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Genomic characteristics of classical swine fever virus strains of bovine origin according to primary and secondary sequence–structure analysis

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Abstract

Background: Classical swine fever virus (CSFV), species member of the family *Flaviviridae*, is generally considered restricted to domestic and wild suids. The circulation of CSFV has been detected in cattle herds in China and India. Natural infection appeared associated with clinical signs in some cases.

Aim: The secondary structures of the internal ribosome entry site in the 5' untranslated region (UTR) were used for the genomic characterization of bovine strains.

Methods: Sequences have been compared to the representative CSFV strains isolated from pigs, vaccines, and contaminants from porcine cell lines and an ovine strain isolated in Spain.

Results: The observed sequences from cattle showed a genetic relatedness with live-attenuated vaccine strains used in pigs. Sequence characteristics of the Chinese strain S171 are genetically distant from the previously reported CSFV genotypes, suggesting a new outgroup in the species, described for the first time, and named CSFV-d. Other Chinese strains were genetically closely related to CSFV genotype a2 (Alfort type) pig strains. Indian strains, reported from the states of Tamil Nadu and Meghalaya, were genetically closely related to CSFV genotype a1 (Brescia type) and a5 pig strains, respectively.

Conclusion: These preliminary observations are new and relevant in countries, where CSFV control and eradication strategies are applied.

Keywords: Cattle, Classical swine fever virus, *Pestivirus*, Secondary structure.

Introduction

The genus *Pestivirus*, family *Flaviviridae*, includes genetically related species recognized as important pathogens in veterinary medicine. The Classical swine fever virus (CSFV) has been eradicated in different countries as in Europe and North America, but still control and eradication strategies are applied against the virus in various other regions. There is a consensus concerning the capacity among pestiviruses to cross species barrier. The wide host range of these viruses may lead to the formation of virus reservoirs in other domestic or wildlife species, presenting a concern for the long-term success of eradication campaigns. Nevertheless, CSFV is generally considered to be restricted to domestic and wild suids. Current knowledge indicates that experimental infections without clinical signs have been reported in cattle, sheep, goats, and deer, but there is no evidence that these species become infected in nature (Biró *et al.*, 1966; Loan and Storm, 1968; Shimizu and Kumagai, 1989; CFSPH, 2015). In only two cases, the species has been identified in sheep (Hurtado *et al.*, 2003; Mohamed, 2004), corroborating these beliefs. One ovine isolate (strain 5440/99), which is similar to CSFV vaccine strains used in the framework of eradication campaigns in pigs, was reported from

Spain (Hurtado *et al.*, 2003). A second isolate (strain 12 Ovine liver 113nt) was obtained during investigations on pestiviruses in the United Arab Emirates (Mohamed, 2004). This *Pestivirus*-like sequence, when aligned with corresponding sequences from a variety of pestiviruses, showed a greatest similarity to CSFV subgenotype 1.1 but raised doubts on correct classification, due to the suboptimal quality of the sequence and its short length (113 nucleotides) (Mohamed, 2004).

On contrary to this apparent epidemiological static nature and apparent inability to cross species barrier in natural conditions, the recent reports indicated the isolation of CSFV strains in cattle (*Bos taurus*) from India and China and contaminants or associated to natural infection with reproductive disorders (abortion, stillbirth, birth defects, repeat breeding, and mastitis) (Zhang *et al.*, 2014; Giangaspero *et al.*, 2017). Twelve strains isolated from cattle aged from 6 months to 2 years have been detected in four farms, out of 19 tested, during an epidemiological survey aiming to identify bovine viral diarrhoea virus (BVDV) persistently infected animals by reverse transcription-polymerase chain reaction (RT-PCR), among BVDV-seropositive enzyme-linked immunosorbent assay (ELISA) cattle and buffalo farms from four districts of the state of Tamil

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Nadu, India (Giangaspero *et al.*, 2017). Four strains were reported from China. *Pestivirus* RNA sequences have been detected from commercial fetal bovine serum batches and designated as strains S171 (KF006974), S173 (KF006975), and S112 (MK118725) (identical to S173) originated from Henan and Jiangsu provinces (Zhang *et al.*, 2014). The attempts to isolate live viruses from some of these samples have been unsuccessful. The strain HEN03 (sequence deposited by Zhang, under accession number KC176778) was detected in cattle from Henan province. In addition, during a survey for the detection and genetic characterization of 5'-untranslated region (UTR) and E2 gene of CSFV from bovine population of the northeastern region of the Indian state of Meghalaya, of 134 cattle serum samples tested, all were positive in RT-PCR for 5'-UTR region, and 10 samples were positive for CSFV antigen by a commercial antigen capture ELISA (Chakraborty *et al.*, 2018). The full-length E2 region of CSFV was amplified from two positive samples. In this study, the 5'-UTR sequences of the reported strains have been considered for genomic characterization according to the secondary structure analysis of the internal ribosome entry site (IRES).

Material and Methods

The 5'-UTR sequences of the 16 reported strains (13 sequences detected in cattle from India and China and three sequences detected in commercial fetal bovine serum batches from China) (Zhang *et al.*, 2014; Giangaspero *et al.*, 2017; and with reference to strain HEN03 sequence deposited by Zhang) have been compared with those from other CSFV strains ($n = 110$) isolated from pigs, vaccines, and contaminants from porcine cell lines and an ovine strain isolated in Spain representative of identified genetic clusters in the species, with particular attention to isolates circulating in Asian countries, including India and China (Table 1), and further compared to 1,300 sequences from other *Pestivirus* species different from CSFV. The two Indian bovine CSFV sequences reported by Chakraborty *et al.* (2018) were evaluated indirectly considering the similarity of their full-length E2 region with other previously deposited pig sequences. Newly described subgenotypes reported from Brazil and Cuba (Postel *et al.*, 2013; Silva *et al.*, 2017) could not be considered, being available only E2 sequences, and apparently specific and restricted to South American and Caribbean regions.

The taxonomical segregation of the genomic sequences was performed according to the genotyping based on the palindromic nucleotide substitution (PNS) method (Harasawa and Giangaspero, 1998; Giangaspero and Harasawa, 2007; Giangaspero and Apicella, 2014), through the qualitative and quantitative evaluation of relevant secondary structure regions from full-length (216–237 nt) 5'-UTR of the viral RNA, the three variable regions, and V1, V2, and V3 genomic sequences in the

IRES. Genotypes were identified according to base-pair (bp) combinations at the level of low-variable positions and ranked with alphabetic nomenclature according to increasing divergence in the species. The classification among CSFV strains according to the PNS analysis based on changes in the secondary structure was compared with those based on the primary structure of the 5'-UTR performed through sequence alignment and construction of phylogenetic trees. A phylogenetic tree was constructed with Clustal X (Chenna *et al.*, 2003) by using the neighbor-joining method (Saitou and Nei, 1987) and visualized with the Newick tree format option (Mega version 7.0.26) (Kumar *et al.*, 2016). In addition, a basic local alignment search tool (BLAST; <http://www.ncbi.nlm.nih.gov>), which is the web-based sequence analysis tool with default values, was used to find the homologous hits for the sequence analysis of strains.

Results

The obtained secondary structures have been aligned (Table 2) for computing genetic distance among sequences, in terms of base-pairing variations at the level of the IRES in the 5'-UTR, to identify genetic groups, ordered according to the increasing values of divergence. Among Indian bovine CSFV sequences, the V2 and V3 loci are all identical. The sequence of 8 India is identical to the sequences 2, 3, 9, and 13. The sequence of 1 India was identical to 4, 5, 6, 7, 10, and 11 (11 India showed only the first nucleotide in V1 locus: adenine instead of guanine). CSFV-c strain Okinawa/86 is identical to the strains P97 and 94.4/IL/94/TWN (difference in V1/1 g-c), CSFV-a1 strain Ibaraki/66 was identical to BresciaX (difference in V2/7 g*u), and CSFV-a1 39 was identical to strains JL1(06), 5NCRCSFMZAI348, Aizawl-09, and RUCSFPLUM (difference in V1/1 g-c).

The sequences have been clustered into four genotypes, from CSFV-a to CSFV-d (Table 3). PNS marker characteristics to the *Pestivirus* CSFV species, genotype, and subgenotypes are shown in Table 4. The secondary sequence analysis of the 5'-UTR sequences of the Chinese and Indian strains suggested their appurtenance to CSFV species, genotype CSFV-a2 (Alfort type) for three of the Chinese strains and genotype CSFV-a1 (Brescia type) for all the Indian strains. The Chinese strain S171 was clustered as a new genotype in the species, named CSFV-d. Only partially related to CSFV-a2 (divergence 62.5%, mean value 9.87), with a marked difference at the level of the V3 locus nucleotide base pairings, S171 was divergent from all other CSFV genotypes (divergence values ranging from 10 to 15). Furthermore, it was significantly less related to border disease virus (BDV) (Fig. 1), a known characteristic of other CSFV strains. Base pairing comparison between strain S171 and strains of the different BDV genotypes scored the high divergence values, ranging from 17 to 25, with a mean value of

Table 1. List of *Pestivirus* strains of CSFV species ($n = 126$) evaluated according to palindromic secondary structure characteristics at the RNA 5'-UTR (PNS method). The nomenclature of identified genotypes is based on divergence in the genus. Clustering according to the primary sequence analysis by depositors is indicated under parenthesis. ND: not determined. Cattle (*B. taurus*); Pig (*Sus scrofa domestica*); Sheep (*Ovis aries*); Wild boar (*Sus scrofa scrofa*).

