



Neutralisation Efficacy of Classical Swine Fever C-strain Specific Vaccine Antibody against Genotype 2

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ABSTRACT

Background: Classical swine fever, a highly endemic pig disease in India, particularly in the NER, spots Assam with the highest outbreaks. Despite available CSF vaccines, concern prevails over protective immune response of C-strain vaccine, as emerging genotype 2 replaces genotype 1 globally, causing outbreak in immunized herds.

Methods: The study assessed the cross-protective and cross-neutralization potency of existing vaccines against additional prevalent genotypes. Archived CSFV samples were reconfirmed by S-ELISA and nRT-PCR. Following E2 full-length genome sequencing and phylogeny (MEGA-X), representative CSFV samples from NER were isolated in PK-15 cell line. Hyper-immune serum was raised to test neutralization and cross-neutralization efficacy.

Result: Of 77 archived samples, 43 were S-ELISA positive and 47 nRT-PCR. Phylogenetic analysis revealed either 1.1 or 2.2 sub-genotypes. Virus titre was 4.49-5.16 log TCID₅₀ per ml in four samples representing two sub-genotypes (1.1 and 2.2) after 5 passages in PK-15 cells. Neutralization assay showed complete neutralization for sub-genotype 1.1, sub-genotype 2.2 showed 84% with C-strain specific antibody. The study revealed prevalence of both sub-genotypes 1.1 and 2.2 in NER with lower neutralization efficacy of vaccine strain antibodies to heterologous genotypes with conserved immunogenic epitope.

Key words: Classical swine fever (CSF), C-strain vaccine, Genotypes, Neutralization.

INTRODUCTION

Classical Swine Fever is a highly contagious WOAHP notifiable viral disease affecting domestic pigs, wild boar (*Sus scrofa*) and pygmy hog (Barman *et al.*, 2012; Blome *et al.*, 2017b). CSFV is an enveloped virus, with single-stranded positive-sense RNA genome and belongs to Pestivirus genus of Flaviviridae family (Lefkowitz *et al.*, 2018). Evolution of viruses categorizes CSFV into three genotypes (1, 2 and 3) and several emerging subgenotypes. Genotype 1 has seven sub-genotypes (1.1, 1.2, 1.3, 1.4, 1.5, 1.6, 1.7) and three sub-genotypes (2.1, 2.2, 2.3) are there within genotype 2 (Silva *et al.*, 2017, Garrido Haro *et al.*, 2018). Genotype 3 has four sub-genotypes (3.1, 3.2, 3.3, 3.4) (Chander *et al.*, 2014, Zhou, 2019). In India notably in North Eastern States this disease is endemic, causing severity and high mortality with great economic losses (Lalremruata *et al.*, 2015, Malik *et al.*, 2020). With the prevalence of historic genotype 1.1 (also the vaccine strain) a shift to genogroup 2 (1.2, 2.1 and 2.2) has been reported globally as well as in NER of India (Roychoudhury *et al.*, 2014; Ahuja *et al.*, 2015; Barman *et al.*, 2020). Reports of immune escape or vaccination failure of pigs immunized with C-strain CSF vaccine is highlighted as the presence of unique variations in the E2 protein, accompanied by antigenic alterations that led to reduced effective neutralization (Nguyen *et al.*, 2022) whereas other reports show higher neutralizing capacity of vaccine than other strains like genotype 2.1, 3.4 (Huang *et al.*, 2020). Thus, cross-protection of C-strain vaccine must be explored. The present study highlights neutralization and cross- neutralization

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efficiency of the vaccine C-strain of CSFV against different circulating geno-groups.

MATERIALS AND METHODS

Samples

Total 77 samples (tissue and lyophilized isolates) collected across Northeastern states were studied during 2020-2022, from the CSFV repository in Department of Veterinary Microbiology, College of Veterinary Science, AAU, Khanapara.

Hog cholera virus infected samples were confirmed using S-ELISA and nRT-PCR (Anonymous, 2020).

