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Characterisation of newly emerged isolates of classical swine fever virus in China, 2014–2015

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Abstract

Introduction: In 2014–2015, the epidemic of classical swine fever (CSF) occurred in many large-scale pig farms in different provinces of China, and a subgenotype 2.1d of CSF virus (CSFV) was newly identified. **Material and Methods:** The phylogenetic relationship, genetic diversity, and epidemic status of the 2014–2015 CSFV isolates, 18 new CSFV isolates collected in 2015, and 43 other strains isolated in 2014–2015 were fully analysed, together with 163 CSFV reference isolates. **Results:** Fifty-two 2014–2015 isolates belonged to subgenotype 2.1d and nine other isolates belonged to subgenotype 2.1b. The two subgenotype isolates showed unique molecular characteristics. Furthermore, the 2.1d isolates were found to possibly diverge from 2.1b isolates. **Conclusion:** This study suggests that the Chinese CSFVs will remain pandemic.

Keywords: swine**,** classical swine fever virus, evolution, subgenotype 2.1d, China.

Introduction

Classical swine fever (CSF), a highly contagious and often fatal disease of pigs, is listed by the Office International des Epizooties (OIE) and remains a significant economic problem of swine industry in numerous regions of the world (5, 18). The disease is caused by CSF virus (CSFV), a member of the *Pestivirus* genus within the *Flaviviridae* family. The genus also includes bovine viral diarrheoa virus types I and II (BVDV I and II) and border disease virus (BDV) (17). Pigs can be infected by other pestiviruses, but these viruses usually do not spread efficiently outside their typical ruminant hosts (20).

CSFV is a single positive-stranded, enveloped RNA virus. The genome is approximately 12.3 kb in length, comprising a single, long open reading frame (ORF) that encodes four structural proteins (C, E^{rns}, E1, and E2) and eight non-structural proteins (N^{pro}, P7, NS2, NS3, NS4A, NS4B, NS5A, and NS5B), flanked by two non-coding regions at the 5 untranslated region (5 UTR) and 3 UTR (16).

Based on the phylogenetic analysis of the sequences of 5 UTR, E2 envelope glycoprotein gene, and NS5B polymerase gene CSFVs were divided into three genotypes (1, 2, and 3) and 10 subgenotypes (1.1–1.3, 2.1–2.3, and 3.1–3.4) (20). Subgenotype 2.1 isolates were further classified into 2.1a and 2.1b and were reported to be epidemic in many countries (4). Recently, subgenotypes 2.1c and 1.4 were reported (10, 23). Four subgenotypes (1.1, 2.1, 2.2, and 2.3) of CSFV isolates have existed in mainland China and contributed to CSFV outbreaks (24, 27). Among these isolates, subgenotype 2.1 isolates, especially 2.1b, have long been the predominant strains (2, 24, 27).

In 2014–2015, CSF outbreaks appeared in many regions of China, especially in Shandong province. A subgenotype 2.1d was newly identified by our laboratory (30). Then other researchers reported similar isolates (8, 15). In the present study, 18 newly emerged

CSFV isolates collected in 2015 are reported for the first time and the phylogenetic relationship, genetic diversity, and epidemic status of all new CSFV isolates collected in 2014–2015 have been fully analysed.

Material and Methods

Sample collection. From August 2014 to July 2015, more than 50 clinical samples were collected, including lungs, spleen, kidneys, and serum samples from suspected CSFV-infected pigs on different large-scale pig farms distributed over six provinces (Shandong, Jilin, Heilongjiang, Jiangsu, Hebei, and Inner Mongolia). The samples were homogenised in Dulbecco's modified Eagle's medium (DMEM, Gibco, USA) using a TissuLyser II (Qiagen, Germany) for RNA extraction.

Genome sequencing. CSFV-positive samples were selected for E2 gene sequencing. The primers used to amplify the E2 gene (E2-forward: GTAAATATGTGTGTGTTAGACCAGA, E2-reverse: GTGTGGGTAATTGAGTTCCCTATCA), methods of RNA extraction, RT-PCR, and genome sequencing were described previously (30).

Phylogenetic analysis. Based on the E2 full-length sequences of 61 new CSFV isolates collected in 2014–2015 and 163 reference CSFV isolates worldwide, phylogenetic trees were constructed using the neighbour-joining method with 1000 bootstraps in MEGA 6.06 software (26). MUSCLE in MEGA software generated multiple sequence alignments (25).

Nucleotide (nt) and amino acid (aa) analysis of E2. The nt and aa sequence homologies between the 61 new CSFV isolates and 11 representative CSFV isolates, including Shimen (1.1), Brescia90 (1.2), CSF0306 (1.3), CSF0705 (1.4), Paderborn (2.1a), HEBZ (2.1b), GDPY.2008 (2.1c), Zj0801 (2.1d), LAL-290 (2.2), Alfort (2.3), and P97 (3.4), were assessed using the Clustal W method of Lasergene (Version 7.1) (DNASTAR Inc., USA). To explore the genetic variation characteristic of the new isolates, the aa sequences of E2 of 38 new 2014-2015 CSFV isolates were fully analysed, together with 27 representative CSFV isolates from China and other countries.

