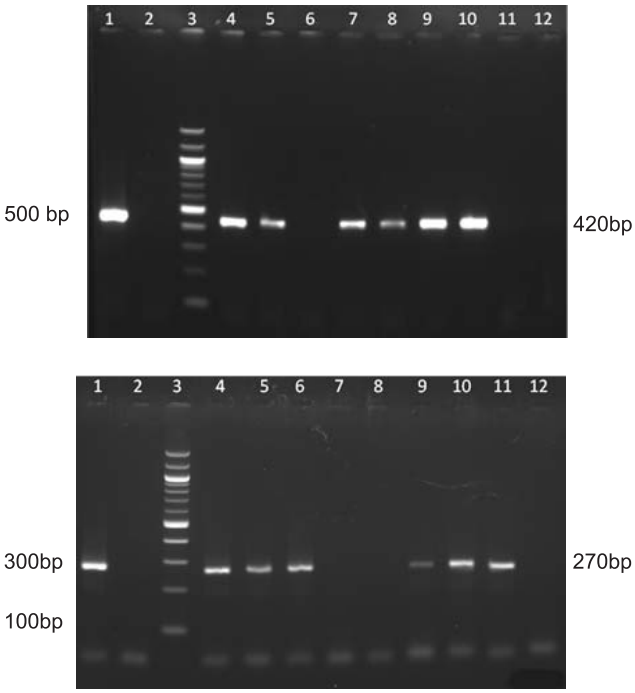

Fig. 1a. 1 (top). Agarose gel (1%) electrophoresis showing 420 bp of 5' UTR outer amplicons. Lane 1: Positive control, Lane 2: Negative control, Lane 3: 100 bp marker, Lane 4, 5, 7– 10: Positive samples, Lane - 6,11,12 : Negative samples. 1a (bottom). Agarose gel (1.5%) electrophoresis showing 271bp 5' UTR inner amplicons. Lane 1: Positive control, Lane 2: Negative control, Lane 3: 100 bp marker, Lane 4–6, 9- 11: Positive samples, Lane - 7,8,12 : Negative samples.

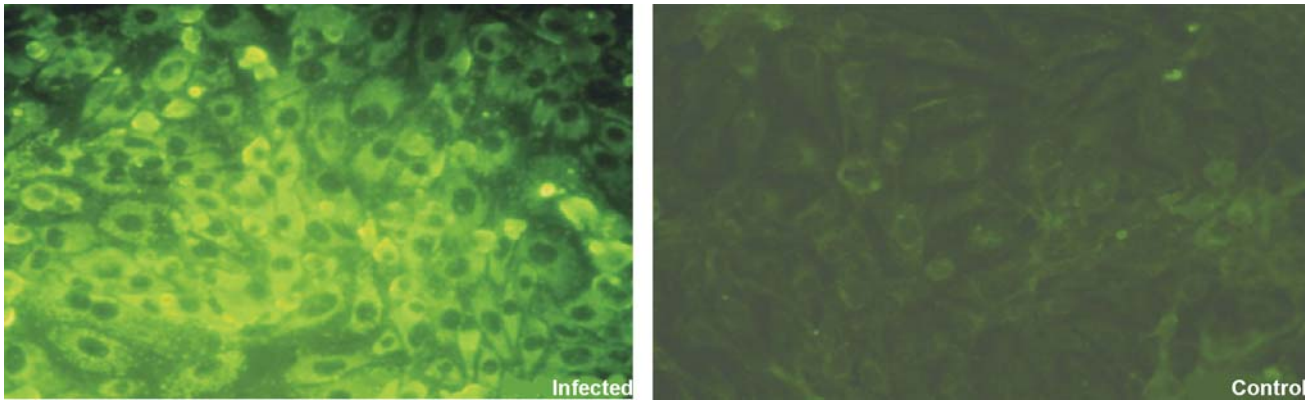

Fig. 2. PK15 cell line infected with the virus showing cytoplasmic fluorescence in indirect immunofluorescent test (400 ×) on the left and control on the right.

