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Epidemiological investigation and phylogenetic analysis of Classical Swine Fever virus in Yunnan province from 2015 to 2021

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ABSTRACT

Background: Classical swine fever virus (CSFV), the causative agent of classical swine fever (CFS), is a highly contagious disease that poses a serious threat to Chinese pig populations.
Objectives: Many provinces of China, such as Shandong, Henan, Hebei, Heilongjiang, and Liaoning provinces, have reported epidemics of CSFV, while the references to the epidemic of CSFV in Yunnan province are rare. This study examined the epidemic characteristics of the CSFV in Yunnan province.
Methods: In this study, 326 tissue samples were collected from different regions in Yunnan province from 2015 to 2021. A reverse transcription-polymerase chain reaction (RT-PCR), sequences analysis, and phylogenetic analysis were performed for the pathogenic detection and analysis of these 326 clinical specimens.
Results: Approximately 3.37% (11/326) of specimens tested positive for the CSFV by RT-PCR, which is lower than that of other regions of China. Sequence analysis of the partial E2 sequences of eleven CSFV strains showed that they shared 89.0–100.0% nucleotide (nt) and 95.0–100.0% amino acid (aa) homology, respectively. Phylogenetic analysis showed that these novel isolates belonged to the subgenotypes 2.1c and 2.1d, with subgenotype 2.1c being predominant.
Conclusions: The CSFV was sporadic in China's Yunnan province from 2015 to 2021. Both 2.1c and 2.1d subgenotypes were found in this region, but 2.1c was dominant.

Keywords: Classical swine fever virus; sequence analysis; subgenotype 2.1c; Yunnan province

INTRODUCTION

The classical swine fever virus (CSFV), belonging to the genus Pestivirus in the family Flaviviridae, is a single positive-stranded, enveloped RNA virus that comprises a genome of approximately 12.3 kb [1]. Classical swine fever (CSF) caused by the CSFV is a highly contagious and fatal disease in pigs. The disease presents acute, subacute, chronic, and asymmetric characteristics, depending mainly on the viral virulence and host factors [2].

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Conflict of Interest
The authors declare no conflicts of interest.

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An outbreak of CSF causes high morbidity and mortality rates and significantly affects the development of the pig industry. Therefore, the occurrence of CSF in the pig population must be reported to the World Organization for Animal Health (OIE) [3].

The genome of the CSFV encodes only one open reading frame, which can be further hydrolyzed into four structural proteins (C, E^{rns}, E1, and E2) and seven non-structural proteins (P7, N^{pro}, NS2, NS3, NS4A, SN4B, NS5A, and NS5B) following co- and post-translational processing [4]. Of the genome of the CSFV, the E2 gene displaying high genetic diversity is considered a standardized molecular marker to investigate the genetic characteristics of the CSFV [5,6].

In recent years, CSFV strains circulating globally have been divided into three genotypes (1-3) and eleven subgenotypes (1.1-1.4, 2.1-2.3, and 3.1-3.4) [7]. The subgenotype 2.1 strains are the main causative agents responsible for outbreaks of CSF in pig populations [8], and they display higher genetic variation (2.1a-2.1j) than the other subgenotypes [9]. More importantly, the prevalence of subgenotype 2.1d strains has been monitored in many C-strain-vaccinated pig farms. Further investigations showed that the C-strain vaccine does not fully protect against the 2.1d strain [4].

CSF is widespread in different regions of the world, and outbreaks have been observed in Japan recently [10,11]. In China, the CSF epidemic remains, despite the wide application of lapinized-attenuated vaccine (C-strain) in pig populations [4,12]. To provide an update on the epidemic status of CSFV in Yunnan province of China, 326 tissue specimens from suspected CSF pigs were collected from this region from 2015 to 2021 to investigate this issue. The genetic characteristics of the E2 sequences from the new CSFV strains obtained were analyzed.

