Molecular typing and phylogenetic analysis of classical swine fever virus isolates from Kerala, India

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Abstract Classical swine fever (CSF) is an economically important disease of pigs caused by CSF virus (CSFV) belonging to the genus *Pestivirus* within the family Flaviviridae. The disease is endemic in many countries including India. A comprehensive study was carried out to assess the type of CSFV circulating in the South Indian state of Kerala. During the period 2013–2014, clinical samples were collected from 19 suspected CSF outbreaks of domestic pigs in different districts of Kerala. The samples were tested using nested reverse transcription PCR (RT-PCR) targeting the E2 gene and RT-PCR for 5'UTR of the virus. Partial 5' UTR and E2 gene regions of six CSFV isolates were sequenced. Phylogenetic analysis revealed that all the CSFV isolates belonged to subgroup 2.2. The isolates showed close resemblance to the other CSFV isolates circulating in India. It was also observed that the CSFV viruses from Kannur district were distinct from those circulating in the other districts as evidenced by their divergence from other Kerala isolates in the phylogenetic tree. Close relationship was seen to the CSFV isolates from South East Asian countries.

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Introduction

Classical swine fever (CSF), an economically important disease of pigs posing a great challenge to the swine industry [37] is caused by CSF virus (CSFV), belonging to the genus *Pestivirus* within the family Flaviviridae [13, 38]. It is an enveloped virus having a single stranded non-segmented positive sense RNA as genome. The first confirmed incidence of CSF was documented in 1833 in USA in [12] where it was known as “hog cholera”. Subsequently, the disease was recognized in Europe where the disease came to be known as European fever [35]. The disease was wiped out from Northern America, Australia and Western Europe and the countries of Eastern Europe, Caribbean, Asia and South and Central America were having the greatest incidence of CSF in the world [17]. The disease has also been reported from the Madagascar islands and South Africa in the African continent [9, 23].

The occurrence of CSF outbreak in India was first established in 1962 in Maharashtra [31] which was followed by outbreaks in Uttar Pradesh [14]. The disease was found to be a principal endemic viral disease of swine in majority of Indian states involved in pig husbandry [36]. The disease has been reported from Punjab [27], the North Eastern states of Arunachal Pradesh, Manipur, Mizoram, Nagaland, and from West Bengal, Kerala [26], Karnataka [3] and Tamil Nadu [4].

Classical swine fever virus can be divided into three genotypes (groups), each containing three or four-subgenotypes (subgroups) [24]. The ancient European, American and Asian CSFV isolates were assigned to group

1 and its subgroups. The present day European and Asian isolates were allocated to group 2 and its subgroups. Asian isolates were categorized in one among the four subgroups within group 3 [22, 29].

Genetic characterization of the CSFV is essential to realize the pattern of evolution, transmission and distribution of viruses and the source of epidemics [5]. Various regions of viral genome are employed in the distinction and molecular characterization of CSFV, including the Npro gene [1], NS5B gene [2], E2 envelope glycoprotein gene [16] and 5'UTR gene region of CSFV [22]. Genetic characterization of CSFV from India have been carried out by many researchers [7, 15, 20]. Since the first report of CSF from Kerala in 2007 [26], there are reports of the occurrence of the disease from the State. However no systematic study has been carried out till date to ascertain the genotype of the virus circulating in the State. This paper reports the findings of a benchmark study undertaken to identify the genotype of CSFV circulating in the State employing sequencing of 5'UTR and E2 regions of the virus.

Materials and methods

Collection of samples

The clinical materials comprised of both, tissues collected from necropsy cases and whole blood from live pigs suspected to be suffering from CSF. A total of 19 samples were collected from pigs belonging to different districts of Kerala viz., Wayanad, Calicut and Kannur. Clinical samples collected included whole blood, tonsil, spleen, lymph nodes and pancreas. The tissue samples were collected in sterile screw capped polypropylene vials using sterile scissors and forceps, and stored at -80°C after proper labeling to prevent RNA destruction till further processing. Whole blood was collected in suitable vacutainers containing EDTA and stored at 4°C .