Virus isolation and titration: Four non cytopathogenic swine fever viruses were isolated along with the lapinized vaccine virus strain in cell culture systems after confirmation in PCR using CSF specific primers. The field isolates from different locations in Tami Nadu, viz. Erode, Karur and Chennai were designated as ERD, KAR and CHN, and the Tirupati isolate of Andra Pradesh as TRP2. Antigenic characterization using CSF specific polyclonal serum in indirect immunofluroscent assay confirmed that all the isolates were of swine fever. The propagated isolates were checked in RT –PCR for the 5'UTR primers at the 5th and 10th passage level. In the present study virus propagation was checked in FAT revealed 60% infectivity at 5th and 10th passage showed increased infectivity about 80% with the presence positive fluorescence in the cytoplasm of the infected cells (Fig. 2), and by 5'UTR nested primers all the isolates yielded a clear single band of size 270bp at 5th passage level (Fig. 3).

Titration of CSF virus was performed by FAVN (fluorescent antibody virus neutralization) test at 10-fold dilution. Titres of the 4 CSF viral isolates and lapinized CSF virus at 5th and 10th passage level are noted in Table 1. The tissue culture infective dose $\log_{10}$ $TCID_{50}$ was calculated by Reed and Muench method. In the analytical

Table1. Titre values of the CSFV isolates at 5th and 10th passage level

Isolates	Titre ($\log_{10}$ $TCID_{50}$)	
	Passage 5	Passage 10
CSF-TRP2 (Tirupati)	2.6	3.7
CSF-KAR(Karur)	2.4	3.2
CSF-ERD (Erode)	2.3	3.5
CSF-CHN (Chennai)	2.4	3.5
Lapinized vaccine virus	3.5	4.5

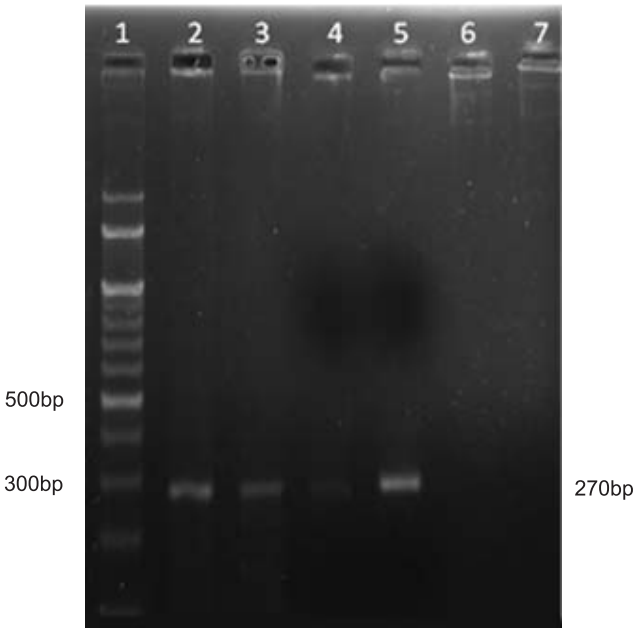

Fig. 3. Agarose gel (1.5%) electrophoresis of 5' UTR nested gene amplicons (271bp). Lane 1: 100bp ladder, Lane 2: Positive control, Lane 3,4,5,6 - CSFV/TRP CSFV/KAR, CSFV/ERD and CSFV/CHN, Lane 7- Negative control.