MATERIALS AND METHODS

Necropsy procedures

All pigs were sacrificed and dissected in strict accordance with the relative standards [13]. Briefly, the pigs were stunned by electric shock and bled to death. The necropsy steps can be summarized as follows: observing the appearance of the corpse; opening the abdominal cavity and removing and examining the abdominal organs; opening the chest cavity and examining the chest organs. These procedures were performed by a veterinarian at different farms.

Cells and reagents

Porcine testis cells (ST) were cultured in Eagle's Minimum Essential Medium (MEM) supplemented with 5% newborn bovine serum (NBCS) free of BVDV-specific antibodies, streptomycin (100 μg/mL), and penicillin (100 IU/mL) at 37°C in a humidified 5% CO_2 incubator. The virulent CSFV Shimen, rescued C-strains, and monoclonal antibody (mAb) against the E2 protein of CSFV were kept in the authors' laboratory.

Collection and pre-treatment of specimens

From March 2015 to April 2021, 326 porcine tissue (tonsils and lymph nodes) samples from 73 pig farms with suspected CSFV infections were collected from different regions of Yunnan province, China (**Table 1**). The specimen homogenates in sterile phosphate-buffered saline underwent three freeze and thaw cycles. The supernatants were collected after being centrifuged with 10,000×g for 10 min at 4°C and stored at −80°C.

Table 1. Detection of the CSFV of the collected samples from different periods in Yunnan province

Year	Total samples	CSFV-positive samples	CSFV-positive rate	Viral isolation
2015	47	1	2.13%	-
2016	63	2	3.17%	1
2017	71	1	1.41%	1
2018	57	4	7.01%	1
2019	36	1	2.78%	-
2020	22	1	4.55%	1
2021	30	1	3.33%	-
Total	326	11	3.27%	4

CSFV, classical swine fever virus.

Detection of CSFV in clinical specimens

The viral RNA genome from the supernatants was extracted using commercial kits (TaKaRa, Japan) according to the manufacturer's protocols. The reverse transcription-polymerase chain reaction (RT-PCR) assay was conducted to detect the presence of CSFV, targeting the NS5B gene using one pair of specific primers [14]. A one-step RT-PCR reaction was performed in the following steps: 50°C for 30 min, 94°C for two minutes; 35 cycles of 94°C for 30 sec, 56°C for 30 sec, 72°C for 40 sec; followed by 7 min at 72°C. An expected DNA band of 449 bp from the PCR products in 1% agarose gel electrophoresis was observed, indicating that the sample was CSFV positive.

Virus isolation and identification of the presence of CSFV in the ST cells

The supernatants of eleven representative CSFV-positive samples were filtered through 0.22-μm filters (Merck Millipore, Cork, Ireland), inoculated on monolayer ST cells, and cultured for 72 h. Finally, the presence of infectious viruses was confirmed by RT-PCR and indirect immunofluorescence assay (IFA).

The CSFV-positive cells were proliferated further with continuous passages. At the tenth passage, the viral titers were determined by IFA using a monoclonal antibody (mAb) against the E2 protein of CSFV, as described previously [15]. In this research, FITC labeled E2 protein was purchased from MEDIAN Diagnostics Company in Korea. FITC labeled goat anti-mouse Ig G (secondary antibody) was purchased from Beijing Boaolong Immunology Technology Co., LTD. The FITC labeled sheep anti-pig secondary antibody was purchased from SBA Company.

Sequence and analysis of the partial E2 genes

RT-PCR assays were performed to amplify the partial E2 genes of eleven CSFV strains obtained in this study with the primers for E2-F: 5-GTAAATATGTGTGTGTTAGACCAGA-3′ and E2-R: 5-GTGTGGGTAATTGAGTTCCCTATCA-3′. After amplification, the purified PCR products were cloned into the pMD19-T vector (TakaRa, Japan). The positive plasmids containing the targeted sequence were confirmed by enzyme digestion identification and sent for sequencing. The nucleotide sequences of these novel CSFV strains were deposited in the GenBank database (**Table 2**).

