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Short communication

Sub-subgenotype 2.1c isolates of classical swine fever virus are dominant in Guangdong province of China, 2018

Chaonan Xing[a,b,1], Zongji Lu[c,1], Jianfeng Jiang[c], Liangzong Huang[c], Jialun Xu[b,d], Desheng He[e], Zelin Wei[c], Haijie Huang[c], Hongren Zhang[e], Cangyao Murong[e], Changchun Tu[a,b], Wenjie Gong[b,*]

[a] College of Veterinary Medicine, Yangzhou University, Jiangsu Co-innovation Center for Prevention, Control of Important Animal Infectious Diseases and Zoonoses, Yangzhou, China
[b] Institute of Military Veterinary, Academy of Military Medical Sciences, Academy of Military Sciences, Changchun, China
[c] School of Life Sciences and Engineering, Foshan University, Foshan, China
[d] College of Veterinary Medicine, Jilin University, Changchun, China
[e] Guangdong Sanshui Chen Ta Kunt Animal Husbandry Development Company, Foshan, China

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ABSTRACT

Classical swine fever (CSF) continues to be a devastating infectious disease for the swine industry in China and commonly exists as wild or atypical types. From June 3rd to October 3rd, 2018, outbreaks of typical CSF cases with mortality rates of 42–86% occurred in 11 swine herds in five cities of Guangdong province, and were confirmed by RT-PCR. Phylogenetic analyses based on the nucleotide sequences of full-length E2 genes showed that the CSFV isolates collected in Guangdong, 2018 grouped into sub-subgenotype 2.1c and formed a separate clade from previously identified 2.1c isolates. Sequence comparison further confirmed the distance between the novel emergent and previously identified 2.1c isolates, with shared 94.5–98.2% and 97.8–99.7% identities at the nucleotide and amino acid levels respectively. Furthermore, 2.1c isolates collected in 2018 from Guangdong province contained a unique amino acid substitution (K174R) in the E2 protein in comparison with other 2.1c representative strains and CSFV 2.1, 2.2, 2.3 strains. Of note, the novel emergent 2.1c isolates are neutralized by sera from C-strain vaccinated sows, indicating that C-strain is still efficacious for protection against field isolates of CSFV.

1. Introduction

Classical swine fever (CSF) is still one of the most devastating infectious diseases for the swine industry in China, featuring high fever, skin bleeding, kidney petechia, and swelling and hemorrhage of lymph nodes (Moennig et al., 2003). Since the successful development of a highly effective vaccine (CSFV C-strain or HCLV) in China, 1954, widespread vaccination has largely controlled the CSF epizootic, although sporadic outbreaks of wild or atypical CSF still occur.

The causative agent, CSF virus (CSFV), belongs to the genus *Pestivirus* within the family *Flaviviridae*. Genetic typing has revealed that CSFV can be grouped into 3 genotypes, and 11 subgenotypes have been further defined (1.1–1.4, 2.1–2.3 and 3.1–3.4) (Paton et al., 2000; Postel et al., 2012; Postel et al., 2013). In the 1990s, subgenotypes 2.1, 2.2, 2.3, and 1.1 circulated within mainland China, with 2.1 and 2.2 subgenotypes playing a dominant role in CSF outbreaks (Tu et al., 2001). Since 2000, subgenotypes 2.2 and 2.3 have become silent, probably due to extensive vaccination with C-strain, and subgenotype 2.1 is now absolutely dominant in China (Chen et al., 2008). At the same time, a rapid transition from genotypes 1 and 3 to genotype 2 occurred in European and other Asian countries (Paton et al., 2000; Deng et al., 2005; Cha et al., 2007). Further analysis of the genetic diversity within subgenotype 2.1 isolates has revealed 10 sub-subgenotypes (2.1a − 2.1j) among which 2.1b is prevalent in most provinces in China, but with 2.1c dominant in south China and some southeastern Asian countries including Thailand, Laos PR and Vietnam (Jiang et al., 2013; Gong et al., 2016a).

In Guangdong province, subgenotypes 2.1, 2.2 and 2.3 were enzootic in the 1990s (Tu et al., 2001), with 2.1 being dominant. Since 2000, subgenotypes 2.2 and 2.3 have not been further detected in this province, although subgenotype 1.1 isolates circulated between 2008 and 2010 (Shen et al., 2011). In 2011, CSFV isolates belonging to sub-

* Corresponding author.
E-mail address: gwj020406@163.com (W. Gong).
[1] Authors contribute equally to the study.