Results

Detection of suspected CSFV samples. More than 40 samples collected from 25 different large-scale pig farms between August 2014 and July 2015 were identified as CSFV-positive by RT-PCR. The E2 genes of 36 positive samples were sequenced. In total, 18 strains isolated in 2014 were reported previously (30). The other 18 strains isolated in 2015 were reported for the first time. In addition, the E2 gene sequences of other 25 strains isolated in 2014–2015 were obtained from NCBI. The detailed information on these isolates was shown in Table 1.

Phylogenetic analysis of 2014-2015 isolates. A total of 224 full-length E2 gene sequences, including 61 isolates identified in 2014–2015, formed the phylogenetic tree (Fig. 1). CSFV isolates were divided into genotypes 1, 2, and 3. Genotype 1 and 2 isolates were further divided into subgenotypes 1.1–1.4 and 2.1–2.3, respectively. In addition, subgenotype 2.1 isolates were further subdivided into 2.1a, 2.1b, 2.1c, and a new group 2.1d.

Of 61 isolates identified between 2014 and 2015, 52 isolates (JLMC1409, HLJTB1409, SDWH11409, SDWH21409, SDWH31409, HLJJY1409, HLJLYG1410, SDWH(F)1410, SDLS(C)1410, SDSG(A)1410, SDSG(B)1410, SDJN(D)1410, SDJN(E)1412, HLJSH1412, JSZL, SDJNa-14, SDLY-14, NMG2015, HB150309, SDLK150320, SDQZ150319, JL150418, NK150425, SDQZ150414, SDZC150416, HB150528, SDZC150514, SDZC150601, HLJSH150609, HLJSH150702, HLJHEB150710, SDJNi2-15, SDJNi3-15, SDLW2-15, SDZB2-15, SDJNi1-15, SDJNi4-15, SDJNi5-15, SDLW1-15, SDLY-15, SDMZ1-15, SDTA2-15, SDTA3-15, SDWK-15, HeN1505) belong to subgenotype 2.1d. The other nine isolates (SDQU1408, SDLW1410, SDZC1411, JL2015, SDZC150526, SDHS9150129, SDHS10150129, SD19-15, SDJNi6-15) belong to subgenotype 2.1b.

The subgenotype 2.1d also include 16 previously reported isolates: SX-04, HuZ2-05, SH2-05, ZJ7.2005, ZS1-08, Zj0801, SDQS11, GD176/2011, GD45/2011, 1-19/HeB-2011, 1-5/HeN-2011, 2-41/HeB-2011, 2-55/HeB-2011, 2-31/HeN-2011, 1-21/HeB-2012 and SDTA1-13. The detailed information on these isolates is shown in Table 2.

Nt and aa analysis of E2 of 2014–2015 isolates. The E2 gene is 1119 nt long and encodes 373 aas. Compared with genotype 2 isolates, including Paderborn (2.1a), HEBZ (2.1b), GDPY.20-08 (2.1c), Zj0801 (2.1d), LAL-290 (2.2) and Alfort (2.3), the nine new 2.1b isolates had 85.8%–94.9% nt and 90.1%–97.3% aa identity, and the 52 new 2.1d isolates showed 86.9%–97.3% nt and 90.6%–98.4% aa identity, which were higher than with genotype 1 isolates of Shimen (1.1), Brescia90 (1.2), CSF0306 (1.3), CSF0705 (1.4), or subgenotype 3.4 isolate of P97. Furthermore, the nine new 2.1b isolates had greater similarity to subgenotype 2.1b isolate of HEBZ than to other genotype 2 isolates of Paderborn (2.1a), GDPY.20-08 (2.1c), or Zj0801 (2.1d). Similarly, the 52 new 2.1d isolates had greater similarity to subgenotype 2.1d isolate of Zj0801 than to other genotype 2 isolates of Paderborn (2.1a), HEBZ (2.1b), or GDPY.20-08 (2.1c). The detailed results are shown in Table 3.

