



Molecular Epidemiological Studies on Classical Swine Fever Virus in Pig Population of Assam, India

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ABSTRACT

Background: Classical swine fever (CSF) continues to be a persistent threat to pig production in North-Eastern states of India, including Assam. This study aimed to investigate the molecular epidemiology of "Classical swine fever virus (CSFV)" by analysing the NS5B gene from field samples collected between 2021 and 2023.

Methods: Tissue specimens from CSF-suspected pigs from various districts of Assam were examined for pathological lesions. Molecular diagnosis was performed using reverse transcription-PCR (RT-PCR) targeting the NS5B gene, followed by sequencing and phylogenetic analysis.

Result: Pathological findings revealed typical CSF-associated lesions, including necrotizing tonsillitis, haemorrhagic pneumonia, renal cortical haemorrhages, lymphoid depletion and splenitis with infarction at the edges. Reverse transcription – PCR assay on tissue samples confirmed the presence of CSFV. Nucleotide sequences and phylogenetic analysis based on the 449 bp fragment of NS5B gene revealed that the circulating CSFV belonged to the genotype 2 and subgroup 2.2. The field isolates shared high nucleotide identity (92.63 to 99.57%) among themselves and clustered closely with known subgroup 2.2 reference strain from India with 94.36% to 95.30% nucleotide homology. This study provides evidence of increasing co-circulation of subgroup 2.2 CSFV strains in Assam and highlights the utility of NS5B-based phylogeny for understanding CSFV evolution and informing control strategies.

Key words: 2.2 subgroup, CSFV, Histopathology, Molecular epidemiology, NS5B gene, Phylogenetic analysis.

INTRODUCTION

Classical swine fever (CSF) is a highly contagious, economically significant viral disease of domestic and wild pigs and is listed as a notifiable disease by the World Organization for Animal Health (WOAH, formerly OIE) due to its transboundary potential and devastating impact on the swine industry worldwide (Blome *et al.*, 2017). The causative agent, Classical Swine Fever Virus (CSFV), is a positive-sense single-stranded RNA virus classified under the genus *Pestivirus* within the family *Flaviviridae*. The CSFV genome is approximately 12.3 kb in length and encodes a single polypeptide, which is co- and post-translationally cleaved into structural and non-structural proteins essential for viral replication and pathogenesis (Zhang *et al.*, 2010).

Molecular epidemiology has become an essential tool in understanding the evolution and transmission dynamics of CSFV. Genotyping of CSFV is primarily based on sequence analyses of conserved genomic regions such as the 5' untranslated region (5' UTR), E2 and NS5B genes. The NS5B gene, encoding the RNA-dependent RNA polymerase, is particularly useful due to its high degree of conservation across strains. Based on E2 gene analysis, CSFV isolates are grouped into three major genotypes (1, 2 and 3), which are further divided into 13 sub-genotypes (1.1-1.4, 2.1-2.5 and 3.1-3.4) (Singh *et al.*, 2017 and Izzati *et al.*, 2021). However, recent studies have proposed a new classification system subdividing genotype 2 further into subtypes up to 2.7, reflecting the increasing genetic diversity of the virus (Izzati *et al.*, 2021 and Rios *et al.*, 2018).

Clinically, CSF presents in acute, subacute, chronic, or subclinical forms, depending on viral virulence and host

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immune status. Highly virulent strains typically cause acute disease, characterized by high fever, anorexia, skin haemorrhages, cyanosis and neurological signs, often culminating in sudden death. Chronic infections, often caused by low-virulent strains, may lead to progressive weight loss, intermittent diarrhoea, respiratory distress, and secondary bacterial infections (Blome *et al.*, 2017). The virulence of recent CSFV isolates has been a topic of debate, with many field strains exhibiting milder clinical signs, making diagnosis difficult and often delayed. In several outbreaks, especially in endemic regions, the disease remained undetected due to its non-specific clinical signs and the lack of differential diagnosis, allowing widespread transmission and significant economic losses. Determining the interplay between viral virulence and host factors such as age, breed, immune competence,

and co-infections remains a challenge (Blome *et al.*, 2017 and Ganges *et al.*, 2020).

