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Molecular characterization and genogrouping of classical swine fever virus isolated from field outbreaks

S CHAKRABORTY[1], B M VEEREGODA[2], B M CHANDRA NAIK[3], D RATHNAMMA[4], S ISLOOR[5], M D VENKATESHA[6], G LEENA[7], H VEERESH[8] and S S PATIL[9]

Karnataka Veterinary, Animal and Fisheries University, Bengaluru, Karnataka 560 024 India

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ABSTRACT

The present study was carried out to characterize classical swine fever virus (CSFV) isolated from field outbreaks in suburban places of bengaluru, India, using molecular techniques and subsequent genogrouping of the virus. Various tissue samples from CSFV affected pigs (12) were collected and subjected to either virus isolation in PK-15 cell line, or RNA extraction. PCR amplification was carried out targeting the 5' NTR gene. Subsequent agarose gel electrophoresis yielded specific amplicons of 421 bp obtained from pooled samples of 3 pigs. The PCR purified products were sequenced and subjected to BLAST analysis and subsequently submitted to GenBank. The obtained nucleotide sequences were aligned using MegAlign programme and further subjected to analysis using MEGA 4 programme. All 3 field isolates were found to be grouped into subgroup 2.2 of group 2.

Key words: Classical swine fever virus, Genogrouping, Molecular characterization, Molecular outbreaks

Classical swine fever (CSF), also known as hog cholera is one of the most dreaded and devastating viral diseases of swine causing serious economic losses. Although eradicated from many countries, CSF continues to cause serious problems in different parts of the world (Edwards *et al.* 2000). The outbreak of CSF, and also the occurrences of classical swine fever virus (CSFV) in the tissues of pigs were reported from India (Sarma and Bostami 2008).

RT-PCR based works to detect different gene fragments of the virus were carried out in India (Singh *et al.* 2005, Ravishankar *et al*. 2007). Sarma *et al*. (2009a) reported phylogenetic analysis of 16 CSFV isolates and grouped them under subgroup 1.1. CSFV Indian isolates (17) recovered during 2006–08 could be grouped into subgroups 1.1 and 2.2 based on partial nucleotide sequencing of the 5´ NTR (Patil *et al.* 2010). Thus, the present study was an attempt to add further to the baseline epidemiological information on CSFV to facilitate the eradication and control programme of the disease.

Present address: [1]M V Sc Scholar (e mail: sandipc_85 @yahoo.com), [2]Assistant Professor, [4] Assistant Professor and Head of Department, [5]Assistant Professor, [7]Assistant. Professor, Department of Veterinary Public Health, Department of Veterinary Microbiology, Bengaluru Veterinary College.

[3]Scientist (Grade-I) (e-mail: dinglychandru@yahoo.com), [6]Joint Director, Institute of Animal Health and Veterinary Biologicals, Bengaluru, Karnataka.

[8]Senior Research Fellow, [9]Principal Scientist, Project Directorate ADMAS, Bengaluru, Karnataka.

MATERIALS AND METHODS

The PK-15 cell line was maintained in MD bottles using DMEM (Dulbecco's Modified Eagle's Medium) containing 5% fetal calf serum. Pooled tissue samples (comprising pieces of affected kidney, spleen, mesenteric lymph node and intestine) were collected from 12 pigs, showing classical symptoms of classical swine fever from Balagere, White Field and Attibele, the suburban places of Bengaluru. Among these samples, 3 were subjected to virus isolation in the PK-15 cell line and 3 passages were given.

Processing of tissue materials: Tissue materials were cut into small pieces of 1-2 g using sterile scissors and grind to a homogenous paste in a mortar and pestle with a small amount of phosphate buffered saline (PBS) and sterile sand. A 20% (w/v) suspension was made by adding balanced salt solution (BSS). One ml of the glutamine-antibiotic stock was added for each 10 ml of suspension. This mixture was held at room temperature for 1 h, centrifuged at 1000 × g for 15 min and the supernatant fluid was collected and stored at –70° C.

For the infection of PK-15 cell line, adsorption method was followed (Rovozzo and Burke 1997). At each passage, duplicate tubes of negative and positive controls were maintained along with test samples. CSFV isolated at

Diagnostic Virology, IAH&VB (Chandranaik *et al.* 2011) was used as positive control during the entire study period.

Remaining 9 pooled tissue samples were directly subjected to RNA extraction. RNA from cell culture supernatant was extracted using the QIAamp viral RNA mini kit,whereas from pooled tissue samples RNA was extracted by using a commercial kit. The concentration and quality of total RNA extracted was determined by diluting the extracted RNA in TE buffer in a quartz microcuvette and the absorbance was measured at a dual wavelength of 260 and 280 nm using spectrophotometer. PCR amplification was carried out targeting the 5´ NTR gene following the one-step RT-PCR kit protocol, using the primer sets of Paton *et al.* (2000a).

The products were analyzed by electrophoresis on 1.2% agarose alongside 100 bp DNA ladder. The amplified products were purified from the gel using gel extraction kit .

