Detection of classical swine fever virus E2 gene in cattle serum samples from cattle herds of Meghalaya

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Abstract The present study focused on the detection and genetic characterisation of 5' untranslated region (5'UTR) and E2 gene of classical swine fever virus (CSFV, family *Flaviviridae*, genus *Pestivirus*) from bovine population of the northeastern region of India. A total of 134 cattle serum samples were collected from organised cattle farms and were screened for CSFV antigen with a commercial antigen capture enzyme linked immunosorbent assay (Ag-ELISA) and reverse transcription-polymerase chain reaction (RT-PCR). A total of 10 samples were positive for CSFV antigen by ELISA, while all of them were positive in PCR for 5'UTR region. Full length E2 region of CSFV were successfully amplified from two positive samples and used for subsequent phylogenetic analysis and determination of protein 3D structure which showed similarity with reported CSFV isolate from Assam of sub-genogroup 2.1, with minor variations in protein structure.

Keywords 5'UTR · CSFV · E2 · I-TASSER · RT-PCR

Introduction

Emerging pathogens that can cross the host species barrier to infect new hosts can profoundly affect human and animal health side by side, as well as a threat for wildlife and the agricultural industries. Although most emerging diseases seemingly result from such a process of cross-species transmission, some viruses seem to rarely jump the species barrier and instead co-diverge with their hosts over long stretches of evolutionary time. For example, long-term virus-host co-divergence has been suggested to play an important role in the evolution of vertebrate herpes viruses over a period of ~ 400 million years [20] and insect baculoviruses over a time-scale of ~ 310 million years [28]. It has been proposed that a number of families of DNA viruses have co-diverged with their hosts over long evolutionary time-scales [10, 11, 31], and do so more frequently than RNA viruses, which in contrast display a combination of co-divergence and host switching [12]. In particular, while phylogenetic trees for some RNA viruses, such as particular retroviruses, are generally congruent with those from their hosts suggesting long-term codivergence [14], for others, such as *Flavivirus*, host jumping appears to be relatively frequent [16]. In the case of *Flavivirus*, this likely in part reflects the fact that many are transmitted by arthropod vectors and characterized by short durations of infection [12].

According to the World organisation of animal health (OIE), classical swine fever virus (CSFV, family *Flaviviridae*, genus *Pestivirus*) is one of the notifiable viruses of swine worldwide. Classical swine fever (CSF) is highly contagious and a lethal disease in pigs and wild boars

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caused by CSFV. CSFV is genetically and antigenically related to other pestiviruses such as bovine viral diarrhoea virus (BVDV) and border disease virus (BDV) [30]. CSFV is an enveloped virus with 12.3–12.5 kb long single-stranded, positive-sense RNA genome flanked by highly conserved 5'NTR and 3'NTR [29].

There are approximately 11 million pigs in India, 40% of which are present in the NE states. Backyard pig farming is the major source of livelihood of about 80% of households in NE region, and pork is a key item in the daily diet [4]. Among various other causes affecting the growth of pig farming, CSFV has emerged as the major obstacle for the development of profitable pig farming in India, particularly in the NE states. Several recent studies revealed occurrence of CSFV among pig herds in Assam and other parts of India [1, 21, 22, 27]. Several outbreaks of CSF have been reported from the region suggesting its endemic nature [3, 5]. Northeast India shares a long porous international boundary with China, Bangladesh, Bhutan and Myanmar. The region is highly prone to the trans-boundary transmission of exotic CSFV strains. Recent studies have shown that sub-genogroup 1.1 is prevalent in India [6, 22], with few reports of genogroup 2 [5, 7].

Integrated farming systems are the mainstay of the agricultural sector of north eastern India. Co-habitation of small livestock holdings is quite common in this region and it is hence very common to see a herd of cattle co-existing with pigs in close proximity. This practice is unique to the region and could well serve in bridging niche-groups leading to cross-species disease transmission.

