Changing pattern of classical swine fever virus genogroup from classical 1.1 to emerging 2.2 in India

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Abstract Classical swine fever (CSF) is one of the most important viral diseases of pigs with high economic impact. The causative agent, Classical swine fever virus (CSFV) is a member of genus *Pestivirus* in family *Flaviviridae* and is structurally and antigenically related to other members of the genus. The identification of virus strains and genotypes can conveniently be used to trace the origin and patterns of virus spread, which contribute substantially in control strategies. In the present study, we have partially sequenced and analysed the 5' untranslated region (UTR) and E2 regions of CSFV clinical samples ($n = 24$) from various parts of the country. Among the samples, the sequence alignment of 5'UTR and E2 regions revealed 96.7–100 and 94.7–100% identities at the nucleotide level, respectively. The samples under study showed the close resemblance to the other CSFV isolates reported in India. In phylogenetic analysis, all the field samples were clustered in subgroup 2.2. Thus the study presents a further phylogenetic evidence for the emergence of subgroup 2.2 CSFV replacing the predominant subgroup 1.1 viruses in India. As the information regarding the molecular epidemiology of CSFV in India is very little, generation of such epidemiological data is warranted to help in comprehending the nationwide disease control program to sustain the growth of pig industry in India.

Keywords CSFV · Untranslated region · Epidemiology · Alignment · Genogroup · Phylogenetic analysis

Introduction

Classical swine fever (CSF) is one of the most important viral diseases of swine with serious socio-economic impact throughout the world including India. The etiological agent—Classical swine fever virus (CSFV) belongs to the genus *Pestivirus* of family *Flaviviridae* [36]. The virus occurs as single serotype and is structurally and antigenically related to other members of the genus like *Bovine viral diarrhoea virus* (BVDV) and *Border disease virus* (BDV) [40]. The viral genomic RNA is neither capped nor polyadenylated and has non/untranslated regions (NTRs/UTRs) at either end (5'UTR and 3'UTR), encompassing a single open reading frame (ORF) with genome size of about 12.3 kb. The single ORF encodes polyprotein of 3898 amino acids that is both co- and post-translationally processed to yield four structural (C, E^{ms}, E1 and E2) and 7–8 nonstructural (Npro, p7, NS2, NS3, NS4A, NS4B, NS5A and NS5B) proteins [26, 42].

Molecular epidemiological studies identified three hyper variable regions: 150 nucleotides (nt) stretch of the 5'UTR, 190 nt stretch of the E2 and 409 nt stretch of the NS5B gene [5, 19]. Based on these regions, CSFV has been divided into three major genotypes (1, 2 and 3) and ten subtypes (1.1, 1.2, 1.3; 2.1, 2.2, 2.3; 3.1, 3.2, 3.3 and 3.4) [15, 26]. The earliest suspected CSF outbreak in India was in 1944 [14] and the first detailed report was in 1962 [30] and thereafter the disease has been reported from majority of the states [1, 3, 4, 20, 28, 29, 33]. The disease is endemic with high prevalence of antibodies and antigen [22]. The Indian outbreak strains were grouped into

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subgroups 1.1 [32], 2.1 [8] and 2.2 [6, 16, 25]. The subgroup 1.1 was reported to be predominant [24, 32]. However, the studies on genetic diversity of different genotypes and the evolutionary rates of CSFV in India are few. Thus, the present study was aimed to analyze the 5'UTR and E2 partial sequences of 24 CSFV isolates collected from outbreaks occurred in different part of the country for phylogenetic relationship with previously reported CSFVs in India and reference isolates of the world.

Materials and methods

CSFV

CSFV suspected tissue samples ($n = 24$) used in the study were available in the CSF repository of Division of Virology, Indian Veterinary Research Institute (IVRI), Mukteswar (Table 1). The lapinised swine fever vaccine virus was obtained from Division of Biological Products,

IVRI, Izatnagar and used as reference positive. All tissue samples were homogenised to form 10% w/v suspension in phosphate buffer saline and kept at -70°C till further use.