Table 1. Characteristics of new CSFV isolates collected in 2014–2015

No.	Virus strain	Collection date	Collection area	Accession no.
1	SDJNa-14 [c]	2014.04	Shandong	KT953589
2	SDQU1408[a]	2014.08	Shandong	Unsubmitted
3	HLJJY1409[a]	2014.09	Heilongjiang	Unsubmitted
4	HLJTB1409[a]	2014.09	Heilongjiang	Unsubmitted
5	JLMC1409[a]	2014.09	Jilin	Unsubmitted
6	SDWH11409[a]	2014.09	Shandong	Unsubmitted
7	SDWH21409[a]	2014.09	Shandong	Unsubmitted
8	SDWH31409[a]	2014.09	Shandong	Unsubmitted
9	HLJLYG1410[a]	2014.10	Heilongjiang	Unsubmitted
10	SDJN(D)1410[a]	2014.10	Shandong	Unsubmitted
11	SDLS(C)1410[a]	2014.10	Shandong	Unsubmitted
12	SDLW1410 [a]	2014.10	Shandong	Unsubmitted
13	SDSG(A)1410[a]	2014.10	Shandong	Unsubmitted
14	SDSG(B)1410[a]	2014.10	Shandong	Unsubmitted
15	SDWH(F)1410[a]	2014.10	Shandong	Unsubmitted
16	SDZC1411 [a]	2014.11	Shandong	Unsubmitted
17	HLJSH1412[a]	2014.12	Heilongjiang	Unsubmitted
18	JSZL [a]	2014.12	Jiangsu	KT119352
19	SDLY-14[c]	2014.12	Shangdong	KT953604
20	SDJN(E)1412[a]	2014.12	Shandong	Unsubmitted
21	SDHS9150129[b]	2015.01	Shandong	Unsubmitted
22	SDHS1015012[b]	2015.01	Shandong	Unsubmitted
23	JL2015 [b]	2015.02	Jilin	Unsubmitted
24	NMG2015 [b]	2015.02	Inner Mongolia	Unsubmitted
25	HB150309[b]	2015.03	Hebei	Unsubmitted
26	SD19-15 [c]	2015.03	Shangdong	KT953603
27	SDJNi2-15 [c]	2015.03	Shangdong	KT953588
28	SDJNi3-15 [c]	2015.03	Shangdong	KT953596
29	SDLK150320[b]	2015.03	Shandong	Unsubmitted
30	SDLW2-15 [c]	2015.03	Shangdong	KT953597
31	SDQZ150319[b]	2015.03	Shandong	Unsubmitted
32	SDZB2-15 [c]	2015.03	Shangdong	KT953590
33	JL150418[b]	2015.04	Jilin	Unsubmitted
34	NK150425[b]	2015.04	Heilongjiang	Unsubmitted
35	SDJNi1-15 [c]	2015.04	Shangdong	KT953587
36	SDJNi4-15 [c]	2015.04	Shangdong	KT953598
37	SDJNi5-15 [c]	2015.04	Shangdong	KT953600
38	SDJNi6-15 [c]	2015.04	Shangdong	KT953601
39	SDLW1-15 [c]	2015.04	Shangdong	KT953593
40	SDLY-15 [c]	2015.04	Shangdong	KT953594
41	SDMZ1-15 [c]	2015.04	Shangdong	KT953599
42	SDQZ150414[b]	2015.04	Shandong	Unsubmitted
43	SDTA2-15 [c]	2015.04	Shangdong	KT953591
44	SDTA3-15 [c]	2015.04	Shangdong	KT953595
45	SDWK-15 [c]	2015.04	Shangdong	KT953602
46	SDZC150416[b]	2015.04	Shandong	Unsubmitted
47	HB150528[b]	2015.05	Hebei	Unsubmitted
48	HeN1505 [c]	2015.05	Henan	KU556758
49	SDZC150514[b]	2015.05	Shandong	Unsubmitted
50	SDZC150526[b]	2015.05	Shandong	Unsubmitted
51	HLJSH150609[b]	2015.06	Heilongjiang	Unsubmitted
52	SDHZ-15 [c]	2015.06	Shangdong	KT953607
53	SDMZ2-15 [c]	2015.06	Shangdong	KT953606
54	SDSK-15 [c]	2015.06	Shangdong	KT953608
55	SDTA4-15 [c]	2015.06	Shangdong	KT953610
56	SDXLS-15 [c]	2015.06	Shangdong	KT953609
57	SDXT-15 [c]	2015.06	Shangdong	KT953611
58	SDZC150601[b]	2015.06	Shandong	Unsubmitted
59	SDZB-15 [c]	2015.06	Shangdong	KT953605
60	HLJHEB15071[b]	2015.07	Heilongjiang	Unsubmitted
61	HLJSH150702 [b]	2015.07	Heilongjiang	Unsubmitted

[a] The isolates reported previously by our laboratory
[b] The isolates first reported in this study
[c] The isolates reported previously by other laboratories

