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Short communication

Molecular characterization of classical swine fever virus isolates from India during 2012–14

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ABSTRACT

Classical swine fever is a highly contagious and economically important viral disease of pigs. Outbreaks of classical swine fever virus (CSFV) were recorded in different places in the Kamrup district of Assam in India between the years 2012 and 2014. The nucleotide sequences of the 10 CSFV isolates were analyzed based on the partial nucleotide sequences of the E2, 5′NTR and NS5B genes. Phylogenetic analysis indicated the dominance of subgroup 2.2 along with 2.1 strains in the northeast part of India. Variation in the nucleotide sequences of E2, 5′NTR and 3′NS5B genes of CSFV allows tracking changes in the virus population over time. The study will provide epidemiological information useful for assessing CSFV circulating genogroups in India.

1. Short communication

Classical swine fever (CSF) is an economically important and highly contagious viral disease of domestic pigs and wild boars. The CSFV infection was once distributed throughout the world. However, countries, including Australia, Canada, New Zealand, USA, and some European Union countries have succeeded in its eradication (Moennig, 2000). The clinical signs of CSF vary with its strain, the age of affected pig and the immune status of the ailing animal (Moennig et al., 2003). Anorexia, lethargy, conjunctivitis, enlarged lymph nodes, respiratory signs and constipation followed by diarrhoea are the initial signs of CSFV infection in pig. In addition, swollen lymph nodes, haemorrhages in the kidney, infarctions of the spleen, severe atrophy of the thymus and depletion of the lymphocytes from lymphoid organs are considered to be pathognomonic for CSF infection (Sato et al., 2000; Tautz et al., 1998).

The causative agent of CSF is classical swine fever virus (CSFV) which belongs to the genus *Pestivirus* in the family *Flaviviridae*. CSFV is genetically and antigenically related to other pestiviruses such as bovine viral diarrhoea virus (BVDV) and border disease virus (BDV) (van Regenmortel et al., 2000). CSFV is an enveloped virus with 12.3 kb to 12.5 kb long single-stranded, positive sense RNA genome flanked by highly conserved 5′NTR and 3′NTR (Thiel et al., 1991). The genome consists of a single open reading frame (ORF) encoding about 3900–4000 amino acid long polyprotein, which is processed to yield different viral proteins (Meyers and Thiel, 1996). The ORF is co- and post translationally processed into 11–12 polypeptides (NH2-Npro-Erns-E1-E2-P7-NS2-NS3-NS4A-NS4B-NS5A-NS5B-COOH) flanking with 5′ and 3′non-polyadenylated non translated regions (NTRs) (Thiel et al., 1991). CSFV has one serotype divided into three major genogroups, which in turn are divided into three to four subgenogroups (Gong et al., 2016; Paton et al., 2000; Postel et al., 2013).

Although the disease has been reported from most of the states in India, Northeastern states due to the higher density of the pig population frequently reports its outbreak. Pig rearing is an important agricultural activity in the Northeastern states of India and has an important role in the socioeconomic development of the states. Of the total, 13.5 million pigs in India, 1.5 million pigs (11.1%) belong to Assam (Sarma et al., 2011). Several outbreaks of CSF have been reported from the region suggesting its endemic nature (Barman et al., 2010; Chakraborty et al., 2011). Northeast India shares a long

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porous international boundary with China, Bangladesh, Bhutan and Myanmar. The region is highly prone to the transboundary transmission of exotic CSFV strains. Recent studies have shown that subgenogroup 1.1 is prevalent in India (Choori et al., 2015; Patil et al., 2010), with few reports of genogroup 2 (Chakraborty et al., 2011; Desai et al., 2010; Kumar et al., 2015). In the present study, outbreaks recorded in different places in district Kamrup, Assam, India during the years 2012 and 2014 were analyzed based on the partial nucleotide sequences of the E2, 5′NTR and NS5B genes with the aim to provide epidemiological information to track the changes in the CSFV genogroup over time.

A total of 210 blood samples of domestic pigs, aged 0–3 months, were collected from different geographical regions of district Kamrup, Assam from December 2012 to May 2014 for CSFV surveillance. All the collected blood samples were properly stored at −70 °C for further analysis. None of the samples were from pigs having the history of vaccination against CSFV. The blood samples were tested for CSFV genome by standard single step TaqMan real-time reverse transcription PCR (RT-PCR) (Hoffmann et al., 2005). Real-time PCR was performed in ABI 7300 Real-Time PCR system (Applied Biosystems, USA). Amplification was carried out in a single step from isolated RNA with "SuperScript™ III Platinum® One-Step Quantitative RT-PCR System" (Life Technologies, Carlsbad, USA) following manufacturer's instructions. Qualitative (positive/negative) responses were recorded and results were analyzed based on threshold cycle (Ct) values. Amplification curves that crossed the threshold ΔRn value were positive while samples showing no Ct were negative. A total of 75 samples were detected as positive for CSFV by real time PCR. Ten representative positive samples originating from different geographic regions of district Kamrup, Assam, India were selected randomly for molecular characterization of CSFV. The samples were subjected for sequencing and phylogenetic analysis using partial genomic regions of E2, 5′NTR and 3′NS5B genes.