Under these circumstances of regular outbreaks of CSFV in pig herds and practice of integrated farming system of piggery and dairy it was highly necessary to survey if CSFV is undergoing of species jump or not. Because being a RNA virus and a species of *Pestivirus*, CSFV may stay sub clinically in cattle population.

Materials and methods

Origin of samples

A total of 134 bovine samples were collected from organised herds and private farms of Meghalaya especially focusing on the places where both piggery and dairy farming is simultaneously practiced. The samples were mainly collected from Indo Danish Project (IDP) farm of East Khasi Hills ($n = 120$), private cattle holds of Umiam of Ri Bhoi district ($n = 3$) and Ramkrishna Mission cattle farm of Sohra Cherrapunjee ($n = 11$). Each sample was aseptically collected from ear veins in a vacutainer using sterile precaution and was transferred in $-20\text{ }^{\circ}\text{C}$ gel packs to the laboratory within 24–48 h. The samples were

collected over a 2 year period (2013–2015). These samples were collected from areas that had integrated farming operations and a mixed livestock population comprising of bovine and porcine. In the north eastern region small holder farms with livestock being reared in close proximity is quite common. Hence the samples were obtained from these farms or zones of integrated farming practice.

CSFV Ag ELISA

The serum samples were then checked for CSFV Antigen (Ag) using Prionics CSFV Antigen capture (sandwich) ELISA kit (Thermo Scientific, USA).

RNA isolation and RT-PCR

The samples which were positive in CSFV Ag ELISA were selected and RNA extraction was done using Qiagen Ultrasens viral DNA and RNA extraction kit (Qiagen, Germany) and cDNA were prepared using Premium first strand cDNA synthesis kit (Thermo Scientific, USA).

These were then subjected to PCR amplification using CSFV and BVDV specific primers listed in Table 1 to confirm the presence of CSFV and/or BVDV. Primary screening for the viral agent was done with PnPest (*Pestivirus* specific), PnCSF (CSFV specific) and PnBVDV (BVDV specific) primer sets (Table 1) to see if the samples positive in Ag capture ELISA were having CSFV genome or not. The cDNA samples showing desired amplification when using PnCSF primers were then screened for full length E2 gene of CSFV (Primers are listed in Table 1).

The cyclic conditions which were same for the three sets of primers (Table 1) were: primary denaturation at $95\text{ }^{\circ}\text{C}$ for 3 min followed by 40 cycles at $95\text{ }^{\circ}\text{C}$ for 30 s, $55\text{ }^{\circ}\text{C}$ for 30 s, $72\text{ }^{\circ}\text{C}$ for 30 s and final extension at $72\text{ }^{\circ}\text{C}$ for 5 min. The cyclic conditions for PCR with full length E2 specific primers was primary denaturation at $95\text{ }^{\circ}\text{C}$ for 3 min followed by 40 cycles at $95\text{ }^{\circ}\text{C}$ for 30 s, $55\text{ }^{\circ}\text{C}$ for 30 s, $72\text{ }^{\circ}\text{C}$ for 1 min 10 s and final extension at $72\text{ }^{\circ}\text{C}$ for 5 min. DreamTaq green PCR mastermix from Thermo-scientific, USA was used for all the PCR reactions.

Sequencing and phylogenetic analysis

The 1119 nt long PCR products (full length E2 gene) from two positive samples were purified with TaKaRa PCR purification kit and purified fragments were then ligated with pTZ57R/T (plasmid) vector (Thermo scientific, USA) and then DH5 α competent cells (prepared with CaCl_2 treatment) were transformed with the plasmid containing E2 gene. Transformed colonies were isolated by blue white screening and plasmids were extracted with plasmid mini

Table 1 List of the primers used to detect and phylogenetic analysis of classical swine fever virus (CSFV) from bovine origin