Isolation and characterization of field isolates

Confirmed CSFV positive samples in S-ELISA and nested RT-PCR were passaged in PK-15 cell line upto 5th passage. Propagated virus was confirmed by immunoperoxidase assay (IPT) and samples representing each NER state were taken for E2 full-length amplification (Table 1). Alfort/187 reference sequences and reported CSFV field isolates from India and abroad in Genbank were used for sequence comparison. Phylogenetic analysis was done with CLUSTAL W and MEGA-X software. The linear conserved immunogenic motif "TAVSPTTLR" in domain A in E2 epitope was checked for identical region with all CSFV strains passaged till P5. TCID₅₀ of the field isolates propagated in PK-15 cell line upto 5th passage and cell culture adapted vaccine virus was done with fluorescent antibody test (FAT) (Anonymous, 2020). Cells with bright cytoplasmic fluorescence were considered positive. Virus isolates representing all genotypes from each state was titrated to determine TCID₅₀. Final virus titre was expressed as 100 TCID₅₀.

Neutralisation and cross- neutralization assay

Hyper-immune sera was raised against C-strain of CSFV, by injecting purified virus in two healthy CSFV antibody free piglets on days 0,14 and 28. Western blot analysis confirmed it as E2 specific (Laemmli, 1970) and serum titre was determined by I-ELISA following the method of EU Manual 2020 (Anonymous, 2020). CSFV field isolates were subjected to immunogenic characterization by cross-neutralization against antibody specific to C-strain of CSFV using FAT. Neutralization assay followed the beta method (constant virus/ varying serum) (Anonymous, 2020), with the endpoint defined as the highest serum dilution showing no FAT-stained cells.

RESULTS AND DISCUSSION

The current study was conducted with CSFV positive samples representing all the North-Eastern states.

Isolation and characterization of field isolates

Overall 77 different samples were collected from domestic pigs (70), wild hogs (2), pygmy hogs (5) of North-eastern states of India including Sikkim. The presence of CSF virus in the tissue samples and lyophilized isolates were confirmed by S-ELISA and nRT-PCR. In S-ELISA, 43 samples out of 77 were positive whereas, 47 out of 77 were positive in nRT-PCR for targeting E2 gene of 271bp product size (Fig 1) from all the NE states. Total 22 samples from Assam were found S-ELISA positive and 24 in RT-PCR out of 38 samples, in Arunachal Pradesh 1 was found RT-PCR

positive out of 4, in Meghalaya, all 11 samples collected were S-ELISA and RT-PCR positive, in Mizoram, out of 6, 1 positive in S-ELISA and 4 in RT-PCR, in Nagaland only 2 samples were S-ELISA positive out of 5, in Sikkim only 1 sample was RT-PCR and S-ELISA positive, out of 2 and in Tripura 6 were positive in both S-ELISA and RT-PCR out of 11. As per OIE, both S-ELISA and nRT-PCR were suitable tools for detection of CSF virus in samples (Anonymous, 2020). Li *et al.* (2018) performed ELISA for detection and confirmation of CSF virus in serum samples making it an appropriate detection assay. RT-PCR and E2 nRT-PCR can be the appropriate approach for screening of CSFV (Rout *et al.*, 2015; Thakuria *et al.*, 2015).

Hence, overall positivity rate was 55.8% in S-ELISA and 61% in nRT-PCR. As RNA viruses gets degraded easily, therefore 100% samples could not be recovered emphasizing development of robust biorepository protocol.

A total 5 CSFV isolates with their known genotypes representing NE states were cultured in PK-15 cell line which showed no CPE (Anonymous, 2020). Isolation represents a classical gold standard method for CSFV diagnosis. PK-15 has been reported to be suitable amongst other cell lines (Basumatary, 2017, Anonymous, 2020). Out of total 5 isolates, one isolate from Assam could not be revived in cell culture. The other four isolates representing Meghalaya, Mizoram, Tripura, Sikkim were successfully revived.