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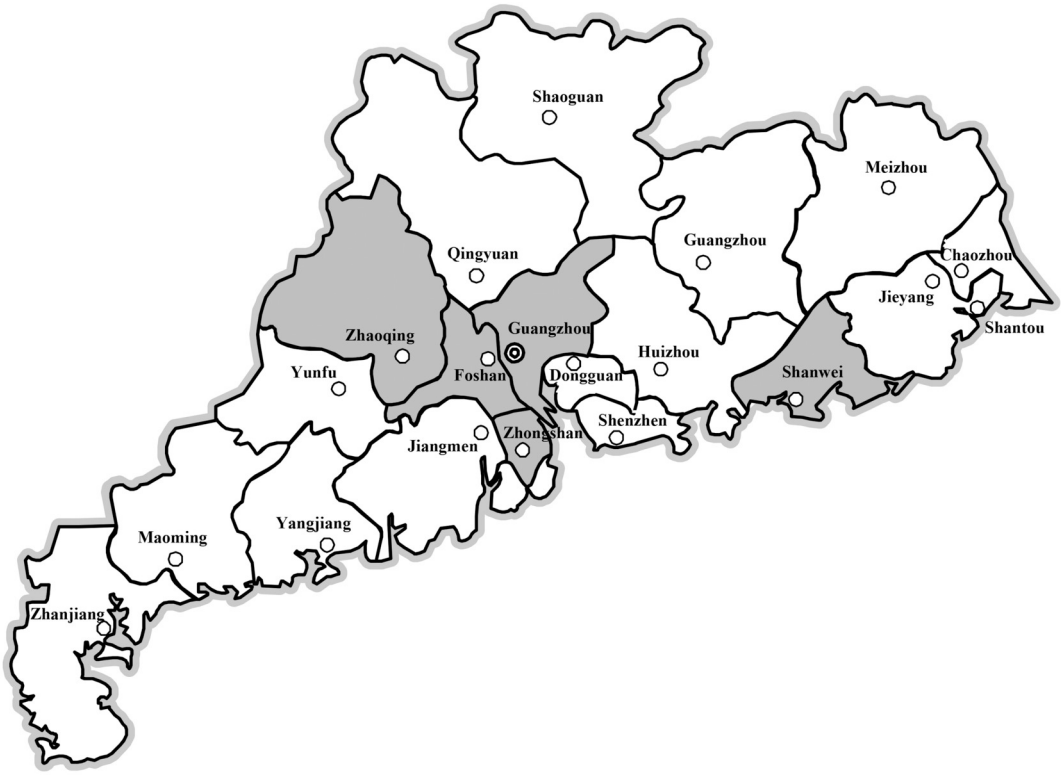

Fig. 1. Geographic distribution of CSF samples collected in Guangdong, 2018. Eleven outbreaks of CSF cases were distributed in five cities (grey) of Guangdong province: Shanwei (Herd 1, GDLF1; Herd 2, GDLH1), Zhongshan (Herd 3, GDZS1), Guangzhou (Herd 4, GDPY1), Zhaoqing (Herd 5, GDGY10), and Foshan (Herd 6, GDFS1; Herd 7, GDFS2; Herd 8, GDFS3; Herd 9, GDFS4; Herd 10, GDFS6; Herd 11, GDFS7).

Table 1
List of CSF cases in Guangdong province from June 3rd to October 3rd, 2018.

Herd	Isolate	Place	Time	Pig group	Herd type	Mortality	GenBank accession No.
1	GDLF1–2018	Lufeng, Shanwei	2018.8.8	Weaned piglet	Farm-bred	150/230 (65%)	MK256361
2	GDLH1–2018	Luhe, Shanwei	2018.10.3	Weaned piglet	Farm-bred	40/80 (50%)	MK256362
3	GDZS1–2018	Tanzhou, Zhongshan	2018.8.9	Weaned piglet	Farm-bred	36/60 (60%)	MK256363
4	GDPY1–2018	Guangzhou	2018.9.3	Weaned piglet	Farm-bred	536/960 (56%)	MK256364
5	GDGY10–2018	Gaoyao, Zhaoqing	2018.7.28	Weaned piglet	Farm-bred	36/42 (86%)	MK256365
6	GDFS1–2018	Sanshui, Foshan	2018.6.11	Weaned piglet	Introduced	50/120 (42%)	MK256366
7	GDFS2–2018	Sanshui, Foshan	2018.6.28	Weaned piglet	Introduced	65/80 (85%)	MK256367
8	GDFS3–2018	Sanshui, Foshan	2018.6.3	Weaned piglet	Introduced	120/200 (60%)	MK256368
9	GDFS4–2018	Sanshui, Foshan	2018.7.11	Weaned piglet	Introduced	360/600 (60%)	MK256369
10	GDFS6–2018	Sanshui, Foshan	2018.8.31	Weaned piglet	Introduced	47/60 (78%)	MK256370
11	GDFS7–2018	Sanshui, Foshan	2018.9.26	Weaned piglet	Introduced	50/80 (63%)	MK256371