Historically, CSF was first recognized in the 1830s in the United States. Since then, the disease has been reported in numerous countries across the America, Asia, and Europe (Dong and Chen 2007). In India, the North-Eastern region is particularly vulnerable, where pig farming is not only a critical source of livelihood but also contributes significantly to nutritional and economic security. Among these states, Assam is a major hub for pig production and consumption. Recurrent CSF outbreaks in Assam have led to significant economic losses, undermining the pig-rearing communities and posing a major challenge to animal health infrastructure and disease control efforts. The present study was undertaken to investigate the pathology and molecular characterization of CSFV strains circulating in Assam during the period between 2021 and 2023. Specifically, we aimed to obtain pathological changes in naturally infected pigs and to genetically characterize the virus through sequencing of the NS5B gene. Phylogenetic analysis based on this gene region were used to assess the genetic relationships of the local isolates with global CSFV strains, providing insights into viral diversity, evolution, and potential epidemiological linkages. This information is critical for informing effective CSF surveillance, control, and vaccination strategies in the North-Eastern region of India with reference to Assam.

MATERIALS AND METHODS

Sample collection

Tissue samples were collected from 23 pigs suspected to have died of CSFV between 2021 and 2023, across seven different districts of Assam including Lakhimpur, Kamrup Metro, Dibrugarh, Jorhat, Dhemaji, Cachar and Baksa. Clinical signs were recorded, and detailed post-mortem examinations were performed on dead animals. Representative tissue samples, including tonsils, lymph nodes, spleen, kidneys, lungs, heart, and intestines were collected and preserved in 10% neutral buffered formalin for routine histopathological processing. For molecular analyses, representative samples were also preserved at -80°C. Formalin-fixed tissues were processed following standard protocol (Bancroft and Gamble 2008) embedded in paraffin and sections were cut to 4-5 µm thickness using a rotary microtome. Histopathological lesions were noted after tissue sections were stained using H and E staining.

Nucleic acid extraction and PCR assay

RNA was extracted using the GeneJet Genomic RNA purification kit (Thermo Scientific, USA) from tissue samples. The extracted RNA was quantified using a Spectrophotometer (Biophotometer, Eppendorf) and subsequently, a 200 ng of total RNA were used for cDNA synthesis by using RevertAid First Strand cDNA Synthesis Kit (Thermo Scientific, USA) as per the manufacturer's recommendations. This cDNA were used for the detection of CSFV by RT-PCR with the help of previously described primer pairs (Björklund *et al.*, 1999) targeting the 449 bp region of NS5B gene. The amplified products were subjected to 1.5% agarose gel electrophoresis at 100V for 30 minutes. The amplicons were stained with ethidium bromide and visualised under UV light against a 100 bp DNA marker (GeneRuler, Thermo Scientific, USA)

Sequencing of NS5B gene and sequence analysis

The amplified 449 bp fragments of NS5B gene were purified by using GeneJET Gel Extraction Kit, K0691, Thermo Scientific, USA) and cloned into PTZ57/T vector by using InstaClone kit, (Thermo Scientific, USA). The clones were sent for Sanger's sequencing at DNA sequencing facility, Delhi University. The obtained sequences were analysed and aligned by using Mega-X software (Kumar *et al.*, 2018) and annotated sequences were submitted to GenBank and the accession numbers are obtained (PV640781, PV640782 and PV640783). NS5B reference sequences were collected from NCBI database encompassing different genotypes, year of sample collection and geo-location. A reference dataset of 57 sequences were created after rigorous curation. The three sequences from this study were combined with the reference dataset and were used for phylogenetic analysis. T92 was identified as the best fit nucleotide substitution model and phylogenetic analysis was performed by Maximum-Likelihood method with a bootstrap-value of 1,000 replicates by using Mega-X software (Kumar *et al.*, 2018). Details of the samples are given in Table 1.