Also, molecular characterization of these selected isolates was done by successful amplification of E2 full-length gene at 1119 bp (Fig 2) as it is highly immunogenic and has neutralizing epitopes. For in-depth characterization of CSFV, E2 full-length gene was preferred to get a high-resolution analysis in order to find out genetic homology, diversity and evolution of the virus according to what the EU and OIE Reference Laboratory recommended for CSFV molecular epidemiology (Postel *et al.*, 2012, Beer *et al.*, 2015). Complete E2 gene sequencing enabled precise phylogenetic analysis, distinguishing closely related CSFV isolates from outbreaks with associated epidemiology (Postel *et al.*, 2012). Previous findings have classified CSFV into three main genotypes- 1, 2 and 3, which have been further divided into various emerging subgenotypes. Within Genotype 1, seven subgenotypes were identified (1.1, 1.2, 1.3, 1.4, 1.5, 1.6, 1.7). Genotype 2, has three subgenotypes (2.1, 2.2, 2.3) (Silva *et al.*, 2017, Garrido Haro *et al.*, 2018). Moreover, Rios *et al.* (2018) reported that the genotype 2 CSFV strains were reorganized into seven subgenotypes (2.1-2.7) due to suggestions to rename the formerly identified subgroups under the subgenotypes 2.1 and 2.2. Genotype 3 was also observed to encompass four subgenotypes (3.1, 3.2, 3.3, 3.4) (Zhou, 2019).

Phylogenetic analysis on the basis of E2 full-length gene sequences formed two distinct clads. Strains CSFV_

Table 1: Primer details for the current study.

Primers	Sequences	Size (bp)	Reference
E2-F	5'CGGAATTCATGCGGCTAGCCTGCAAGGAAGATTAC 3'	1119	Kumar <i>et al.</i> (2016)
E2-R	5'CCGCTCGAGTCAACCAGCGGCGAGTTGTTCTG 3'	1119	

AAU_As01, CSFV_AAU_Sk01 and CSFV_AAU_Tr01 from Assam, Sikkim and Tripura, respectively were phylogenetically close at nucleotide level to the CSFV sequences from already submitted sequences to GenBank from Assam, India (MT232843.1, Pig/India-Assam/96/2017-CSFV) and to sequence from Uttarakhand, India (KC851953.1, CSFV IND-UK-LAL-290) and clustered under Genotype 2.2. Also, CSFV_AAU_Mg01 and CSFV_AAU_Mz01 from Meghalaya and Mizoram shared close phylogenetic relationship with the standard reference sequence (KM262189.1) CSFV/IVRI/VB-131 from India and (X87939.1) Alfort/187 that belonged to genotype 1.1 (Fig 3). The current findings reveal that three isolates out of five belonged to genotype 2.2 indicating about the shift from genotype 1.1 to 2.2. The genogroup 1 (historical CSFV strain) is highly virulent causing acute cases; genogroup 2 and 3 strains are moderately virulent causing sub-acute and chronic cases (Chander *et al.*, 2014, Bajwa *et al.*, 2015). This study too reveals that the isolates from Assam, Sikkim and Tripura are clustered under Genotype 2.2. Various studies indicated that genogroups other than genogroup 1.1 of CSFV strains have emerged in India too. Since 1980s, China reported emergence of genotype 2.1 (Gong *et al.*, 2016). The shift from genotype 1 to 2 was observed globally at different time periods, as it was reported from Europe, China, Columbia and Taiwan (Malik *et al.*, 2020). In India too, genogroup 2.2 viruses spread recently and replaced subgroup 1.1 viruses (Patil *et al.*, 2012). NE India too notified genotype 2.2 outbreak from wild hog and domestic pigs in nearby sanctuary (Barman *et al.*, 2014). The per cent nucleotide sequence similarity analysis showed that the isolates from Meghalaya and Mizoram belonging to genotype 1.1 revealed 99% nucleotide identity when compared with the standard Alfort/187 strain. Whereas nucleotide identity of isolates from Assam, Sikkim and Tripura belonging to genotype 2.2 revealed 84% nucleotide identity when compared. However, pairwise identity matrices revealed 99% nucleotide identity within the genotypes (Fig 4). The complete genome-based nucleotide for Indian 1.1 strains showed 92.075%-96.38%, similarity and complete genome of Indian 2.2 strains showed 83.425- 84.99% similarity, which corroborates the present findings in the study (Malik *et al.*, 2020). The above-mentioned isolates representing each state of north-east and belonging to different genotypes were further selected for isolation in PK-15 cell line.

