Phylogenetic analysis of Classical swine fever virus from archival formalin fixed clinical tissues reveals vietnamese origin of the isolates

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Abstract Detection of Classical swine fever virus (CSFV) nucleic acid in archival formalin fixed tissue samples and their use for phylogenetic analysis was investigated. Ten samples were examined for the presence of CSFV nucleic acid by reverse transcriptase polymerase chain reaction (RT-PCR) amplification of 5'UTR and *E2* gene. RT-PCR was found positive for 5'UTR fragment in eight samples while only one tissue samples showed amplification for *E2* gene target fragment. For molecular epidemiology of the disease, 5'UTR PCR product of sample from Darbhanga (Bihar), was cloned and sequenced. The sequence was compared with the sequences available in database. The phylogenetic analysis reveals that the isolate belongs to subgroup 2.2 sharing 98.7% nucleotide identities with Vietnamese isolate (CaTh05-1, AB252170), indicating towards the possible origin of genogroup 2.2 CSFV isolates involved in the outbreak from Vietnam. From the study, it can be concluded that the tissue samples collected and stored in buffer formalin for years can be used to detect CSFV nucleic acid. Results are also suggestive of that the 5'UTR region of genome is more suitable target for RT-PCR based detection of CSFV in archival formalin fixed specimens. The study also indicates the potential of archival formalin fixed tissues for molecular epidemiology and genotyping of the CSF virus.

Keywords CSFV · Detection · Formalin · Retrospective epidemiology · Tissues

Classical swine fever virus (CSFV) belongs to genus *Pes-tivirus* in the family *Flaviviridae*, causes severe hemorrhagic disease in pigs which is often fatal [1, 2]. The disease is a serious problem to pig welfare and pork production worldwide and is one among the top five viral diseases of livestock in India [3]. Rapid and accurate detection of the virus is critical for disease containment while epidemiological information is vital for formulating the prophylactic strategies [4]. Classical swine fever (CSF) can be diagnosed by various conventional techniques but highly sensitive and specific nucleic acid based methods are ideal for detection in tissue samples. In developing countries where facilities are limited, sometimes formalin is the only available option for collection and storage of clinical specimens. These specimen reserves represent the largest available source of biological materials which could be of extreme importance for retrospective epidemiological and genetic studies. Advances in molecular methods have facilitated genetic typing of CSF virus which is useful for classification, to trace the origin and patterns of virus transmission and exposing the weaknesses in control measures. Moreover, the genetic comparison of the isolates obtained from successive outbreaks with known linkages can be used to validate and to interpret the genetic typing to determine the rate of virus evolution in the field. Three regions of the CSFV genome, i.e., 5'UTR (150 nt), *E2* envelope glycoprotein gene (190 nt) and *NS5B* polymerase gene (409 nt), have been most widely used and recommended for classifying CSF virus into groups and subgroups [5, 6]. To date, however, there have been no studies reporting use of archival formalin fixed tissues for

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Table 1 Details of the sequences used in the study

S. No.	Isolate	Acc. No.	Country/place	Year ^c
1	Alfort/187	X87939	France	1987
2	HCLV-INDIA (Vaccine)	EU857642	India	<u>2008</u>
3	CSF 11-89	AJ605584	Andaman and Nicobar Island	<u>2003</u>
4	CSF 23-02	AJ605585	Uttarakhand (India)	2002
5	Paderborn	AY072924	Germany	1996
6	HNSD-2012	JX218094	China	2012
7	GXF29/2013	KP233070	China	2013
8	CSFV/UK/LAL-290 ^a	KC595507	Lalkuan (India)	2012
9	169/Lokhara	EF185782	Lokhara (India)	<u>2006</u>
10	Patna(BR1/06)	EU567082	Patna (India)	2006
11	Hisar	EU567079	Hisar (India)	<u>2008</u>
12	Mathura	EU567081	Mathura (India)	<u>2008</u>
13	372.wb	KF551933	Kaziranga (India)	2008
14	CSF/As/430	KJ645982	Assam (India)	2009
15	Bih-Sup/09	GU722590	Supol (India)	2009
16	WF2	HM138669	Karnataka (India)	2009
17	CSFV-UP-GZ-NVD-11	JQ861548.2	Uttar Pradesh (India)	2011
18	CSFV/UP/RAM/494/2012 ^a	KJ197312	Rampur (India)	2012
19	Prambi ^b	KT239105	Parambikulam (India)	2014
20	CaTh05-1	AB252170	Vietnam	2003
21	94.4 IL 94 TWN	AY646427	Taiwan	1994
22	Kanagawa/74	AB019166	Japan	<u>1998</u>

