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Phylogenetic Diversity of Classical Swine Fever Virus (CSFV) Field Isolates from Outbreaks in China Between 2008 and 2011

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ABSTRACT

Classical swine fever virus (CSFV) causes severe economic losses to the swine industry worldwide. To gain insight into the molecular epidemiology of classical swine fever in China, we analyzed a 190 bp N-terminal fragment of the E2 gene of 103 Chinese CSFV isolates. Clinical samples were collected between January 2008 and March 2011. CSFV was detected in 103 of these samples by RT-nested PCR and were selected for sequencing. Further analysis based on the E2 fragment sequences revealed that all of the Chinese isolates belonged to subgroups 1.1, 2.1 and 2.2. CSFV isolate of genogroup 3 was not found. The most significant observation between genetic and geographical distribution for the isolates in the study, especially for the subgroup 2.1 strains, was that they occupied the widest area since these viruses existed throughout mainland China. These results enhance our knowledge of the phylogenetic diversity of Chinese CSFV isolates and may contribute to the development of reliable diagnostic tests, epidemiological surveillance and effective strategies for disease control.

Key words: Classical swine fever virus, phylogenetic diversity, glycoprotein E2, genetic analysis, Chinese isolates

INTRODUCTION

Classical Swine Fever (CSF) is a highly contagious worldwide disease that has significant economic ramifications. CSF is a notifiable disease to the OIE and its eradication programs have been implemented in most countries (Paton and Greiser-Wilke, 2003). The causative agent of this disease is Classical swine fever virus (CSFV), a member of the *Pestivirus* genus within the Flaviviridae family (Becher *et al*., 2003). CSFV is a primary swine health concern causing significant economic losses to the pig industry worldwide. In particular, a mild, atypical form of the disease with a long duration, subclinical signs and relatively low morbidity rate has often been

observed since the late 1970s, even in a certain proportion of vaccinated pigs (Tu *et al*., 2001). The long-lasting chronic form, which has often been ignored, poses a threat for commercial trade with CSF-free countries.

The CSFV genome is a positive single-stranded RNA that is approximately 12.3 kb long. The genome consists of 5'- and 3'-untranslated regions flanking a single ORF that encodes four structural (core, E^{rns}, E1, E2) and eight non-structural (N^{pro}, p7, NS2-NS5B) proteins (Meyers *et al*., 1996). The molecular epidemiology (5'UTR, E2, NS5B) (Blacksell *et al*., 2004, 2005; Pereda *et al*., 2005; Sabogal *et al*., 2006; Patil *et al*., 2010) of various regions has been studied and despite minor identity differences, grouping of the isolates is essentially conserved with the most commonly analyzed regions (Vilcek *et al*., 1997; Paton *et al*., 2000). These studies have resulted in the classification of CSFV isolates into three groups (Paton *et al*., 2000; Deng *et al*., 2005). Partial CSFV sequencing from all over the world has enabled the delineation of global phylogenetic relatedness (Hofmann *et al*., 1994; Vilcek *et al*., 1996; Lowings *et al*., 1999; Widjojoatmodjo *et al*., 1999; Hurtado *et al*., 2003; Pereda *et al*., 2005; Sabogal *et al*., 2006; Patil *et al*., 2010, 2011).

The CSFV envelope glycoprotein E2 (gp55) is highly immunogenic and induces neutralizing antibodies (Paton *et al*., 1992; Van Rijn *et al*., 1994). Comparative sequence analyses of the E2 gene provide invaluable epidemiologically relevant information for tracing the possible outbreak origin and virus spread in the field, understanding and predicting viral evolution and identifying viral reservoirs. Furthermore, phylogenetic analyses based on the 190 nucleotides spanning the highly variable N-terminal half of the E2 protein have proved useful for discriminating among the genetic groups of different isolates (Paton *et al*., 2000). Several studies of E2 sequence comparisons of CSFV isolates from different epizootic areas in the world have been reported and the genetic relationships and molecular epidemiology of CSF outbreaks in different regions were established (Paton *et al*., 2000; Biagetti *et al*., 2001; Blacksell *et al*., 2005; Pereda *et al*., 2005; Sabogal *et al*., 2006; Cha *et al*., 2007; Blome *et al*., 2010).

CSF was recognized in the 1920s in China, where it remained endemic for nearly a century. Although no large epizootics have occurred in China for several decades, yearly CSF outbreaks that result in severe economic losses in the swine industry are still being recorded in different parts of the country. In addition, few domestic reports have described CSFV genogroups (Tu *et al*., 2001; Chen *et al*., 2008, 2010; Luo *et al*., 2011; Shen *et al*., 2011). In this study, the N-terminal fragment of the E2 glycoprotein gene sequences of 103 CSFV isolates in 15 provinces from 2008-2011 were sequenced and phylogenetic analysis was then performed in an effort to fully understand the extent of the genetic diversity of CSFV in China.