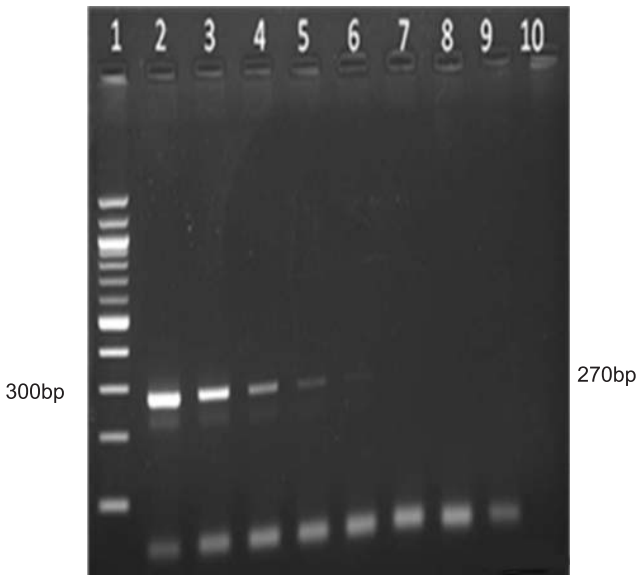

Fig. 4. The detection sensitivity of RT-PCR for CSFV in TCF samples using 5'UTR inner nested primers. Lane: 1-100bp, Lane: 2 to 10 represent PCR products from 10 fold serial dilution of CSFV-TS2 isolate respectively.

sensitivity of RT-PCR for the detection of CSFV in tissue culture fluid, the detection limit observed was 10^{-5} for 5' UTR.

PK15 cell line was used for isolating swine fever virus as has been done earlier (Choi *et al.* 2003). Since CSFV is noncytopathogenic, CSFV specific antibodies were used for detecting the virus in cell culture. Fluorescent antibody technique was used by several workers in detecting CSFV antigen in tissues sections as well as in infected cell lines. Although virus propagation and FAT staining are time consuming process, it is considered as a gold standard for confirming CSF outbreaks (Gresier – Wilkie *et al.* 2007). In our study, out of the 9 outbreak samples, 4 isolates were obtained and confirmed both at 5th and 10th passage, with the 10th passage infected cells showing more than 80% infectivity with intense cytoplasmic fluorescence. There was significant increase in titre of all the isolates adapted in PK15 cells with the lapinized vaccine virus showing the maximum titre of 4.5, indicating the suitability of PK15 cells for virus isolation and adaptation.

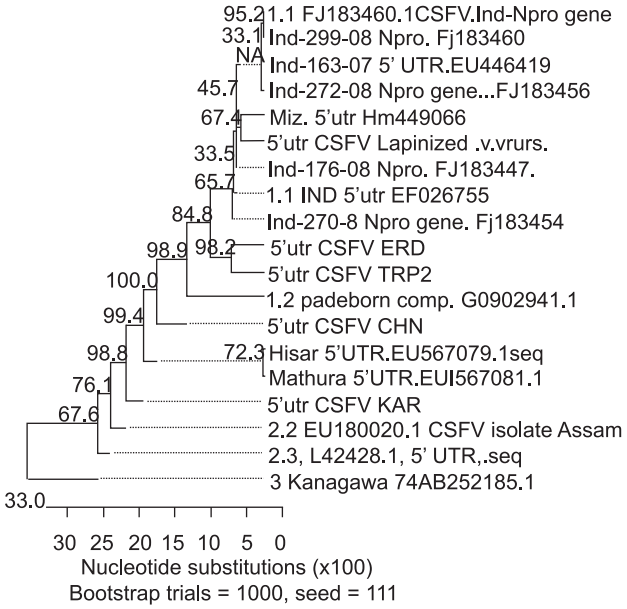

Fig. 5. Phylogenetic analysis of CSFV isolate based on 150 nt bp of 5'UTR region in comparison with the other isolates available in the genebank, along with their accession number.

In analytical sensitivity of 5'UTR based RT-PCR for the detection of CSFV in cell culture fluid, the detection limit observed was 10^{-5} which correspond to 0.36 $TCID_{50}$/ml. Paton *et al.* (2000) reported that the sensitivity of detection limit for 5'UTR in swine fever infected serum was between 10^{-3}– 10^{-4} when compared to detection sensitivity from infected samples as 10^{-6} – 10^{-7}. Hence, 5'UTR gene based RT –nPCR appeared to be rapid and sensitive for the detection of CSFV from field samples.

Genetic typing

Genetic typing of CSFV isolates based on nucleotide sequence comparisons has been used very effectively in tracing the origin and spread of outbreaks, establishing genetic relationship between virus isolates and elucidating the genetic diversity (Chen *et al.* 2008). Three regions of the genome, 5' UTR (150 nucleotides), E2 (190 nt) and NS5B (409 nt), have been found useful in classifying CSFV (Paton *et al.* 2000)). Group 1 viruses include most of the historical isolates, while group 2 and 3 contains most of the currently circulating viruses in various parts of the world (Paton *et al.* 2000, Deng *et al.* 2005).