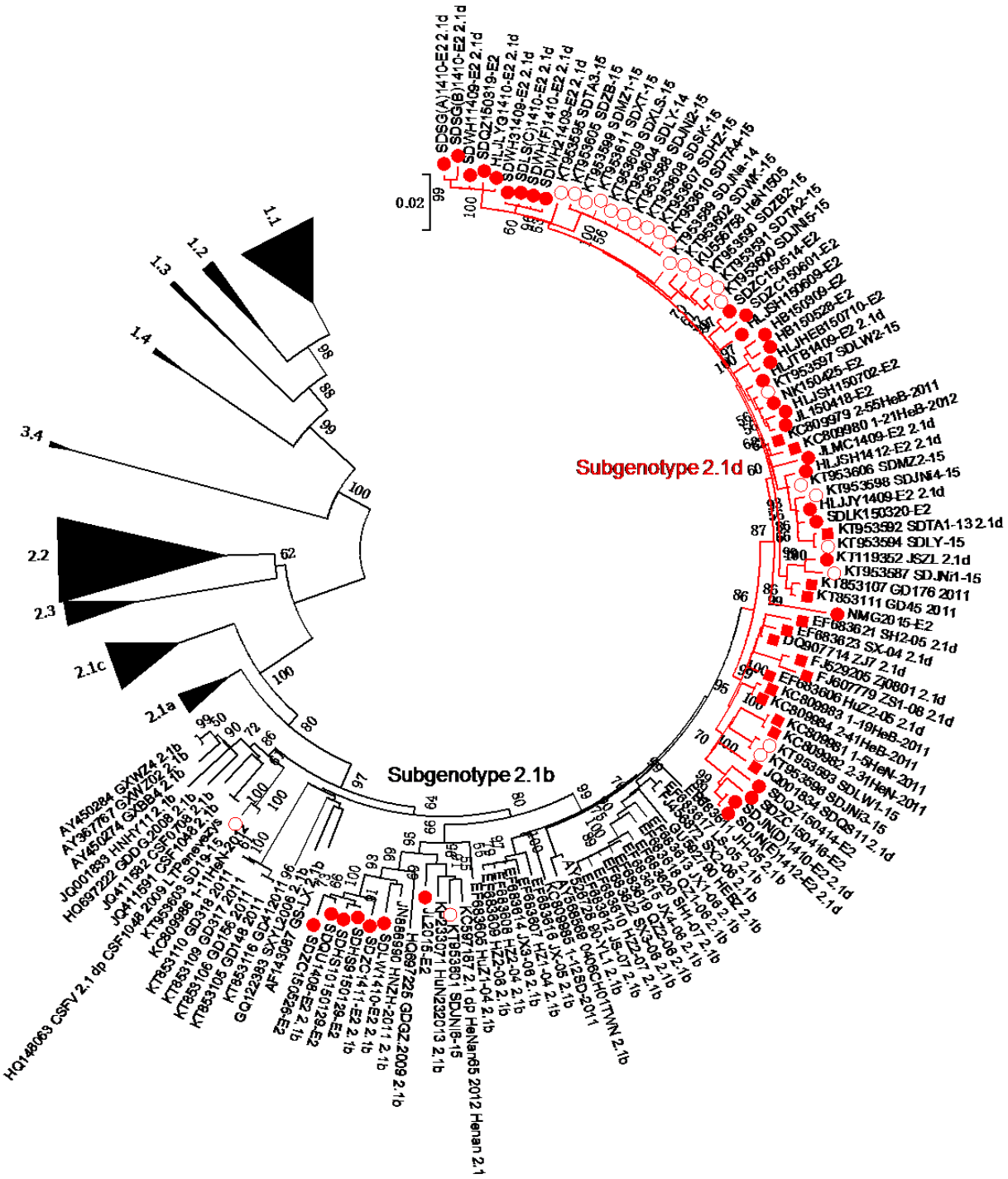

Fig. 1. Phylogenetic analysis of the 61 new isolates collected in 2014–2015 and other 163 reference CSFV isolates based on the full-length E2 gene sequences. For the new 2014–2015 isolates, 37 strains (●) were isolated by our laboratory and 25 strains (○) were isolated by others. Red lines indicate the subgenotype 2.1d, including 52 strains (● or ○) isolated in 2014–2015 and 16 strains (■) isolated between 2004 and 2013

According to the results of aa analysis of E2, the subgenotype 2.1d isolates, including 52 isolates collected in 2014–2015 and 16 previous isolates, showed several unique molecular characteristics, including aa R at position 31 (R^{31}), S^{34}, K^{205}, K^{303} and A^{331}, compared with all other isolates. Some subgenotype 2.1d isolates also showed unique aa substitutions, including $G/D/N^{36}S$, $D^{97}N$, $V/M^{168}A$, $Q/V/P^{200}L$, $E/N^{213}G$, and $D^{244}N$. In addition, most subgenotype 2.1d and 2.1b isolates shared four consistent aa substitutions at positions $T^{56}I$, $I/S^{108}T$, $L^{182}W$, and $T/K/A^{197}M$ compared with other subgenotype isolates. We also found that most new subgenotype 2.1b isolates collected in 2014–2015

showed unique molecular characteristics, including aa D at positions D^{192}, V^{195}, Y^{210}, E^{228}, K^{278}, I^{283}, VLA^{364}, and I^{365} (Fig. 2).