MATERIALS AND METHODS

Clinical specimens: From January 2008 to March 2011, clinical specimens, including tonsils, spleens, lymph nodes, livers, kidneys and blood serum, were collected from suspected CSF cases in geographically different swine herds of 15 provinces (Anhui, Beijing, Fujian, Guangxi, Hebei, Henan, Hubei, Hunan, Jiangsu, Jiangxi, Shandong, Shanghai, Tianjin, Xinjiang and Zhejiang) in China.

RNA extraction, RT-nested PCR (RT-nPCR): Tissue samples were homogenized. Total RNA was extracted using TRIzoL Reagent (Invitrogen, Inc.) according to the manufacturer's instructions, dissolved in nuclease-free water and kept at -70°C until further use.

The reverse transcription reaction mix contained the following components: 10 μL of total RNA, 4 μL of 5×RT buffer, 4 μL 0.25 mM dNTPs, 1 μL 10 pmol of primer SR2898 and 1 μL Reverse Transcriptase XL (Takara, Inc.). The reaction mixtures were incubated at 42°C for 1 h and kept on ice for 2 min.

A set of previously designed primers Sabogal *et al.* (2006) was used to amplify a 671-nt fragment of the E2 gene of CSFV [SF2228: 5'AGR CCA GAC TTG CCN TAY GA 3' (2228-2250 nt) and SR2898: 5'TTY ACC ACT TCT GTT CTC A 3' (2898-2880 nt)]. The internal primers par selected defined of a 271 nt of the E2 gene and were used to analyze 190 bp as previously described by Lowings *et al.* (1996) [SF2477: 5'TCR WCA ACC AAY GAG ATA GGG 3' (2477-2497 nt) and SR2848: 5'CAC AGY CCR AAY CCR AAG TCA TC 3' (2748-2726 nt)].

In vitro amplification of the gene was carried out as follows: 2 μL RT products, 12.5 μL 2H PCR *Taq*mix (Tiangen, Inc.) and 0.5 μL 10 pmol of each SF2228 and SR2898 primer adjusted to a final volume of 25 μL with double-distilled water. Cycling conditions included initial denaturation at 94°C for 3 min, followed by 35 cycles at 94°C for 45 sec, 55°C for 1 min and 72°C for 1 min. The final elongation step was at 72°C for 5 min. The nPCR was the same except for use of primers SF2477 and SR2848. All of the nPCR products were analyzed in 2% agarose gel stained with ethidium bromide.

Cloning and sequencing: For sequencing, the PCR products were purified using a QIAquick PCR Purification Kit (Qiagen, Inc.) and cloned in a pGEM-T vector (Promega, Inc.). Positive recombination plasmids (pGEM-T-E2) were sequenced by Invitrogen using SP6 or T7 prime.

The 103 sequences reported in this paper were deposited in GenBank under the accession numbers JN009106, JN009107 and JF908818-JF908918.

Phylogenetic analysis and homology analysis: All of the gene sequence data were edited and compiled using the Lasergene sequence analysis software package (DNASTAR, Inc.). Multiple sequence alignment was performed using the CLUSTAL W Program. The unrooted phylogenetic tree was generated by the distance-based neighbor-joining method using MEGA software version 5.05 (http://www.megasoftware.net/). Bootstrap values were calculated on 1000 replicates of the alignment. The corresponding amino acid sequences were aligned using Lasergene Megalign software with those of C and Shimen strains.

RESULTS

Detection of CSFV in clinical samples: In the present study, 103 clinical samples were first detected as positive by RT-nPCR and then selected for further sequencing analysis (Table 1). These clinical samples were collected in 15 provinces covering approximately 3.5 milhon square km, which equates to 36% of Chinese territory (Fig. 1).

Homology analysis of the sequences: The 103 gene fragments of the CSFV isolates were sequenced and their homologies were determined by comparison with sequences available in GenBank (Table 2). The accession numbers of our isolates are shown in Table 1. The shared identities among the 103 Chinese isolates in all the three subgroups were 77.9-100% and 79.4-100% for the nucleotide sequences and the amino acid sequences, respectively. These isolates had 77.4-99.5% nucleotide sequence identity and 81.0-100% amino acid sequence identity with C-strain. All of our Chinese isolates demonstrated 78.4-95.3% nucleotide sequence identity and 79.4-93.7% amino acid sequence identity with the reference Shimen strain.