Species/Genotype	Strain	Origin	Year	Country	Accession	Reference
CSFV-a1	1 India	Cattle	2016	India	MG859286	Giangaspero <i>et al.</i> , 2017
CSFV-a1	10 India	Cattle	2016	India	MK105823	Giangaspero <i>et al.</i> , 2017
CSFV-a1	11 India	Cattle	2016	India	MG859287	Giangaspero <i>et al.</i> , 2017
CSFV-a1	13 India	Cattle	2016	India	MK105824	Giangaspero <i>et al.</i> , 2017
CSFV-a1	2 India	Cattle	2016	India	MK105825	Giangaspero <i>et al.</i> , 2017
CSFV-a1	39	Pig	2001	China	AF407339	Wu <i>et al.</i> , unpublished
CSFV-a1	3 India	Cattle	2016	India	MK105826	Giangaspero <i>et al.</i> , 2017
CSFV-a1	4 India	Cattle	2016	India	MK105820	Giangaspero <i>et al.</i> , 2017
CSFV-a1	5 India	Cattle	2016	India	MK105821	Giangaspero <i>et al.</i> , 2017
CSFV-a1	5NCR/CSF/MZ/AIZ/348	Pig	2011	India	JX975460	Rajkhowa <i>et al.</i> , unpublished
CSFV-a1	5NCR/CSF/MZ/AIZ/352	Pig	2011	India	JX975461	Rajkhowa <i>et al.</i> , unpublished
CSFV-a1	6 India	Cattle	2016	India	MK105822	Giangaspero <i>et al.</i> , 2017
CSFV-a1	7 India	Cattle	2016	India	MK109913	Giangaspero <i>et al.</i> , 2017
CSFV-a1	8 India	Cattle	2016	India	MG813566	Giangaspero <i>et al.</i> , 2017
CSFV-a1	9 India	Cattle	2016	India	MK105827	Giangaspero <i>et al.</i> , 2017
CSFV-a1 (1.1)	Alfort 187	Pig		France	X87939	Ruggli <i>et al.</i> , 1995
CSFV-a1	Alfort A19	Pig		France	U90951	Smondack <i>et al.</i> , unpublished
CSFV-a1	Aizawl-09	Pig	2009	India	HM449066	Rajkhowa <i>et al.</i> , unpublished
CSFV-a1	Bangalore Ind-163/07	Pig	2007	India	EU446419	Patil <i>et al.</i> , unpublished
CSFV-a1 (1.1)	Brescia	Pig		Italy	M31768	Moorman <i>et al.</i> , 1990
CSFV-a1 (1.2)	BRESCIAX	Pig		Italy	AY578687	Risatti <i>et al.</i> , 2005
CSFV-a1	BV-P	Pig	2005	Hungary	DQ314582	Farsang <i>et al.</i> , unpublished
CSFV-a1	C strain	Vaccine	1994	China	Z46258	Moorman <i>et al.</i> , 1996
CSFV-a1 (1.1)	CAP	Pig		Switzerland	X96550	Tratschim <i>et al.</i> , unpublished
CSFV-a1	cF114	Pig	2001	China	AF333000	Mingxiao <i>et al.</i> , unpublished
CSFV-a1	CSF/MZ/KOL/73	Pig	2009	India	JX094153	Rajkhowa, unpublished
CSFV-a1	CSF/MZ/SAI/76	Pig	2009	India	JX094154	Rajkhowa, unpublished
CSFV-a1 (1.1)	CSFV/1.1/dp/CSF0382/Koslov	Pig		Czech Republic	HM237795	Leifer <i>et al.</i> , 2010
CSFV-a1 (2.3)	CSFV/2.3/wb/0608/2005/Euskirchen	Wild boar	2005	Germany	GU233732	Leifer <i>et al.</i> , 2010
CSFV-a1 (2.3)	CSFV/2.3/wb/CSF1046/2009/Hennef	Wild boar	2009	Germany	GU233733	Leifer <i>et al.</i> , 2010
CSFV-a1 (2.3)	CSFV/2.3/wb/CSF1045/2009/Roesrath	Pig	2009	Germany	LT593749	Hoepfer, unpublished
CSFV-a1 (1.1)	CSFV212L-13	Pig	2013	India	KY860615	Tomar <i>et al.</i> , unpublished

Table 1. (Cont'd)

Species/Genotype	Strain	Origin	Year	Country	Accession	Reference
CSFV-al (1.1)	CSFV-GZ-2009	Pig	2009	China	HQ380231	Shen <i>et al.</i> , 2011
CSFV-al (1.1)	CSFV/IVRI/VB-131	Pig	2009	India	KM262189	Kamboj <i>et al.</i> , 2014
CSFV-al (1.1)	CSFV-PK15C-NG79-11	Contaminant	2011	India	KC503764	Tomar <i>et al.</i> , 2015
CSFV-al	Eystrup	Pig		Germany	AF326963	Meyers <i>et al.</i> , 1999
CSFV-al	GPE (-)	Vaccine		Japan	AB019152	Harasawa and Giangaspero 1999
CSFV-al (1.1)	HCLV	Vaccine		India	AF091507	Wang <i>et al.</i> , unpublished
CSFV-al (1.1)	HVCAD22/14	Contaminant	2008	India	U606028	Desai <i>et al.</i> , unpublished
CSFV-al	Hokkaido/66	Pig	1966	Japan	AB019154	Harasawa and Giangaspero 1999
CSFV-al	Ibaraki/66	Pig	1966	Japan	AB019156	Harasawa and Giangaspero 1999
CSFV-al	Ibaraki/81-115	Pig	1981	Japan	AB019158	Harasawa and Giangaspero 1999
CSFV-al	Ibaraki/81-20	Pig	1981	Japan	AB019160	Harasawa and Giangaspero 1999
CSFV-al	Ibaraki/81-38	Pig	1981	Japan	AB019162	Harasawa and Giangaspero 1999
CSFV-al	Ibaraki/81-40	Pig	1981	Japan	AB019164	Harasawa and Giangaspero 1999
CSFV-al	Ind-173/08	Pig	2008	India	FJ183444	Patil <i>et al.</i> , 2010
CSFV-al	Ind-174/08	Pig	2008	India	FJ183445	Patil <i>et al.</i> , 2010
CSFV-al	Ind-175/08	Pig	2008	India	FJ183446	Patil <i>et al.</i> , 2010
CSFV-al	Ind-176/08	Pig	2008	India	FJ183447	Patil <i>et al.</i> , 2010
CSFV-al	Ind-239/08	Pig	2008	India	FJ183449	Patil <i>et al.</i> , 2010
CSFV-al	Ind-243/08	Pig	2008	India	FJ183452	Patil <i>et al.</i> , 2010
CSFV-al	Ind-272/08	Pig	2008	India	FJ183456	Patil <i>et al.</i> , 2010
CSFV-al (1.1)	JL1(06)	Pig	2006	China	EU497410	Qiu <i>et al.</i> , unpublished
CSFV-al	KC	Vaccine		Russia	AF099102	Grebennikova <i>et al.</i> , 1999
CSFV-al	LK-VNIVViM	Vaccine		Russia	KM522833	Zhou <i>et al.</i> , 2014
CSFV-al (1.1)	LOM	Pig		Japan	AB019655	Sakoda <i>et al.</i> , 1999
CSFV-al	Miyazaki/81	Pig	1981	Japan	AB019168	Harasawa Giangaspero 99
CSFV-al	Nakamura/66	Pig	1966	Japan	AB019170	Harasawa Giangaspero 99
CSFV-al (1.1)	NFP/AS-1	Pig	2011	India	KC617749	Roychoudhury <i>et al.</i> , unpublished
CSFV-al (1.1)	NFP/ML-2	Pig	2011	India	KC617761	Roychoudhury <i>et al.</i> , unpublished
CSFV-al (1.1)	NFP/ML-4	Pig	2011	India	KC617750	Roychoudhury <i>et al.</i> , unpublished
CSFV-al	Rovac	Vaccine	1994	USA	KJ1873238	Zhou <i>et al.</i> , 2014
CSFV-al (1.2)	RUCSFLUM	Vaccine		USA	AY578688	Risatti <i>et al.</i> , 2005
CSFV-al	Shimen	Pig	1999	China	AF092448	Huang <i>et al.</i> , unpublished

Table 1. (Cont'd)

Species/Genotype	Strain	Origin	Year	Country	Accession	Reference
CSFV-a1	SWH	Pig	2004	China	DQ127910	Li <i>et al.</i> , 2006
CSFV-a1	Vac A	Pig		USA	L42435	Stadejek <i>et al.</i> , 1996
CSFV-a1	Yamanashi/69	Pig	1969	Japan	AB019182	Harasawa and Giangaspero 1999
CSFV-a2	17-93	Pig		Poland	L42413	Stadejek <i>et al.</i> , 1996
CSFV-a2 (2.3)	Alfort/Tubingen	Pig		France	J04358	Meyer <i>et al.</i> , 1989
CSFV-a2	Chiba-80	Pig		Japan	AB019659	Sakoda <i>et al.</i> , 1999
CSFV-a2 (2.3)	CSFV/2.3/dp/CSF0821/HR/Novska	Pig	2002	Croatia	HQ148061	Leifer <i>et al.</i> , unpublished
CSFV-a2 (2.3)	CSFV/2.3/dp/CSF857/Borken	Pig	2006	Germany	GU233731	Leifer <i>et al.</i> , 2010
CSFV-a2 (2.3)	CSFV/2.3/dp/CSF864/BG/Jambul	Pig	2007	Bulgaria	HQ148062	Leifer <i>et al.</i> , unpublished
CSFV-a2 (2.3)	CSFV/2.3/wb/XXX0609/Uelzen	Wild boar	2004	Germany	GU324242	Leifer <i>et al.</i> , 2010
CSFV-a2 (2.3)	CSFV/2.3/SRB/1264/2005	Pig	2005	Serbia	KY849593	Petrovic <i>et al.</i> , unpublished
CSFV-a2 (2.3)	CSFV/2.3/SRB/6168/2006	Pig	2006	Serbia	KY849594	Petrovic <i>et al.</i> , unpublished
CSFV-a2	CSF/wb/FR57/2004/0964-03	Pig	2004	Germany	LT158404	Hoepfer, unpublished
CSFV-a2	CSF/wb/FR67/2003/0647-19	Pig	2003	Germany	LT158502	Hoepfer, unpublished
CSFV-a2	CSF/wb/FR67/2005/0018-06	Pig	2005	Germany	LT158405	Hoepfer, unpublished
CSFV-a2	CSF/wb/FR67/2005/0125-05	Pig	2005	Germany	LT158406	Hoepfer, unpublished
CSFV-a2	CSF/wb/FR67/2005/0238-02	Pig	2005	Germany	LT158407	Hoepfer, unpublished
CSFV-a2	CSF/wb/FR67/2006/0199-01	Pig	2006	Germany	LT158409	Hoepfer, unpublished
CSFV-a2	CSF/wb/FR67/2006/060003-06	Pig	2006	Germany	LT158408	Hoepfer, unpublished
CSFV-a2	CSF/wb/FR67/2007/0192-01	Pig	2007	Germany	LT158410	Hoepfer, unpublished
CSFV-a2 (2.1g)	GD19/2011	Pig	2011	China	KU504339	Gong <i>et al.</i> , 2016
CSFV-a2	HEN03	Cattle	2012	China	KC176778	Zhang <i>et al.</i> , unpublished
CSFV-a2 (2.1)	HNLV-2011	Pig	2011	China	JX262391	Jiang <i>et al.</i> , 2013
CSFV-a2 (2.1)	HNSD-2012	Pig	2012	China	JX218094	Jiang <i>et al.</i> , 2013
CSFV-a2	Osaka/51	Pig	1951	Japan	AB019174	Harasawa and Giangaspero 1999
CSFV-a2	Osaka/71	Pig	1971	Japan	AB019176	Harasawa and Giangaspero 1999
CSFV-a2	Pader	Pig		Germany	AY072924	Uttenthal <i>et al.</i> , 2001
CSFV-a2	Paderborn	Pig		Germany	GQ902941	Rasmussen <i>et al.</i> , 2010
CSFV-a2	S112	Contaminant	2014	China	MK118725	Zhang <i>et al.</i> , 2014
CSFV-a2	S173	Contaminant	2014	China	KF006975	Zhang <i>et al.</i> , 2014
CSFV-a2	Shizuoka/73	Pig	1973	Japan	AB019180	Harasawa and Giangaspero 1999
CSFV-a2 (2.1)	SKCDK	Pig	2009	China	GQ923951	Li <i>et al.</i> , unpublished