Some aa sites of E2 protein were further analysed. In position 205 of E2 protein, most new 2.1d isolates collected in 2014–2015 showed aa K, and other 2.1d isolates were aa R, which was consistent with the 2.1b and other subgenotype isolates. In addition, most 1.1 isolates showed the same aa S, K, and K with 2.1d isolates in positions 34, 205, and 303 of E2 protein, respectively. In position 331 of E2 protein, all the 2.1d isolates showed aa A, and the other subgenotype isolates showed aa V, except for subgenotype 1.1 isolate HCLV, a Chinese lapinised vaccine strain (C-stain), which showed the same aaA with 2.1d isolates (Fig. 2).

Geographical distribution of subgenotype 2.1d isolates. The 52 new 2014–2015 subgenotype 2.1d isolates were distributed over seven provinces (Shandong, Jilin, Heilongjiang, Jiangsu, Hebei, Henan, and Inner Mongolia) of China. However, most strains were isolated in Shandong province. The other 16 subgenotype 2.1d strains were isolated in six provinces (Hebei, Henan, Hunan, Zhejiang, Shanghai, and Guangdong) between 2004 and 2013. These provinces in which the emergence of the 2.1d isolates were confirmed formed a large, relatively connected area on the map of China (Fig. 3).

Table 2. Characteristics of the subgenotype 2.1d CSFV isolates collected between 2004 and 2013

No.	Virus strain	Collection date	Collection area	Accession no.
1	SX-04	2004	Zhejiang	EF683623
2	HuZ2-05	2005	Zhejiang	EF683606
3	SH2-05	2005	Shanghai	EF683621
4	ZJ7.2005	2005	Zhejiang	DQ907714
5	ZS1-08	2008	Zhejiang	FJ607779
6	Zj0801	2008.03	Zhejiang	FJ529205
7	SDQS11	2011	Hunan	JQ001834
8	GD176/2011[a]	2011	Guangdong	KT853107
9	GD45/2011[a]	2011	Guangdong	KT853111
10	1-19/HeB-2011	2011.07	Hebei	KC809983
11	1-5/HeN-2011	2011.10	Henan	KC809981
12	2-41/HeB-2011	2011.10	Hebei	KC809984
13	2-55/HeB-2011	2011.10	Hebei	KC809979
14	2-31/HeN-2011	2011.11	Henan	KC809982
15	1-21/HeB-2012	2012.03	Hebei	KC809980
16	SDTA1-13	2013.12	Shangdong	KT953592

[a] CSFV strains were isolated in Guangdong province by other labs

Table 3. Analysis of nucleotide (nt) and amino acid (aa) identity of E2 gene between the 61 new isolates (including 9 2.1b isolates and 52 2.1d isolates) collected in 2014–2015 and other 11 representative CSFV isolates (%)

Identities		Shimen (1.1)	Brescia 90 (1.2)	CSF0306 (1.3)	CSF0705 (1.4)	Paderborn (2.1a)	HEBZ (2.1b)
2.1b	nt	82.8-84.1	81.8-83.4	83.5-84.4	83.8-85.2	93.2-94.8	93.5-94.9
	aa	87.8-89.9	88.5-91.2	89.0-91.2	88.5-90.6	94.6-97.3	95.2-97.3
2.1d	nt	83.1-84.4	82.4-83.6	82.5-83.5	82.9-84.1	93.1-94.3	95.1-96.5
	aa	89.1-91.0	89.0-90.9	88.5-90.3	88.7-90.3	94.6-97.3	95.4-97.6

Identities		GDPY.20-08 (2.1c)	Zj0801 (2.1d)	LAL-290 (2.2)	Alfort (2.3)	P97 (3.4)
2.1b	nt	90.3-92.1	93.2-94.6	85.8-86.7	87.7-88.2	80.9-82.3
	aa	94.4-96.8	94.4-96.5	90.1-92.0	92.2-93.3	87.9-90.1
2.1d	nt	90.3-91.4	95.8-97.3	85.3-86.4	86.9-88.0	81.7-82.4
	aa	94.1-96.8	96.2-98.4	89.5-91.7	90.6-92.8	88.7-90.3