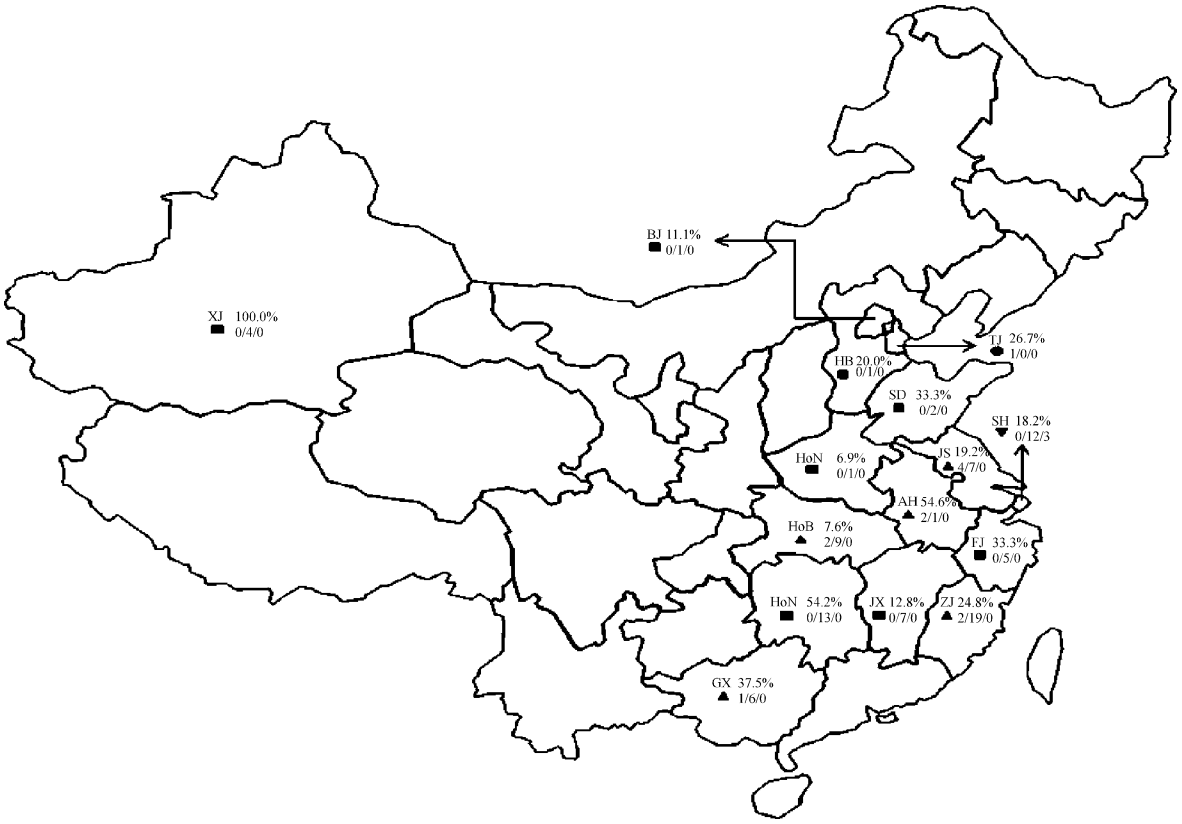

Fig. 1: Geographic distribution of Classical swine fever virus (CSFV) subgroups in China in 2008-2011, percentages indicate the CSFV positivity rate of the province, ●: Infected by subgroup 1.1 CSFV only, ■: Infected by subgroup 2.1 CSFV only, ▲: Multi-infected by subgroups 1.1 and 2.1 CSFV, ▼: Multi-infected by subgroups 2.1 and 2.2 CSFV, x/y/z: Number of subgroup 1.1, 2.1 and 2.2 CSFV in the province, respectively

Table 1: Origin of the CSFV isolates

No.	Isolate	Province	Year	Subgroup	Accession No.	No.	Isolate	Province	Year	Subgroup	Accession No.
1	GX0889	Guangxi	2008	2.1	JF908909	53	ZJ0924	Zhejiang	2009	2.1	JF908828
2	GX08102	Guangxi	2008	2.1	JF908904	54	ZJ0926	Zhejiang	2009	2.1	JF908827
3	GX08140	Guangxi	2008	2.1	JF908903	55	ZJ0927	Zhejiang	2009	2.1	JF908826
4	HuN0803	Hunan	2008	2.1	JF908890	56	ZJ0928	Zhejiang	2009	2.1	JF908825
5	HuN0805	Hunan	2008	2.1	JF908889	57	ZJ0929	Zhejiang	2009	2.1	JF908824
6	HuN0816	Hunan	2008	2.1	JF908888	58	ZJ0930	Zhejiang	2009	2.1	JF908823
7	HuN0819	Hunan	2008	2.1	JF908887	59	ZJ0933	Zhejiang	2009	2.1	JF908822
8	HuN0822	Hunan	2008	2.1	JF908886	60	ZJ0934	Zhejiang	2009	2.1	JF908821
9	HuN0825	Hunan	2008	2.1	JF908885	61	ZJ0935	Zhejiang	2009	2.1	JF908820
10	HuN0833	Hunan	2008	2.1	JF908884	62	ZJ0936	Zhejiang	2009	2.1	JF908819
11	HuN0835	Hunan	2008	2.1	JF908883	63	ZJ0958	Zhejiang	2009	2.1	JF908818
12	HuN0836	Hunan	2008	2.1	JF908882	64	ZJ0959	Zhejiang	2009	2.1	JF908843
13	HuN0837	Hunan	2008	2.1	JF908881	65	AH1001	Anhui	2010	1.1	JF908916