Reverse transcription-polymerase chain reaction (RT-PCR)

Total RNA was isolated from tissue suspensions using Tri Reagent[®] (Sigma-Aldrich, USA) as per the manufacturer's instructions. Reverse transcription was performed with RevertAidTM first strand cDNA synthesis kit (Thermo Scientific). The obtained cDNA was subjected to PCR amplification using reference primers targeting 421 nucleotides segment of 5'UTR {5'UTR (Ext) F- 5'CTAGC CATGCCWAYGTAGG3' (94–113, Alfort/187); 5'UTR (Ext) R- 5'CAGCTTCARYGTTGATTGT3' (514–496, Alfort/187) [11]} and 272 nucleotides or 504 nucleotides segment of E2 gene {E2 (Int) F- 5'TCRWCAA CCAAYGAGATAGGG3' (2477–2497, Alfort/187); E2 (Int) R- 5'CACAGYCCRAAYCCRAAGTCATC3'}

Table 1 Details of tissue samples used in the present study

State	Repository ID	ID used in study	Organ	Year of collection	Accession No.	
					5'UTR	E2
Maharashtra	CSF/MH/PUN/219	V1	Spleen	2009	KJ197325	KF751905
	CSF/MH/PUN/207	V2	Spleen	2009	KJ197326	KF751906
	CSF/MH/PUN/181	V3	Spleen	2009	KJ197327	KF751907
	CSF/MH/PUN/172	V4	Kidney	2007	KJ197328	KF751908
	CSF/MH/PUN/183	V5	Spleen	2009	KJ197329	KF751909
Bihar	CSFV/BR/PAT/072	V6	Kidney	2009	KJ197330	KF751910
	CSFV/BR/PAT/073	V7	Spleen	2009	KJ197331	KF751911
	CSFV/BR/SUP/081	V8	Spleen	2009	KJ197332	KF751912
	CSFV/BR/DAR/038	V9	Spleen	2009	KJ197333	KF751913
Madhya Pradesh	CSF/MP/BHO/202	V11	Liver	2009	KJ197315	KF751895
	CSF/MP/BHO/229	V12	Spleen	2009	KJ197316	KF751896
	CSF/MP/BHO/230	V13	Kidney	2009	KJ197317	KF751897
	CSF/MP/REW/233	V14	Spleen	2009	KJ197318	KF751898
	CSF/MP/REW/234	V15	Lymph node	2009	KJ197319	KF751899
Karnataka	CSF/KN/BAN/163	V16	Spleen	2005	KJ197320	KF751900
	CSF/KN/BAN/164	V17	Kidney	2005	KJ197321	KF751901
	CSF/KN/BAN/165	V18	Spleen	2005	KJ197322	KF751902
	CSF/KR/KER/228	V19	Liver	2009	KJ197323	KF751903
	CSF/TN/THR/175	V20	Spleen	2007	KJ197324	KF751904
Uttarakhand	CSF/UK/DDN/365	V21	Kidney	2012	KJ197304	KF751890
	CSF/UK/DDN/373	V22	Kidney	2012	KJ197305	KF751891
	CSF/UK/DDN/380	V23	Kidney	2012	KJ197306	KF751892
	CSF/UK/LAL/293	V24	Kidney	2012	KJ197307	KF751893
	CSF/UK/LAL/304	V25	Kidney	2012	KJ197308	KF751894

Details of tissue samples used in the present study

(2748–2726, Alfort/187) [19]; E2-5 Fwd-5′CAACCACGG CATTCTCATYTG3′ (2345–2366, Alfort/Tubingen); E2-5 Rev-5′TGACACCCGTCCACCCTATTG3′ (4848–4828, Alfort/Tubingen) [23]]. PCR amplification of target genome fragments of CSFV was carried out in 25 µL reaction volume as follows: 2 µL cDNA, 2.5 µL of 10X PCR Buffer, 2 µL of 25 mM MgCl₂, 1 µL of 10 mM dNTPs, 1.25 U Taq DNA Polymerase and 1 µL 10 pMole of each primer adjusted to final volume with nuclease free water. PCR reaction composition was same for all the three targets except the use of primers specific for each case. Stepwise cyclic conditions employed were same as used by Singh et al. [34], Barman et al. [3] and Pan et al. [23] for amplification of 5′UTR, E2 (int) and E2-5, respectively. Positive control and non template control was included in each run performed. All PCR products were analyzed by electrophoresis using 1.5% agarose gel stained with ethidium bromide along with molecular weight marker.