The E2 nucleotide sequences of the eleven CSFV isolates obtained, and their corresponding reference strains from other regions/countries in the GenBank database were aligned using DNA Star version 7.0 software to analyze the genetic characteristics. A phylogenetic tree based on the E2 nucleotide sequences was constructed using the neighbor-joining (NJ) method with 1000 bootstrapping in MEGA 7.0 software.

Table 2. Detailed information on the eleven CSFV isolates collected in this study

Herd	Isolate	Place	Time	Pig group	Immunization	GenBank accession no.
1	YNWS-2021	Wenshan, Yunnan	2021.3	Nursery pig	Yes	OK169300
2	YNDL-2020*	DaLi, Yunnan	2020.11	Nursery pigs	Yes	OK169301
3	YNKM-2019	Kunming, Yunnan	2019.9	Weaned piglet	Yes	OK169302
4	YNLP-2018	Luoping, Yunnan	2018.7	Nursery pigs	Yes	MW392291
5	YNMZ-2018*	Mengzi, Yunnan	2018.6	Weaned piglet	Yes	MW392292
6	YNHP-2018	Huaping, Yunnan	2018.7	Fattening pig	Yes	MW392289
7	YNDL-2018	Dali, Yunnan	2018.3	Nursery pigs	Yes	MW392288
8	YNYS-2017*	Yongsheng, Yunnan	2017.6	Weaned piglet	Yes	MW392294
9	YNKD-2016*	Kedu, Yunnan	2016.8	Nursery pigs	Yes	MW392290
10	YNQJ-2016	Qujing, Yunnan	2016.5	Weaned piglet	Yes	OK169299
11	YNSM-2015	Songming, Yunnan	2015.8	Weaned piglet	Yes	MW392293

*Represent the strain we isolated successfully.

Ethics approval and consent to participate

All experiments related to animals in this research have been approved by Yunnan Tropical and Subtropical Animal Virus Diseases Laboratory, Yunnan Animal Science & Veterinary Institute, Yunnan, China.

RESULTS

Pathological changes

In this study, obvious pathological lesions of CSFV infection were observed from a necropsy, such as an ecchymosis on the skin (**Fig. 1A and B**), tonsil ulcers (**Fig. 1C**), petechiae of the lymph nodes (**Fig. 1D**), petechiated hemorrhage on the kidneys (**Fig. 1E**), infarction of the spleen (**Fig. 1F**), necrosis of the stomach and intestine (**Fig. 1G and H**) and button-shaped ulcers in the ileocecal valves (**Fig. 1I**).

CSFV detection

PR-PCR was performed to confirm the infection of the CSFV. **Table 1** lists the detailed detection results from 2015 to 2021. The total number of samples was 326; 11 were CSFA-positive, accounting for 3.27%. Importantly, four strains of CSFV were isolated successfully. **Fig. 2** was the agarose gel electrophoresis analysis of PCR products of partial NS5B gene sequence of CSFV strains, and positive bands with the size of 449 bp were observed.

Phylogenetic tree based on partial E2

The genetic characteristics of CSFV strains in Yunnan province were examined by amplifying and sequencing the partial E2 gene sequences of eleven novel CSFV strains in this study, which were submitted to the GenBank database. **Table 2** lists the detailed relative information of the eleven CSFV isolates collected. In addition, the neighbor-joining phylogenetic tree based on the E2 gene nucleotide sequences was constructed. As shown in **Fig. 3**, only one CSFV strain (YN-DL/2020) belonged to subgenotype 2.1d. The remaining ten (YNWS-2021, YNKM-2019, YNLP-2018, YNMZ-2018, YNHP-2018, YNDL-2018, YNYS-2017, YNKD-2016, YNQJ-2016, and YNSM-2015) prevalent in Yunnan province, obtained in this study, were clustered into subgenotype 2.1c.

Proliferation characteristics of the CSFV isolated

The proliferation characteristics of these CSFV strains from Yunnan province were investigated by inoculating the monolayer ST cells with the supernatants of the CSFV-positive specimens.