Table 1: Continue

No.	Isolate	Province	Year	Subgroup	Accession No.	No.	Isolate	Province	Year	Subgroup	Accession No.
14	HuN0838	Hunan	2008	2.1	JF908880	66	AH1004	Anhui	2010	1.1	JF908915
15	HuN0839	Hunan	2008	2.1	JF908879	67	FJ1001	Fujian	2010	2.1	JF908914
16	HuN0840	Hunan	2008	2.1	JF908918	68	FJ1002	Fujian	2010	2.1	JF908913
17	SD0805	Shandong	2008	2.1	JF908859	69	FJ1003	Fujian	2010	2.1	JF908912
18	SH0802	Shanghai	2008	2.2	JF908857	70	FJ1004	Fujian	2010	2.1	JF908911
19	SH0804	Shanghai	2008	2.2	JF908856	71	FJ1005	Fujian	2010	2.1	JF908910
20	SH0805	Shanghai	2008	2.2	JF908855	72	GX1001	Guangxi	2010	2.1	JF908908
21	SH0806	Shanghai	2008	2.1	JF908854	73	GX1002	Guangxi	2010	2.1	JF908907
22	SH0807	Shanghai	2008	2.1	JF908853	74	GX1003	Guangxi	2010	2.1	JF908906
23	SH0808	Shanghai	2008	2.1	JF908852	75	GX1004	Guangxi	2010	1.1	JF908905
24	SH08103	Shanghai	2008	2.1	JF908849	76	HuB1024	Hubei	2010	2.1	JF908901
25	SH08104	Shanghai	2008	2.1	JF908848	77	HuB1031	Hubei	2010	2.1	JF908900
26	SH08105	Shanghai	2008	2.1	JF908847	78	HuB1032	Hubei	2010	2.1	JF908899
27	SH08112	Shanghai	2008	2.1	JF908846	79	HuB1034	Hubei	2010	2.1	JF908898
28	SH08134	Shanghai	2008	2.1	JF908845	80	HuB1035	Hubei	2010	1.1	JF908897
29	SH08136	Shanghai	2008	2.1	JF908844	81	HuB1037	Hubei	2010	2.1	JF908896
30	SH08137	Shanghai	2008	2.1	JF908878	82	HuB1039	Hubei	2010	2.1	JF908895
31	ZJ0801	Zhejiang	2008	2.1	JF908835	83	HuB1044	Hubei	2010	2.1	JF908894
32	AH0921	Anhui	2009	2.1	JF908917	84	HuB10104	Hubei	2010	2.1	JF908892
33	BJ0904	Beijing	2009	2.1	JN009106	85	HuB10121	Hubei	2010	2.1	JF908891
34	HB0901	Hebei	2009	2.1	JF908902	86	JS1018	Jiangsu	2010	1.1	JF908877
35	HeN0903	Henan	2009	2.1	JN009107	87	JS1094	Jiangsu	2010	2.1	JF908876
36	JX0901	Jiangxi	2009	2.1	JF908866	88	JS1096	Jiangsu	2010	2.1	JF908875
37	JX0905	Jiangxi	2009	2.1	JF908865	89	JS1097	Jiangsu	2010	2.1	JF908874
38	JX0909	Jiangxi	2009	2.1	JF908864	90	JS1098	Jiangsu	2010	2.1	JF908873
39	JX0922	Jiangxi	2009	2.1	JF908863	91	JS1099	Jiangsu	2010	2.1	JF908872
40	JX0926	Jiangxi	2009	2.1	JF908862	92	JS10102	Jiangsu	2010	1.1	JF908871
41	JX0933	Jiangxi	2009	2.1	JF908861	93	JS10103	Jiangsu	2010	2.1	JF908870
42	JX0946	Jiangxi	2009	2.1	JF908860	94	JS10106	Jiangsu	2010	2.1	JF908869
43	XJ0901	Xinjiang	2009	2.1	JF908839	95	JS10107	Jiangsu	2010	1.1	JF908868
44	XJ0902	Xinjiang	2009	2.1	JF908838	96	JS10113	Jiangsu	2010	1.1	JF908867
45	XJ0903	Xinjiang	2009	2.1	JF908837	97	SD1017	Shandong	2010	2.1	JF908858
46	XJ0904	Xinjiang	2009	2.1	JF908836	98	SH1031	Shanghai	2010	2.1	JF908851
47	ZJ0912	Zhejiang	2009	2.1	JF908834	99	SH1056	Shanghai	2010	2.1	JF908850
48	ZJ0913	Zhejiang	2009	2.1	JF908833	100	ZJ1001	Zhejiang	2010	1.1	JF908842
49	ZJ0914	Zhejiang	2009	2.1	JF908832	101	ZJ1014	Zhejiang	2010	1.1	JF908841
50	ZJ0915	Zhejiang	2009	2.1	JF908831	102	HuB1141	Hubei	2011	1.1	JF908893
51	ZJ0916	Zhejiang	2009	2.1	JF908830	103	TJ1129	Tianjin	2011	1.1	JF908840
52	ZJ0921	Zhejiang	2009	2.1	JF908829						