Primer name	Primer sequence	Reference
E2 full	F: GCC AAC CGA ACA TTC CTT CTG A R: GTC TCG TTG AGC GAC GAC CA	In this study
PnPest	F: TAG CCA TGC CCT TAG TAG GAC T R: TGT GCC ATG TAC AGC AGA GAT T	[26]
PnCSFV	F: CGT CAG TAG TTC GAC GTG AGC A R: TAT CAG GTC GTA CCC CCA TCA C	[26]
PnBVDV	F: AAC AGT GGT GAG TTC GTT GGA T R: CAC CCT ATC AGG CTG TAT TCG T	[26]

kit (Thermo scientific, USA) and then sequencing was done with ABI Big Dye Terminator v3.1 cycle sequencing kit (Applied Biosystems, USA) in ABI 3500 × 1 genetic analyser. The sequences were assembled using Seqman software (DNASTAR Inc., Madison, USA). Additional CSFV and BVDV sequences were retrieved from NCBI GenBank and used in subsequent analysis. Both the sequences were submitted to the Gen Bank during March 2017 and provisional accession numbers were obtained (KY860531 and KY860532). These sequences were used for building the phylogenetic tree and to derive protein data. Phylogenetic data analysis and tree construction was done with molecular evolutionary genetics analysis (MEGA) software, version 7 [17], based on full length E2 sequences. The bootstrap [9] values were based on 1000 replicates using the neighbour-joining method [25], and the evolutionary distances were determined using the Kimura two-parameter method [15].

Protein structure prediction

One full length E2 gene sequence obtained from cattle (KY860531) was translated to protein sequence using online translation tools and one previously reported sequence of full length E2 from porcine isolate was retrieved from NCBI database and translated to protein sequence by using EditSeq software (Lasergene ver.6). Both the protein sequences were then submitted to iterative threading assembly refinement (I-TASSER) [24] protein structure prediction server to obtain protein 3D structure to assess if there were some differences in the structure because E2 glycoprotein helps virus to enter the host cell. Structural differences because of host change will further help us to understand if the virus is in the process of adaptation. These structures were again compared with BVDV full length E2 protein which was derived from NCBI database and converted to 3D protein structure using I-TASSER. The structures were chosen comparing the C-score and the structures with the highest C-score were taken for analysis.

Result

Detection of CSFV by Ag-ELISA

A total of 10 (7.46%) out of 134 bovine samples were found positive in ELISA. Among these, 8 were from IDP farm, East Khasi Hills and 2 from Umiam farm, Ri Bhoi. However, no positive samples were found among those from Ram Krishna Mission Cherrapunjee.

Detection of CSFV by RT-PCR targeting 5'UTR and E2 region

All the 10 ELISA positive serum samples were clearly showing the presence of CSFV (PnCSFV)-specific amplification whereas there was no amplification in BVDV (PnBVDV) lanes of agarose gel (Fig. 1). None of the samples gave an amplification with Pn Pesti primers, presumably as serum samples were used and the viral load in such samples were low in quantity. Two full length E2 were amplified from sample ID CS911 and AF_C among 10 primary CSFV positive bovine serum samples (Fig. 1). The source of those two samples was IDP farm and Umiam farm, respectively. The detailed result is shown in Table 2.

Quantification of CSFV by real time RT-PCR (qRT-PCR)

10 Ag-ELISA positive bovine serum samples were also positive in qPCR using standard curve method (absolute quantification). The results are shown in Fig. 3 (supplementary) and in Table 3 (supplementary). All the serum samples showed CSFV specific amplification and interestingly two samples CS911 and AF.C that showed lower Ct values were the ones that could amplify the E2 gene. The remaining 8 samples were positive only for 5'UTR and not for E2.

