Virulence evaluation of classical swine fever virus subgenotype 2.1 and 2.2 isolates circulating in China

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ABSTRACT

Classical swine fever (CSF) remains an important pig disease in China, where it usually presents with mild or atypical clinical manifestations, with large scale outbreaks rarely seen. This has led to speculation about the possible circulation of viral strains of low virulence. To investigate this possibility, five field isolates within the predominant genotype 2 (2.1b, 2.1c, 2.1 h and 2.2) were evaluated and compared by experimental infection of naturally farrowed but colostrum-deprived piglets. All infected piglets displayed clinical signs, including persistent high fever, depression, anorexia, dyspnea, conjunctivitis, constipation, and hesitant gait. Typical pathological lesions, including pulmonary edema, hemorrhagic or cellulosic exudation, and swelling and hemorrhage of lymph nodes, were observed. Viremia and E^{rns} protein expression in the blood of all infected animals were detectable from 3 to 5 days post infection (DPI), their presence correlating with the onset of fever, clinical signs and leukopenia. E2 antibody did not develop in any of the field CSFV-infected piglets during the disease course, while E^{rns} antibody was detectable in 4–56% of infected animals at various time points. Mortalities ranged from 20 to 80% within 21 DPI, progressing to 100% by 43 DPI. Based on clinical scores and fatalities within 21 DPI, 2 of the 5 field isolates were classified as of moderate virulence and 3 of high virulence; *i.e.*, no field isolates of low virulence were identified. The study has provided data supporting the use of these isolates as challenge viruses to evaluate the efficacy of current CSF vaccines.

1. Introduction

Classical swine fever (CSF) is an OIE notifiable swine disease causing significant economic and sociological impact worldwide. The causative agent is CSF virus (CSFV), which belongs to the genus *Pestivirus* within the family *Flaviridae* together with bovine viral diarrhea virus (BVDV) and border disease virus (BDV) (Simmonds et al., 2017). Typical clinical signs and pathological lesions of CSFV-infected pigs are high fever, leukopenia, extensive hemorrhage, convulsion, and constipation or diarrhea (Moennig et al., 2003). While the disease has been endemic in China for about a century, current epidemics are sporadic with only mild and atypical symptoms (Luo et al., 2014).

Suckling and weaning pigs are currently the main affected populations, while asymptomic sows with CSFV persistent infection are a major transmission source (Wang and Tu, 2015). This has raised the question as to whether CSFV field strains of low virulence are circulating in China, which has prompted our evaluation of the virulence and pathogenicity of current field CSFVs.

Worldwide, CSFVs are divided into three genotypes (1, 2, and 3), 11 subgenotypes (1.1–1.4, 2.1–2.3 and 3.1–3.4) (Paton et al., 2000; Postel et al., 2012, 2013). In China in the 1990s there existed 4 subgenotypes (1.1, 2.1, 2.2, and 2.3) with 2.1 and 2.2 being dominant (Sun et al., 2001). However, in recent decades, subgenotypes 2.3 and 2.2 have become silent, while subgenotype 2.1 has played a predominant role in

outbreaks in the country. Increasingly, studies have shown that this subgenotype rapidly evolves and has generated considerable genetic diversity with 10 sub-subgenotypes (2.1a-2.1 j) identified up to date (Chen et al., 2008; Jiang et al., 2013; Gong et al., 2016a). Of these, subgenotypes 2.1b, c and h are dominant, and subgenotype 2.1b is currently the most widely prevalent in China as well as in some neighboring countries, including Korea, Mongolia and Russia (Gong et al., 2016a).