^a Isolation source is pork from backyard pigs kept for human consumption at local shops

^b Isolation source is wild boar

^c Year underlined correspond to year of submission of sequences wherever collection date is not available

molecular epidemiology of CSFV. Keeping this in view, archival formalin fixed ten tissue samples were subjected to RT-PCR amplification of 5'UTR and E2 gene fragments using recommended primers to compare the target genome segment detection in archival formalin fixed specimens. Further, phylogenetic analysis was carried out to access the invaluable information about the possible origin of outbreak and the movement of the virus in the field. All the samples taken for study were positive for the presence of CSFV antigen in ELISA collected in 2009 from an outbreak and stored in the CSF repository at Indian Veterinary Research Institute, Mukteswar, Uttarakhand, India.

Formalin fixed tissues samples were bathed overnight in running tap water followed by three washing in phosphate buffer saline (PBS, pH 7.2) for 1 h each. All the tissues were triturated to produce 10% (w/v) suspension in PBS for extraction of total RNA using Tri Reagent® (Sigma Aldrich, USA) which was reverse-transcribed using RevertAid™ First Strand cDNA Synthesis Kit (Thermo Scientific, USA) following the manufacturer's instructions. The cDNA obtained were subjected to the amplification of housekeeping β -actin gene fragment (275 bp) using primers and amplification conditions as described by Collins

et al. [7] as RNA extraction and reverse-transcription control. After positive amplification for β -actin, the cDNA of all the samples were subjected to amplification of 5'UTR (421 bp) and E2 gene (272 bp) as described by Greiser-Wilke et al. [8] and Lowings et al. [5], respectively. CSFV vaccine used in country was used as reference positive for PCR amplification. Specific amplification was observed in eight samples for 5'UTR target while one of the tissues shows amplification for E2 gene target fragment. Similarly, Singh et al. [9] yielded specific amplicons in three out of ten archival samples subjected to RT-PCR targeting 172 bp of E2 gene. Amplification in less number of samples can be attributed to decrease in the sensitivity of RT-PCR due to formalin fixation process [10]. The false negative results in prolong formalin fixed tissue were also observed previously for other viruses also [11, 12]. Amplification of E2 gene target in one out of ten formalin fixed tissues indicates that the amplification of 5'UTR region of genome is more suitable target for RT-PCR based detection of CSFV in case of formalin fixed tissue samples. In a report it has been quoted that 5'UTR specific primers were considered as more sensitive than E2 region primers for RT-PCR based detection of CSFV genome in non-degraded samples [13].

Fig. 1 Unrooted phylogenetic tree reconstructed using 150 nucleotide within 5'UTR of 23 CSFV isolates employing neighbour-joining algorithm with Kimura 2-parameter. Bootstrap values are shown as

percentage near the node. The isolate analyzed in this work is labeled with *solid square*. Group and subgroup are labeled as proposed by Paton et al. (2000). Bar represent the number of substitution per site