RESULTS AND DISCUSSION

Clinical disease and pathology of CSFV affected pigs

A broad spectrum of clinical and pathological progression was observed among the affected pigs during the period of field investigation. Most of the animals infected with (CSF) exhibited general clinical signs including, elevated body temperatures ranging from 104 to 106°C, marked lethargy,

Table 1: Details of the sample used in this study with the GenBank accession numbers.

Isolate	Accession numbers	Source	Vaccination status	Year of collection
CSFV/ASM/IND/L1	PV640781	Large Black, 6 months old, female	Not vaccinated	12-Sep-2021
CSFV/ASM/IND/S1	PV640782	Duroc, 3 months old, female	Not vaccinated	22-Jun-2022
CSFV/ASM/IND/S2	PV640783	Large white Yorkshire (LWY), 15 months old, male	Not vaccinated	12-Jul-2023

severe depression, and complete loss of appetite. Erythematous lesions were observed on ears, abdomen and legs (Fig 1A). The pigs were often seen huddling and piling up in corners. Neurological symptoms were evident in some cases, including incoordination, posterior paresis and convulsions. Detailed post mortem examination revealed, pinpoint to petechial haemorrhages on the subcapsular cortical surface of the kidney. These lesions conferred a characteristic “turkey egg” appearance, which is considered pathognomonic for (CSF). In most cases, the kidneys appeared swollen and congested, with the presence of focal pale areas (Fig 1B). Upon cross-sectioning, prominent cortico-medullary congestion was observed. The palatine tonsils showed moderate to severe congestion and petechial haemorrhages (Fig 1C). The liver was markedly enlarged, congested, and haemorrhagic, often with rounded margins. Mesenteric blood vessels showed severe congestion. The mesenteric lymph nodes were mottled, severely congested and haemorrhagic, and in some cases, it was oedematous and pale. Spleen showed haemorrhagic infarcts on the edges and was enlarged (Fig 1D).

Histopathology of the tonsils and other lymph nodes showed marked lymphoid depletion, follicular necrosis, interfollicular mononuclear infiltration, mild congestion, and focal haemorrhages. Renal lesions included interstitial nephritis with mononuclear inflammatory infiltration, cortico-medullary congestion, tubular epithelial degeneration, and

focal necrosis (Fig 2A, B). Glomerulonephritis with glomerular hypercellularity and inflammatory cell infiltration was also observed. The spleen exhibited lymphoid depletion, follicular atrophy, multifocal necrosis, red pulp haemorrhages, and sinusoidal congestion (Fig 2C). Inguinal lymph nodes showed marked depletion of lymphocytes from the lymphoid follicles, and follicular necrosis along with extensive haemorrhage in the para-follicular region (Fig 2D). Liver sections showed centrilobular congestion, sinusoidal dilation, fatty and vacuolar degeneration, and focal necrosis (Fig 2E). Lungs demonstrated interstitial pneumonia with septal thickening, alveolar capillary congestion, bronchiolar epithelial hyperplasia, lymphoid follicle formation, and areas of haemorrhage and emphysema (Fig 2F). Intestinal mucosa revealed villous atrophy, epithelial necrosis, crypt degeneration, haemorrhages, and in severe cases, mucosal sloughing and submucosal involvement.