The 103 CSFV isolates obtained from field outbreaks in China during 2008-2011 in this study

Table 2: Reference CSFV sequences

GenBank accession No.	Isolate	Country/region	Year	Subgroup
AJ781101	09/Baco	Brazil	1995	1.1
AJ781103	1121	Mexico	1991	1.1
D49532	ALD	Japan	na	1.1
U90951	Alfort A19	France	na	1.1

Table 2: Continue

GenBank accession No.	Isolate	Country/region	Year	Subgroup
X87939	Alfort/187	France	1987	1.1
AY382481	C-strain	China	na	1.1
X96550	CAP	France	na	1.1
AJ781109	Casilda	Argentina	1978	1.1
AF333000	cF114	China	2001	1.1
AY535801	Col814-03	Colombia	2003	1.1
AF099102	CS	Russia	na	1.1
AF326963	Eystrup	Germany	1964	1.1
EU915211	flc-LOM	Korea	na	1.1
U45478	Glentorf	Germany	1968	1.1
D49533	GPE-	Japan	na	1.1
EU857642	HCLV-India	India	na	1.1
EU497410	JL 1 (06)	China	2006	1.1
AF352565	LPC	Taiwan	na	1.1
U45477	Riems	Germany	na	1.1
AY578688	RUCSFPLUM	USA	2001	1.1
AF092448	Shimen	China	1945	1.1
DQ127910	SWH	China	2004	1.1
EU490425	Thiverval	France	1978	1.1
AY422081	Weybridge	Australia	1954	1.1
AJ781111	39-Cuba	Cuba	na	1.2
AF091661	Brescia	Italy	1945	1.2
AY578687	BRESCIAX	USA	na	1.2
AJ704817	Margarita	Cuba	1958	1.2
AY571083	03/TN/01/TWN	Taiwan	2001	2.1
AY568569	0406/CH/01/TWN	Taiwan	2001	2.1
AY554397	96TD	Taiwan	1996	2.1
AF143091	GS-LT	China	1999	2.1
AY367767	GXWZ02	China	2003	2.1
AY027672	Italy	Italy	na	2.1
AY283667	L119	Lao PDR	1998	2.1
AY072924	Paderboru	Germany	1996	2.1
AF407339	39-China	China	2001	2.2
AY283658	L175	Lao PDR	1998	2.2
AJ312876	PR/98/dp	Italy	1998	2.2
J04358	Alfort/Tuebingen	Germany	na	2.3
L36169	n5W	Italy	1991	2.3
AJ312857	OR/98/dp	Italy	1998	2.3
FJ265020	Sp01	Spain	2001	2.3
AF521708	JJ9811	Korea	1998	3.2
AF521710	YI9908	Korea	1999	3.2
AF241628	CBR93	Thailand	1993	3.3
AF241635	NKP95/5	Thailand	1995	3.3

Virus sources and subgroups information for reference CSFV used in this study, na: Not available

Within subgroup 2.1, the percent identity among the 88 isolates was 91.6-100% for the nucleotide sequences and 84.1-100% for the amino acid sequences. GXWZ02, which was reported to be in subgroup 2.1 by (Pan *et al*., 2005), was considered closely related to most of the Chinese

isolates. The Chinese isolates in our study demonstrated 93.7-95.8% nucleotide sequence identity and 90.5-96.8% amino acid sequence identity with the reference strain GXWZ02, which was also isolated from China (Pan *et al*., 2005).

Phylogenetic analysis: To assess the relationships between isolates in China and those in other parts of Asia, Europe and South and Central America, phylogenetic analysis was performed based on the 103 gene sequences and other representative sequences. As shown in Fig. 2, these 103 strains belong to group 1 and 2 and are classified to subgroups 1.1, 2.1 and 2.2. CSFV was not found in group 3.

Twelve isolates (11.7% of the total) belonged to subgroup 1.1 along with some Chinese original strains, such as C-strain, Shimen and some CSFV reference strains from Europe, North and South