subgenotypes 2.1b, c, g, h, i, and j were detected in Guangdong province, indicating that CSFV isolates circulating in Guangdong province keep changing.

In the present study, typical CSF cases in 11 swine herds from five cities in Guangdong province, 2018 were found to be caused by CSFV sub-subgenotype 2.1c isolates, while other sub-subgenotypes of 2.1, including 2.1b, were not detected, thereby indicating the dominant role of 2.1c in outbreaks of CSF cases in this province.

2. Materials and methods

2.1. Clinical features of pigs with suspected CSF

From June 3rd to October 3rd, 2018, suspected CSF cases occurred in 11 swine herds from five cities within Guangdong province, 6 of which were located in Sanshui district, Foshan city, > 72 km from the other 5 swine herds in the four other cities (Fig. 1). Most ill pigs were weaned piglets, either bred within the farms (5 farms) or introduced from elsewhere (6 farms) (Table 1). In the farm-bred herds, sows but not the weaned piglets had received CSFV vaccination. The piglets introduced from elsewhere became ill within 7–10 days of purchase and had not received any vaccinations. The ill pigs displayed multiple symptoms, including hyperpyrexia (> 40 °C), anorexia, mental depression, hesitant gait, cough, reddened conjunctivitis, constipation or diarrhea, and pinpoint skin hemorrhages. Following onset of clinical signs, 42–86% of affected pigs were dead within 1–3 weeks, and typical pathological lesions of CSF were observed upon necropsy, including hemorrhage in the stomach and large intestine, button-shaped ulcers in the ileocecal valves, pulmonary edema, consolidated or cellulosic exudation, swelling and hemorrhage of lymph nodes, and individual or multiple petechiae in the kidneys (Fig. 2).