Molecular confirmation of CSFV in the clinical samples

Total RNA was isolated from the tissue samples and blood by using GeneipureTM Total RNA isolation kit (Bangalore Genei, India) as per the manufacturer's protocol. The eluted RNA was stored at -80°C until used. Complementary DNA (cDNA) was made by employing Thermo Scientific RevertAid H Minus First Strand cDNA Synthesis Kit (Thermo Scientific, USA) using random hexamer primers and M-MuLV reverse transcriptase by following the manufacturer's protocol. The cDNA was stored at -20°C for further use.

Detection of CSFV by RT-PCR using 5'UTR and E2 gene specific primer

Presence of CSFV genome in the clinical samples was detected by nested RT PCR using primers specific to E2 envelope glycoprotein gene and 5'UTR as described previously [22]. The primers employed were E2F (5'AGR CCA GAC TGG TGG CCN TAY GA 3') and E2R (5' TTY ACC ACT TCT GTT CTC A 3') (outer primer set); E2NF (5'TCR WCA ACC AAY GAG ATA GGG 3') and E2NR (5'CAC AGY CCR AAY CCR AAG TCA TC 3') (nested primer set); 5' UTR FP (5'CTA GCC ATG CCC WYA GTA GG 3') and 5'UTR RP (5'CAG CTT CAR YGT TGA TTG T 3'). The products were electrophoresed in 1.5 % agarose gels prepared in 0.5X TBE buffer and ethidium bromide was added to a final concentration of 0.5 $\mu\text{g}/\text{ml}$. The gel was visualized to identify the size of the product and photograph was taken in a gel documentation system under UV illumination.

Cloning and sequencing of the PCR product

The amplicons generated by PCR targeting 5' UTR and E2 gene regions were purified from the gel by using GeneJET gel extraction kit (Thermo Scientific, USA) and cloned into pJET1.2/blunt cloning vector using CloneJET PCR cloning kit (Thermo Scientific, USA) as per the manufacturer's protocol. Competent cells (*E.coli* DH5 α) were prepared and transformed with the ligated plasmid as described previously [30].

Confirmation of the recombinant plasmid DNA by PCR

About two to three colonies were picked up from the LB agar plate with sterile microtips and inoculated into 5 ml of LB broth containing 100 $\mu\text{g}/\text{ml}$ ampicillin and incubated overnight at 37°C in a shaker incubator at 150 rpm. After overnight incubation, 1 ml of the broth was centrifuged at 10,000 rpm for 5 min and the broth was pipetted out. The bacterial pellet was resuspended in 50 μl of NFW and kept in a boiling water bath for 5 min. Mixed the suspension by pipetting and centrifuged at 10,000 rpm for 5 min. One microliter of the supernatant was used as template in the PCR for confirmation of recombinant plasmid, using pJET1.2 forward and reverse sequencing primers (supplied along with the kit), which was carried out as per the protocol provided with the kit.

Isolation of plasmid DNA and sequencing of the clones

From the overnight broth culture found to be positive for the recombinant *E. coli*, the plasmid DNA was isolated

using Thermo Scientific GeneJET Plasmid Miniprep Kit as per the manufacturer's protocol. The plasmids were sequenced by using T7 promoter sequencing primer at SciGenom Lab Private Limited, Cochin, India.

Analysis of the nucleotide sequences by BLAST

The chromatograms received after sequencing were analyzed using Chromas Lite v2.01 software (<http://www.technelysium.com.au>) and sequence reading errors if any were edited. BLAST was performed with the sequences within the non-redundant nucleotide database (<http://www.ncbi.nlm.nih.gov/Blast>) to confirm the presence of the gene specific to CSFV and then compared with nucleotide sequences of other CSFV isolates available in the GenBank database.

Phylogenetic analysis

Phylogenetic and molecular evolutionary analyses of the sequences were done using MEGA v6 software [34]. A 150 bp region corresponding to positions 200–349 and a 190 bp region corresponding to positions 2518–2707 of Alfort 187 (X87939) were selected for phylogenetic analysis as described previously [22]. The sequences obtained during this study and those downloaded from GenBank were aligned using the Clustal W program of MEGA v6 software. The sequences were then trimmed to correspond to the regions given above. The evolutionary history was inferred by using neighbor joining (NJ) [28] method. The bootstrap consensus tree inferred from 1000 replicates [8] was taken to represent the evolutionary history of the sequences analyzed. The evolutionary distances used to infer the phylogenetic trees were computed using the Tamura 3-parameter method [33]. The details of sequences employed for the phylogenetic analysis are given in Supplementary Tables 1 and 2 for 5'UTR and E2 gene regions respectively.