Fig. 1 Detection of CSFV genes in bovine serum by RT-PCR. **a** Partial CSFV 5'UTR region(133 bp) in bovine serum samples. Sample IDs from lane 1 to lane 5 are CS911, CS946, CS1046, AF.C, AF.C/2 respectively. **b** Full length E2 region of CSFV from bovine

serum samples. Sample IDs from lane 1 and lane 2 are CS911 (KY860532), AF.C (KY860531) respectively (1119 bp). For both (**a**, **b**) M is 100 bp plus DNA marker (Thermoscientific, USA), P is CSFV known positive sample (Porcine origin), N is non template control

Table 2 Detailed result of bovine sample screened for classical swine fever virus (CSFV)

Sample ID	Place (district)	Ag ELISA	RT-PCR		
			PnCSFV	PnBVDV	E2 full
CS909	East Khasi Hills	Positive	Positive	Negative	Positive
CS910		Positive	Positive	Negative	
CS911		Positive	Positive	Negative	
CS946		Positive	Positive	Negative	
CS997		Positive	Positive	Negative	
CS1021		Positive	Positive	Negative	
CS1033		Positive	Positive	Negative	
CS1046		Positive	Positive	Negative	
AF_C	Ri Bhoi	Positive	Positive	Negative	Positive
AF_C/2		Positive	Positive	Negative	

Phylogenetic analysis on the basis of full length E2

The full length E2 sequences were first edited with DNASTAR software by Lasergene and matched with other full length CSFV sequences available online with the help of BLAST tool of NCBI and they were showing 100% match with CSFV 2.1 genotype. Since the full length E2 glycoprotein encoding region (1119 nt) is the most suitable for phylogenetic analysis of CSFV [23] a phylogenetic tree was constructed taking the full length E2 gene [Fig. 4 (supplementary)]. The tree revealed closeness among the cattle isolates of CSFV and the isolates belonged to 2.1 genotype which was earlier reported by in 2015 [1] from a naturally infected pig belonging to Assam, India.

Analysis of CSFV E2 protein sequences

Protein sequences were derived from the genetic sequences by using EditSeq software (Lasergene ver.6) and they were aligned with other CSFV and BVDV full E2 protein sequences derived from NCBI [Fig. 5 (supplementary)].

The 3D structure (Fig. 2a–c) which were predicted using I-TASSER and was chosen by the highest C score revealed that both the protein structures derived from b and porcine isolates were almost similar with some dissimilarities which suggested that the virus may be adopting in the new host system.

A total of 16 amino acid variation was there in the E2 protein sequence with respect to the CSFV 1.1, 2.1, 2.2 and 2.3 genogroups and there is a vast difference with BVDV E2 protein sequence. Amino acids at the position no. 18 (I–T), 207 (C–R), 238 (S–P) and 252 (G–W) was changed in case of CSFV E2 found in cattle with respect to other CSFV E2 found in pigs. Amino acids at some positions were the same in case of the cattle isolate and IND/AS/GHY/G4 (reported from Assam, India by Ahuja et al. [1] but different in other genotypes, such as, 121 (N–S), 158 (E–G), 170 (T–I), 242 (N–S), 244 (D–G), 272 (G–P) and 282 (V–I); (Fig. 5). This result suggested that the cattle isolate is similar to the already circulating CSFV isolates in north eastern India. Although both the CSFV E2 found in cattle are coming under 2.1 genogroup according to the

Fig. 2 CSFV E2 protein 3D structure obtained by using I-TASSER server. **a** Obtained from AF.C (cattle origin), **b** obtained from IND/AS/GHY/G4 sequence (porcine origin) (gi 694219975). **c** obtained from full E2 of BVDV (gi 1129879657). The 3D structures were selected on the basis of highest C-score given by I-TASSER

phylogenetic tree (Fig. 4) but there are 11 amino acid changes in the protein sequence from 2.1 genogroup.