To assess the potential of formalin fixed tissues for molecular epidemiology of CSFV, the 5'UTR amplicon of a sample (Repository I.D. CSFV/BR/DAR/039) was cloned into pJET 1.2/blunt vector (Thermo Scientific, USA) and sequenced (GenBank Accession no. KJ197334). The homology of CSFV-BR-DAR-039/2009 (KJ197334) isolate was determined by comparison with twenty-two similar sequences of field isolates of domestic pig, wild boar and the pork origin available in Genbank. While selecting sequences for analysis, emphasis was given to include sequence of isolates representing all the genotypes reported in the country from different locations and vaccine strain used in the country along with reference sequences of all the genotypes of CSFV (Table 1). CSFV-BR-DAR-039/2009 isolate was found to have 88–94, 94.6–99.3 and 87.3–90.5% identity at nucleotide level among the isolates of genogroup 1, 2 and 3, respectively and 94% nucleotide identity with lapinised vaccine strain used in the country for control of the disease. Within the subgroup 2.2, the

identity ranges from 95.3 to 99.3%. The identity/divergence analysis also reveals that the CSFV-BR-DAR-039/2009 isolate share highest identity with Hisar (EU567079), Kaziranga (KF551933) and Assam (KJ645982) isolate with divergence of 0.7% with difference of only single nucleotide at position 84 of the aligned sequence corresponding to position 283 of Alfort/187, which is unique. Lokhra (EF185782), Mathura (EU567081), Patna (EU567082) and Supol (GU722590) isolates share 98.7% nucleotide identity with difference of two nucleotides at different positions. With the isolates of wild boar and pork origin, the identity was found to be 97.3% with Prambi (KT239105) and Lalkuan (KC595507) and 98% with Rampur (KJ197312) isolates, respectively. Vietnam isolate (CaTh05-1, AB252170) which belongs to subgroup 2.2 was considered closely related to Indian isolates having nucleotide percent identity ranging 94.6–98.7 among the subgroup 2.2 with 98.7% identity with (EU567079), Kaziranga (KF551933) and Assam (KJ645982) isolate and

98% with Lokhra (EF185782), Mathura (EU567081), Patna (EU567082), Supol (GU722590) and Darbhanga (KJ197334) isolate.

The relationship between CSFV isolates circulating in India and its neighboring countries was determined by reconstructing the phylogenetic tree using 150 nt of 5' end of 5'UTR sequence of test isolate along with twenty-two reference sequences as described earlier [14]. Multiple sequence alignment was carried out using Clustal W algorithm [15] and Nucleotide divergence was calculated using MegAlign programme from LaserGene Biocomputing software Package (DNASTAR Inc.). The phylogenetic tree was reconstructed using neighbor joining method in the MEGA software version 6 [16]. Bootstrapping was carried out with 1000 replications [17]. 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 [18] 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. [6]. The resultant phylogenetic tree clustered the isolates under analysis to three major genogroups 1, 2, and 3 (Fig. 1) and the topology of tree obtained was similar to that seen in several previously published phylogenies [19, 20]. The test isolate CSFV-BR-DAR-039/2009 was segregated to genogroup 2.2 along with Hisar, Kaziranga, Assam, Mathura and Patna isolates and do not seem to be related to the vaccine stains (genogroup 1.1) used in the country which indicate no reversal of the live vaccine virus strain being used in the country. The subgroup 2.2 CSFV isolates prevailing in many European countries in the past [21], emerged recently in India and several Asian countries, has been identified as one of the most important genogroup responsible for majority of epizootics reported in recent years [22, 23]. The test isolate CSFV-BR-DAR-039/2009 and the other isolates of subgroup 2.2 used in the analysis were clustered with CaTh05-1 isolate from Vietnam, which indicates the likely introduction of genogroup 2.2 isolates in India is from the neighboring countries. A similar finding of subgroup 2.2 CSFV incursion from China was also reported by Patil et al. [3]. Therefore an epidemiological link between the CSF outbreaks of subgroup 2.2 in India and its neighboring countries cannot be ignored. This also clue for the international transmission of CSFV and indicated the possible outbreak trend in the future rooted in the economic trade of pig populations introduced from overseas. In conclusion, the data presented in this study is suggestive of that in resource limited setting, collection of tissue samples in buffer formalin can be adopted for detection of CSFV nucleic acid by RT-PCR amplification of 5'UTR and can be used for molecular

epidemiology and typing of the virus. Also, the study providing a basis for in-depth studies for utilizing the valuable large reserves of archival formalin fixed clinical samples for retrospective epidemiology and better understanding of the evolution and diversity of virus.

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