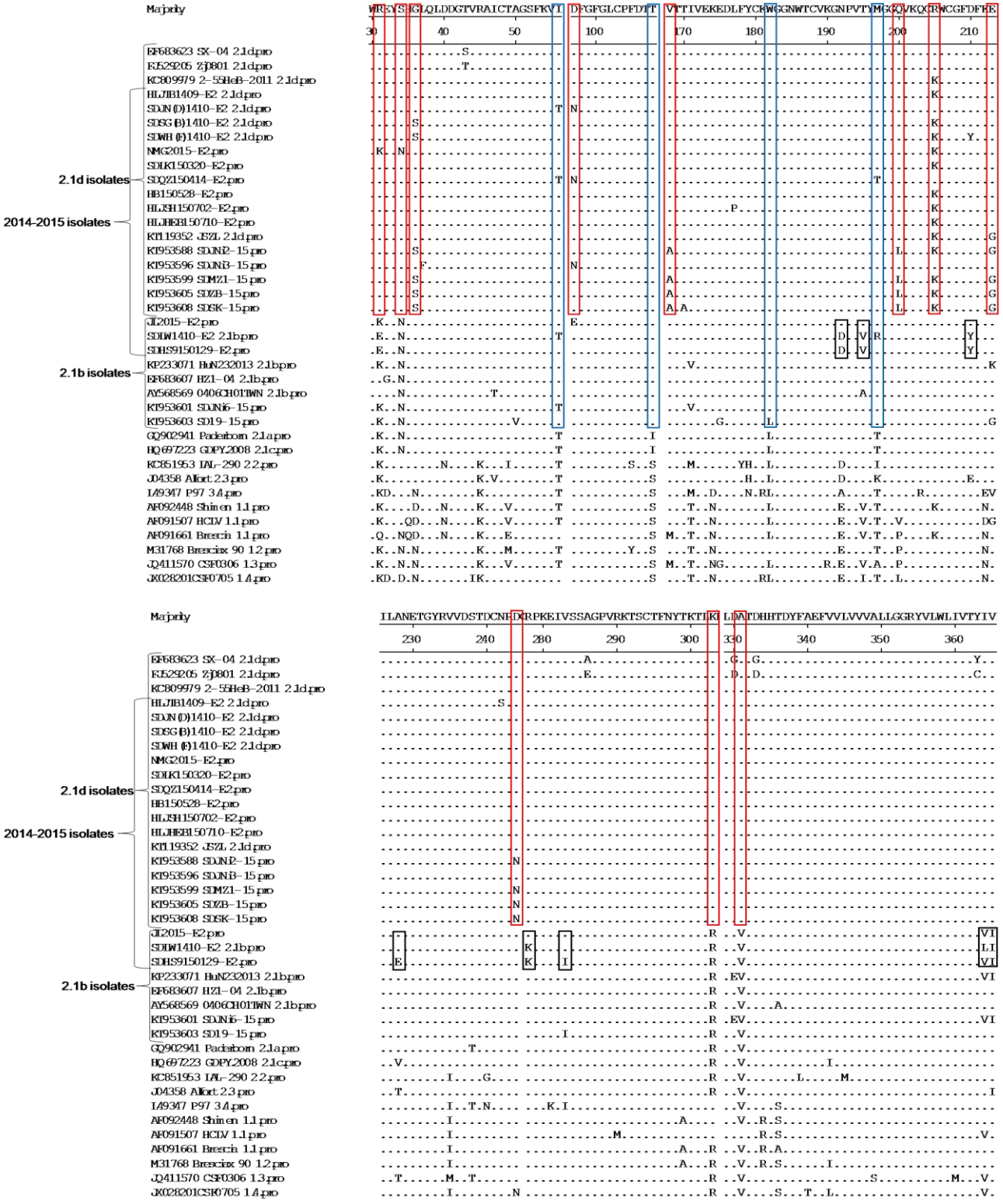

Fig. 2. Amino acid sequence alignments of E2 genes of 38 isolates collected in 2014–2015 and 27 reference CSFV strains. A–C – the unique molecular characteristics (R^{31}, S^{34}, S^{36}, N^{97}, A^{168}, L^{200}, K^{205}, G^{213}, N^{244}, K^{303}, and A^{331}) of 2.1d isolates are indicated by red boxes (□). The consistent molecular characteristics (T^{56}, T^{108}, W^{182}, and M^{197}) of 2.1d and 2.1b isolates are indicated by blue boxes (□).The unique molecular characteristics (D^{192}, V^{195}, Y^{210}, E^{228}, K^{278}, I^{283}, V/L/A^{364}, and I^{365}) of the new 2.1b isolates collected in 2014–2015 are indicated by black boxes (□)