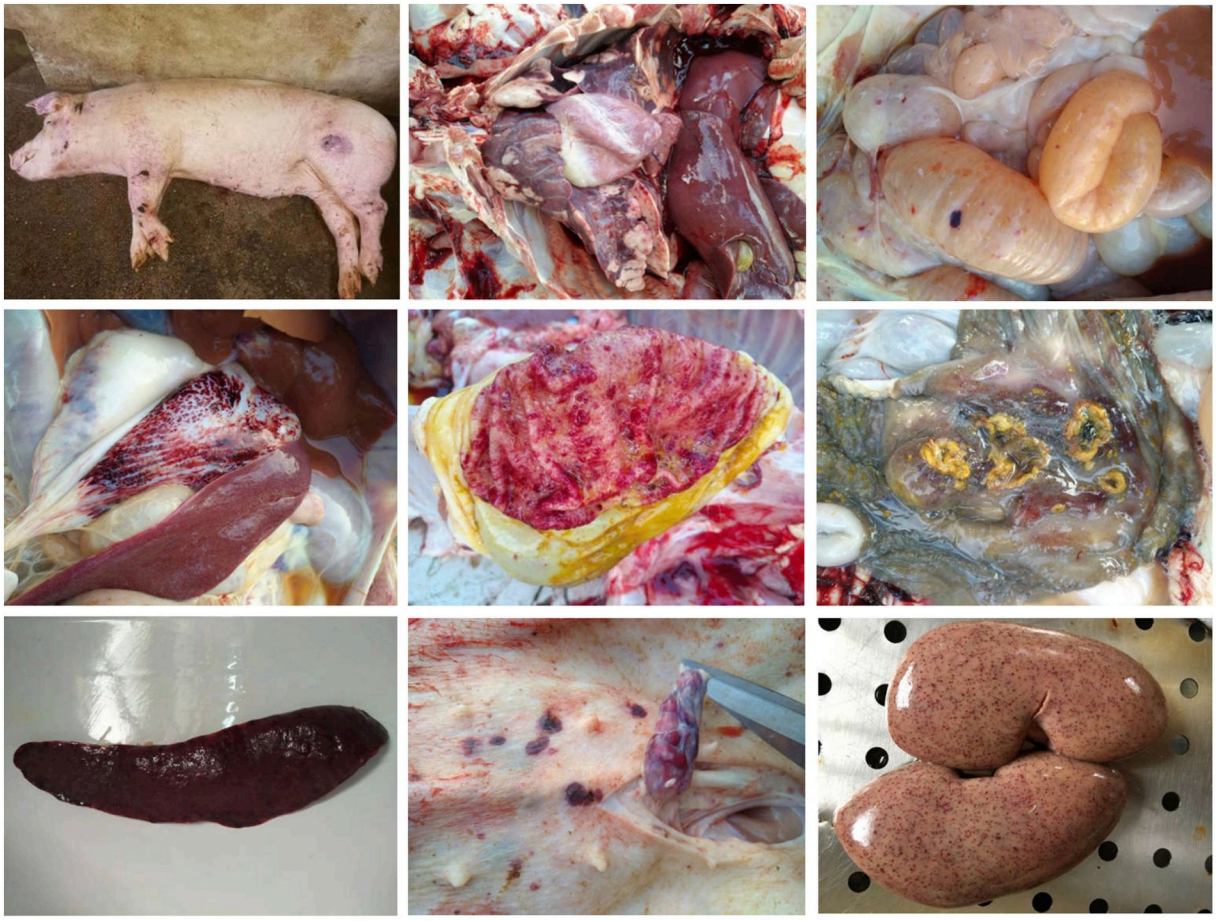

Fig. 2. Typical pathological lesions were observed in CSFV-infected pigs. Hemorrhage in the skin, stomach, large intestine and lymph nodes, button-shaped ulcers in ileocecal valves, pulmonary consolidation and cellulosic exudation, and multiple petechiae in kidneys were observed in pigs infected by CSFV.

2.2. Sample preparation

Tissues from lymph nodes, kidneys, spleens, lungs, and tonsils of necropsied animals were collected for laboratory diagnosis. Ten% tissue homogenates were prepared and, following clarification by centrifugation at 12,000 rpm for 10 min, supernatants were subjected to RNA extraction with Trizol reagent (Invitrogen, USA) according to the manufacturer's instructions. Sera were obtained from ill pigs and from clinically healthy sows from a swine herd located in Gaoyao township, Zhaoqing city.

2.3. RT-PCR, amplification, sequencing, and evolutionary analysis of E2 gene

Assay of clinical samples for CSF by RT-PCR, and amplification and sequencing of full-length E2 genes to obtain consensus sequences was performed as previously described (Gong et al., 2016a). Alignments of nucleotide and deduced amino acid sequences of CSFV full-length E2 genes were conducted using CLC sequence viewer 7.6.1 (Qiagen, Germany). Phylogenetic trees were constructed with MEGA 6.0, and the Maximum-likelihood method with 1000 bootstrap replicates was used to strengthen the robustness of the results.

2.4. Virus isolation, neutralization assay, and detection of E2 antibody

Isolation of virus from positive samples was performed by incubating supernatants of 10% tissue homogenates with PK-15 cells, with cell and viral passages as previously described (Gong et al., 2016b). The infectious titer of the resulting cell-adapted CSFV was determined prior to neutralization assay (Gong et al., 2016b), and the ND_{50}s of sera from infected pigs and clinical healthy sows in CSF-infected herds were determined as described previously (Floegel-Niesmann et al., 2009). E2 antibody titers were determined using the CSFV Antibody Detection Kit (IDEXX, USA) according to the manufacturer's instructions, and the cutoff of the blocking rate for positive is 40%: positive, ≥40%; doubtful, < 40% and ≥ 30%; negative, < 30% .

3. Results and discussion

3.1. Sub-subgenotype 2.1c isolates are dominant in Guangdong province, 2018

Tissue samples of sick weaned piglets from the 11 swine herds presenting with typical clinical signs and pathological lesions of CSF were all determined to be CSFV positive by RT-PCR. Sequence analysis of their full-length E2 genes revealed that these CSFV isolates are