Analysis of conserved linear immunogenic region of E2 gene was done by comparing the amino acid sequence analysis to a standard reference virus Alfort/187, the linear immunogenic conserved motif of CSFV isolates ("TAVSPTTLR") using CLUSTAL W. The analysis revealed that the immunogenic component remained unchanged for all the five isolates following its propagation and passage till 5 passages in PK-15 cell line (Fig 5). This result corroborated with the findings of Kumar *et al.* (2015), Anindita (2018). As per reports, the linear immunogenic

epitope of E2 "TAVSPTTLR" remains highly conserved (Wang *et al.*, 2015), although the N- terminal half of E2 gene is considered unstable (Chen *et al.*, 2010). But the 16% difference in pairwise identity of genotype 2.2 in comparison with genotype 1.1, in the present finding brings concern to check for the nucleotide alteration if any, which might lead to divergence of the genotypes. Further, extensive studies will be required to determine the amino acid arrangements and alterations in the immunogenic domain of E2 gene. Furthermore, the linear epitope "TAVSPTTLR" located in domain A, which has been employed in the development of epitope-based vaccines (Reimann *et al.*, 2010; Tarradas *et al.*, 2011), has been extensively documented to exhibit high conservation among various CSFV strains but not in other pestiviruses.

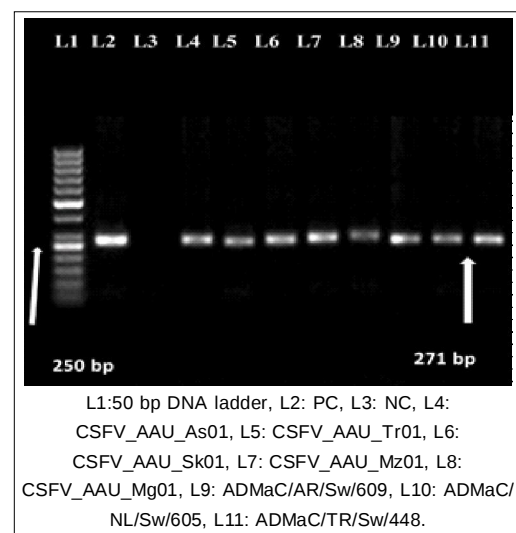


Fig 1: E2-nested RT-PCR of samples from each of the NE states.

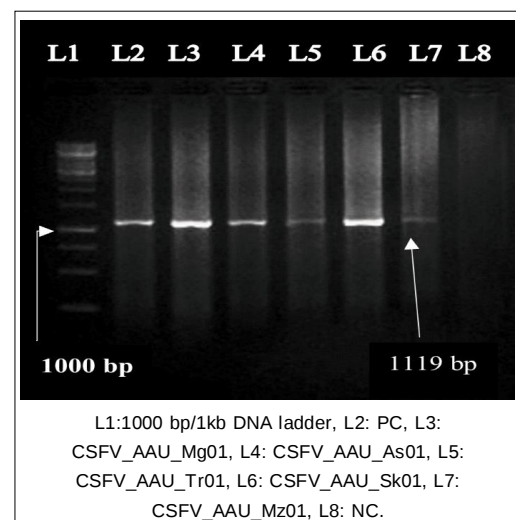


Fig 2: Full-length amplification of E2 gene.