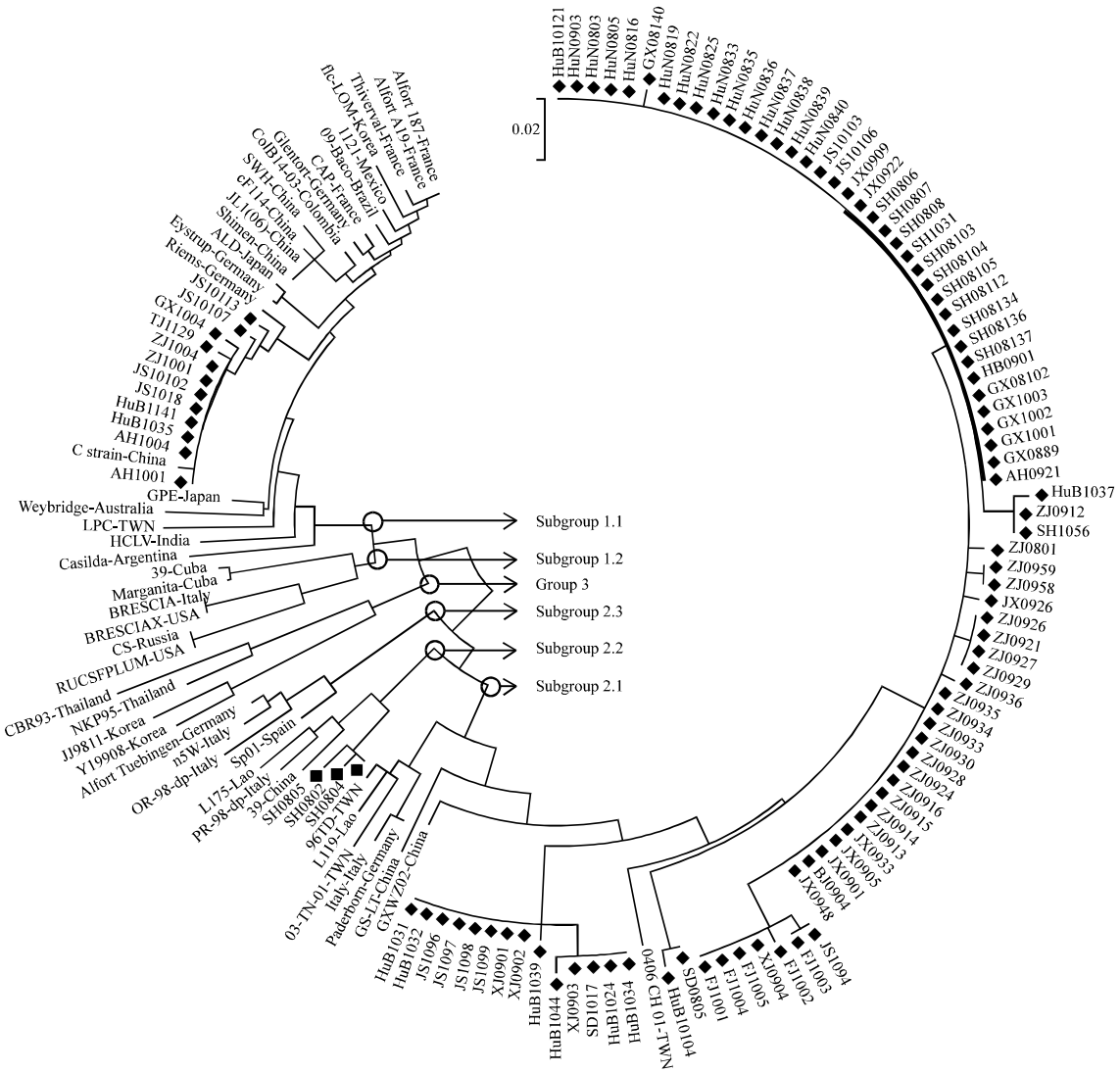

Fig. 2: Phylogenetic analysis of Chinese Classical swine fever viruses (CSFV) and CSFV isolated in other countries based on the 190 bp sequence of the E2 glycoprotein gene, the 103 isolates obtained in this study are marked with filled diamonds

America, Oceania and other regions of Asia such as Riems (Germany), Alfort/187 (France), 1121 (Mexico), Col814-03 (Columbia), 09-Baco (Brazil), Casilda (Argentina), Weybridge (Australia), flc-LOM (Korea), GPE- (Japan) and LPC (Taiwan).

Ninety-one isolates (88.3% of the total) belonged to group 2 and were further divided into two subgroups. Eighty-eight isolates (85.4% of the total) were classified as subgroup 2.1, along with the reference strains GXWZ02 (China), Italy (Italy), Paderborn (Germany), 96TD (Taiwan) and L119 (Lao). Only 3 isolates (2.9% of the total) fell into subgroup 2.2, along with 39 (China), L175 (Lao) and PR-98-dp (Italy).

Taken together, the phylogenetic analysis results revealed that three (1.1, 2.1 and 2.2) CSFV subgroups were involved in the CSFV epizootics in China from 2008-2011, which indicated that CSFV surveillance should be given high priority, especially that of the predominant subgroup 2.1.