Table 1. (Cont'd)

Species/Genotype	Strain	Origin	Year	Country	Accession	Reference
CSFV-a2	Sp01	Pig	2001	Spain	FJ265020	Mena <i>et al.</i> , unpublished
CSFV-a2	Switzerland 1/93	Pig	1993	Switzerland	AF045068	Hofmann and Bossy, 1998
CSFV-a2	Switzerland 2/93'	Pig	1993	Switzerland	AF045069	Hofmann and Bossy, 1998
CSFV-a2	Switzerland 3/93/1'	Pig	1993	Switzerland	AF045070	Hofmann and Bossy, 1998
CSFV-a2	Switzerland 3/93/2'	Pig	1993	Switzerland	AF045071	Hofmann and Bossy, 1998
CSFV-a2	Switzerland 4/93'	Pig	1993	Switzerland	AF045072	Hofmann and Bossy, 1998
CSFV-a2	Venhorst	Pig		Netherlands	AF084049	Widjoatmodjo <i>et al.</i> , 1999
CSFV-a2	VR14762	Pig		Malaysia	L42437	Stadejek <i>et al.</i> , 1996
CSFV-a2 (2.3)	Wingene	Pig	1994	Belgium	JQ595295	Haegeman <i>et al.</i> , unpub
CSFV-a3	Saitama/81	Pig	1981	Japan	AB019178	Harasawa and Giangaspero 1999
CSFV-a4 (2.2)	179/MIB/2014	Pig	2014	India	KR350485	Bhaskar <i>et al.</i> , 2015
CSFV-a4 (2.2)	181/MIB/2014	Pig	2014	India	KR350486	Bhaskar <i>et al.</i> , 2015
CSFV-a4 (2.2)	211/MIB/2014	Wild boar	2014	India	KR350487	Ravishankar <i>et al.</i> , unpublished
CSFV-a4 (2.2)	23/MIB/2014	Pig	2014	India	KR149284	Bhaskar <i>et al.</i> , 2015
CSFV-a4 (2.2)	322/MIB/2013	Pig	2013	India	KR350488	Bhaskar <i>et al.</i> , 2015
CSFV-a4 (2.2)	99/MIB/2014	Pig	2014	India	KR350483	Bhaskar <i>et al.</i> , 2015
CSFV-a4 (2)	CSFV-UP-BR-757-09	Pig	2009	India	KC533785	Tomar <i>et al.</i> , unpublished
CSFV-a4	Parambi	Wild boar	2014	India	KT239105	Chandramohan <i>et al.</i> , unpublished
CSFV-a5	IND/AS/GHY/G4	Pig	2014	India	KM362426	Ahuja <i>et al.</i> , 2015
CSFV-a6	Fukuoka/72	Pig	1972	Japan	AB019150	Harasawa and Giangaspero 1999
CSFV-a6	Honduras	Pig		Honduras	L42426	Stadejek <i>et al.</i> , 1996
CSFV-b	5440/99	Sheep	1999	Spain	AY159514	Hurtado <i>et al.</i> , 2003
CSFV-c (3)	94.4/IL/94/TWN	Pig	1994	Taiwan	AY646427	Lin <i>et al.</i> , 2007
CSFV-c (3.4)	Kanagawa/74	Pig	1974	Japan	AB019166	Harasawa and Giangaspero 1999
CSFV-c	Okinawa/86	Pig	1986	Japan	AB019172	Harasawa and Giangaspero 1999
CSFV-c	Okinawa-86-2	Pig	1986	Japan	AB019663	Sakoda <i>et al.</i> , 1999
CSFV-c (3)	P97	Pig		Taiwan	L49347	Liu, unpublished
CSFV-d	S171	Contaminant	2014	China	KF006974	Zhang <i>et al.</i> , 2014
CSFV nd (2.2)	Sch180	Pig		Germany	JQ411560	Postel <i>et al.</i> , 2012
CSFV nd (1.3)	VRI 41167	Pig		Malaysia	JQ411570	Postel <i>et al.</i> , 2012

Table 2. Alignment of genus *Pestivirus* CSFV species genotypes variable loci 5'-UTR RNA secondary structure sequences, segregated according to types of base pair combinations. Base pairing combinations were identified for genotype characterization ($n = 37$) and considered for identification marker definition. The different types are ordered according to increasing divergence in the species (*), and divergence in the genus (**), was expressed in a number of divergent base pairs, with reference to most common base pairs in the prevalent positions. Highly conserved base pair positions are excluded. Positions 13 in V1 locus and 2, 7 and 8 in V3 locus are species specific; positions 15 in V1 locus, 5 and 7 in V2 locus and 1 in V3 locus are genotype specific. Y: C or U; ND: not determined.

Variable locus	V1																		
	1	2	3	5r	6	7	8	9	12	13	14	15	16	17	18	19	20	21	22
Prevalent base pairs	GY	UA	GC	UG	A	UA	CG	GY		CG	UA	AC	GC	CG	AA				
CSFV	GY	UA	AU	UG	A	UA	CG	GC	GC	UA	GC	AC	GC	CG	AA				
CSFV-a.1																			
39	GA	-	-	-
HCLV	GA	-	-	-
Ibaraki/66	GA	-	-	-
Ibaraki/81-115	GA	-	-	-
CSFV/2.3/Hennef	CC	-	-	-
CSFV/2.3/Roesrath	CU	-	-	-
8 India	.	.	.	GU	GA	-	-	-
5NCR/CSF/MZ/AIZ/352	AC	GA	-	-	-
Alfort 187	.	.	.	UU	GA	-	-	-
Brescia	GA	-	-	-
1 India	AU	.	GU	GA	-	-	-
CSFV-a.2																			
Alfort	CU	-	-	-
Osaka/51	CG	-	-	-
Pader	GG	-	-	-
Switzerland 1/93	CC	-	-	-
SKCDK	GU	-	-	-
Sp01	CC	-	-	-
CSFV/2.3/SRB/6168/2006	CC	-	-	-
CSFV/2.3/Jambul	UG	CU	-	-	-
17-93	GC	CU	-	-	-
Chiba-80	CU	-	-	-

Table 2. (Cont'd)

Variable locus	V1																					
	1	2	3	5r	6	7	8	9	12	13	14	15	16	17	18	19	20	21	22			
Position	GY	UA	GC	UG	A	UA	CG	GY	GC	UA	GC	AC	GC	CG	AA							
Prevalent base pairs	GY	UA	GC	UG	A	UA	CG	GY	GC	UA	GC	AC	GC	CG	AA							
CSFV	GY	UA	AU	UG	A	UA	CG	GC	GC	UA	GC	AC	GC	CG	AA							
S173	GG						
HEN03	GG						
CSFwbFR672006060003-06	CU						
CSFV-a.3							
Saitama/81	GA						
ND Sch180	GA						
CSFV-a.4							
Parambi	GA						
211/MIB/2014	GA						
322/MIB/2013	GA						
181/MIB/2014	GA						
CSFV-UP-BR-757-09	CA						GA						
CSFV-a.5							
IND/AS/GHY/G4	AG						
CSFV-a.6							
Fukuoka/72	GA						
Honduras	UG	AU	GC				UG						
ND VRI 4167	CA	AU	CU				
CSFV-b							
5440/99	AG	UA					
CSFV-c							
Okinawa/86	GA						
Kanagawa/74	AU						GA						
CSFV-d							
S171	CA						GG						

Table 2. (Cont'd)

Variable locus	V2										V3														
	1		2		3		4		5		6		7		8		9		10		11		12		
	AU	CG	YG	CG	UA	CG	UA	YG	CG	UA	GY	UA	GC	CG	UA	CG	UA	GC	CG	UA	CG	UA	GC	CG	
CSFV-a.1																									
39	.				AU																				
HCLV	.				AU																				
Ibaraki/66	.				AU																				
Ibaraki/81-115	.				AU																				
CSFV/2.3/Hennef	.				AU																				
CSFV2.3/Roesrath	.				AU																				
8 India	.				AU																				
5NCR/CSF/MZ/AIZ/352	.				AU																				
Alfort 187	.				AU																				
Brescia	.				AU																				
1 India	.				AU																				
CSFV-a.2																									
Alfort	.				GU																				
Osaka/51	.				GC																				
Pader	.				GC																				
Switzerland 1/93	.				GU																				
SKCDK	.				GC																				
Sp01	.				GU																				
CSFV/2.3/SRB/6168/2006	.				GU																				
CSFV/2.3/Jambul	.				GU																				
17-93	.				GU																				
Chiba-80	.				GU																				
S173	.				GC																				
HEN03	.				GC																				
CSFVwbFR672006060003-06	.				GU																				
CSFV-a.3																									
Saitama/81	AC				GU																				
ND Sch180	AC				GU																				
CSFV-a.4																									
Parambi	.				GU																				
211/MIB/2014	.				GU																				
322/MIB/2013	.				GU																				
181/MIB/2014	.				GU																				