Neutralisation and cross-neutralisation assay based on 100 TCID₅₀

Representing each NE states with different genogroup, total 5 isolates were propagated in PK-15 cell line and confirmed in-situ by indirect IPT test with reddish brown cytoplasm and unstained nucleus confirming replication of the non-CPE producing virus in the cytoplasm (Fig 6). For neutralization assay, hyperimmune sera was raised which showed 1:800 and 1:1600 titres in I-ELISA. Western blot analysis was done by coating with E2 protein, which reacted strongly with hyperimmune sera giving a sharp band at 40 kDa size (Fig 7). This confirmed presence of E2 specific antibodies in the hyperimmune sera raised against C-strain vaccine.

Using this hyperimmune serum, virus titration of each sample was performed by Fluorescent Antibody Virus Neutralization (FAVN) (Anonymous, 2020). CSFV infected PK-15 cell cytoplasm exhibited bright fluorescence (Fig 8).

Neutralization efficacy was evaluated by FAVN. Immunofluorescence test was preferred due to its recognized sensitivity and specificity compared to immunoperoxidase staining (Jafari *et al.*, 2015; Zhang *et al.*, 2017). Although CSF virus neutralization can be done following any of the two methods namely Neutralization Peroxidase Linked Assay (NPLA) and FAVN. 100 TCID₅₀ was determined to perform neutralization assay. Infectivity titre at 5th passage varied in the range of 4.49 to 5.16 among the field isolates. Highest log TCID₅₀ was recorded in CSFV_AAUMg01 of Meghalaya - 10^{5.16} log TCID₅₀ per ml for CSFV (Table 2). The dilution factors for representative isolates were determined to make the final concentration of 100 TCID₅₀ per 100 µl volume required for neutralization test.

One way cross- neutralisation titre of CSFV field isolates were compared with hyperimmune sera raised against CSFV vaccine by FAT following standard protocols