Cloning and sequencing

RT-PCR gel purified products were cloned into a pJET1.2/blunt vector (Thermo scientific, USA) and competent *Escherichia coli* Top 10α cells were transformed with the recombinant plasmid. The recombinant clones were identified by gene-specific PCR and Restriction endonuclease (RE) digestion using *Bgl* II endonuclease (data not shown). Plasmid DNAs from the positive recombinant clones were sequenced commercially. Sequences thus obtained were purified using NCBI-BLAST to get the desired sequence of 5′UTR and E2 fragments and were submitted to GenBank (Table 1).

Phylogenetic analysis

The sequences were trimmed to obtain the 150 nucleotides (201–350 nt position of HCLV India; EU857642) and 190 nucleotides (2520–2709 nt position of HCLV India; EU857642) fragments of 5′UTR and E2 regions, respectively. Thirty-eight sequences were retrieved from GenBank database (Table 2) to achieve a data set that represents a higher variety of genotypes and subgenotypes distributed in India and the world. Multiple sequence alignment was carried out using Clustal W algorithm [37] and Nucleotide divergence was calculated using MegaAlign programme from LaserGene Biocomputing software Package (DNASTAR Inc., Madison, USA). Phylogenetic tree was reconstructed using Maximum Likelihood method in the MEGA software version 6 [35] keeping 1000 bootstrap value [9]. The tree is drawn to scale, with branch lengths in the same units as those of the evolutionary distances used to infer the phylogenetic tree. The evolutionary distances were computed using the Kimura 2-parameter method [13] and are in the units of the

number of base substitutions per site. The rate variation among sites was modeled with a gamma distribution. The nomenclature of the groups and subgroups was done as per Paton et al. [26].

Results and discussion

Identity and homology of sample sequences

Genetic typing of CSFV isolates and comparison with those originating from nearby and far countries are important to determine the genetic diversity and to establish a control strategy. Phylogenetic analysis of CSFV has been useful in tracing the pathways of virus transmission between outbreaks and the origin of the spread. This also helps in determining the divergence; which may indicate the epidemiological link or independent pathway.

Sequence alignment of 150 nucleotide fragment of 5′UTR region showed that all the isolates (V1–V25) were sharing maximum sequence homology with Meghalaya (D), Hisar, CSFV-UP-GZ-NVD-11 and Patna (BR1/06) isolates. In pairwise distance analysis, sequence identity ranged from 96.7 to 100% among them and 94.0–99.3% identity with subgroup 2.2 viruses at nucleotide level. Among the groups, it ranged from 91.3–94.0, 92.0–99.3 and 88.0–90.0% for viruses of genogroup 1, 2 and 3, respectively. Genetic analysis based on 190 nucleotides fragments of E2 gene at nucleotide level exhibited 94.7–100% percent identity among the samples, whereas 91.0–100% sequence identity was found among subgroup 2.2 virus and samples sequenced in this study. Comparison between the gene of viruses of different genogroups and that of the samples of the present study showed sequence identity ranging from 82.0–84.7, 85.2–100 and 79.4–82.5% for genogroup 1, 2 and 3 viruses, respectively. Also, in E2 sequence all the isolates under study were found to have close sequence identity with CSF1060 isolate (JX162241) of Nepal with highest percent identity 97.9% with isolate V-8 (CSFV/BR/SUP/081-KF751912) followed by V-7 (CSFV/BR/PAT/073-KF751911) and V-25 (CSFV/UK/LAL/304-KF751894) with 97.4 and 96.8% identity, respectively. The Nepal isolate was having highest divergence from V-9 (CSFV/BR/DAR/038-KF751913) among all the samples under study. None of the samples showed close homology with the virus off apinized vaccine (genogroup 1.1) used presently in the country for controlling the disease; which shows that there is no reversion of vaccine virus in the field.