Discussion

The present study involves the detection of CSFV genome in b serum samples that were positive for CSFV antigen and a detailed analysis of these isolates. It has been earlier reported that BVDV in pigs is increasingly prevalent and has been found to affect the diagnosis of CSFV. However, detection of CSFV gene/(s) in samples of bovine origin has so far not been reported. It needs to be mentioned that the present study does not provide evidence of an active CSFV infection in bovines but only the presence of CSFV genome as detected by differential primers directed against the 5'UTR of both the viruses, viz. CSFV and BVDV. The incidence of such detection was also very low, i.e. only in two out of 10 samples, we could obtain full length E2 amplification. The sequencing of the full length E2 revealed the relatedness to the CSFV 2.1 genogroup. The isolate was close to a CSFV isolate reported from Assam in 2015. The most interesting aspect was that the bovine samples that tested positive were mostly from farms that were in close proximity to pig farms. In farms that had no access to porcine, no such prevalence was noted. Protein level analysis showed no significant amino acid changes [Fig. 5 (supplementary)], only four amino acids were differing, however the three dimensional folding revealed more aspartate and glutamate residues at the C-terminal region (Fig. 2a, b). Regarding the C-terminal end of E2, five proximal cysteine residues at positions 180, 188, 204, 207, and 241 are critical for the structural integrity of the C-terminal conformational epitopes [18].

There is an amino acid substitution at position 207 from C to R in the CSFV E2 gene found from cattle serum. Hence it could be a result of a compromise in the structural integrity of the E2 domain and thus affect the replication ability of the virus. Cellular attachment and entry is the first step of viral infection of host cells. *E^{gns}* plays a role in virus attachment [13], whereas E2 is involved in both virus attachment to and entry into target cells [13], thereby determining the cell tropism of *Pestivirus* [19]. The sequences and structures of E2 proteins are presumed to be involved in *Pestivirus* host specificity at the level of cell entry [2]. The cell surface receptor of the BVDV E2 glycoprotein is bovine CD46 [19], and inhibition of BVDV infection by CSFV E2 suggests that CSFV E2 and BVDV E2 share an identical receptor [13]. Indeed, both porcine CD46 and HS were recently shown to be the primary components that drive CSFV attachment and entry [8]. In case of CSFV adaptation to bovines, the nature of receptor binding can only be established after a stable genotype of virus is identified. A change in the position 207 that affects the conformational stability of the E2 glycoprotein may indicate adaptation of the virus to either HS or CD46

receptors of cattle origin. In light of the above observations, it merits attention to observe whether more of such finding of CSFV genome are encountered in cattle and their molecular diversity is worth examine.

Another observation is that only two out of 10 serum samples showed E2 amplification using established primers. Defects in the way of deletion mutations/insertions/substitutions in the E2 gene may have rendered detection of E2 negative in these samples. It could also indicate a putative virus that may be adapting to a new host as the E2 molecule is critical in virus attachment and entry.

We have detected presence of CSFV in bovine serum by antigen immunoassay, qRT-PCR detection of 5' UTR gene, RT-PCR detection of 5' UTR and E2 (full length), Sequencing of the E2 full length gene and phylogenetic analysis of the same. We are yet to ascertain the nature and viability of the virus detected in cattle serum. Considering the initial data obtained it would be worthwhile to state that the detection of CSFV genome amplicons along with immunological demonstration of antigen in bovine serum needs further studies on the subclinical nature of infection if any. Such studies would be relevance if experimental infections of calves with the virus are done, viremia monitored and periodic samples collected to ascertain the viability of the virus if present. In case isolation has to be attempted it is essential that controlled animal trials are carried out instead of using serum samples from random collections. The main drawback in using such samples is that they would contain very low titers of antigen and secondly presence of cross reactive BVDV antibodies would be detrimental to such isolation attempts.

The study is first of its kind regarding the genetic profiling of CSFV full length E2 gene from bovine in a north eastern state of India where co-existence of pig and cattle in small holder farms is common. From antigen capture ELISA and primary detection by PCR with the CSFV specific primers we got an initial indication about the presence of CSFV gene in bovine serum samples. The initial idea was then confirmed with the amplification of full length E2 genes. The phylogenetic study revealed that both full E2 gene sequences were very much close with the earlier reported CSFV belonging to 2.1 genotype and distantly related to CSFV genotype 2.3. Phylogenetic tree further revealed that the isolates were distantly related to the bovine pestivirus BVDV. Protein structure prediction was also showing similarity with CSFV genotype 2.1 but with a little change. Surveillance for CSFV genome in cattle thus needs to be done in areas where there is cohabitation or close contact of cattle with pigs.

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