Here we used the genotyping that could be used for sequence comparison across the pestivirus genus. In the present report, phylogenetic of analysis of 150 bp nucleotide sequences typed the 3 isolates into group 1 and 1 isolate in 2.2 subgroup (Fig 4). Based on genetic typing the historic isolates over the world belonged to group 1. There are evidences that the recent isolates from different regions in Europe, Asia belonged to group 2, which further subdivided into 2.1, 2.2 and 2.3.

Percent Identity

Divergence

	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	
1		98.5	85.9	95.4	90.7	95.4	94.1	99.2	99.2	99.1	99.2	100.0	93.8	95.4	97.2	97.8	88.1	89.3	94.9	1
2	1.6		81.9	94.9	90.0	93.9	94.1	99.3	99.5	99.7	99.0	98.5	93.8	95.2	96.5	98.5	88.3	87.9	95.0	2
3	15.6	20.7		97.7	90.7	96.4	97.4	82.2	82.0	80.6	82.2	85.9	97.1	82.4	92.9	94.4	78.0	73.7	70.6	3
4	4.7	5.4	2.3		92.4	95.6	99.4	94.9	94.9	94.9	94.9	95.4	98.9	94.3	94.4	94.9	94.9	94.9	94.3	4
5	10.3	11.1	10.2	8.1		89.3	89.3	90.0	90.0	90.0	90.0	90.7	88.7	90.0	86.7	90.0	90.0	90.0	89.3	5
6	4.8	6.4	3.7	4.5	11.6		96.1	95.0	94.8	94.2	95.0	95.4	95.7	93.9	94.3	94.4	86.1	93.6	95.0	6
7	6.1	6.2	2.6	0.6	11.8	4.0		94.5	94.5	94.5	94.5	94.1	99.6	95.2	96.2	95.6	95.6	95.0	95.2	7
8	0.8	0.7	20.3	5.4	11.1	5.2	5.7		100.0	100.0	99.8	99.2	94.1	95.7	97.5	98.9	88.6	87.9	95.7	8
9	0.8	0.5	20.6	5.4	11.1	5.4	5.7	0.0		100.0	99.8	99.2	94.1	96.0	97.8	98.9	91.2	87.9	95.6	9
10	0.9	0.3	22.4	5.4	11.1	6.1	5.7	0.0	0.0		99.7	99.1	94.1	96.0	97.5	98.9	95.1	87.9	95.2	10
11	0.8	1.0	20.3	5.4	11.1	5.2	5.7	0.2	0.2	0.3		99.2	94.1	96.0	97.5	98.9	88.6	87.6	95.5	11
12	0.0	1.6	15.6	4.7	10.3	4.8	6.1	0.8	0.8	0.9	0.8		93.8	95.4	97.2	97.8	88.1	89.3	94.9	12
13	6.5	6.6	3.0	1.2	12.6	4.4	0.4	6.2	6.2	6.2	6.2	6.5		94.9	95.7	95.2	95.2	94.6	94.9	13
14	4.8	5.0	20.1	6.0	11.1	6.4	5.0	4.4	4.1	4.1	4.2	4.8	5.4		95.8	96.7	85.5	85.2	93.8	14
15	2.9	3.6	3.3	5.8	15.1	6.0	4.0	2.5	2.3	2.5	2.5	2.9	4.4	4.3		97.9	88.7	97.5	82.3	15
16	2.3	1.5	5.8	5.4	11.1	5.8	4.6	1.1	1.1	1.1	1.1	2.3	5.0	3.4	2.2		100.0	100.0	99.6	16
17	13.0	12.7	26.0	5.4	11.1	15.5	4.6	12.4	9.4	5.1	12.4	13.0	5.0	16.1	12.3	0.0		91.6	87.0	17
18	11.6	13.2	32.3	5.4	11.1	6.8	5.2	13.2	13.2	13.2	13.6	11.6	5.6	16.6	2.5	0.0	9.0		86.1	18
19	5.4	5.2	37.3	6.0	11.9	5.2	5.0	4.4	4.6	5.0	4.7	5.4	5.4	6.5	16.0	0.4	14.3	15.4		19
	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	