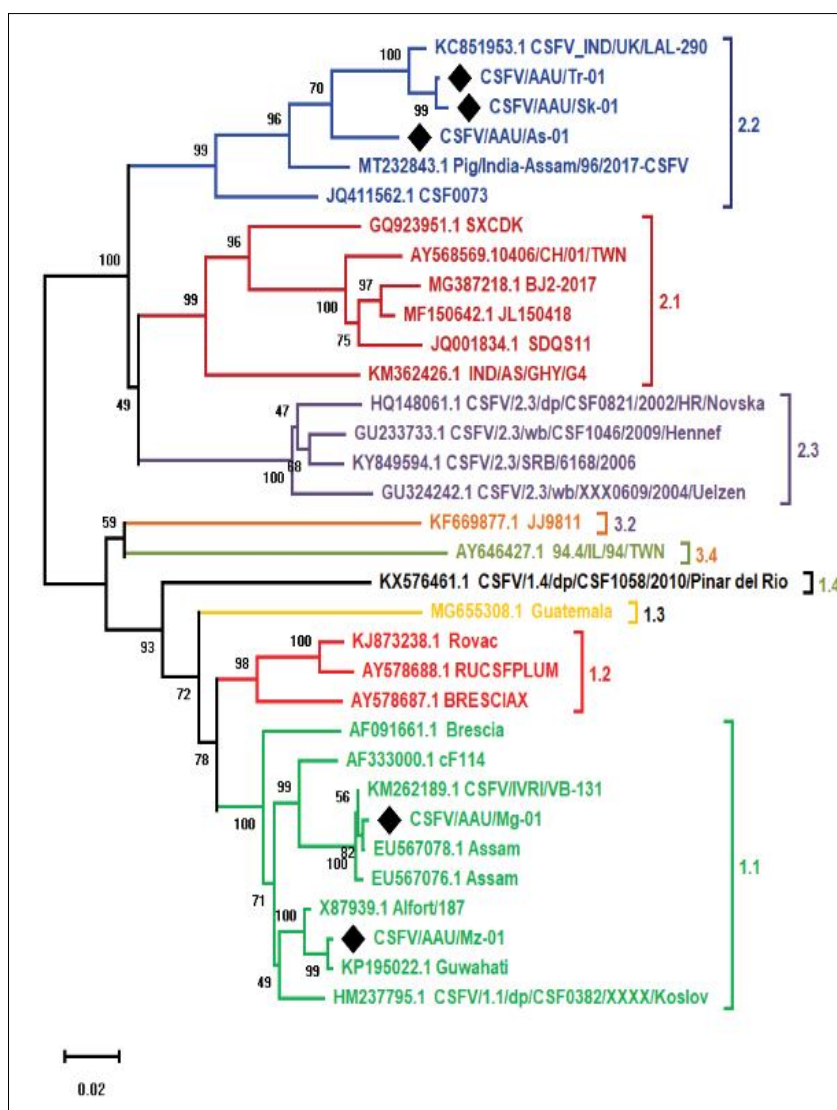


Fig 3: Phylogenetic tree constructed based on CSFV full-length E2 gene by MEGA-X software.

(Anonymous, 2020). The virus-antibody mixture failed to produce any infection showing unstained cell cytoplasm in PK-15 cell. The end point of 50% neutralization titre of the hyperimmune serum was found to range from 1/133 to 1/158 when assayed against the different viruses (Table 2). In between 1:160 and 1:320, 50% neutralization can be seen as 1-3 wells out of five wells showed fluorescence of the infected monolayer. Upon comparison of the field isolates with vaccine virus it was found that CSFV_AAU_Mg01, CSFV_AAU_Mz01 of genotype 1.1 reacted equally with the homologous vaccine virus. Whereas, the vaccine virus antibodies showed lower neutralizing titre for the isolates CSFV_AAU_Sk01,

CSFV_AAU_Tr01 of genotype 2.2. Despite retention of protective titre, neutralization assay using an antibody specific to the C-strain vaccine, alongwith cross-neutralization assay revealed that genotype 1.1 was completely neutralized (100%), whereas 84% neutralization was observed in genotype 2.2. Genotype 2.2 depicted 16% difference in neutralization efficacy. This might also indicate an alteration in the hyper-variable region of E2 region between genogroup 1 and 2 (Liao *et al.*, 2016). An alteration in the nucleotide sequence will reflect change in protein expression of 'TAVSPTTLR'. Further studies are required for distinguishing the antigenic variation between the different genotypes. Chen *et al.* (2010) evaluated the

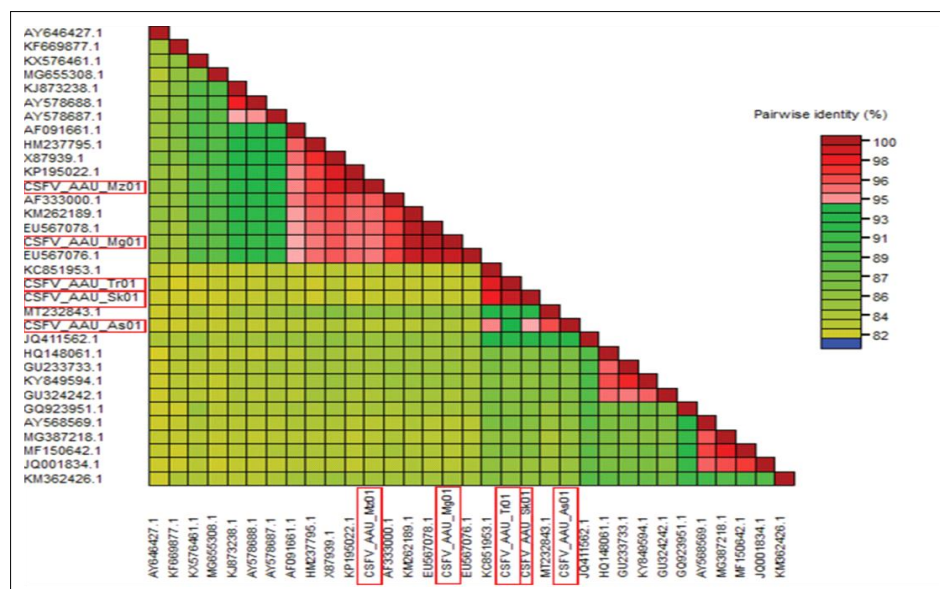


Fig 4: Pairwise identity matrices of CSFV isolates on the basis of full-length E2 gene in comparison with reference sequences.