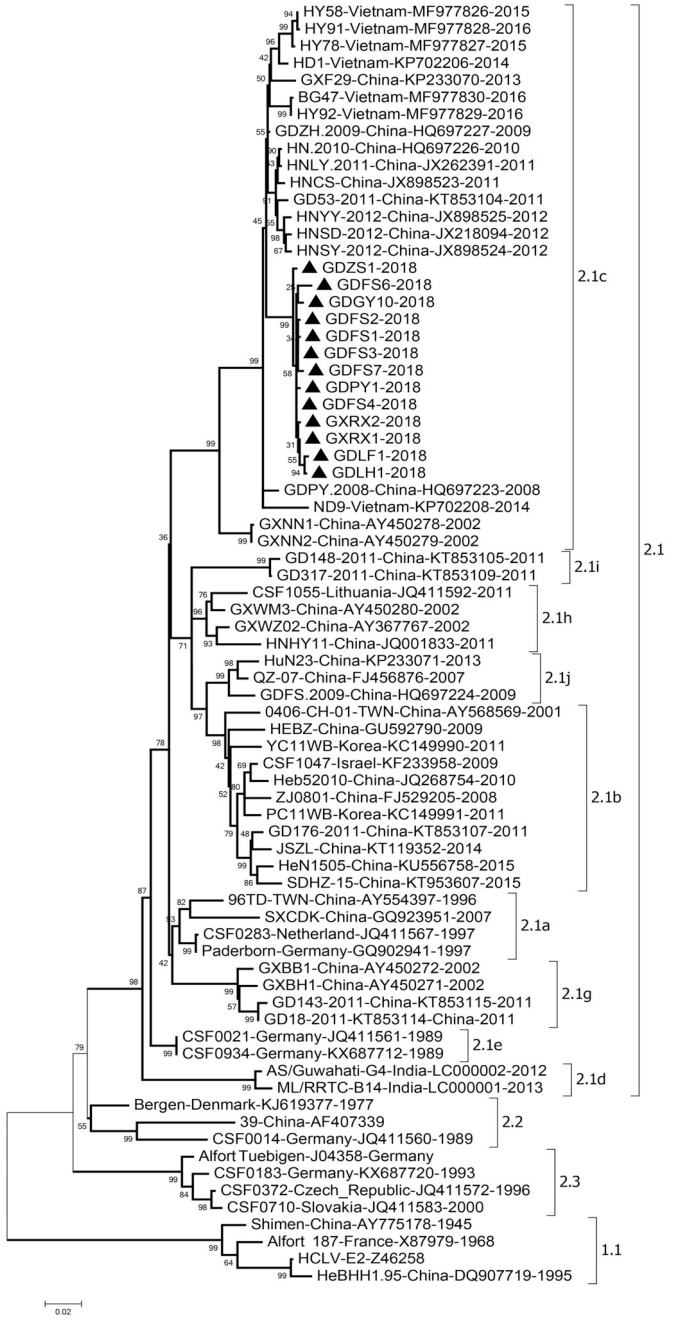

Fig. 3. CSFV isolates from Guangdong, 2018 belong to sub-subgenotype 2.1c. Phylogenetic analysis based on the nucleotide sequences of CSFV full-length E2 genes were performed with Mega 6.06 software. Sub-subgenotype 2.1c isolates from Guangdong, 2018 are identified by black triangles.

closely related to each other, sharing identities of 99.0–99.9% and 99.2–100% at the nucleotide and amino acid levels respectively. Phylogenetic analyses based on the nucleotide sequences revealed that, while the Guangdong, 2018 isolates grouped into sub-subgenotype 2.1c, they formed a separate clade from previously identified 2.1c isolates (Fig. 3). Sequence comparison further confirmed the distance between the novel emergent CSF viruses and previously identified 2.1c isolates from south China (Guangdong, Guangxi, and Hunan) and Vietnam, sharing 94.5–98.2% and 97.8–99.7% identities at the nucleotide and amino acid levels respectively. Furthermore, 2.1c isolates collected in 2018 from Guangdong province contained a unique amino acid substitution (K174R) in the E2 protein, not found in other 2.1c representative strains and CSF viruses of subgenotypes 2.1, 2.2, 2.3, and the amino acid residue in this site of C-strain (HCLV) is asparagine (N) (Fig. 4). This substitution is located in the antigenic domain A1 (86–176) containing neutralizing antigenic epitopes (Wensvoort, 1989). Thus, whether this amino acid substitution altered antigenic epitopes and escape neutralization should be resolved in future. The above results indicate that the novel emergent 2.1c isolates have become dominant in Guangdong province.