Molecular detection sequence analysis and phylogenetic analysis

A total of 23 pigs suspected of CSF were included in this study of which, six cases tested positive. The RT-PCR amplicons targeting the NS5B region of the CSFV genome (449 bp) from three field isolates were used for characterization, followed by genotypic classification based on sequence analysis. According to the phylogenetic

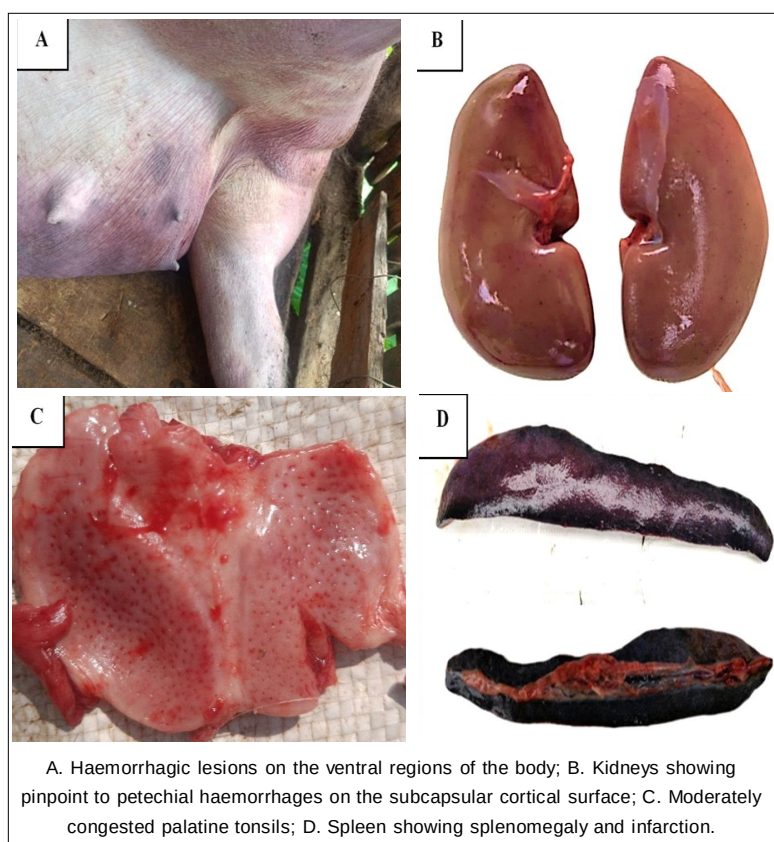


Fig 1: Gross pathology of CSF affected animals.

analysis of nucleotide sequence of full NS5B gene, all three of the CSFV isolates that were collected in 2021-2023 were placed in the genotype 2 and subgroup 2.2 (Fig 3). The nucleotide sequence similarity among the three isolates ranged from 92.63 to 99.57% indicating a high degree of genetic homogeneity among themselves (Fig 4). When compared with reference sequences representing various CSFV genogroups, the three isolates showed sequence similarity ranging from 94.36% to 95.30% with the highest identity observed with subgroup 2.2 viruses, particularly with the other Indian isolates (MK405702, KC533776, JQ861548). Despite being grouped under the same phylogenetic subgroup, the Indian isolates formed a distinct clade, suggesting region-specific evolution. No clear association was observed between the geographical

origin of the isolates and their positions in the phylogenetic tree. These results confirm that the circulating CSFV strains in the study area belong to subgroup 2.2 of genotype 2 and exhibit localized genetic variation within the NS5B region, further supporting the role of NS5B as a reliable marker for molecular epidemiological studies.

CSF remains one of the most economically significant viral diseases of pigs in India, with serious implications for the swine industry (Barman *et al.*, 2016). The disease is notifiable to the (WOAH) due to its highly contagious nature, rapid transmission, and potential to cause devastating mortality and productivity losses. In regions like Assam, where pig farming plays a critical socio-economic role, CSF outbreaks present a substantial threat to rural livelihoods.