Table 2. (Cont'd)

Variable locus	V2										V3									
	1	2	3	4	5	6	7	9	1	2	3	4	5	6	7	8	9	10	(*)	(**)
Position	YG	YG	YG	UA	GY	GU	GC	GC	GC	UA	CG	CG	CG	UA	UA	UA	UA	UA	A	A
Prevalent base pairs	AU	CG	CG	CG	UA	GU	GC	GC	GC	UA	CG	CG	CG	UA	UA	UA	UA	UA	A	A
CSFV																				
CSFV-UP-BR-757-09						GU								AC	CA	A	-	-	2	8
CSFV-a.5																				
IND/AS/GHY/G4	AC					AU								GU	CA	U	-	-	3	8
CSFV-a.6																				
Fukuoka/72						AU								AC	CA	C	-	-	2	8
Honduras						AU		AC						AC	CA	C	-	-	4	8
ND VRI 4167						AU		AC						AC	CA	C	-	-	ND	ND
CSFV-b																				
5440/99					AU	GC	AC							UC	UA	A	-	-	4	9
CSFV-c																				
Okinawa/86						GC		AC	AG					AU	CA	U	-	-	5	10
Kanagawa/74						GC			AG					AU	CA	U	-	-	6	9
CSFV-d																				
S171	AA					GC		UC	UU	CC	AC	CA	A-	CA	C	-	-	7	12	

20.31. These values were very high in confront of the mean divergence value of 14.57 obtained compared to the other CSFV strains with BDV strains (Table 5). Divergence from BDV-2 tentative species is also higher with CSFV-d (divergence values ranging from 21 to 22; mean divergence value 21.33), in confront of scores obtained with other CSFV genotypes CSFV-a, CSFV-b, and CSFV-c (divergence values ranging from 15 to 20; mean divergence value 17.06) (Table 5). No relation was observed between CFSV and any other *Pestivirus* species (Fig. 2).

By phylogenetic analysis of E2 region, the Indian bovine strains CS/ML/911/IDP/13 (KY860532) and CS/ML/AF/Umiyam/14 (KY860531) (Ahuja *et al.*, 2015; Chakraborty *et al.*, 2018) were similar to pig isolates as IND/AS/GHY/G4 (KM362426) (Ahuja *et al.*, 2015), originated from Assam, a neighboring state. By BLAST, the E2 sequence of both strains CS/ML/911/IDP/13 and CS/ML/AF/Umiyam/14 showed 99% and 92% nucleotide identity with strain IND/AS/GHY/G4 (CSFV-a5) and reference strain Paderborn (GQ902941) (CSFV-a2), respectively. Taking into account the PNS evaluation of Paderborn and IND/AS/GHY/G4 5'-UTR, both bovine strains CS/ML/911/IDP/13 and CS/ML/AF/Umiyam/14 were clustered into CSFV-a5. Similar to type Parambi (CSFV-a4), also this cluster was the characteristic of India. A schematic phylogenetic tree, based on secondary structure base pair variation and divergence values at the three variable loci, is shown in Figure 3. At the species level, the observed taxonomic status of the examined strains corresponded to the estimation obtained by phylogenetic tree based on the primary sequence analysis of the 5'-UTR constructed from the alignment with the representative strains from the identified genogroups (Fig. 4).

Discussion

On contrary to other pestiviruses, as BVDV-1 which accounted for much more genotypic variants (Giangaspero and Apicella, 2018), the CSFV species was low heterogeneous. Only two genotypes, CSFV-c and CSFV-d, both of Asian origin, were highly divergent. Taking into account the high divergence in the species based on the primary sequence analysis, the strain Kanagawa/74 (CSFV-c) (Japan, 1974; Dreier *et al.*, 2007; Beer *et al.*, 2015) was proposed, similarly to congenital Tremor (United Kingdom, 1964), as harmonized outgroup strain for phylogenetic analyses (Paton *et al.*, 2000). Similar to the strain Kanagawa/74, the Chinese bovine strain S171 (CSFV-d) was divergent in the species (Fig. 4), suggesting its consideration as another outgroup of the species with even more enhanced divergence.

The other bovine Chinese strains are less divergent, showing common genomic trait characteristic of the known reported CSFV-a2 strains, indicating the concomitant circulation of typical CSV types with others showing atypical characteristics, possibly related

Table 3. Clustering into genotypes of *Pestivirus* CSFV species strains ($n = 126$) evaluated according to the PNS method at the 5' untranslated region of RNA. Nomenclature according to primary sequence analysis is indicated under parenthesis. ND: not determined.

Species genotypes	Strains	Distance within species
CSFV-a	CSFV-a1 (1.1; 1.2): 39; 5NCR/CSF/MZ/AIZ/348; 5NCR/CSF/MZ/AIZ/352; 1 India; 2 India; 3 India; 4 India; 5 India; 6 India; 7 India; 8 India; 9 India; 10 India; 11 India; 13 India; Alfort 187; Alfort A19; Aizawl-09; Bangalore Ind-163/07; Brescia; BRESCIAX; BV-P; C strain; CAP; cF114; CSFV212L-13; CSFV-GZ-2009; CSFV-PK15C-NG79-11; CSFV/IVRI/VB-131; CSFV/1.1/dp/CSF0382/XXXX/Koslov; CSF/MZ/KOL/73; CSF/MZ/SAI/76; CSFV/2.3/wb/CSF1046/2009/Hennef; CSFV/2.3/wb/XXX0608/2005/Euskirchen; CSFV/2.3/Roesrath; Eystруп; GPE (-); HCLV; HCVCAD22/14; Hokkaido/66; Ibaraki/66; Ibaraki/81-115; Ibaraki/81-20; Ibaraki/81-38; Ibaraki/81-40; Ind-173/08; Ind-174/08; Ind-175/08; Ind-176/08; Ind-239/08; Ind-243/08; Ind-272/08; JL1(06); KC; LK-VNIVViM (V1 GC); LOM; Miyazaki/81; Nakamura/66; NFP/AS-1; NFP/ML-2; NFP/ML-4; Rovac; RUCSFPLUM; Shimen; SWH; Vac A; Yamanashi/69.	0.54
	CSFV-a2 (2.1; 2.3): 17-93; Alfort; Chiba-80; CSFV/2.3/dp/CSF857/2006/Borken; CSFV/2.3/dp/CSF864/2007/BG/Jambul; CSFV/2.3/dp/CSF0821/2002/HR/Novska; CSFV/2.3/wb/XXX0609/2004/Uelzen; CSF/wb/FR5720040964-03; CSF/wb/FR672006060003-06; CSF/wb/FR6720070192-01; CSF/wb/FR6720030647-19; CSF/wb/FR6720060199-01; CSF/wb/FR6720050238-02; CSF/wb/FR6720050125-05; CSF/wb/FR6720050018-06; CSFV/2.3/SRB/1264/2005; CSFV/2.3/SRB/6168/2005; CSFV/2.3/SRB/6168/2006; GD192011; HEN03; HNLV-2011; HNSD-2012; Osaka/51; Osaka/71; Pader; Pader born; S112; S173; Shizuoka/73; SKCDK; Sp01; Switzerland 1/93; Switzerland 2/93'; Switzerland 3/93/1'; Switzerland 3/93/2'; Switzerland 4/93'; Venhorst; VRI4762; Wingene.	0.46
	CSFV-a3 (2.2): Saitama/81.	1
	CSFV-a4 (2.2): Parambi; 179/MIB/2014; 181/MIB/2014; 211/MIB/2014; 23/MIB/2014; 322/MIB/2013; 99/MIB/2014; CSFV-UP-BR-757-09.	1.4
	CSFV-a5 (2.1): IND/AS/GHY/G4.	3
	CSFV-a6 (1.3): Fukuoka/72; Honduras.	3
CSFV-b	5440/99.	4
CSFV-c (3)	94.4/IL/94/TWN; Kanagawa/74; Okinawa/86; Okinawa-86-2; P97.	5.5
CSFV-d	S171.	7
CSFV ND	Sch180; VRI 4167.	

to the host species adaptation or a result of geographic isolation. Such occurrences have been suggested also for BVDV-1 isolates in China (Xue *et al.*, 2010; Wang *et al.*, 2014). Similarly, Indian bovine strains were genetically closely related to common CSFV genotype a1 strains and corresponded to circulate *Pestivirus* pig strains reported in the country. Between 2009 and 2011, Rajkhowa *et al.* reported three CSFV isolates from pigs (Aizawl-09, 5NCR/CSF/MZ/AIZ/348, and 5NCR/CSF/MZ/AIZ/352) (accessions numbers HM449066, JX975460, and JX975461, respectively), from the Indian state of Mizoram, located at the extreme northeastern border of Myanmar, well distant from Tamil Nadu, the area where CSFV was detected in cattle. Pig strains from Mizoram are very close to the bovine strains, belonging to the genotype CSFV-a variant 1. A distinctive guanine uracile base pairing in position 3 in the V1 locus was found only in the bovine isolates originated from Tamil Nadu. This

peculiarity was different from all other CSFV strains, which present a conserved A–U pairing in this position. Interestingly, G–C or G*U pairings are conserved only among strains belonging to the BVDV species types 1, 2, and 3 (only five exceptions, out of more than 1,000 considered sequences). In the CSFV species, three genetic clusters referred specifically to Asian countries. The CSFV genotype a variant 4 (type Parambi) included only pig and wild boar strains from India [Bhaskar *et al.*, 2015; and sequences deposited by Ravishankar *et al.* (unpublished); Tomar *et al.* (unpublished); and Chandramohan *et al.* (unpublished)]. CSFV genotype C (type Okinawa) was reported only in pigs originating from Japan and Taiwan (Harasawa and Giangaspero, 1999; Sakoda *et al.*, 1999; Lin *et al.*, 2007; and strain P97 deposited by Liu). Furthermore, the sequence characteristics of strain S171 (Zhang *et al.*, 2014) (CSFV-d) were observed only in China.