The purified PCR products were got sequenced commercially and compared with published sequences using the BLAST programme. To understand the genetic relatedness among CSFV field isolates in the present investigation, the isolates were subjected for sequence pair distance analysis and phylogenetic analysis along with 16 published sequences of CSFV isolated from domestic pigs during 2005–07 in different districts of Asom and 17 CSFV isolates collected from 10 outbreaks comprising 6 different states of India during 2006–08. The tree was constructed with the modules of MEGA 4 programme. Phylogenic tree was visualised using the same programme.

RESULTS AND DISCUSSION

None of the samples subjected for virus isolation could yield any cytopathic effect (CPE) even after the third passage. The absence of CPE in the cell culture could be due to the expression of N – terminal protease (N^{pro}) and lack of accumulation of protein NS3 in the cell culture system (Ruggli *et al.* 2003). These findings fall in agreement with Singh *et al.* (2006) wherein, they reported that, none of the 26 FAT positive samples showed CPE in the PK-15 cell line, and with Sarma *et al.* (2009b), who found that, none of the 22 CSFV isolated in PK-15 cell line showed CPE, wherein, all these isolates were confirmed by sandwich ELISA, immunoperoxidase test and RT-PCR.

Out of 9 pooled tissue samples and 3 cell culture fluid, 2 pooled tissue samples, 1 each from Balagere and Attibele (Whitefield) and 1 cell culture fluid (the original tissue sample being collected from Whitefield, Bengaluru from an animal showing symptoms of CSF) were confirmed for CSFV by RT-PCR and subsequent agarose gel electrophoresis as intense bands just above 400 bp could be seen in the gel. No other bands were seen below and above the amplified DNA of these 3 isolates indicating the specific amplicons of size 421 bp. There are several genes like E2, NS5B and 5´ NTR targeted for PCR amplification of CSFV. But, the PCR amplification of 5´ NTR gene finds its agreement with Ravishankar *et al.* (2007) and Tiwari *et al.* (2009). They opined that the PCR amplification of this region is highly specific and sensitive tool for detecting the presence of the virus in the field samples. Moreover, this region is highly conserved, so that the PCR primers used for its amplification can also readily be applied to other pestiviruses, enabling sequence comparisons to be made across the genus. This genetic stability also makes the region a favourite target for

Table. 1. Sequence pair distance analysis of classical swine fever virus field isolates KVAFSU CSFV B1, KVAFSU CSFV WF1, KVAFSU CSFV WF2 with some of the published sequences of Sarma *et al.* (2009) (Slow/Accurate, IUB)

Per cent identity

Divergence

	1,	2	3	4	5	6	7	8	9		
1		83.9	83.4	84.4	82.9	83.9	94.0	95.0	95.0	1	Asom
2	3.1		97.9	99.1	97.5	98.3	59.1	56.0	55.8	2	Boko
3	3.7	2.2		99.2	98.3	99.6	95.3	90.3	90.3	3	Dudhnoi
4	2.4	1.1	0.9		98.3	99.6	95.3	90.3	90.3	4	Goalpara
5	3.1	1.7	0.9	0.9		98.7	95.3	89.8	89.8	5	Marigaon
6	3.1	1.7	0.4	0.4	0.4		95.8	90.7	90.7	6	Sonitpur
7	5.3	19.5	5.4	20.4	5.0	4.9		98.0	97.1	7	KVAFSU CSFV B1
8	5.2	21.5	7.7	22.4	7.2	7.2	1.5		98.7	8	KVAFSU CSFV WF1
9	5.2	20.0	5.2	20.9	4.7	4.7	1.6	1.1		9	KVAFSU CSFV WF2
	1	2	3	4	5	6	7	8	9		

Table 2. Sequence pair distance analysis of classical swine fever virus field isolates KVAFSU CSFV B1, KVAFSU CSFV WF1, KVAFSU CSFV WF2 with some of the published sequences of Patil *et al.* (2010) (Slow/Accurate, IUB).

Per cent identity

Divergence	1	2	3	4	5	6	7	8	9		
1		94.5	95.4	95.5	845	94.5	89.0	87.1	88.1	1	Bangalore-294
2	5.8		99.7	100.0	94.1	100.0	88.1	85.2	87.4	2	Ind-173-08
3	4.8	0.3		99.7	88.4	99.7	85.6	83.5	82.3	3	Ind-187-08
4	4.7	0.0	0.3		89.3	100.0	86.2	84.2	83.1	4	Ind-239-08
5	6.8	0.3	0.3	0.3		99.7	91.9	91.7	90.8	5	Ind-240-08
6	5.8	0.0	0.3	0.0	0.3		94.4	88.7	91.1	6	Ind-273-08
7	3.9	8.9	6.4	6.5	8.1	8.9		98.0	97.1	7	KVAFSU CSFV B1
8	4.4	9.5	7.1	7.2	8.3	9.1	1.5		98.7	8	KVAFSU CSFV WF1
9	2.7	8.0	5.1	5.4	6.0	7.7	1.6	1.1		9	KVAFSU CSFV WF2
	1	2	3	4	5	6	7	8	9		

diagnostic PCR, where a broad specificity of viral recognition is important. The assured efficacy of these diagnostic PCR primers is indeed one reason for continuing to sequence viruses in this region (Paton *et al.* 2000a).