Phylogenetic analysis

Reconstruction of phylogenetic tree based on 150 nucleotides sequences of 5′UTR region and 190 nucleotides

Table 2 Details of reference sequence used in sequence analysis

CSFV isolate	GenBank accession no.		Virus isolation/collection#	
	5'UTR	E2	Country/region	Year
Subgenotype 1.1				
Eystrup	AF326963	AF326963	Germany	1964
India	EU857642	EU857642	India	2011
Subgenotype 1.2				
Brescia	M31768	M31768	Italy	1945
Subgenotype 1.3				
CSF0306	JQ411570		Malaysia	1986
CSF0650	JX028200	JX028200	Guatemala	2013
Subgenotype 1.4				
CSF1057 santiago de cuba		JX028203	Cuba	2011
Subgenotype 2.1				
Paderborn	AY072924	AY072924	Germany	1997
HNLV-2011	JX262391	JX262391	China	2011
Subgenotype 2.2				
CSF1060		JX162241	Nepal	2011
CSFV-UP-GZ-NVD-11	JQ861548	JQ861548	India	2011
Hisar	EU567079	EU567075	India/Hisar	2008
CSFV/UK/LAL-290	KC595507	KC851953	India/UK	2012
CSFV-UP-BR-757-09	KC533785		India	2009
Meghalaya(D)	EU180017		India/Meghalaya	2007
Patna (BR1/06)	EU567082		India/Bihar	2008
MG 214	EF210360		India/Meghalaya	2007
169/Lokhara	EF185782		India/Assam	2007
Mizoram		EU567072	India/Mizoram	2008
34MIB2009_E2NRV		HM768900	India/Kerala	2009
Meghalaya 214 K		EU567073	India/Meghalaya	2005
Mathura		EU567077	India/UP	2008
Subgenotype 2.3				
Alfort/Tuebingen	J04358	J04358	Germany	1989
Sp01	FJ265020	FJ265020	Spain	2001
Subgenotype 3.4				
94.4/IL/94/TWN	AY646427	AY646427	Taiwan	1994
P97	L49347	L49347	Taiwan	2006

Details of reference sequence used in sequence analysis

#Wherever year of collection is not available, year of submission of publication has been taken

of E2 gene region segregated the strains into three major groups (groups 1–3) with various subgroups. The 5'UTR sequence based phylogenetic tree grouped all the field isolates under study into two different clusters in subgroup 2.2 (Fig. 1). The majority of the field isolates were in one cluster with Meghalaya (D) and CSFV/UK/LAL-290 isolates; whereas isolate V8, (Supol), V11 (Bhopal) and V25 (Lalkuan) were segregated with Patna (BR1/06), Hisar, CSFV-UP-GZ-NVD-11, CSFV-UP-BR-757-09 isolate in other clusters. Phylogenetic tree reconstructed using 190 nt of E2 gene revealed similar genetic typing results as found in the 5'UTR region but segregated the isolates under study

into three clusters within subgroup 2.2 (Fig. 2). All the CSFV isolates under study along with other subgroup 2.2 virus included in analysis were clustered with isolate from Nepal supported by high bootstrap value.

Phylogenetic analysis revealed that all the sequences in this study in either of the phylogenetic trees fell under subgroup 2.2 of genogroup 2 along with earlier published Indian CSFV isolates and the reference isolates. These findings corroborate with the study of Chakraborty et al. [6] wherein all 3 field isolates were found to be grouped into subgroup 2.2 of group 2. Work of Sarma et al. [31] and Patil et al. [24] are suggestive of the