Results

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

Out of the 19 samples tested, six samples were found to be positive for CSFV nucleic acid by RT-PCR targeting the 5'UTR and E2 gene region as evidenced by the appearance of 421 and 272 bp PCR products respectively.

Cloning and sequencing of the RT-PCR products

All the six 5'UTR and E2 PCR products generated were cloned and sequenced. When the sequences were subjected

to BLAST analysis similarity was found to CSFV subgroup 2.2 isolates from India and abroad. The sequences of the E2 and 5' UTR gene regions of the isolates were submitted to GenBank. The accession numbers obtained for E2 gene sequences were KR350476, KR350477, KR350478, KR350479, KR350480 and KR350482, and that for 5'UTR were KR149284, KR350483, KR350484, KR350485, KR350486 and KR350488.

Analysis of the CSFV sequences by BLAST

When the sequences were analysed by BLAST, the sequences obtained in the study showed identity with sequences of other Indian and foreign isolates of CSFV. As the maximum identity was to subgroup 2.2 isolates, the isolates of the study were also designated as 2.2. In case of both 5'UTR and E2 sequences the identity with Indian sequences ranged from 96 to 98 %. The sequences generated during the study showed maximum similarity to foreign isolates from Thailand, Taiwan and China.

Phylogenetic analysis of CSFV gene sequences

The phylogenetic analysis was carried out using 5'UTR and E2 gene sequences. In both the analyses, the isolates clustered with Indian subgroup 2.2 viruses (Figs. 1, 2). Isolates 23/MIB/14 (KR350476 and KR149284) and 181/MIB/14 (KR350480 and KR350486), both from Kannur district, even though placed in subgroup 2.2, were distinct from the other isolates of the subgroup. The Kannur isolates were similar to isolates from Karnataka state with which the district shares common boundaries. The bootstrap values obtained for the phylogenetic tree created using E2 sequences was higher when compared to that for 5'UTR sequences (Fig. 2). The isolate 99/MIB/14 (KR350477) was found to be closely related to isolate 31/MIB/2009 (HM768899.1) which was obtained from a CSF outbreak which occurred in 2009 in Kerala (Fig. 2). However, two other isolates obtained from Kerala in 2009 (34/MIB/2009 and 56/MIB/2009) were distinct from 31/MIB/2009 and formed a separate branch in the phylogenetic tree.

Discussion

Classical swine fever is one among the five significant viral diseases affecting livestock in India [21]. The occurrence of the disease is notifiable to the OIE and it has a devastating effect on the swine population. The high mortality and reduced production due to the disease cause severe economic loss to the farmers involved in pig farming. Outbreaks of highly contagious diseases like CSF can be

Fig. 1 Neighbour joining tree constructed using 150 nucleotides (corresponding to nucleotide positions 200–349) of 5'UTR region of CSFV isolates. Legend for figure 1 Filled triangle isolates obtained during this study. Boot strap replicates = 1000. Evolutionary

distances between sequences were calculated by Tamura 3 parameter method. Branch length are given as 0.05 substitutions per position according to scale bar underneath the tree

effectively controlled and managed only by the timely detection of the disease in the field. The extreme variation in the clinical signs of CSF hampers the diagnosis based on clinical signs. Hence definitive and accurate diagnosis of CSF is vital in implementing suitable treatment and control measures. Reverse transcriptase PCR is a considered as an appropriate emerging strategy for checking and confirmation of clinical cases suspected of CSF due to the high sensitivity and rapidity of the test [11]. As RT-PCR detects CSFV nucleic acid, the chances of obtaining positive results are more in situations where negative results are obtained by other assays and thus ensures high sensitivity [19]. Also the test is reported to be used for the preclinical identification of the infectious agent [6].

Molecular epidemiological studies on CSF are performed by assessing the genetic dissimilarities of the different CSFV isolates. Amplification of the CSFV nucleic acid by RT-PCR and subsequent nucleotide sequencing and genetic typing are the approaches commonly employed to accomplish the analysis of the viral isolates. The different regions of the CSFV genome as, E2, 5' UTR, and NS5B are commonly employed for the construction of phylogenetic trees and subsequent typing [10].