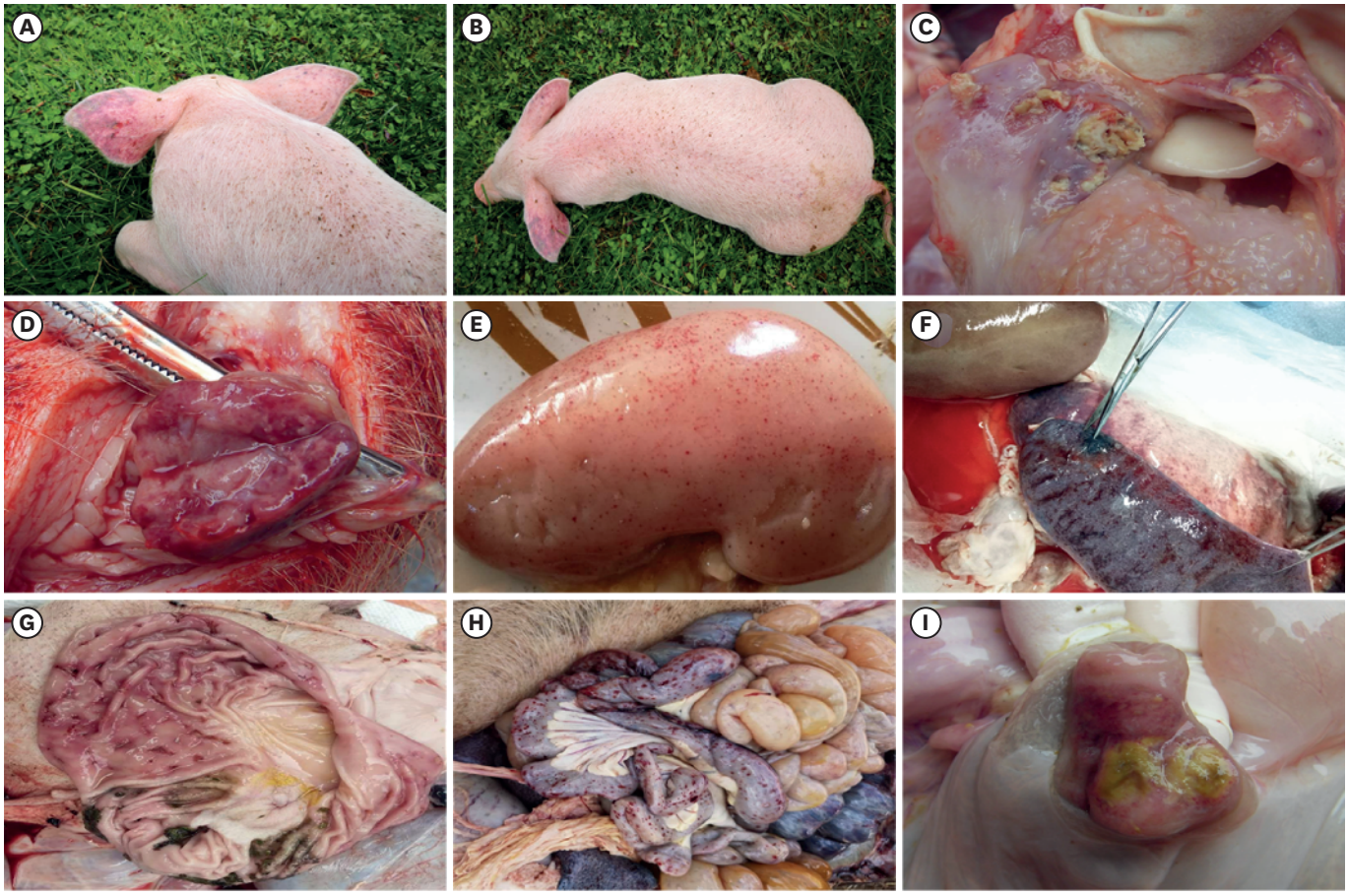

Fig. 1. Typical clinical and pathological signs were observed in CSFV-infected pigs, such as bleeding points on the skin (A, B), tonsil ulcers (C), welling and hemorrhage of lymph nodes (D), hemorrhagic spots on the kidneys (E), infarction of spleen (F), necrosis of intestine (G, H) and button-shaped ulcers in the ileocecal valves (I).
CSFV, classical swine fever virus.