Amino acid analysis: To determine the amino acid difference among all three subgroup isolates, the related E2 amino acid sequences of our 103 representative CSFV isolates were aligned with C-strain and the Shimen strain (Fig. 3). Present investigation showed that glycoprotein E2 of the CSFV isolates from China shows conspicuous genetic diversity. To differentiate these isolates among different genetic groups, the specific or concurrent amino acid substitutions were observed. Three specific substitutions were found in the CSFV isolates of group 2, including $V^{49}\rightarrow T^{49}$, $K^{72}\rightarrow R^{72}$ and

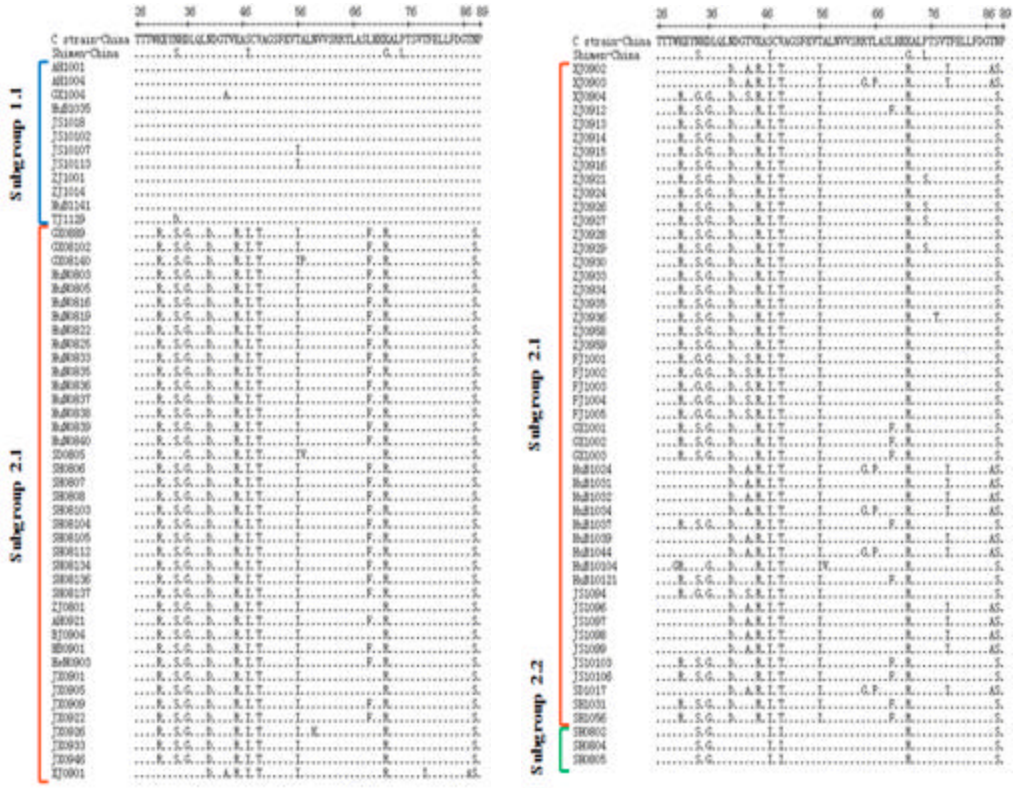

Fig. 3: Alignment of the deduced amino acid sequences of the *E2* gene of 103 Chinese isolates with two Chinese reference strains (C-strain and Shimen strain). The sequences are clustered by subgroup. Dots indicate the same amino acids as in C-strain, while substitutions are indicated by the amino acid letter codes

N^{88}→S^{88}. Two specific substitutions were found in subgroup 2.1: N^{40}→D^{40} and K^{45}→R^{45}. The concurrent amino acid substitutions T^{43}→A^{43} and T^{56}→I^{56} were also found to contain heterogenetic mutations between groups 1 and 2.

DISCUSSION

Determination and comparison of nucleotide sequences of different regions of the CSFV genome can be used to evaluate the relationships among different isolates in epidemiological studies. In this study, the genetic diversity of CSFV E2 in China was analyzed based on the same primers that were used in previous phylogenetic analysis (Lowings *et al.*, 1996). Therefore, the results obtained here may be directly compared to previously published data and contribute to the big picture of the CSFV phylogenetic tree.

Represented by reference strain Brescia from Italy, group 1 previously comprised isolates from Europe, North America, South and Central America, Oceania and Asia. Twelve of our isolates belonged to group 1 and showed 91.1-92.1% nucleotide sequence identity and 88.9-90.5% amino acid sequence identity with the Brescia strain. In contrast, these 12 isolates had 98.4-99.5% nucleotide sequence identity and 98.4-100% amino acid sequence identity with the old Chinese domestic isolate C-strain.

There were two standard CSFV reference strains used in China: Shimen, a virulent strain isolated in 1945 and Hog Cholera Lapinized Virus (HCLV), an attenuated vaccine strain (Tu *et al.*, 2001). The HCLV, referred to as C-strain, was considered one of the safest and most effective live vaccines (Moormann *et al.*, 1996) and was genetically stable (Moormann *et al.*, 1996; Chenut *et al.*, 1999). Both C-strain and the Shimen strain were used as references and fell into group 1.1 with the 12 isolates. Interestingly, the group 1 strains in our research were all isolated after 2010. We did not find a group 1 strain in 2008-2009, although, isolates in these two years comprised 62.1% of the 103 strains. However, CSFV in group 1 comprises 30.8% of the isolates in 2010-2011, which is obviously higher than that of 2008-2009. This finding suggests the possible risk of group 1 CSFV re-emergence.