Fig. 1 Unrooted phylogenetic tree reconstructed using 150 nucleotide within 5'UTR of 43 CSFV isolates employing neighbour-joining algorithm with Kimura 2-parameter using MEGA6. Bootstrap values are shown as percentage near the node. The isolates analyzed in this work are labeled with solid diamond (*filled diamond*). Group and subgroup are labeled as proposed by Paton et al. [26]. Bar represent the number of substitution per site

predominance of group 1 viruses in India. But, the Ind-294B/08 (bangalore-294) strain isolated from an outbreak in Harohalli, Karnataka fell under subgroup 2.2 of group 2

[24]. Of late, a study from Kerala of 19 suspected CSF outbreaks during the period 2013–2014, also revealed the circulation of subgroup 2.2 [29] and so all the samples

Fig. 2 Unrooted phylogenetic tree reconstructed using 190 nucleotide within E2 gene of 43 CSFV isolates employing neighbour-joining algorithm with Kimura 2-parameter using MEGA6. Bootstrap values are shown as percentage near the node. The isolates analyzed in this work are labeled with solid diamond (*filled diamond*). Group and subgroup are labeled as proposed by Paton et al. [26]. Bar represent the number of substitution per site

of the present study. From the phylogenetic tree as well as from aligned nucleotide sequences, it can be inferred that CSFV isolates exhibited higher divergence among themselves and with other members of subgroup 2.2 in the E2 region compared to the conserved 5'UTR region. Grouping of these isolates within the subgroup 2.2 has held true, even when the phylogenetic trees were constructed by UPGMA methods (data not shown). Recently, a study based on the NS5B gene indicated the plausible Chinese origin of

subgroup 2.2 virus in India and provided indirect evidence of routes of CSFV movement within South East Asia region [25]. Similarly, analysis of full-length E2-encoding sequences revealed that two isolates from Nepal belonged to CSFV subgenotype 2.2 and had the highest genetic similarity to Indian isolates indicating Nepal and India as one epidemiological unit for CSFV [27]. This also holds true for the isolates of the this study as they along with the other Indian isolates had clustered with the Nepal isolate in

the E2 sequence based phylogenetic tree with a high bootstrap value. The unrestricted movement of animals and illegal trafficking of pork that occurs across the border might be one of the reasons of the introduction and spread of the viruses across the countries. Also, the wild boars which maintains the virus among the dense populations having large numbers of susceptible young animals [12] cannot be overlooked as a source of virus transmission across the borders. Domestic pigs get infected either due to direct contact [41] or by indirect contact via feeding of contaminated wild boar meat [17, 18]. It was also reported that 59% of primary outbreaks of CSF in Germany over a decade were caused by contact between wild boar and domestic pigs [10, 21]. In India, Barman et al. [2] reported the CSFV genome detection in several organs and tissues collected from wild hogs in three National parks, namely Kaziranga National Park, Manas National Park and Jaldapara National Park. In addition, CSF-specific antibodies were detected in two wild hogs and eighteen feral pigs from the same locations. The phylogenetic analysis of the partial E2 protein gene and 5' untranslated region of CSFV isolates from the wild hog showed identities with genotype 2.2 of the Indian isolates [2].

To conclude the study, the phylogenetic analysis comprising of isolates from different part has further proved the emergence of subgroup 2.2 in India. As the genogroup 2 viruses are mostly of moderate virulence and, therefore, can mutate and adapt better, similar emergence of genogroup 2 viruses has been also observed in Europe, China, and Taiwan [7, 26, 38]. It is also been reported that a vaccines based on the recombinant E2 protein produced from the Brescia (genogroup 1) was not able to protect pigs perfectly against the possible infection of the Paderborn strain (subgroup 2.1) [39]. So with this scenario, even though genogroup 1 viruses are predominant in India but the increasing reports confirming the genogroup 2.2 outbreaks is noteworthy as this may prove to be a more devastating threat to pig industry in the near future. As the information regarding the molecular epidemiology the CSFV in India is very little and the status of the disease in wild boars is lacking, more studies, involving large number of isolates both from wild and domestic pigs covering wider geographic zones are warranted for further resolving the genogroups circulating as well as the tracing the spread of virus for framing better disease control strategies.

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