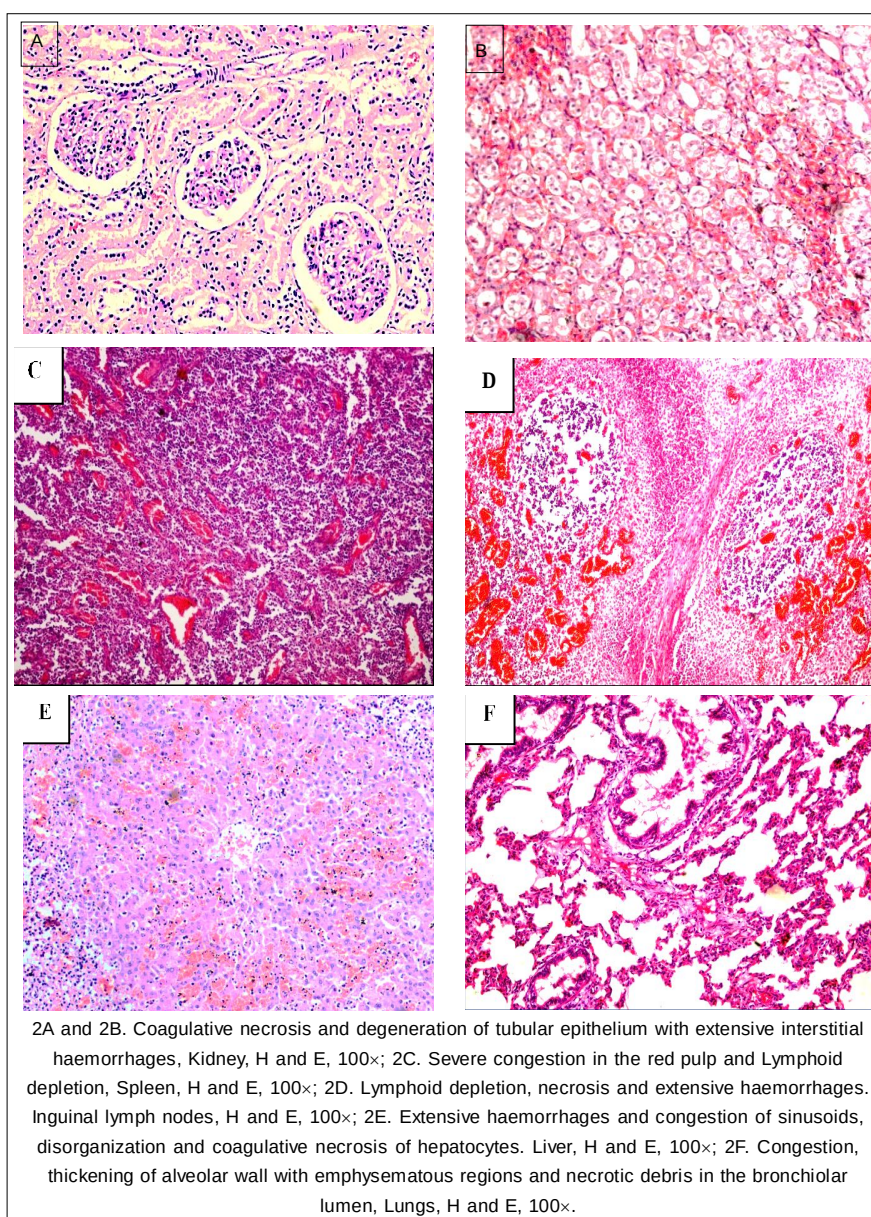


Fig 2: Histopathological lesions in CSFV affected pigs.