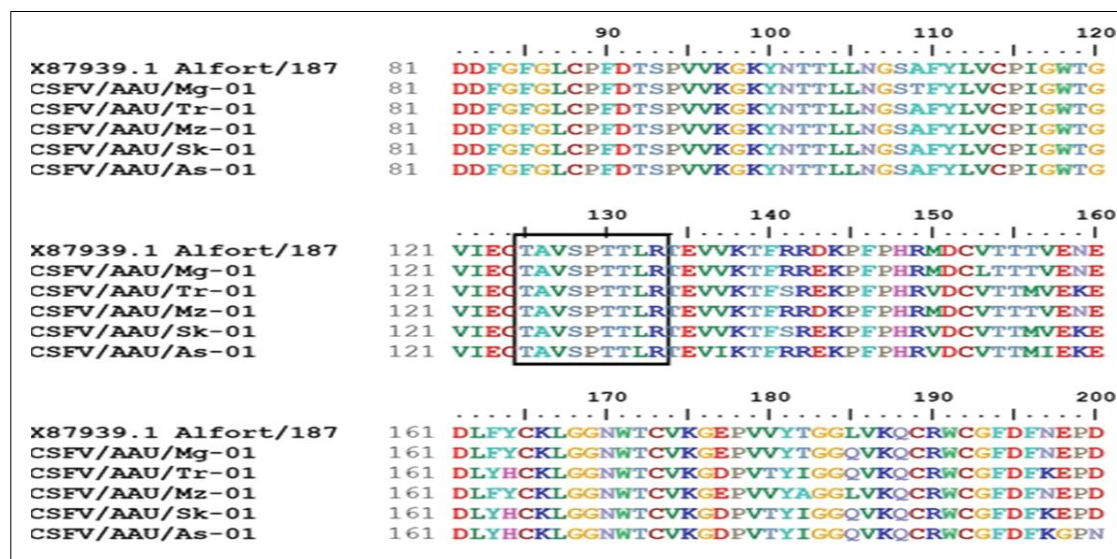
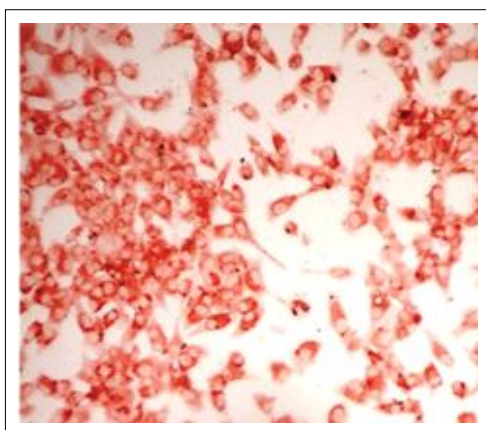
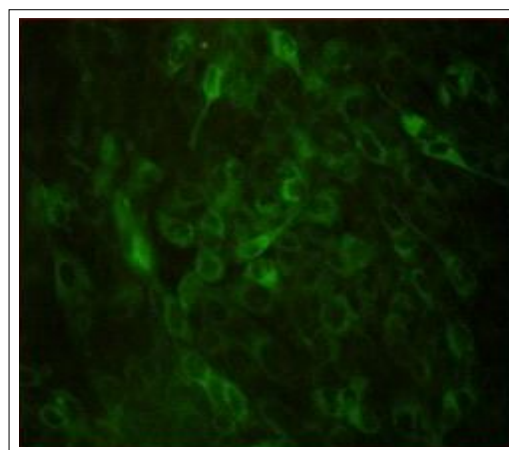
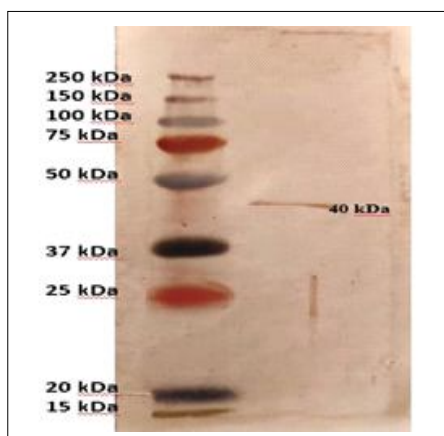