Although the geographic distance between the infected swine herds in the most westerly (Zhaoqing, herds 1 and 2) and easterly (Shanwei, herd 5) regions is > 400 km, the outbreaks of CSF in these farms were caused by closely related CSFV isolates. This may reflect the possibility that the novel 2.1c isolates are circulating widely within Guangdong province. Of note, two CSFV isolates with mortalities of 75–90% collected in 2018 from two herds in Rong township, Guangxi province, which is about 200 km from swine herd 5 in Zhaoqing city of Guangdong province, shared 99.0–99.8% nucleotide sequence identity of full-length E2 genes with the novel emergent 2.1c isolates and also contained the K174R substitution in the E2 proteins (Fig. 3 and 4). Cross-border transportation of pigs between these two neighboring provinces may have promoted the dissemination of the newly identified CSFV 2.1c isolates.

In addition, the nucleotide sequences of the full-length E2 genes of the novel emergent 2.1c isolates obtained in this study have been deposited in GenBank under accession numbers MK256361-MK256373 (Table 1).

3.2. Detection of E2 and neutralizing antibodies

Levels of E2 and neutralizing antibodies determine the outcome of CSFV infection, and therefore they were tested in the sera of ill animals. Results showed that sera collected from 10 ill piglets from 4 farms were all positive for CSFV by RT-PCR. Of the 10, 8 were negative or doubtful for E2 antibody, with the titer of the positive two samples (GDLF-W8 and GDLH1) being only slightly higher than the positive cutoff (≥ 40%) (Table 2).

To further investigate the involvement of neutralizing antibody, assays were carried out using CSFV field isolates adapted to growth in PK-15 cells as challenge. Seven such cell-adapted 2.1c viruses had been obtained after 13 passages, with infectious titers of $10^{6.16–6.33}$/ml. Neutralization assay with one of these isolates (GDLF1–2018) revealed that the ND_{50} titers of sera from CSFV-infected piglets ranged from < 5 to 7.5, levels which would not provide the infected piglets with protection.

To investigate the possibility that the CSFV in the infected piglets came from sows with persistent infection, sera from 10 of the 14 sows in Zhaoqing swine herd 5, all of which had been vaccinated with C-strain, were collected for determining the levels of E2 and neutralizing antibodies. Results showed that only one sow was CSFV positive by RT-PCR, with a positive (41%) blocking rate of E2 antibody but with a very low (7.5) ND_{50}. The infected sow may therefore be the source of infection of the piglets. In the farms with piglets from external sources, clinical signs appeared 7–10 days following their introduction and were therefore likely to have been infected in the donor farms through vertical transmission from persistently infected sows or by contact with