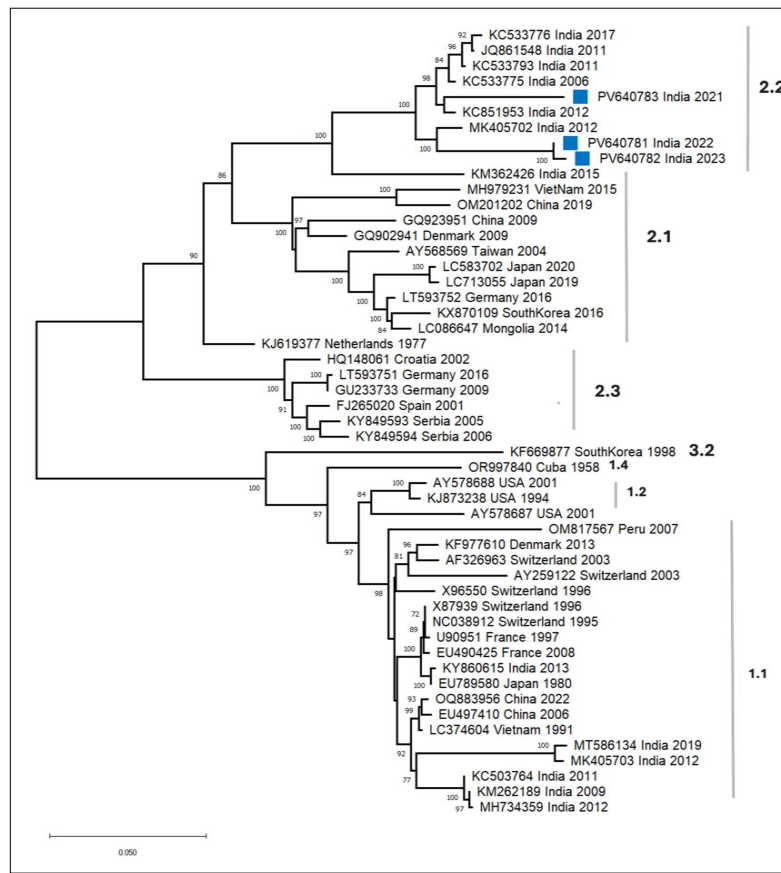


Fig 3: Phylogenetic tree based on analysis of NS5B gene showing close grouping of the field isolates (Blue square marked) in the subgroup 2.2 of genotype 2 of CSFV.

Sl No	Sequences	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19
1	MK405703 India 2012 1.1																			
2	OR195698 China 2022 1.1	99.920																		
3	AF326963 Switzerland 2003 1.1a	99.934	99.957																	
4	KC503764 India 2011 1.1b	99.938	99.947	99.965																
5	NC038912 Switzerland 1995 1.1c	99.940	99.953	99.975	99.970															
6	AY578688 USA 2001 1.2	99.923	99.939	99.956	99.943	99.957														
7	OR997840 Cuba 1958 1.3	99.899	99.919	99.924	99.917	99.932	99.930													
8	OM201207 China 2018 1.4	99.815	99.829	99.836	99.825	99.835	99.828	99.820												
9	GQ902941 Denmark 2009 2.1	99.823	99.835	99.838	99.831	99.842	99.837	99.835	99.946											
10	AY554397 Taiwan 2004 2.1a	99.813	99.831	99.837	99.827	99.837	99.833	99.834	99.941	99.968										
11	AY568569 Taiwan 2004 2.1b	99.817	99.834	99.837	99.823	99.839	99.831	99.829	99.963	99.950	99.949									
12	JQ861548 India 2011 2.2	99.809	99.813	99.828	99.821	99.825	99.820	99.815	99.885	99.903	99.895	99.888								
13	PP209410 India 2021 2.2	99.813	99.829	99.837	99.825	99.834	99.826	99.817	99.873	99.887	99.881	99.875	99.951							
14	PP209411 India 2022 2.2	99.821	99.835	99.861	99.843	99.848	99.839	99.832	99.876	99.888	99.884	99.881	99.944	99.926						
15	PP209412 India 2023 2.2	99.823	99.839	99.866	99.846	99.851	99.844	99.835	99.874	99.885	99.882	99.876	99.940	99.930	99.996					
16	LC388757 Vietnam 2013 2.2	99.811	99.819	99.829	99.818	99.824	99.825	99.809	99.894	99.909	99.900	99.898	99.912	99.893	99.897	99.893				
17	HQ148061 Croatia 2002 2.3	99.820	99.842	99.843	99.832	99.842	99.841	99.829	99.879	99.889	99.883	99.885	99.872	99.857	99.863	99.860	99.886			
18	LT593757 Germany 2016 2.3	99.816	99.840	99.842	99.834	99.841	99.841	99.830	99.884	99.888	99.881	99.891	99.874	99.855	99.865	99.862	99.888	99.972		
19	KF669877 SouthKorea 1998 3.2	99.862	99.874	99.890	99.887	99.893	99.890	99.883	99.827	99.825	99.823	99.831	99.815	99.818	99.824	99.827	99.817	99.826	99.829	