Prior to this study, most of the Chinese CSFV isolates belonged to group 2. Our data indicate that these 103 isolates included members of subgroups 2.1 and 2.2. The most significant correlation between the genetic and geographical distribution of the isolates, especially for the subgroup 2.1 strains, was that they occupy the widest area since these viruses existed throughout mainland China. Although, a relationship between temporal and genetic distance was not found for this subgroup, the CSFV in subgroup 2.1 has spread to almost all of the pig-producing provinces of China for more than a decade. Homologous analysis of the above revealed that the subgroup 2.1 strains might have a different origin. Our data also indicate that the subgroup 2.1 CSFV remains predominant in China. Interestingly, a similar tendency was described by other reports in the CSFV endemic areas, where the currently dominating group 2 has replaced the historical groups. Pan *et al.* (2005) and Deng *et al.* (2005) observed a dramatic switch from subgroup 3.4 to subgroup 2.1 in Taiwan and there has been a switch from subgroup 3.2 to subgroup 2.1 in Korea Cha *et al.* (2007). A switch from group 1 to group 2 has also been reported in Europe (Paton *et al.*, 2000). It is worth mentioning that even in the mid-East, CSF re-emerged in Israel in February 2009 after a 62-year absence. This outbreak occurred on a domestic pig farm in northern Israel and affected domestic pigs and wild boars (David *et al.*, 2011). Phylogenetic characterization indicated that the Israeli CSFV strain belonged to genotype 2.1 and exhibited the highest genetic homology to a Chinese CSFV strain, which may suggest a common origin of these two strains (David *et al.*, 2011).

Only three isolates (SH0802, SH0804 and SH0805) obtained in 2008 were classified into subgroup 2.2. The isolates demonstrated 95.3-95.8% nucleotide sequence identity and 98.4% amino acid sequence identity with the 39-China strain. He *et al.* (2007) reported that 39-China is a naturally homologous recombinant isolate and identified its two putative parental-like strains Shimen (subgroup 1.1) and GXWZ02 (subgroup 2.1), suggesting that the exchange of different genomic regions by recombination is an important mechanism in the evolution of CSFV. This kind of change offers the possibility that high-virulence CSFV could involve into low-virulence CSFV and indicates that subgroup 2.2 should be given significant focus in the evaluation of CSFV evolution in China. Furthermore, multi-infection by different CSFV genogroups in the same herd was found in some herds-such as G×1001 (subgroup 2.1) multi-infected with G×1004 (subgroup 1.1), HuB1031 (subgroup 2.1) multi-infected with Hu-1035 (subgroup 1.1) and JS10103 (subgroup 2.1) multi-infected with JS10102 (subgroup 1.1)-and is an essential step in recombination, which suggests that the evolving risk remains.

Subgroup 2.3 in China was first reported by Tu *et al.* (2001). Nine viruses in subgroup 2.3 were restricted to South China, including Guangdong, Hainan, Guangxi, Fujian, Hubei and Hunan, which comprised 8.2% of their isolates. The isolation dates of these nine viruses were 1986-1999. However, this subgroup has not been reported in the studies performed in China after 2001 (Chen *et al.*, 2008, 2010; Luo *et al.*, 2011; Shen *et al.*, 2011), including this study. It might be a hint that subgroup 2.3 has been gradually declining in the evolution of CSFV in China.

An earlier study indicated that amino acid site mutations at positions 16, 21, 24, 40 and 45 of E2 could induce immune escape (Van Rijn *et al.*, 1993). The amino acid sites at positions 40 and 45 were investigated in our research. For C-strain, these two sites referred to above are N and K. When the 103 E2 sequences were compared with C-strain, specific changes in these two sites were found in all subgroup 2.1 isolates: $N^{40}\rightarrow D^{40}$ and $K^{45}\rightarrow R^{45}$. If these mutations in the E2 region occurred in vivo, the subgroup 2.1 isolates could at least relate to immune escape and escape from the neutralizing antibodies. This might be one of the possible mechanisms of the mild CSF that circulates in China. Whether mutation of the 40 and 45 amino acid positions affects induction of the protective immune response in swine as well as the viral virulence or pathogenesis requires further investigation.

In summary, the phylogenetic analysis conducted in this study showed that the recent Chinese CSFV could be clustered into three subgroups. The majority of the Chinese isolates belonged to subgroup 2.1, but the isolates of subgroups 1.1 and 2.2 were still involved in CSFV outbreaks in the field. In any case, our results provide valuable data that contribute to the understanding of the molecular diversity of CSFV strains circulating in China that can be of great significance in the development of diagnostic tests, epidemiological surveillance and effective disease control strategies.

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