Table 4. PNS characteristics to the *Pestivirus* CSFV species with related genotypes and genotype variants. The position of base pairings is defined by numbering from the bottom of the variable locus. Y= C or U.

CSFV species	Locus	Characteristic PNS markers
	V1	U-A in position 13 (exception U*G or C-G; C A bulge);
	V3	U-A in position 2; C A or U-A in position 7; U or C in position 8 (exception A).
CSFV genotypes	Locus	Characteristic PNS markers
CSFV-a	V1	A C bulge in position 15 (exceptions G:Y and A-U);
	V2	U-A in position 5; G:Y in position 7;
	V3	A-U in position 1 (exception A G bulge).
CSFV-b	V1	G:Y in position 15; A G bulge in position 19; U-A in position 20;
	V2	A-U in position 5; A C bulge in position 7;
	V3	A-U in position 1; U C bulge in position 6.
CSFV-c	V1	A-U in position 15;
	V2	U-A in position 5; G:Y in position 7;
	V3	A G bulge in position 1.
CSFV-d	V1	A C bulge in position 15;
	V2	U-A in position 5; G:Y in position 7;
	V3	U U bulge in position 1.
CSFV genotype variants	Locus	Characteristic PNS markers
CSFV-a1	V1	AC in position 15;
	V2	A-U in position 1; A-U in position 6;
	V3	C or U in position 8.
CSFV-a2	V1	AC in position 15 (exception G-C);
	V2	A-U in position 1; G:Y in position 6;
	V3	A-U in position 6; U or C in position 8.
CSFV-a3	V1	AC in position 15;
	V2	AC in position 1; G:Y in position 6;
	V3	A in position 8.
CSFV-a4	V1	G:Y in position 15;
	V2	A-U in position 1; G:Y in position 6;
	V3	AC or UC in position 6; A in position 8 (exception C).
CSFV-a5	V1	A-U in position 15;
	V2	AC in position 1; A-U in position 6;
	V3	U in position 8.
CSFV-a6	V1	G:Y in position 15;
	V2	A-U in position 1; A-U in position 6;
	V3	C in position 8.

In general, apart nomenclature difference, the comparison between primary and secondary structure analyses revealed corresponding main genomic groups, defined by phylogenetic analysis, dividing CSFVs into three major lineages (Brescia, Alfort and Kanagawa/Okinawa types) and their sublineages (Lin *et al.*, 2007). Furthermore, the phylogenetic trees showed such similarity (Figs. 3 and 4). Genotype CSFV-a variant 1 (type Brescia) corresponded to 1.1 and 1.2, CSFV-a

variant 2 (type Alfort/Tübingen) to 2.1 and 2.3, CSFV-a variant 4 to 2.2, and genotype CSFV-c to 3 and 3.4. The CSFV 2.2 strain Sch180 (JQ411560) from Germany and the CSFV 1.3 strain VRI 4167 (JQ411570) from Malaysia could not be determined due to incomplete V1 locus, but their other sequence fragments were compatible with PNS genotype variants CSFV-a3 (type Saitama) and CSFV-a5 (type Honduras), respectively (Table 2). However, as expected, the

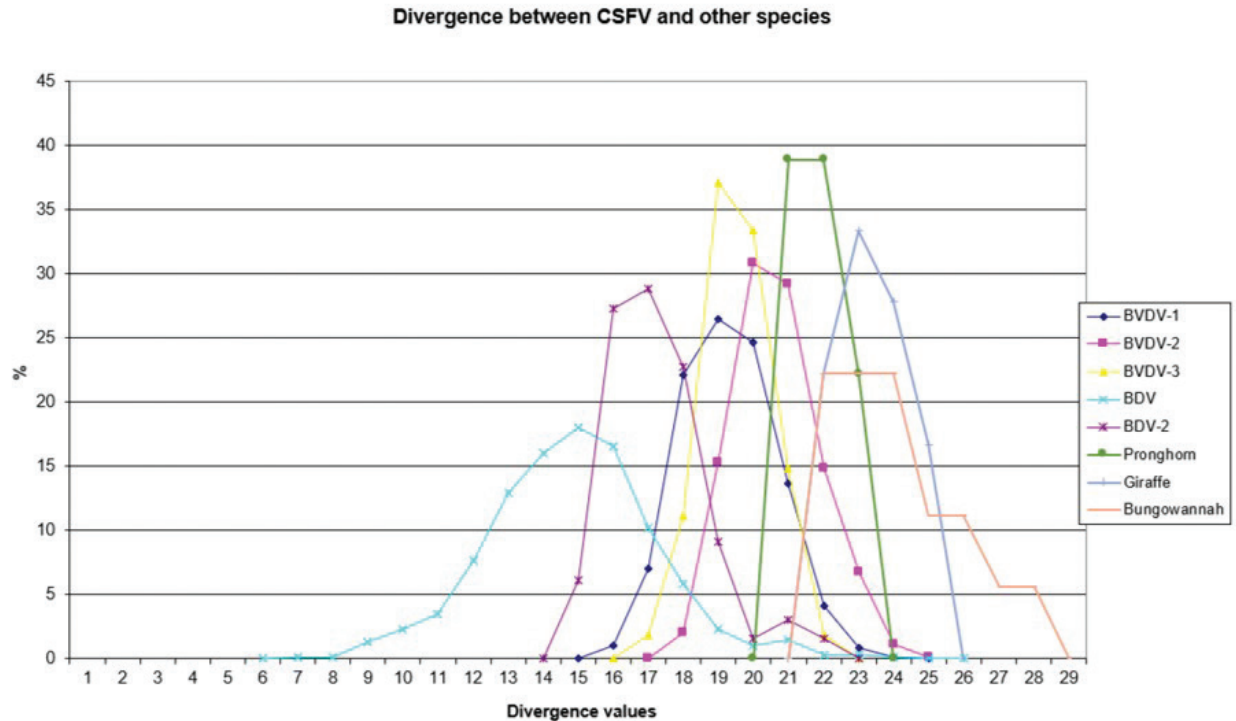


Fig. 1. Graphical representation of PNS quantitative evaluation. CSFV shows close genetic relation to BDV and a lesser extent with BDV-2.

specific differences observable at the level of the E2 gene sequence fragment (Risatti *et al.*, 2005; Postel *et al.*, 2012) were not necessarily related to secondary structure 5'-UTR clustering. Similarly, PNS genotype CSFV-c included variants as 3.4 not discriminable from other members of genotype 3 (Lin *et al.*, 2007). Similar discrepancies may occur comparing other regions, as between 5'-UTR and Npro.

With concern to the relationship between CSFV detection and clinical observations, the strain HEN03, the only among the Chinese strains for which clinical anamnestic data could be retrieved, was associated with reproductive disorders observed in many animals in the herd: abortion, stillbirth, and reduced milk yield. Furthermore, the mortality rate was high. However, concomitant infection with BVDV-1 and BVDV-2 was revealed. It is particularly interesting that also all the Indian bovine CSFV strains were isolated in herds suffering from reproductive disturbances, abortions, birth defects, repeat breeding, and mastitis. Serological positivity for bovine herpesvirus 1 (IBR) and *Brucella* sp. was absent or very low, manifestly not correlated to observed pathologies, and no BVDV has been isolated (Giangaspero *et al.*, 2017).

Hypothetically, CSFV bovine strains detected in China and India might find their origin from vaccine prophylaxis performed in pig populations. CSFV-a1 comprises a CSFV-lapinized vaccine strain from India (EU857642–AF091507), which showed a high

homology with Indian cattle strains (99% identities according to the results obtained by BLAST). Investigations on vaccination protocols applied in the research area showed that none of the cattle farms investigated in Tamil Nadu showed to have contact with pigs, and no immunization program was carried for BVDV (Giangaspero *et al.*, 2017). However, CSFV vaccine prophylaxis is carried out in the swine industry in India. At present, anti-BVDV-killed virus vaccines are available in China, but, for a long time, there were no commercial BVDV vaccines in the Chinese market. In China, and particularly in Tibet and Qinghai provinces, prophylaxis against BVDV in cattle and yaks was based on the wide use of the live-attenuated hog cholera lapinized vaccine (HCLV), administered to animals at a triple dose. This followed the experimental demonstration of efficacy and safety of the prophylactic use of HCLV for BVDV in lactating and pregnant cows, calves, and yaks undertaken by Yuan Qingzhi in 1957 (Liu *et al.*, 2003). For a long time, the HCLV was the only authorized vaccine against CSFV in China. In Tibet, the long-term use of live-attenuated HCLV not only reduced losses caused by BVDV infection but also gave the opportunity for vaccinal strain adaptation in cattle, allowing as a possible consequence of a subsequent natural diffusion. Tibet is historically a high-quality breeding region managed by a traditional seminomadic agropastoral system and appreciated by traders who seasonally purchase and move live

Table 5. PNS quantitative evaluation. CSFV versus BDV and BDV-2 tentative species secondary structure sequence comparison divergence values ($n = 2,178$). Scores exceeding species determination divergence limit value (13) indicate genetic distance and different species clustering.