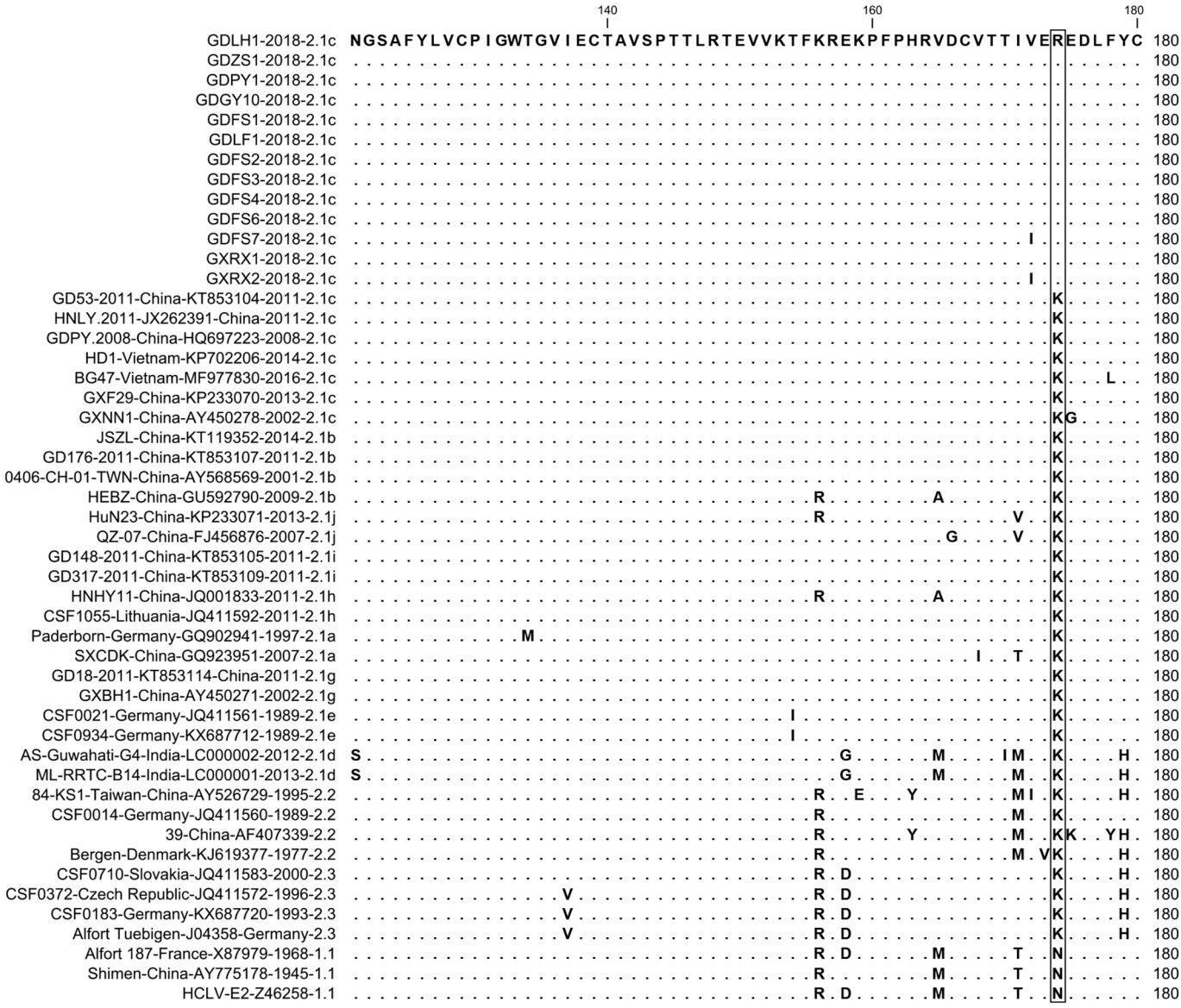

Fig. 4. CSFV sub-subgenotype 2.1c isolates from Guangdong, 2018 have a unique substitution (K174R) in E2 proteins. Multiple alignments of deduced amino acids of E2 proteins of CSF viruses, including sub-subgenotype 2.1c isolates collected from Guangdong, 2018 and representative strains of subgenotypes 2.1, 2.2, 2.3, were carried out using CLC sequence viewer 7.6.1. The unique amino acid substitution in the E2 proteins of the newly identified 2.1c isolates is identified within a box.

infected pigs. Transportation from one herd to another, together with other environmental stresses, may have reduced the body resistance of the weaned piglets resulting in rapid onset of symptoms in infected animals. Thus, screening for and elimination of CSFV carrier sows are very important for prevention and control of CSF in China, with strict quarantine necessary before introduction of weaned piglets into fresh herds.

Although the novel emerged 2.1c isolates are genetically distant from vaccine C-strain with shared identities of only 81.4–81.9% (nt) and 89.0–89.5% (aa) in their full-length E2 genes, these novel viruses can be neutralized by the sera from sows vaccinated with C-strain (Table 2), and levels of E2 antibody in the 9 CSFV-negative sows in swine herd 5 ranged from 91 to 95%, the ND_{50}s were from 320 to 1920 (Table 2), indicating that C-strain is still efficacious for protection against them. Thus, high mortality in the swine herds caused by infection with the newly identified 2.1c isolates can be prevented through vaccination with C-strain.

Conflict of interest

The authors declare no conflict of interest.

Table 2
E2 and neutralizing antibodies against CSFV in the sera of pigs from 4 swine herds.

Herd	Sample	Pig group	Place	RT-PCR	E2 antibody	ND_{50}
1	GDLF-W5	Weaned piglet	Shanwei	+	-2	< 5
1	GDLF-W6	Weaned piglet	Shanwei	+	37	7.5
1	GDLF-W8	Weaned piglet	Shanwei	+	48	7.5
1	GDLF-W9	Weaned piglet	Shanwei	+	20	< 5
1	GDLF-W10	Weaned piglet	Shanwei	+	37	5
2	GDLH1	Weaned piglet	Shanwei	+	68	5
3	GDZS1	Weaned piglet	Zhongshan	+	32	5
5	GDGY-W1	Weaned piglet	Zhaoqing	+	20	< 5
5	GDGY-W2	Weaned piglet	Zhaoqing	+	17	< 5
5	GDGY-W3	Weaned piglet	Zhaoqing	+	24	< 5
5	GDGY1	Sow	Zhaoqing	−	94	640
5	GDGY2	Sow	Zhaoqing	−	96	1280
5	GDGY3	Sow	Zhaoqing	−	95	1280
5	GDGY4	Sow	Zhaoqing	−	93	640
5	GDGY5	Sow	Zhaoqing	−	95	1920
5	GDGY6	Sow	Zhaoqing	−	93	320
5	GDGY7	Sow	Zhaoqing	−	95	320
5	GDGY8	Sow	Zhaoqing	−	92	480
5	GDGY9	Sow	Zhaoqing	−	91	480
5	GDGY10	Sow	Zhaoqing	+	41	7.5

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References

Cha, S.H., Choi, E.J., park, J.H., Yoon, S.R., Kwon, J.H., Yoon, K.J., Song, J.Y., 2007. Phylogenetic characterization of classical swine fever viruses isolated in Korea between 1988 and 2003. Virus Res. 126, 256–261.