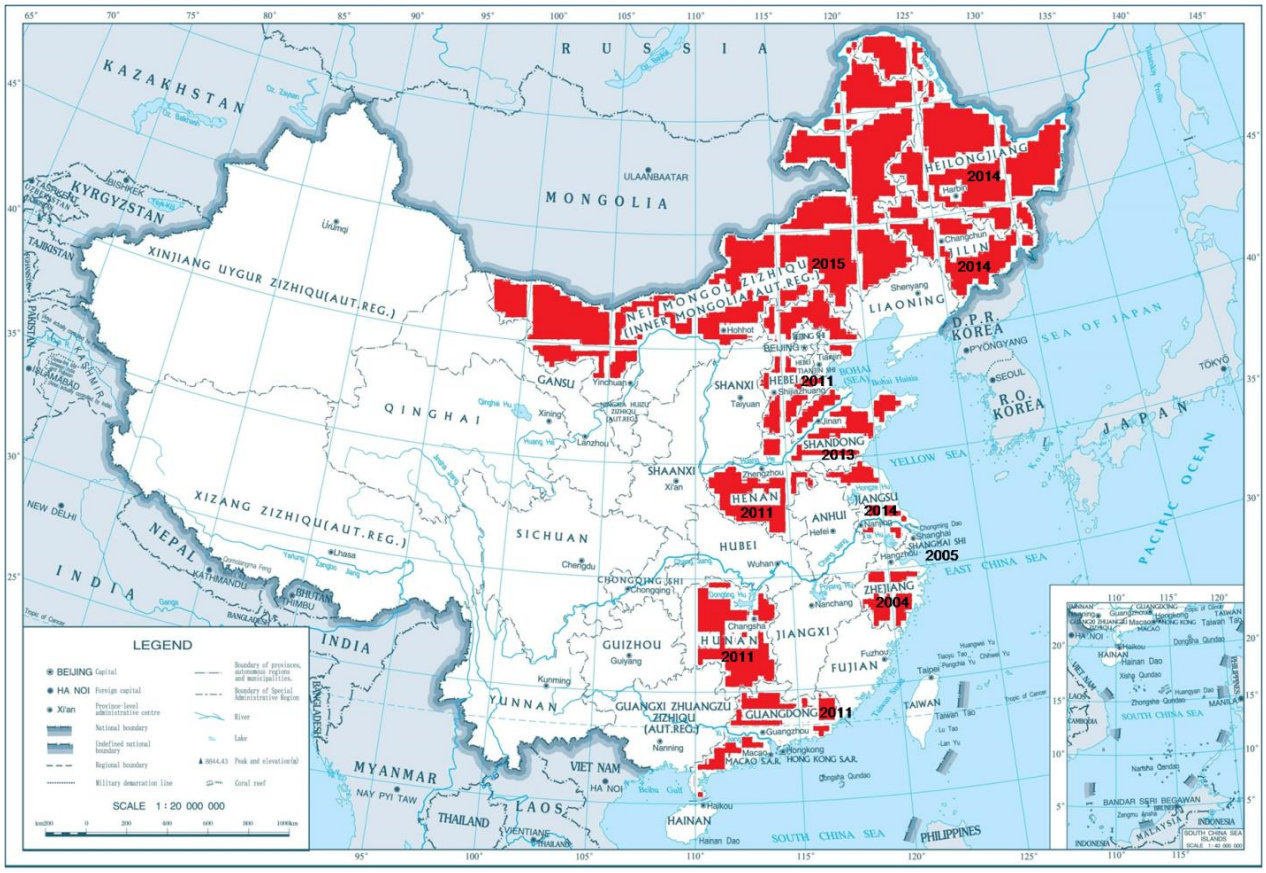

Fig. 3. Distribution and emergence time of the CSFV subgenotype 2.1d isolates in China. The 2.1d isolates emerged in the provinces marked red

Discussion

CSF is a devastating, highly contagious, often fatal disease of swine, causing huge economic losses to pig farms in numerous areas of the world (5, 18). In 1954, the C-strain vaccine was successfully developed. The vaccine was used in many endemic countries, and large CSF outbreaks had been rare (14). However, although the intensive control programmes have been implemented for over 20 years, CSF has not been completely controlled since it is sporadic or endemic in many countries of the world (1, 3, 13, 19). Recently, a new cluster of 2.1c virus subgenotype was identified in South China (10). Our laboratory also reported a new subgenotype 2.1d in China last year (30). The 2.1d isolates were epidemic in Shandong province, which has caused heavy economic losses since 2014 (8). In the present study, we reported 18 new CSFV isolates collected in 2015. The phylogenetic relationship, genetic diversity, and epidemic status of all the new CSFV isolates collected in 2014–2015 were fully analysed.

The full-length E2 sequence can be used for classification of CSFV isolates (22). Therefore, the phylogenetic tree was constructed based on the 224 full-length E2 gene sequences, including 61 isolates collected in 2014–2015 and 163 reference CSFV strains (Fig. 1). The result showed that CSFV isolates could be divided into three genotypes (1, 2, and 3) and several subgenotypes (1.1–1.4, 2.1a–2.1d, 2.2, 2.3 and 3.4). Among the 61 new CSFV isolates, 52 isolates belong to subgenotype 2.1d and the other 9 isolates belong to 2.1b. The 2.1d isolates were clearly in an independent branch, close to 2.1b isolates, and were located at the end of the evolutionary tree. Interestingly, the C-strain, HCLV, belong to the 1.1 subgenotype, located at the other end of the evolutionary tree. This result was consistent with a previous report, which showed that the prevalent CSFV isolates shifted away from the vaccine strain (2). This might have resulted from their adaptive abilities to the selection forces within the host (2). Recently, Gong *et al*. (6) reported that subgenotype 2.1 isolates of CSFV could be divided into 10 sub-subgenotypes (2.1a–2.1j) and the 2.1d–2.1j were newly identified (6). However, in this report, many 2.1 isolates, especially the newly emerged 2014–2015 isolates, were not included. So we do not agree with this classification completely.