CSFV	39	Alfort 187	8 India	Brescia	HCLV	Ibaraki/66	Ibaraki/81-115	17-93	Alfort	Chiba-80	Osaka/51	Pader	S173	HEN03	Switzerland 1/93	Saitama/81	Fukuoka/72	Honduras	5440/99	Okinawa/86	Kanagawa/74	S171	
Border disease virus (BDV)																							
a1 A841/1	14	13	15	15	14	14	14	13	13	14	12	13	14	14	13	14	15	12	14	16	12	20	Divergent
a1 V1414	15	14	16	16	15	15	15	14	14	15	13	14	15	15	14	15	16	13	15	16	12	21	Divergent
a1 135 661	14	13	15	15	14	14	15	14	14	15	13	13	14	14	14	14	15	12	16	15	11	20	Divergent
a1 170 337	15	14	16	16	15	15	15	14	14	15	13	14	15	15	14	15	16	13	16	16	12	21	Divergent
a1 G1305	16	15	17	17	16	16	16	15	15	16	14	15	16	16	15	16	16	14	17	16	12	22	Divergent
a1 G2048	14	13	15	15	14	14	14	13	13	14	12	13	14	14	13	13	15	12	15	15	11	20	Divergent
a1 D1586/2	14	13	15	15	14	14	14	13	13	14	12	13	14	14	13	14	15	12	15	16	12	20	Divergent
a1 K1729/3	15	14	16	16	15	15	15	14	14	15	14	14	15	15	14	15	16	14	16	16	12	21	Divergent
a1 1505744	16	15	17	17	16	16	16	15	15	16	14	14	16	16	14	16	16	15	17	16	12	22	Divergent
a1 1502304	15	14	16	16	15	15	14	13	13	14	12	13	15	15	12	15	16	14	15	16	12	21	Divergent
a1 1062689	15	14	16	16	15	15	15	14	14	15	13	13	15	15	13	15	16	14	16	16	12	21	Divergent
a2 X818	14	15	15	15	14	14	14	12	13	14	13	13	14	14	13	14	13	12	15	15	11	20	Divergent
a2 Moredun ncp	12	13	17	13	12	12	12	10	11	12	10	11	12	12	11	12	11	9	13	13	9	18	Related
a2 T1789/1	12	13	17	13	12	12	12	10	11	12	10	11	12	12	11	12	11	9	13	13	9	18	Related
a2 Q1673/2	13	14	18	14	13	13	13	11	12	13	11	12	13	13	12	13	12	10	14	14	10	19	Related
a2 JH2816	13	15	18	14	13	13	13	11	12	13	11	12	13	13	12	13	12	10	13	14	10	19	Related
a2 Lyon2	12	13	17	13	12	12	12	10	11	12	9	10	12	12	10	12	11	9	13	13	9	18	Related
a2 L991	12	13	17	13	12	12	12	10	11	12	10	11	12	12	11	12	11	11	13	14	11	18	Related
a2 Moredun cp	13	14	18	14	13	13	13	11	12	13	11	12	13	13	12	13	12	10	14	14	11	19	Related
a2 A1870	12	13	17	13	12	12	12	10	11	12	10	11	12	12	11	12	11	9	13	13	10	18	Related
a2 8320-22NZ	13	14	18	14	13	13	13	11	12	13	11	12	13	13	12	13	12	10	14	14	10	19	Related
a2 1376527	13	14	17	14	13	13	13	11	12	13	12	12	12	12	12	13	12	13	14	15	12	18	Related
a2 8320-31NZ	13	14	18	14	13	13	13	11	12	13	11	12	13	13	12	13	12	10	14	14	10	19	Related
a2 FNK2012-1	13	14	18	14	13	13	13	11	12	13	10	11	13	13	11	13	12	10	14	14	10	19	Related
a2 1118212	13	14	14	13	13	13	13	11	12	13	10	11	13	13	11	13	12	10	14	14	10	19	Related

Table 5. (Cont'd)

CSFV	Alfort 187	8 India	Brescia	HCLV	Ibaraki/66	Ibaraki/81-115	17-93	Alfort	Chiba-80	Osaka/51	Pader	S173	HEN03	Switzerland 1/93	Saitama/81	Fukuoka/72	Honduras	5440/99	Okinawa/86	Kanagawa/74	S171	
a2	Ch1Es	15	16	16	15	15	13	14	15	13	14	15	15	14	15	14	12	15	16	12	21	Divergent
a2	Coos Bay 5 c	15	16	15	15	15	13	14	14	12	13	15	15	13	15	14	12	13	16	12	21	Divergent
a3	BD31	15	15	16	15	15	15	14	15	14	14	15	15	14	15	16	15	17	17	13	21	Divergent
a4	LA/5909/09	15	16	16	15	15	13	14	14	12	13	15	15	13	14	14	13	14	16	13	21	Divergent
b	Genzkow 701	13	14	14	13	13	12	12	13	12	12	13	13	12	13	13	12	15	12	10	18	Related
b	Reitner Rudolph	14	15	15	14	14	13	13	14	13	13	14	14	13	14	14	13	16	13	9	19	Divergent
b	BT2305	14	15	15	14	14	13	13	14	13	13	14	14	13	14	14	13	16	13	9	19	Divergent
b	ST1405	14	15	15	14	14	13	13	14	13	13	14	14	13	14	14	13	16	13	9	19	Divergent
b	ST1507	14	15	15	14	14	13	13	14	13	13	14	14	13	14	14	13	16	13	9	19	Divergent
b	J1004	14	15	15	14	14	13	13	14	13	13	14	14	13	14	14	13	16	13	9	19	Divergent
c1	CH-BD3	13	14	14	13	13	13	13	14	12	12	14	14	12	13	13	13	16	13	11	19	Related
c1	CH-BD4	15	16	16	15	15	14	14	15	12	13	15	15	13	15	15	14	17	15	13	20	Divergent
c1	Italy-103761	15	16	16	15	15	14	14	13	13	12	14	13	13	15	15	12	16	13	13	19	Divergent
c1	Italy-58987	17	18	18	17	17	16	16	17	15	15	17	17	15	17	17	16	18	17	15	22	Divergent
c1	Chamois-VdA-2	18	19	19	18	18	17	17	18	16	16	18	18	16	18	18	17	19	18	16	23	Divergent
c2	90-F-6335	16	17	17	16	16	15	15	16	15	15	16	16	15	14	16	15	17	16	14	21	Divergent
c2	06-F-0299/357	16	17	17	16	16	15	15	16	15	15	16	16	15	14	16	15	17	16	14	21	Divergent
c2	92-F-7119	16	17	17	16	16	15	15	16	15	15	16	16	15	14	16	15	17	16	14	21	Divergent
c2	10F03356	16	17	17	16	16	15	15	16	14	14	16	16	14	14	16	15	17	16	14	21	Divergent
c2	RUPI-05	17	18	18	17	17	16	16	17	15	15	17	17	15	15	17	16	18	17	15	21	Divergent
d1	AH12-01	14	15	15	14	14	12	12	13	12	12	14	14	12	13	14	13	16	14	12	19	Divergent
d1	AHX15	14	15	15	14	14	13	13	14	12	12	14	14	12	13	14	13	16	14	12	19	Divergent
d2	297	16	17	17	16	16	15	15	16	14	14	16	16	14	15	16	15	16	16	14	20	Divergent
e1	C27	17	18	17	17	17	16	16	17	15	16	16	16	15	16	16	15	15	18	16	21	Divergent
e2	ZAI-1115	17	18	19	17	17	15	16	17	16	16	18	18	16	16	16	15	16	18	16	23	Divergent
f1	0501209-052GI	14	15	15	14	14	13	13	14	12	13	14	14	13	14	14	12	15	14	12	20	Divergent

Table 5. (Cont'd)

CSFV	39	Alfort 187	8 India	Brescia	HCLV	Ibaraki/66	Ibaraki/81-115	17-93	Alfort	Chiba-80	Osaka/51	Pader	S173	HEN03	Switzerland I/93	Saitama/81	Fukuoka/72	Honduras	5440/99	Okinawa/86	Kanagawa/74	S171	
f1	16	17	17	16	16	16	16	15	15	16	15	15	16	16	15	15	16	14	17	16	14	21	Divergent
f1	17	18	18	17	17	17	17	16	16	15	14	15	16	15	15	16	17	13	18	15	15	21	Divergent
f1	17	18	18	17	17	17	17	16	16	15	16	16	16	15	16	16	17	13	18	15	15	21	Divergent
f1	17	18	18	17	17	17	17	16	16	15	16	16	16	15	16	16	17	13	18	15	15	21	Divergent
f1	18	19	18	18	18	18	18	17	17	16	15	16	17	16	16	17	18	14	19	16	16	22	Divergent
f2	18	17	19	18	18	18	18	19	19	20	18	19	20	20	19	19	18	17	20	20	16	25	Divergent
f2	16	15	17	16	16	16	16	17	17	18	16	17	18	18	17	17	16	15	18	18	17	23	Divergent
f2	17	16	18	17	17	17	17	18	18	19	17	18	19	19	18	18	17	16	19	19	16	24	Divergent
f3	17	18	18	17	17	17	17	16	16	17	16	16	16	17	16	16	17	15	17	17	15	22	Divergent
f3	16	17	17	16	16	16	16	17	17	18	16	17	18	18	17	17	16	15	18	18	17	23	Divergent
f3	16	17	17	16	16	16	16	17	17	18	16	17	18	18	17	17	16	15	18	18	17	23	Divergent
f3	17	18	18	17	17	17	17	16	16	17	16	16	16	17	16	16	17	15	17	17	15	22	Divergent
f4	15	16	15	16	15	15	15	16	16	17	16	16	16	16	16	16	15	14	18	17	16	21	Divergent
f4	14	15	15	14	14	14	14	15	15	16	13	14	16	16	14	15	14	13	17	16	15	21	Divergent
f4	14	15	15	14	14	14	14	15	15	16	15	15	16	16	15	16	14	13	17	16	15	21	Divergent
f4	14	15	15	14	14	14	14	15	15	16	13	14	16	16	14	15	14	13	17	16	15	21	Divergent
f4	15	16	16	15	15	15	15	16	16	17	14	15	17	17	15	16	15	14	18	17	16	22	Divergent
f4	16	17	16	17	16	16	16	17	17	16	17	17	16	15	17	17	16	13	19	16	17	21	Divergent
f4	15	16	16	16	15	15	15	16	16	15	15	16	16	15	16	16	15	12	18	15	16	21	Divergent
f4	15	16	16	16	15	15	15	16	16	15	15	16	16	15	16	16	15	12	18	15	16	21	Divergent
f4	14	15	15	14	14	14	14	15	15	14	14	14	15	14	14	15	14	12	17	14	15	20	Divergent
g1	12	13	13	13	12	12	11	9	10	11	9	10	12	12	9	12	11	10	11	12	10	18	Related
g1	13	14	14	14	13	13	12	10	11	12	10	11	13	13	10	13	12	11	12	13	11	19	Related
g1	12	13	13	12	12	11	9	7	8	9	7	9	11	11	7	12	11	10	9	11	9	18	Related
g1	12	13	13	12	12	11	10	8	9	10	9	8	10	10	9	12	11	10	11	11	9	17	Related
g1	11	12	12	12	11	11	10	10	11	12	10	11	13	13	10	13	10	9	11	13	11	19	Related