The field isolates were given the laboratory names, viz. KVAFSU CSFV B1, KVAFSU CSFV WF1 and KVAFSU CSFV WF2 respectively. It was noted that at nucleotide level, KVAFSU CSFV B1, KVAFSU CSFV WF1 and KVAFSU CSFV WF2 had shown maximum identities with Asom isolates and least divergence with Ind-294B/08, the Bangalore isolate (Tables 1, 2). The nucleotide sequence pair distance analysis findings are in correlation with the work of Li *et al.* (2006), wherein they compared the genome of a novel classical swine fever virus isolated in China in 2004 (SWH/CA/2004) with other CSFV strains and found a pair wise identity of 80.4–99.8% between the corresponding segments of SWH/CA/2004 and other reported strains at the nucleotide level, Sarma *et al.* (2009a) wherein they compared the nucleotide sequence of 16 CSFV isolates collected from different outbreaks in Asom during 2005–07 and found that the isolates shared pair-wise identity between 98.3– 99.3% with the other isolates of the same subgroup from Europe; and Patil *et al.* (2010) wherein they compared the nucleotide sequences of CSFV isolates collected from 10 outbreaks comprising 6 states of India during 2006–08 and found that an isolate named IND-294B/08 collected from Harohalli, Karnataka shared a pair-wise identity of 97.4% and divergence of 2.6% at the nucleotide level from the other isolates of the same subgroup from Europe.

The phylogenetic analysis revealed that all the 3 field isolates fall under subgroup 2.2 under group 2 along with

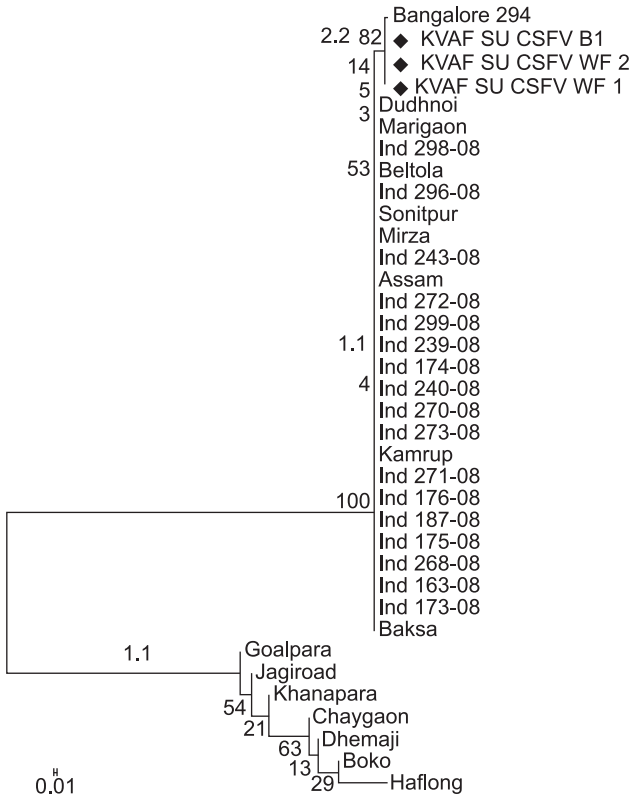

Fig. 1. Phylogenetic tree showing clustering of CSFV isolates KVAFSU CSFV B1, KVAFSU CSFV WF1 and KVAFSU CSFV WF2 along with 33 other published sequences of Indian isolates, constructed based on the nucleotide sequence of 5´ NTR by bootstrap test of phylogeny using neighbor-joining method.

reference strain Ind-294B/08 (bangalore-294) whereas rest of the Indian isolates fall under subgroup 1.1 under group 1 (Fig. 1). The works of Sarma *et al*. (2009a) and Patil *et al*. (2010) are suggestive of the predominance of group 1 viruses in India. But recently the Ind-294B/08 (bangalore-294) strain isolated from an outbreak in Harohalli, Karnataka fell under subgroup 2.2 of group 2 (Patil *et al.* 2010) and so also the 3 field isolates in the present study, viz. KVAFSU CSFV B1, KVAFSU CSFV WF1 and KVAFSU CSFV WF2. This suggests the common evolution of these viruses. These findings also revealed that even though group 1 viruses are predominant in India, but the group 2 viruses are also rapidly spreading and may form a major threat in future. Reports from Taiwan (Deng *et al.* 2005), Columbia (Sabogal *et al*. 2006) and Germany (Leifer *et al.* 2010) are also suggestive of the predominant involvement of group 2 and 3 viruses in CSF outbreaks worldwide. This trend is quiet noteworthy in the present study as well.

It is important to harmonise genetic typing methods so that the sequence data obtained in different laboratories are comparable. Providing a large collection of sequences from one or more specific target regions increases the likelihood of such regions being used in future studies also. Studies on phylogeny of current isolates of CSFV are a useful means to reveal the origin and diversity of CSFV. More importantly, phylogenetic studies prove to be a useful approach to aid control and eradication programme of CSF.

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