Whole blood collected from piglets were processed for detection of CSFV specific nucleic acid by single step TaqMan RT-PCR targeting 5′NTR region of CSFV (Hoffmann et al., 2005). Total RNA was extracted from whole blood using the QIAamp® Viral RNA Mini spin protocol (QIAgen, Hilden, Germany), following the manufacturer's instructions and stored at −20 °C until further use. CSFV nucleic acid was amplified following the reverse transcriptase polymerase chain reaction technique from the extracted RNA (Applied Biosystems, USA). RT-PCR was carried out using CSF genome specific primers for three genomic regions (Table 1) viz., E2 (Lowings et al., 1996), 5′NTR (Greiser-Wilke et al., 1998) and NS5B (Björklund et al., 1999). The purified PCR products were sequenced using the CSFV gene specific primers commercially through Bangalore Genei Ltd, India. Sequences were analyzed by comparison with sequences of different CSFV strains available in GenBank using the online BLAST server. The multiple sequence alignment of CSFV isolates were carried by the CLUSTAL W algorithm of DNASTAR software (Wisconsin, USA). Phylogenetic analysis of the CSFV sequences was conducted using MEGA5 software (Tamura et al., 2011). Congenital tremor and Kanagawa strain were used as outgroup for the present study.

All the samples specifically showed amplification of 271 bp of E2, 271 bp of 5′NTR and 449 bp of the 3′NS5B region (Fig. 1). Annotated sequences were submitted to GenBank (Table 2). 190 nucleotide (nt) of E2, 150 nt of 5′NTR and 409 nt of NS5B were used to carry out a phylogenetic analysis of the Kamrup samples of CSFV. Phylogenetic analysis of all Kamrup samples showed similar genogroup based on partial E2, 5′NTR and NS5B nucleotide sequence analysis (Fig. 2). Eight CSFV samples were classified into subgroup 2.2 and two were classified in subgroup 2.1. The E2 sequences of all the field samples of CSFV were aligned with the available genogroup sequences from GenBank using the ClustalW algorithm (Fig. 2A). Eight out of ten field samples (CSF/As/620, CSF/As/637, CSF/As/651, CSF/As/654, CSF/As/659, CSF/As/678, CSF/As/681& CSF/As/688) in the genogroup 2.2 were more closely related to the Indian isolates than with Thailand and Bergen. Similarly, two sequences (CSF/As/666 & CSF/As/680A) belonging to the 2.1 genogroup showed close similarity with the isolates from North East India. Similarly, phylogenic analysis using 3′NTR (Fig. 2B) and 3′NS5B (Fig. 2C) nucleotide sequences of all the CSFV isolates showed its clustering to genogroup 2.1 and 2.2. Inclusion of the third genomic region, i.e., 3′NS5B of CSFV for phylogenetic analysis can better discriminate minor differences among the isolates (Björklund et al., 1999). All the CSFV field samples from Kamrup district showed close identity with sequences from Indian origin in the phylogenetic tree.

Pairwise distance analysis of the E2 sequences of all the CSFV samples showed identity of 95.3–100% within the genogroup 2.2 and the divergence of 5% (Table 3A). CSFV isolates CSF/As/666 and CSF/As/680A showed 100% identity with each other among genogroup 2.1 viruses. Distance analysis of the E2 sequences of the CSFV samples when compared to representative sequences from 2.2 and 2.1 phylogenetic group showed the maximum sequence identity of 95.8–98.4% and 99.5%, respectively. Least identity was calculated for CSFV strain congenital tremor (3.1) with identity of 81.6–82.6% and divergence of 20.2–21.8% (Fig. 3A).

Pairwise distance analysis of the 5′NTR sequences of all the CSFV samples showed very close identities, ranging from 97.4% to 100% within the genogroup 2.2 and the divergence ranged up to 2.7%. CSFV isolates CSF/As/666 and CSF/As/680A showed 99.4% identity with each other and divergence of 0.7% within the genogroup 2.1 viruses. Distance analysis of the 5′NTR sequences of the all CSFV field samples when compared to representative sequences from 2.2 and 2.1 viruses showed the maximum sequence identity of 97.4–100% and 96.1–96.8%, respectively. Least identity was evident with Kanagawa (3.4) and congenital tremor (3.1) viruses with an identity up to 79.4% and divergence from 5.7% to 15% (Fig. 3B).

Pairwise distance analysis of the 3′NS5B sequences of the 10 field samples showed high identity, ranging from 88.9% to 99.8% within the genogroup 2.2 (Table 3B). CSFV isolates CSF/As/666 and CSF/As/680A of 2.1 genogroup showed 98.5% identity with each other and divergence of 1.5%. Distance analysis of the 3′NS5B sequences of the 10 field samples when compared to representative sequences from 2.2 and 2.1 phylogenetic group showed the maximum sequence identity ranging between 96.6% to 99%. Least identity could be observed when compared with congenital tremor (3.1) viruses with an identity upto 83.9% and divergence of 19% (Fig. 3C).

The Northeastern region (NER) of India, owing to its unique geographical location sharing five international borders, bears a constant threat to the country's livestock for incursions of exotic as well as transboundary diseases. The region shares highly porous and sensitive frontiers with neighboring countries. The NER shares about 4500 km international boundaries with Myanmar, Bangladesh, Bhutan, Nepal and China. Uncontrolled migration of animals from neighboring countries, therefore, is a great threat for spreading of emerging and transboundary diseases to the region. Among the livestock available in NER of India, pig rearing is an important agricultural activity. In addition, pork is the preferred meat for the non-vegetarian population. Pig population in NER constitutes nearly 40% of the total pig population of India. However, limited infectious agents cripple this prosperous industry.

Phylogenetic analysis of CSFV can depict the origin and spread of the virus prevailing in domestic pigs as well as wild boars in this region. Outbreaks recorded in different places in district Kamrup, Assam between the years 2012 and 2014 were analyzed based on the partial nucleotide sequences of the E2, 5′NTR and NS5B genes. Molecular epidemiology based on nucleotide sequence diversity is a useful tool for tracing the virus spread and for developing disease