The E2 nt and aa sequences of the 2014–2015 isolates were compared with 11 representative CSFV isolates, including Shimen (1.1), Brescia90 (1.2), CSF0306 (1.3), CSF0705 (1.4), Paderborn (2.1a), HEBZ (2.1b), GDPY.20-08 (2.1c), Zj0801 (2.1d), LAL-290 (2.2), Alfort (2.3), and P97 (3.4) (Table 3). The results indicated that the nine new 2.1b isolates and 52 2.1d isolates showed the highest identity with 2.1b isolate HEBZ and 2.1d isolate Zj0801

respectively, which suggests the accuracy of the classification of these new isolates. In the present study, the 2.1d isolates showed many unique molecular characteristics in E2 protein (R^{31}, S^{34}, S^{36}, N^{97},A^{168}, $L^{200}G^{213}$, N^{244}, K^{205}, K^{303}, and A^{331}), and most of these characteristics were reported previously (8, 30) (Fig. 2). Furthermore, the 2.1d isolates also shared the same aa with the 2.1b isolates in some positions of E2 (T^{56}, T^{108}, W^{182}, and M^{197}) (Fig. 2). Previous reports have found that natural recombination occurrs in CSFV isolates (7, 9, 29). In this study, we did not find evidence that the 2.1d isolates are derived from recombination between 2.1b and 1.1 or other subgenotype isolates after recombination analysis by the SimPlot programme (12) (data not shown). Although we speculated that the newly emerged 2.1d isolates may have diverged from 2.1b isolates, the internal evidence needs further exploration. In addition, we also found some unique molecular characteristics (D^{192}, V^{195}, Y^{210}, E^{228}, K^{278}, I^{283}, and V/L/A^{364} and I^{365}) of the new 2.1b isolates (Fig. 2). Whether these isolates will diverge into a new subgenotype is worthy of attention and vigilance.

As we all know, E2 is the most antigenic protein of CSFV and is involved in virus neutralisation. Four antigenic domains, A (86–176aas), B (1–83aas), C (1–110aas), and D (86–110aas), have been mapped on E2 (28). Domain A was subdivided into A1, A2, and A3.The aa substitutions reported in the present study were located in all these four domains. Whether these substitutions could affect the structure and function of E2 needs to be further studied. However, the six cysteines at positions 4, 48, 103, 129, 139, and 167, which were essential for binding by monoclonal antibodies of the four domains, had no variation in E2 protein of the 2014–2015 isolates (28). In addition, the potential N-glycosylation sites in E2 protein of these isolates were consistent with previous isolates.

Previous reports showed that several CSFV subgenotypes (1.1, 2.1, 2.2, and 2.3) existed in mainland China, and subgenotype 2.1b had become predominant within the last 10 years (2, 27). In 2014–2015, the new 2.1d isolates were epidemic in some districts of China (8, 30). All the 2.1d isolates were distributed over 11 provinces (Shandong, Jilin, Heilongjiang, Jiangsu, Hebei, Inner Mongolia, Henan, Hunan, Zhejiang, Shanghai, and Guangdong), and these areas together formed a defined region on the map of China (Fig. 3). In addition, the epidemic regions of 2.1d isolates seem to be grossly undetermined because most cases were not notified by farmers, and some 2.1d strains isolated by other laboratories were not reported in a timely manner. If all these new strains were isolated and reported, the epidemic areas would be larger and the trend of geographical cluster could be more obvious. Furthermore, most 2.1d isolates, together with some 2.1b isolates, were isolated simultaneously. The earliest subgenotype 2.1d strain, SX-04, was isolated in 2004. This indicated that the 2.1d isolates emerged more than 10 years ago, evolved continuously with subgenotype 2.1b isolates, and were epidemic until recently. Taken together, the presented results indicate that the 2.1d isolates may have diverged from 2.1b isolates.

It is well known that the C-strain, which belonged to subgenotype 1.1, was widely used in China. The vaccination may influence the divergence of CSFV through recombination or point mutation (9). In addition, positive selection pressure may act on the divergence of CSFV under C-strain vaccination, and several positively selected sites are found in E2 protein (8, 9, 21). Previous reports indicated that the surface structural proteins of CSFV of the vaccine-related groups contain more positive sites than other proteins of the vaccine-related groups and all proteins of the non-vaccine-related groups, suggesting that the difference was from immune selection (9, 11). In the present study, we did not find the evidence that the aa substitution of the new isolates was related to C-strain immune pressure. However, considering the fact that all clinical samples were collected from C-strain immunised pig farms, we speculated that the C-strain may promote the divergence and appearance of these new isolates. Of course, the internal evolution mechanism needs further research.

In summary, we analysed the phylogenetic relationship, genetic diversity, and epidemic status of the new 2014–2015 CSFV isolates. Most of these isolates belong to 2.1d and others belong to 2.1b. The 2.1d isolates and new 2.1b isolates showed unique molecular characteristics. The 2.1d isolates appeared more than 10 years ago, evolved continuously with subgenotype 2.1b isolates, and were epidemic until recently. We speculate that the 2.1d isolates might have diverged from 2.1b isolates. This comprehensive analysis may provide new insights into the prevention and control of CSF.

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