The ancient European, American and Asian CSFV isolates were assigned to group 1 and its subgroups. The present day European and Asian isolates were allocated to group 2 and its subgroups. The Asian isolates were categorized into group 3 and its four sub-groups. The group 3

Fig. 2 Neighbour joining tree constructed using 190 nucleotides (corresponding to nucleotide positions 2518–2707) of E2 gene region of CSFV. Legend for figure 2 *Filled triangle* isolates obtained during this study. *Filled circle* isolates obtained from Kerala in 2009. Boot

strap replicates = 1000. Evolutionary distances between sequences were calculated by Tamura 3 parameter method. Branch length are given as 0.2 substitutions per position according to *scale bar* underneath the tree

viruses were isolated primarily from Korea, Japan, Taiwan, Thailand and U.K [22, 29]. Type 3.2 and 3.3 forms a novel genetic group distinct from the pre-existing genotypes. Genotype 3.2 was isolated from Korea and 3.3 from Taiwan. Kanagawa type represented sub-group 3.4. Subgroups 3.2, 3.3 and 3.4 have not been isolated from anywhere else in the world.

In this study, by the phylogenetic analysis of the 5'UTR and E2 gene all the six isolates of CSFV were assigned to subgroup 2.2 and were grouped along with the other Indian 2.2 isolates. The isolates 99/MIB/14, 168/MIB/14, 179/MIB/14 and 322/MIB/13, though from different districts, were more closely related. The Kannur isolates, 23/MIB/14 and

181/MIB/14 were slightly distinct from the other isolates even though placed in subgroup 2.2. These isolates were more similar to isolates from the neighbouring state of Karnataka with which Kannur shares boundaries. A recent study reported the prevalence of subgroup 2.2 CSFV in Karnataka [32] whereas earlier reports indicated a prevalence of subgroup 1.1 viruses in the State [20]. A similar study conducted in Assam based on the 5'UTR and E2 gene regions could detect subgroup 1.1 viruses only. The prevalence of CSFV genotypes 2.1 and 1 in north eastern India has also been reported [7].

In the study, the isolate 99/MIB/14 was found to be closely related to isolate 31/MIB/2009 (HM768899.1)

which was recorded in 2009 from Kerala. The close similarity of these isolates obtained after a gap of 5 years indicates that this virus has been circulating in Kerala since 2009. In a separate study conducted in the neighbouring state of Tamil Nadu, the clustering of CSFV isolates from that state with Kerala isolates 31/MIB/2009 and 56/MIB/2009 (HM768899.1 and HM768901.1) was reported [25]. Isolates 23/MIB/14 and 181/MIB/14 sequenced during this study were slightly distinct from the other isolates even though placed in subgroup 2.2. These isolates were more similar to isolates from the neighbouring state of Karnataka where a prevalence of subgroup 2.2 was reported [32]. Thus the predominant viruses currently circulating in the South Indian states of Kerala, Tamil Nadu and Karnataka seem to be of the subgroup 2.2. This may be due to the movement of animals across the state boundaries through animal trade.

When the isolates of the present study were subjected to BLAST analysis, the foreign isolates to which greatest similarity was observed was from Thailand, China and Taiwan. Hence it is prudent to believe that the CSFV virus circulating in Kerala now have entered India from South East Asian countries through the animal movement or trade. Similar inference on the origin of Indian CSFV viruses have been reported previously also [21].

On comparison of the phylogenetic tree constructed from the nucleotide sequences of 5'UTR and E2 gene it was seen that even though genotyping results were similar, the E2 glycoprotein gene was found to ensure superior distinction of the isolates studied. This was also supported by higher bootstrap values. These findings were similar to those reported previously [10]. Better differentiation was ensured among the related isolates also by analysis based on E2 region. Also E2 gene region is largely employed in genetic typing studies as more relevant records are available in this region and also due to reliable differentiation of the isolates [18].

The results of the present study have indicated that the CSFV subgroup 2.2 viruses are circulating in Kerala. The vaccine being used in the Kerala is derived from the C strain, which belongs to subgroup 1.1. More studies have to be carried out to ascertain whether this strain of CSFV provides complete protection against the currently circulating CSF viruses.

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