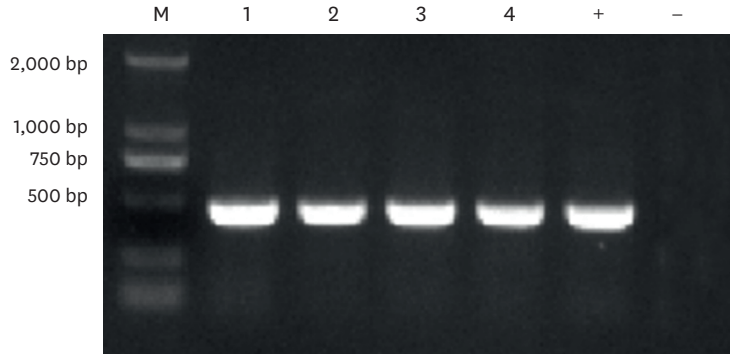

Fig. 2. Agarose gel electrophoresis analysis of PCR products of partial NS5B gene sequence of CSFV strains. "+" and "–" were regarded as positive and negative controls during PCR amplification, respectively. 1–4 represents the samples from CSFV-infected pigs.

Finally, four CSFV strains (YNDL-2020, YNKD-2016, YNMZ-2018, and YNYS-2017) were isolated successfully. The unique fluorescence signal of the CSFV was visualized using the mouse monoclonal antibodies against the E2 protein of CSFV in an IFA assay (**Fig. 4**). In addition, the cell-adapted viruses after 15 passages were obtained with infectious titers of $10^{6.75}$, $10^{6.375}$, $10^{7.875}$, and $10^{5.75}$ $TCID_{50}$/mL, respectively.

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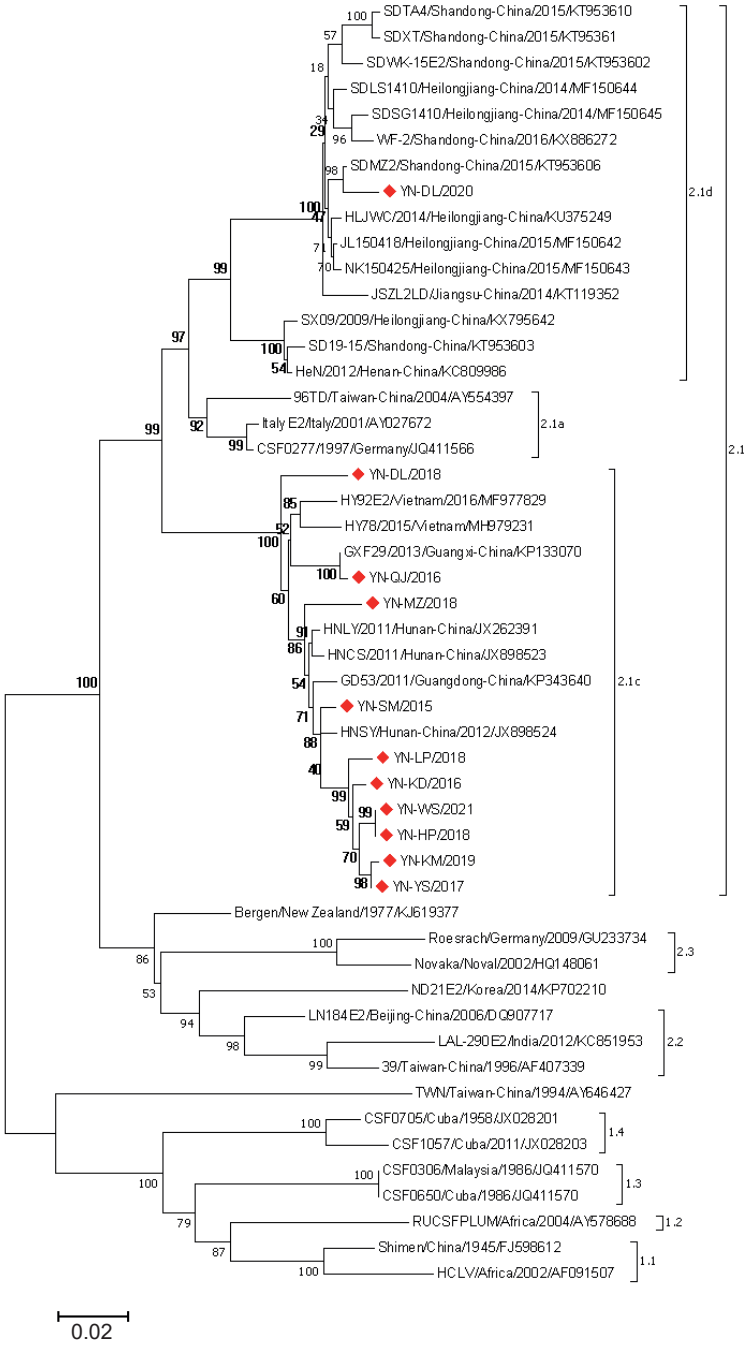