Table 5. (Cont'd)

CSFV	39	Alfort 187	8 India	Brescia	HCLV	Ibaraki/66	Ibaraki/81-115	17-93	Alfort	Chiba-80	Osaka/51	Pader	S173	HEN03	Switzerland I/93	Saitama/81	Fukuoka/72	Honduras	5440/99	Okinawa/86	Kanagawa/74	S171	19	Related	
g1	91-F-6731	13	14	14	13	13	12	11	9	10	11	10	11	12	12	10	13	12	11	12	8	19	20	Divergent	
g2	37A	13	14	14	15	14	14	15	13	14	15	14	13	14	14	14	14	12	11	14	12	20	21	Divergent	
g2	BM01 isolate 5	15	16	16	16	15	15	14	12	13	14	13	14	15	15	13	15	14	13	14	15	11	21	Divergent	
g2	33S	14	15	15	15	14	14	15	13	14	15	14	13	14	14	14	14	13	13	14	16	13	20	20	Divergent
h	JS12/04	16	17	17	17	16	16	16	15	15	14	14	15	14	13	15	16	13	18	14	14	14	18	18	Divergent
h	JSL12-01	15	16	16	16	15	15	15	14	14	13	13	14	13	12	15	15	12	17	13	13	13	18	18	Divergent
h	JSYZ15	15	16	16	16	15	15	15	13	13	14	13	13	14	13	15	15	14	17	15	13	13	19	19	Divergent
i1	TR-13	18	19	19	19	18	18	18	16	17	18	16	16	18	18	16	18	17	16	18	19	16	22	22	Divergent
i2	TR-14	17	18	18	18	17	17	17	15	16	17	15	15	17	17	15	17	16	15	17	18	16	21	21	Divergent
j1	Ind 830-09	13	14	14	14	13	13	13	11	12	13	11	11	13	13	10	13	12	11	14	14	12	18	18	Related
j2	CH-BD1	15	16	16	16	15	15	15	14	14	15	12	13	15	15	13	15	14	14	17	15	14	20	20	Divergent
j2	06-F-0083	15	16	15	16	15	15	15	14	14	15	13	14	16	16	14	15	15	15	17	16	15	20	20	Divergent
j2	85-F-588	14	15	14	15	14	14	14	14	13	14	12	13	15	15	13	14	15	15	17	17	16	19	19	Divergent
j2	90-F-6227	15	16	15	16	15	15	15	14	14	15	13	14	16	16	14	15	15	16	17	16	15	20	20	Divergent
j2	LA6442110	17	18	16	18	17	17	17	16	16	17	14	15	17	17	15	17	16	16	18	17	15	21	21	Divergent
j2	06M0150	16	17	15	17	16	16	16	15	15	16	14	14	16	16	14	16	15	16	17	16	15	20	20	Divergent
j2	89-F-5374	15	16	15	16	15	15	15	14	14	15	14	14	16	16	14	15	15	16	17	16	15	20	20	Divergent
j3	Gifhorn	14	15	14	15	14	14	14	12	13	14	13	13	15	15	13	14	13	14	15	16	15	20	20	Divergent
j3	B30006	16	17	15	17	16	16	16	14	15	16	14	14	16	16	14	16	14	15	16	17	16	21	21	Divergent
j3	CH-BD2	17	18	16	18	17	17	17	15	16	15	15	15	16	15	15	17	15	14	17	16	17	21	21	Divergent
k	BDV/Burdur/05-TR	17	18	16	18	17	17	17	15	16	17	16	16	17	17	16	17	16	16	17	18	15	23	23	Divergent
k	BDV/Aydin/04-TR	17	18	18	18	17	17	17	15	16	17	16	16	17	17	16	17	16	16	16	18	15	23	23	Divergent
Border disease virus type 2 (BDV-2)																									
BDV-2	712/02	17	18	17	18	17	17	17	16	16	17	16	16	16	16	16	17	18	18	19	18	21	21	21	Divergent
BDV-2	TO/121/04	17	18	17	18	17	17	16	15	15	16	15	16	16	15	16	17	18	17	18	17	19	21	21	Divergent
BDV-2	LA/91/05	18	19	18	19	18	18	17	16	16	17	16	17	17	16	17	18	19	18	19	20	19	22	22	Divergent

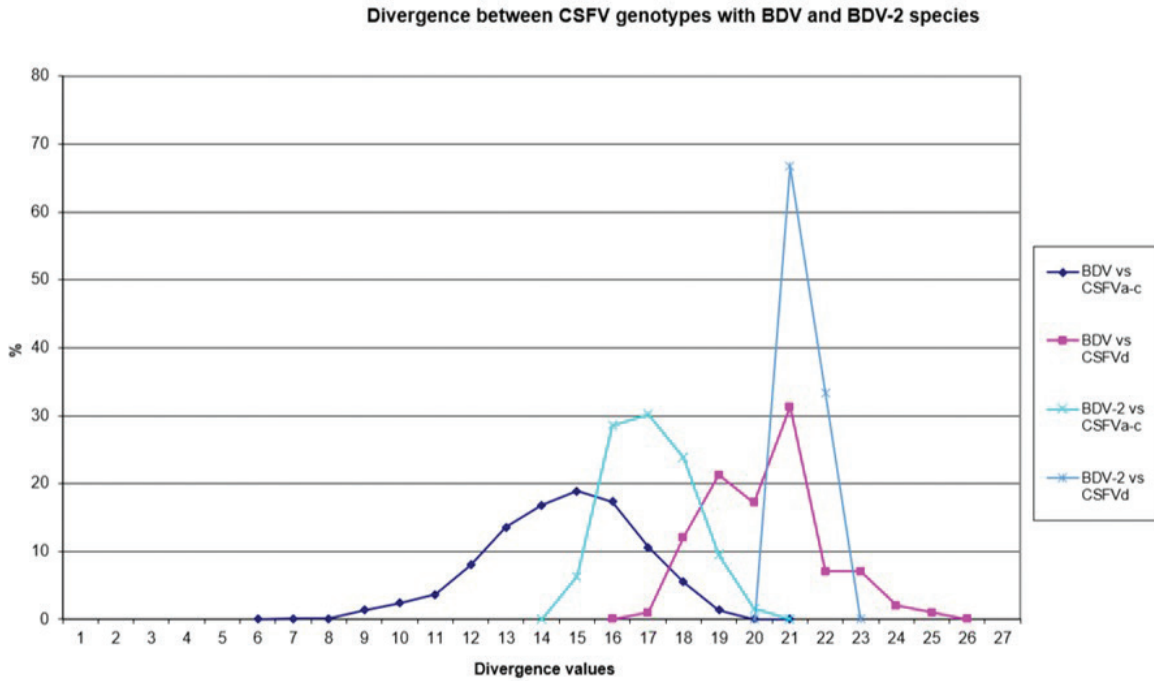


Fig. 2. Graphical representation of PNS quantitative evaluation. Genotype CSFV-d is not related to BDV or BDV-2.

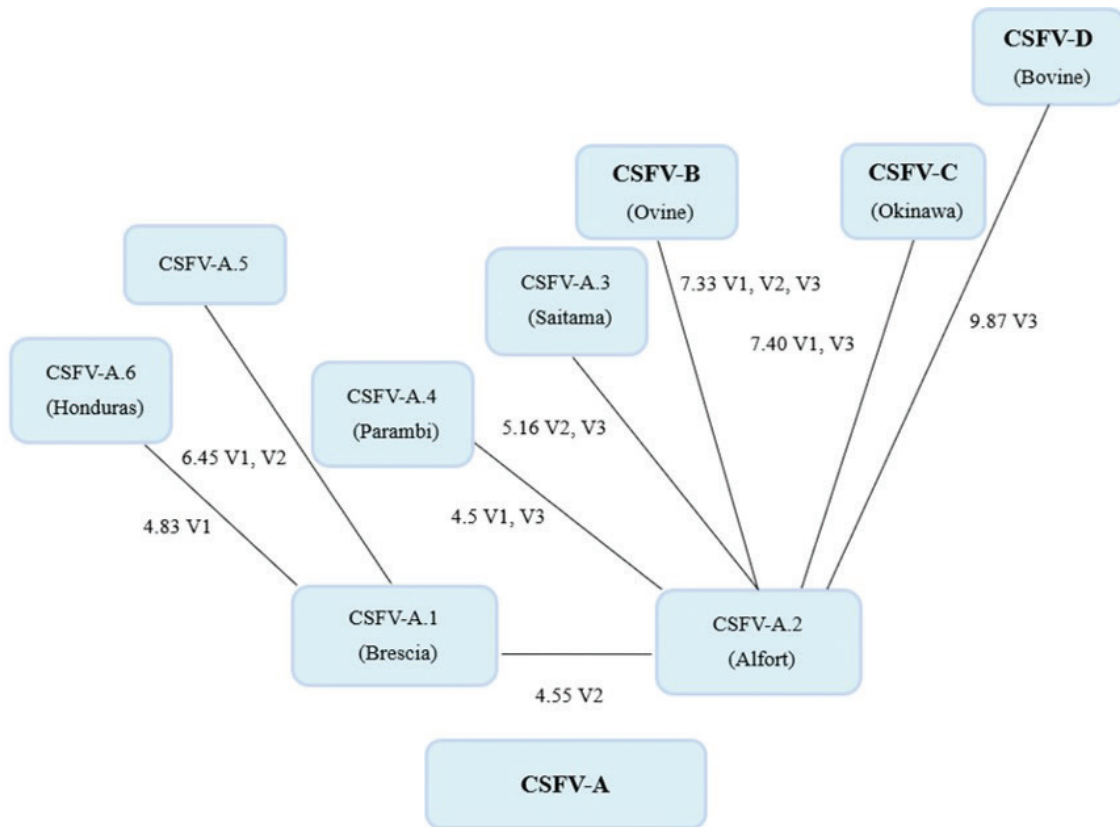


Fig. 3. Schematic phylogenetic tree based on nucleotide changes in the secondary structure of classical swine fever virus genotypes. Mean values of base-pair divergence and variable locus location indicate the evolutionary changes.