1 1.1 FJ183460.1CSFV.Ind-Npro gene, cds.s
2 1.1 IND 5 'utr EF026755.1.seq
3 1.2 padeborn comp. GQ902941.1.seq
4 2.2 EU180020.1 CSFV isolate Assam.seq
5 2.3 Kanagawa 74AB252185.1.seq
6 2.3, L42428.1, 5' UTR,.seq
7 Hisar 5' UTR.EU567079.1.seq
8 Ind-163-07 5' UTR.EU446419.1.seq
9 Ind-176-08 Npro . FJ183447.1.seq
10 Ind-270-08 Npro gene.FJ183454.1.seq
11 Ind-272-08 Npro gene...FJ183456.1.seq
12 Ind-299-08 Npro .FJ183460.1.seq
13 Mathura 5' UTR.EU567081.1.seq
14 Miz. 5'utr HM449066.1.seq
15 5'utr CSFV KAR.seq
16 5'utr CSFV CHN.seq
17 5'utr CSFV ERD.seq
18 5'utr CSFV TRP2.seq
19 5'utr CSFV Lap.vac. virus.seq

Fig. 6. Sequence distance analysis of CSFV in comparison with the other isolates available in the genebank, along with their accession number. Revealed maximum variability between 11.1 and 15.1% with genotype 2.3 Kanagawa group of viruses and lowest divergence of about 1.1% with genotype 1 group of viruses.

As far India is concerned, group 1 viruses are predominant as per the reports of Sarma *et al.* (2009) and Desai *et al*. 2010. The recent outbreaks from Assam involved the group 2 viruses, similarly Patil *et al.* (2010) reported one isolate from Karnataka belonged to 2.1 virus.

In our study we reported the involvement of both 1.1 and 2.2 viruses from the outbreaks reported during 2009–2011 suggesting the spread of group 2 viruses in the Southern part of the country, which are also prevalent in other parts of country (Patil *et al.* 2010) as well as worldwide (Sabogal *et al.* 2006). It was shown from the phylogenetic tree that the South Indian isolates are placed separately in a different group from the other members of the Indian isolates belonging to subgroup 1.1 that would have been originated from European isolates as previously opined by suggested by Patil *et al.* (2010).

The sequence distance comparative analysis performed between 4 CSF field isolates and representative isolates form different swine fever phylogenetic groups revealed maximum variability between 11.1 and 15.1% with genotype 2.3 Kanagawa group of viruses and lowest divergence of about 1.1% with genotype 1 group of viruses (Fig. 5). The CSF Karur had minimum divergence of 2.3% with Hisar isolate and maximum of 15.1% with the group 3 Kanagawa strain. The CSF-CHN revealed an average maximum of 99% identity with 1.1 subgroup viruses, also CSF ERD showed maximum divergence of 16% with MIZ HM449056 isolate and minimum variation of 5% with Mathura isolate (EU567081).

The nucleotide sequence alignment (Fig. 6) indicated that the 4 CSF isolates had a base change of 'G' in place of 'C' at 250nt position of the genome which was also noted in other Indian isolates (IND/163/07, IND/176/08,IND/270/08 and IND/299/08). Further the CSF/KAR isolate had three base modifications one at 303 nt position from 'A' to 'G' base, the second change from 'A' to 'G' at 328nt position and the third change of 'T' to 'C' at 332 nt position which was present in group 2 viruses. The above results suggested the dominance of group 1 viruses and also the involvement of group 2 viruses from one outbreak at Karur, Tamil Nadu.

Our findings concerning the typing of isolates in subgroup 1.1 and the 2.2 emphasizes the circulation of group 1 and 2 viruses in Southern parts of India. Though group 1 virus which was present in earlier years in larger population continues to dominate the outbreaks, circulation of group 2 virus is of concern indicating their spread. However, intense molecular epidemiological studies with more isolates need to be analyzed for a better picture.

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