Fig 4: Per cent nucleotide identity of the NS5B gene among CSFV isolates from Assam, India, and reference sequences from GenBank.

Accurate and rapid diagnosis is essential for effective control of CSF. Clinical signs alone are often insufficient for conclusive diagnosis due to their variable presentation, ranging from per acute death to chronic wasting. In the present study, the clinical signs, gross and microscopic lesions observed were consistent with findings reported in earlier studies (Rahman *et al.*, 2001; Rajkhowa *et al.*, 2013 and Barman *et al.*, 2016). Molecular detection using RT-PCR is a highly sensitive and specific method for CSFV detection, and it enables confirmation even in preclinical stages (Depner *et al.*, 2006). The RT-PCR targeting the NS5B gene has confirmed the presence of the virus in total of 6 field samples collected from different districts of Assam between 2021 and 2023. Phylogenetic analysis of the NS5B gene revealed that all the three isolates belonged to subgroup 2.2, clustering with previously reported Indian subgroup 2.2 isolates of genotype 2. This suggests continued circulation and genetic stability of this subgroup in North-Eastern India. Prior studies conducted in southern states such as Kerala and Karnataka, also reported the increased circulation of CSFV subgroup 2.2 viruses (Shivaraj *et al.*, 2014 and Bhaskar *et al.*, 2015). Earlier studies from Assam reported subgroup 1.1 as the prevalent genotype (Desai *et al.*, 2010) indicating a possible shift in circulating genotypes in the region over time. This could be attributed to factors like pig trade and transboundary animal movement, as Assam shares porous borders with other North-Eastern states and neighbouring countries. In our study, some of the field isolates from this study showed close similarity with foreign strains from Southeast Asia especially from China and Vietnam as revealed by phylogenetic analysis. This raises the possibility that the current Indian subgroup 2.2 viruses may have originated from Southeast Asia, similar to previous hypotheses suggesting cross-border introduction and spread of CSFV (Patil *et al.*, 2012).

Among the CSFV genomic regions used for molecular epidemiological studies, the E2 and NS5B regions are frequently targeted due to their variability and utility in genotyping (Moennig *et al.*, 2003 and Sarma *et al.*, 2011). While E2 gene-based phylogeny is often preferred due to higher discriminatory power and availability of sequence data, our analysis confirms that the NS5B gene can also effectively differentiate Indian subgroup 2.2 isolates, though broader comparisons may be limited due to fewer available reference sequences in public databases.

Historically, genotype 1.1 was predominant in India and therefore the C-strain vaccine is being used as the backbone for control and prevention of the disease. However, the increasing prevalence of subgroup 2.2 raises concerns about cross-protective efficacy. Although no immediate evidence of vaccine failure was documented, the genetic divergence between vaccine and field strains underscores the need for future studies to evaluate cross-protection and potentially consider genotype-matched vaccines.

CONCLUSION

In conclusion, this study provides updated molecular and pathological insights into the current status of CSFV in Assam. The findings suggest that subgroup 2.2 of genotype 2 continues to circulate in the region, with pathological evidence confirming the systemic nature of the infection. Continued molecular surveillance and genetic characterization are vital for informed control strategies, including vaccine evaluation and implementation of biosecurity measures to restrict the spread of the disease.

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Conflict of interest

The authors declare that there is no conflict of interest regarding the publication of this manuscript.

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