Fig. 3. Phylogenetic tree based on the partial *E2* gene sequence of the CSFV strains obtained in this study and available in the GenBank database generated by the neighbor-joining method in MEGA 7.0 software. The black and red diamonds represented CSFV strains obtained here and vaccine strains, respectively.
*Represents the strain isolated successfully.

DISCUSSION

A CSFV infection causes severe clinical symptoms in pigs, including hyperpyrexia, vomiting, constipation or diarrhea, and cyanosis or bleeding points on the skin. Indeed, noticeable pathological lesions (**Fig. 1**) were observed in the necropsy. In this study, only eleven samples

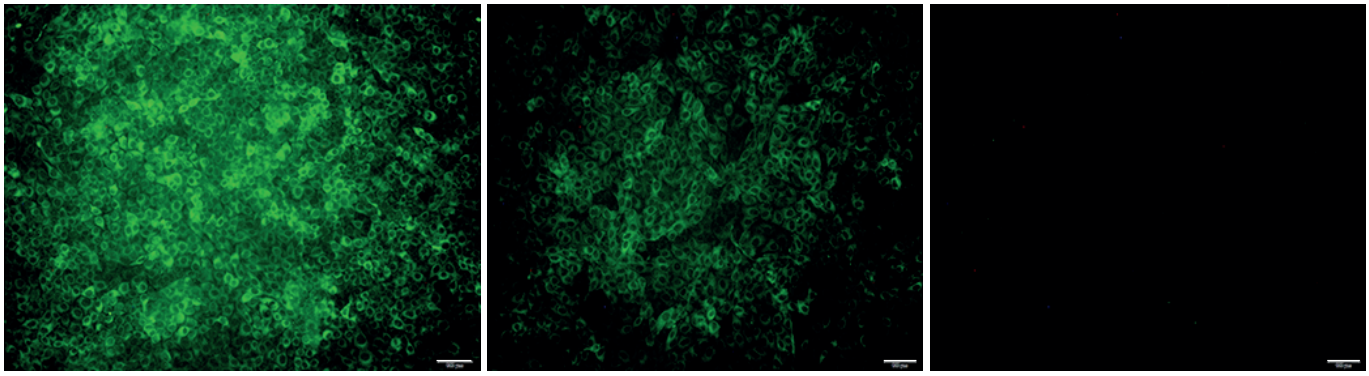

Fig. 4. Indirect immunofluorescent assay for the detection of the CSFV in ST cells using anti-CSFV *E2* primary antibodies. Scale bar = 100 μm.
CSFV, classical swine fever virus.