Chen, N., Hu, H.X., Zhang, Z.F., Shuai, J.B., Jiang, L.L., Fang, W.H., 2008. Genetic diversity of the envelope glycoprotein E2 of classical swine fever virus: recent isolates branched away from historical and vaccine strains. Vet. Microbiol. 127 (3–4), 286–299.

Deng, M.C., Huang, C.C., Huang, T.S., Chang, C.Y., Lin, Y.J., Chien, M.S., Jong, M.H., 2005. Phylogenetic analysis of classical swine fever virus isolated from Taiwan. Vet. Microbiol. 106, 187–193.

Floegel-Niesmann, G., Blome, S., Gerβ-Dülmer, H., Bunzenthal, C., Moennig, V., 2009. Virulence of classical swine fever virus isolates from Europe and other areas during 1996 until 2007. Vet. Microbiol. 139, 165–169.

Gong, W.J., Wu, J.M., Lu, Z.J., Zhang, L., Qin, S.B., Chen, F.L., Peng, Z.C., Wang, Q., Ma, L., Bai, A.B., Guo, H.C., Shi, J.S., Tu, C.C., 2016a. Genetic diversity of subgenotype 2.1 isolates of classical swine fever virus. Infect. Genet. Evol. 41, 218–226.

Gong, W.J., Lu, Z.J., Zhang, L., Xie, X.M., Jiang, D.L., Jia, J.J., Guo, H.C., Shi, J.S., Tu, C.C., 2016b. *In vitro* adaptation and genome analysis of a sub-subgenotype 2.1c isolate of classical swine fever virus. Virus Genes 52, 651–659.

Jiang, D.L., Gong, W.J., Li, R.C., Liu, G.H., Hua, Y.F., Ge, M., Wang, S.Q., Yu, X.L., Tu, C.C., 2013. Phylogenetic analysis using E2 gene of classical swine fever virus reveals a new subgenotype in China. Infect. Genet. Evol. 17, 231–238.

Moennig, V., Floegel-Niesmann, G., Greiser-Wilke, I., 2003. Clinical signs and epidemiology of classical swine fever: a review of new knowledge. Vet. J. 165, 11–20.

Paton, D.J., McGoldrick, A., Greiser-Wilke, I., Parchariyanon, S., Song, J.Y., Liou, P.P., Stadejek, T., Lowings, J.P., Björklund, H., Belák, S., 2000. Genetic typing of classical swine fever virus. Vet. Microbiol. 73, 137–157.

Postel, A., Schmeiser, S., Bernau, J., Meindl-boehmer, A., Pridotkas, G., Dirbakova, Z., mojzis, M., Becher, P., 2012. Improved strategy for phylogenetic analysis of classical swine fever virus based on full-length E2 encoding sequences. Vet. Res. 43, 50.

Postel, A., Schmeiser, S., Perera, C.L., Rodríguez, L.J.P., Frias-Lepoureau, M.T., Becher, P., 2013. Classical swine fever virus isolates from Cuba form a new subgenotype 1.4. Vet. Microbiol. 161, 334–338.

Shen, H.Y., Pei, J.J., Bai, J.L., Zhao, M.Q., Ju, C.M., Yi, L., Kang, Y.M., Zhang, X.T., Chen, L.J., Li, Y.G., Wang, J.Y., Chen, J.D., 2011. Genetic diversity and positive selection analysis of classical swine fever virus isolates in south China. Virus Genes 43, 234–242.

Tu, C.C., Lu, Z.J., Li, H.W., Yu, X.L., Liu, X.T., Li, Y.H., Zhang, H.Y., Yin, Z., 2001. Phylogenetic comparison of classical swine fever virus in China. Virus Res. 81, 29–37.

Wensvoort, G., 1989. Topographical and functional mapping of epitopes on hog cholera virus with monoclonal antibodies. J. Gen. Virol. 70 (11), 2865–2876.