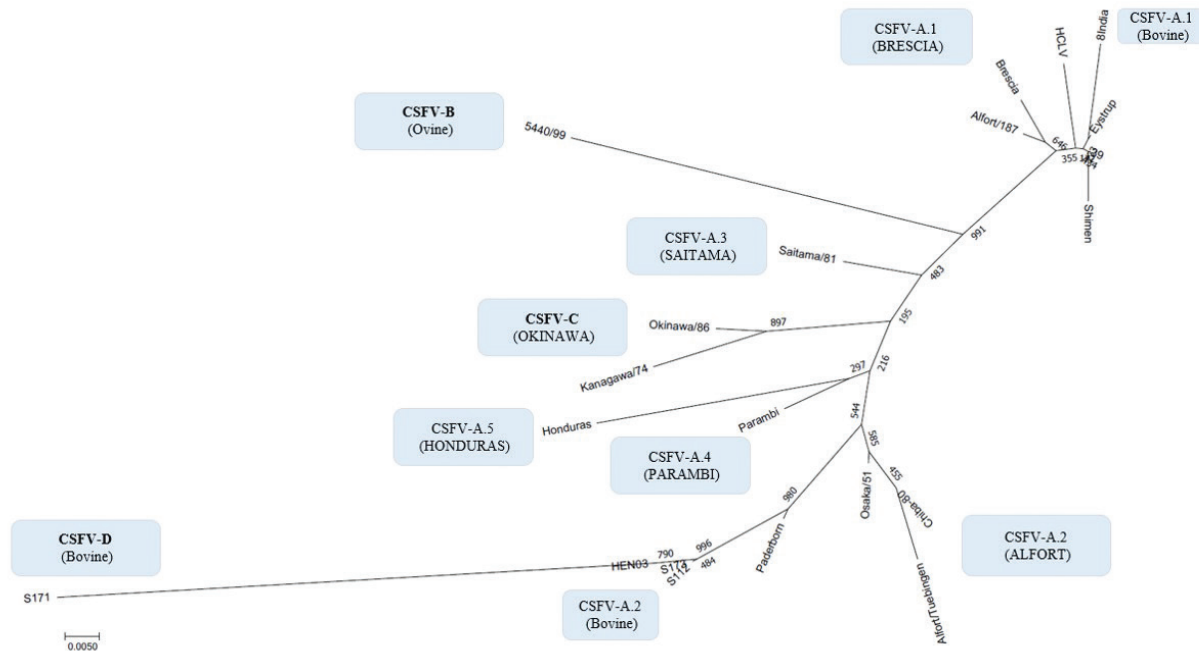


Fig. 4. Phylogenetic tree based on the 5'-UTR comparison, showing taxonomic relationships of the CSFV strains in the *Pestivirus* genus. Strains Brescia (M31768), 39 (AF407339), Eystrup (AF326963), Alfort/187 (X87939), HCLV (AF091507), Shimen (AF092448), and the bovine strain 8 India (MG813566) are references for the genotype CSFV-a variant 1. Strains Alfort/Tuebingen (J04358), Chiba-80 (AB019659), Osaka/51 (AB019174), Paderborn (AY072924), and the Chinese bovine strains S173 (KF006975), HEN03 (KC176778), and S112 (MK118725) are references for the genotype CSFV-a variant 2. Strain Saitama/81 (AB019178) is the reference for the CSFV-a variant 3, and strains Parambi (KT239105), IND/AS/GHY/G4 (KM362426), and Honduras (L42426) are the references for the CSFV-a variants 4, 5, and 6, respectively. The Spanish ovine strain 5440/99 (AY159514) is the reference for the genotype CSFV-b. The Japanese strains Kanagawa/74 (AB019166) and Okinawa/86 (AB019172) are references for the genotype CSFV-c. The Chinese bovine strain S171 (KF006974) represents the genotype CSFV-d. Distances were computed using Clustal X, version 1.8, using the neighbor-joining method. Scale bar indicates 10 nucleotide substitutions per 100 nucleotides. Nomenclature of identified genotypes is based on divergence in the genus.

animals and may be possibly responsible for the spread of pestiviruses, especially through immunotolerant persistently infected subjects. In Spain, the reported ovine strain 5440/99 showed the sequence similarities with CSFV vaccine strains used in pigs, suggesting spillovers from prophylactic campaigns (Hurtado *et al.*, 2003). In Belgium, some fetal calf sera positive for CSFV-like 5'-UTR sequences, experimentally not infecting or seroconverting pigs, have been detected during the 90s (Dr Koenen, Sciensano, Belgium, personal communication), suggesting circulation of unidentified virus strains with sequence similarity to CSFV within the 5'-UTR. However, in Belgium, since the last occurrence of CSFV was reported in 1997, thus, in this period, wild and vaccinal strains were present in the country, and it cannot be excluded from the occurrence of possible spillovers in cattle from CSFV-infected or immunized pigs.

The recent taxonomy of CSFV is based on the analysis of the E2 genomic region (Postel *et al.*, 2012). Due to the relation to envelope glycoprotein (the main immunogen of CSFV, thus essential for the development of

vaccines) (Perez *et al.*, 2012) and the longer size (1,119 nucleotides), the full-length E2 encoding sequences were recommended for accurate phylogenetic analyses, assessing the viral types and subtypes. Based on the primary sequence analysis, three CSFV genotypes, each with three or six subgroups, have been identified (Paton *et al.*, 2000; Dreier *et al.*, 2007; Postel *et al.*, 2013; Beer *et al.*, 2015; Silva *et al.*, 2017). However, despite a limiting factor for the differentiation of closely related isolates (Postel *et al.*, 2012), short-length target fragments in other regions of the viral genome as 5'-UTR or NS5B have been largely applied for genotyping purposes and molecular epidemiology (Lowings *et al.*, 1996; Paton *et al.*, 2000; Beer *et al.*, 2015). Phylogenetic analysis of the sequences based on a 190-nucleotide (nt) fragment of the E2 glycoprotein proved very useful for the genetic typing of isolates in the case of new outbreaks (Dreier *et al.*, 2007). In any case, laboratory testing international standards are indicated only by the World Organization for Animal Health (Office International des Epizooties - OIE). According to the Terrestrial Manual of the World Organization for Animal Health (Office International

des Epizooties—OIE) (Chapter 2.8.3 CSFV; Paragraph 1.1.5.) (OIE, 2014), as the E2 major glycoprotein gene (190 nucleotides), the fragment 5'-UTR of the CSFV genome (150 nucleotides), targeted for molecular characterization of the bovine sequences in the present study, is admitted too for genetic typing and molecular epidemiology of CSF, based on the comparison of genetic differences between virus isolates. According to the Terrestrial Code (Chapter 15.2; Article 15.2.28, Paragraph 3, Virological surveillance) (OIE, 2017) and the Terrestrial Manual (Chapter 2.8.3; Paragraph 1.1.4.) (OIE, 2014) of the World Organization for Animal Health, the RT-PCR amplification of CSFV RNA followed by nucleotide sequencing, applied for the obtention of the sequences evaluated in the present study (Zhang *et al.*, 2014; Giangaspero *et al.*, 2017), originally focused on investigations of BVDV, is admitted among recommended methods for CSFV agent detection, confirmation of clinical cases, and differentiation from other pestiviruses. It is clear that the sequence of the gene encoding the E2 glycoprotein is preferred in the recent taxonomy of the species (Postel *et al.*, 2012), but the consideration of 5'-UTR was the only possible approach to perform a comparative evaluation of CSFV with sequences of the other different species, otherwise not feasible taking into account that the majority of *Pestivirus*-deposited sequences are 5'-UTR (Yesilbag *et al.*, 2017; Giangaspero *et al.*, 2018). The secondary structure–sequence characterization allowed an exhaustive comparative evaluation with genomic variants in the genus, about 1,400 *Pestivirus* strain sequences. The particularity of the PNS method is the exclusive consideration of strategic genomic sequences corresponding to the 5'-UTR IRES, which is responsible for translational, transcriptional, and replicational events in pestiviruses. Thus, at this level, stable nucleotide variations assume a high importance in terms of virus evolutionary history. The PNS method has been successfully applied for the characterization of pestiviruses, including atypical species as Giraffe, Pronghorn, or Bungowannah (Harasawa *et al.*, 2000; Giangaspero and Harasawa, 2008; 2011), thus adequate for the scope of the present study. The presence of CSFV 5'-UTR sequences in bovines from India and China appeared atypical. However, another recent report from India (Chakraborty *et al.*, 2018) strongly corroborated the previous observations, amplifying also full-length E2 region of CSFV in two positive samples, and suggesting a potential emerging health risk in cattle. Furthermore, the possible occurrence of similar circulation in cattle in CSFV endemic environment in Kenya is currently under evaluation (Prof VanLeeuwen, University of Prince Edward Island, Canada, personal communication).

Conclusions

These preliminary observations are new and relevant in countries, where CSFV control and eradication strategies

are applied. Further investigations will be necessary to confirm adaptation and diffusion of the virus in cattle as well as provide a clear picture of genetic characteristics, in particular, to evaluate other genomic regions, E2, and eventually full-length genome, and apply other internationally recognized tests. Furthermore, it will be important to clarify the clinical suspicions and related virus differential diagnosis for correct notification obligations in compliance with the World Organization for Animal Health reference norms.

Conflict of interest

Authors declare that there is no conflict of interest.

Authors' contribution

M. Giangaspero and S. Zhang contributed equally to the present study.

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