were confirmed to be CSFV-positive by RT-PCR (**Fig. 2**, **Table 1**), yielding an average prevalence of 3.37% (11/326), which is lower than those in Shandong province (11.1%, 188/4,866) [16], and other regions of China, including Henan, Hebei, Heilongjiang, and Liaoning provinces (6.0%, 21/350) [17]. Therefore, these results suggest that despite the low epidemic tendency of CFSV in Yunnan province, this pathogen is widespread in China. This phenomenon can be explained. First, different provinces have different epidemic prevention and control policies; the comparative lower positive rate in Yunnan province might be related to the better prevention rules and vaccination. More importantly, Yunnan is a mountainous province covered by extensive forest areas providing an excellent natural barrier in Yunnan province, which is beneficial for the epidemic prevention. Many farms in Yunnan province are located at some places with poor transportation systems and the farms are sparsely distributed.

The CSFV strains prevalent in China mainly belong to genotype 2, which could be divided further into three subgenotypes, namely 2.1, 2.2, and 2.3. Subgenotypes 2.2 and 2.3 are less prevalent in China than subgenotype 2.1 [2], which can be divided further into 2.1a, 2.1b, 2.1c, and 2.1d. In particular, the prevalence of subgenotypes 2.1 strains in different regions of China presents high diversity [4,6,9]. For example, subgenotype 2.1d of the CSFV is mainly dominant in the Shandong province of China [18-20], while CSFV strains circulating in Guangdong province of China belong to subgenotype 2.1c [6]. This study examined the genetic characteristics of the CSFV strains. There were two subgenotypes (2.1c and 2.1d) of CSFV prevalent in Yunnan province, China, with subgenotype 2.1c being predominant (**Fig. 3**, **Table 2**), which is similar to Guangdong province.

The lengths of the amplified E2 gene sequences of the CSFV strains obtained in this study were 1343 bp, encoding 435 amino acids. Further genetic analyses showed that the eleven strains displayed identities of 89.0–100.0% and 95.0–100.0% at the nt and aa levels, respectively. In addition, the E2 nucleotide sequences of eleven CSFV strains obtained in this study were compared with six reference strains, including Shimen (1.1), 96TD (2.1a), HNLY (2.1c), SDMZ2 (2.1d), LAL-290E (2.2), and Novaka (2.3). The results showed that the eleven new strains shared 90.0–98.7% and 89.6–99.0% identity with subgenotypes 2.1c and 2.1d, respectively. Moreover, they exhibited 85.1–93.0% sequence identity with other subgenotypes 2 and 3 isolates, including 2.1a, 2.1b, 2.2, and 2.3. On the other hand, these new strains displayed lower sequence identity with subgenotype 1.1 (Shimen), 82.2–83.0%, suggesting that all CSFV strains had different genetic relationships with vaccine strains.

The proliferation characteristics of these CSFV strains from Yunnan province were investigated. Four CSFV strains were isolated and were specially recognized by the IFA assay (**Fig. 4**), demonstrating that the CSFV collected can proliferate well in the monolayer ST cells. Intriguingly, the viral titer of the YNMZ-2018 strain was ten times higher than these of the other strains. SA sequence comparison showed that the YNMZ-2018 strain contained a series of unique amino acids substitution in the E2 protein, such as the T to I change at the position of 49. Moreover, K71R, D97G, K174N, N192S, and T197S were also identified in this novel strain. Nevertheless, whether these amino acid substitutions would influence the proliferation characteristics of CSFV strain in vitro needs further investigation.

Historically, owing to the extensive application of effective vaccines against CSF in Chinese pig populations, this infectious disease appears to have been eradicated in some regions of China. Moreover, prevention has been neglected because of the occurrence or prevalence of other infectious diseases, such as porcine reproductive and respiratory syndrome, porcine circovirus disease, and porcine epidemic diarrhea. In the current research, a low prevalence of CSFV (3.37%, 11/218) in pigs was confirmed in Yunnan province of China, even though these pigs had been vaccinated against CSF. This suggests that this infectious disease should not be neglected, and corresponding measures for the prevention of CSF need to be conducted. Further genetic analyses showed that all CSFV strains obtained here belonged to subgenotypes 2.1c and 2.1d, with 2.1c being the dominant subgenotypes in China's Yunnan province.

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