Fig 5: Comparison of Alfort/187 reference E2 sequence of CSFV around the TAVSPTTLR region (marked) with that of test isolates by Clustal W, Bioedit, Dnastar software.

Table 2: Cross-neutralisation titre of CSFV field isolates based on 100 TCID₅₀ with CSFV vaccine strain specific hyperimmune serum.

Lab I.D. of virus isolates	Genotypes	Log TCID ₅₀ /ml at 5 th passage	Neutralisation of virus infection in PK-15 cell monolayer against local isolate at different dilution					50% neutralization titre
			1:20	1:40	1:80	1:160	1:320	
CSFV_AAU_Mg01	1.1	5.16	0/5	0/5	0/5	1/5	3/5	1/158
CSFV_AAU_Mz01	1.1	4.49	0/5	0/5	0/5	1/5	3/5	1/158
CSFV_AAU_Sk01	2.2	4.75	0/5	0/5	0/5	2/5	3/5	1/133
CSFV_AAU_Tr01	2.2	5.00	0/5	0/5	0/5	2/5	3/5	1/133
Vaccine virus	1.1	5.50	0/5	0/5	0/5	1/5	3/5	1/158

**Fig 6:** CSFV infected PK-15 cells showing reddish brown cytoplasm in IPT post 72 hours incubation, x200.**Fig 8:** PK-15 cell infected with CSFV in FAT (post 72 hours incubation, x200).**Fig 7:** Western blot analysis depicting raised hyperimmune sera as E2 specific, sharp band at 40 kDa.

neutralization efficiency of monoclonal antibodies that targeted both the vaccine C-strain and heterologous subgroup 2.1 viruses which revealed reduced efficiency in binding to and neutralizing subgroup 2.1 strains. In a cross-neutralization experiment, it was observed that the anti-C-strain serum significantly diminished its capacity to neutralize RecC-HZ-E2 and QZ-14 (a 2014 subgroup 2.1d field isolate) and conversely, the efficacy of RecC-HZ-E2 and QZ-14 in neutralization was also reduced (Liao *et al.*,

2016). Therefore, present study findings demonstrated the emergence of genotype 2.2 that might overcome the neutralizing efficacy of the vaccine antibodies. However, an extensive study should be undertaken with large field samples from different geographical locations as well as at different time points. Cross-neutralization must be done in both ways along with challenge study for reconsideration of new candidate against CSF virus.

CONCLUSION

This study highlights: (i) CSFV genogroups 1.1 and 2.2 circulates in NER, India. (ii) Their sequences showed 98% and 84% nucleotide identity with the Alfort strain, but 99% within genogroups. (iii) Field isolates of both genogroups revived in the first passage and reached a TCID₅₀ titre (4.49-5.16) similar to the vaccine strain at 5th passage. In neutralization assays, genotype 1.1 showed 100% efficacy, while 2.2 showed 84%. However, extensive E2 whole genome characterization is necessary along with spatial-temporal analysis throughout NER states to depict real shifting of CSFV genogroups and cross-neutralization evaluation should be done uptaking a challenge study to identify potent vaccine candidate.

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Disclaimers

The observations and conclusions expressed in this article are solely those of the authors and are responsible for the accuracy and completeness of the information provided, but do not accept any liability for any direct or indirect losses resulting from the use of this content.

Informed consent

The use of experimental animals in test procedure was approved by the Institutional Animal Ethics Committee of Assam Agricultural University, Khanapara, Assam, India.

Conflict of interest

The authors have no conflicts of interest.

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