Although CSF has been endemic in China since the early 20th century, virulence of field CSFVs has not been systematically evaluated to understand their pathogenicity. A decade ago, experimental infections to evaluate the virulence of field CSFVs were reported in Europe and other areas, in which 13 CSFV field isolates collected between 1945 and 2007 (subgenotype 1, 3; subgenotype 2.3, 7; subgenotype 2.2, 1; subgenotype 2.1, 2) were characterized as moderately or highly virulent based on clinical signs and pathological lesions (Mittelholzer et al., 2000; Floegel-Niesmann et al., 2003, 2009). In east Asia, virulence of two field subgenotype 2.1b isolates from south Korea and Mongolia were recently evaluated. Results showed that the south Korean isolate, from a wild boar, caused death of all experimentally infected domestic pigs by 16 DPI, but pigs infected with the Mongolian isolate displayed no overt clinical signs and all survived the challenge during a 21-day observation period (Enkhbold et al., 2017; Lim et al., 2017). Most recently in China two laboratories have reported the virulence evaluation of two field CSFV isolates of subgenotype 2.1b from Heilongjiang province (Luo et al., 2017; Zhang et al., 2018). Based on clinical scores results showed that both isolates were moderately virulent; however, other factors associated with virulence and pathogenicity, such as leucopenia, viremia, E[rns] protein and antibody levels as well as E2 antibody in serum were not analyzed, and the different isolates were not compared. The results therefore cannot be considered to reflect the overall situation in the country. To provide a more detailed assessment, the present study was conducted to systematically characterize and compare the virulence and pathogenicity of predominant subgenotype 2 CSFVs presently circulating in China.

2. Materials and methods

2.1. Virus strains

Five CSFV field viruses isolated from sporadic outbreaks in weaning piglets in China were used for the study: two subgenotype 2.1b viruses (HuB16 and JL23) respectively isolated from Hubei and Jilin provinces in 2015; one subgenotype 2.1c virus (GD23) from Guangdong province in 2017; one subgenotype 2.1 h virus (HuB12) also from Hubei province in 2015 and one subgenotype 2.2 virus (AH1) from Anhui province in 1999. Full-length E2 genes of these isolates were amplified and sequenced with methods previously described (Gong et al., 2016a), and the obtained sequences have been deposited in GenBank under the accession numbers MK691765-MK691769. These isolates were cultured in PK-15 cells as described previously (Gong et al., 2016b), and passage 6 of HuB16, passage 14 of JL23, passage 12 of GD23, passage 10 of HuB12, and passage 14 of AH1 were used for the evaluation. Previous studies utilizing sequencing and analysis of full-length E2 and E[rns] genes, had shown that high genetic stability was maintained in such isolates during adaptation to PK-15 cells (Gong et al., 2016b). Blood stock of the highly virulent Shimen strain of CSFV (passage F115) was used as a positive control. All isolates had been tested and found free of contamination with BVDV, PRRSV, PCV2, PCV3, PRV, and PPV by RT-PCR or PCR (Gong et al., 2016b; Sun et al., 2018).

2.2. Animal experiments

Thirty-four naturally farrowed, colostrum-deprived piglets at 45-days old were housed in the negative pressure facility of Tecon Biology Joint Stock Company Ltd. (Urumqi, China). They were randomly divided into 5 experimental groups (n = 5), 1 negative control (NC, n = 5, mock-infected), and 1 positive control (PC, n = 4, infected with Shimen strain). All piglets were allowed free access to standard diet and water. Prior to the experiments, all were tested and found to be CSF antibody negative by IDEXX ELISA kit (see Section 2.4) and CSF virus as well as other swine viruses (BVDV, PRRSV, PCV2, PCV3, PRV, and PPV) by RT-PCR or PCR (Gong et al., 2016b; Sun et al., 2018). Piglets were given an intramuscular injection of 10^5 $TCID_{50}$ CSFV field isolates or Shimen strain. The animal study plan was reviewed and approved by the Experimental Animal Use and Care Committee, Academy of Military Medical Sciences.

2.3. Evaluation of clinical signs and pathological lesions

Rectal temperatures and clinical signs in the experimental piglets were recorded daily. The clinical score (CS) system developed by Mittelholzer et al. (2000) was used with a minor modification. Ten parameters were measured: liveliness, body tension, body shape, breathing, walking, skin, eyes, appetite, defecation and fever, and graded according to the following scoring system: 0, normal; 1, slightly altered; 2, showing distinct clinical symptoms; and 3, showing severe CSF symptoms. Fever was scored as follows: 0 ($\leq 39.5°C$), 1 (39.6–40.0°C), 2 (40.1 − 40.9°C) and 3 ($\geq 41.0°C$). The mean clinical score was derived from the highest score of each piglet within a group during a 21-day observation period. Case fatality was defined by numbers of deaths at 21 DPI as in the previously described scoring system (Floegel-Niesmann et al., 2009): no deaths (0), 1–40% (1), 41–80% (2) and > 81% (3). Points scored in case fatalities of individual groups were combined with the mean clinical score of the corresponding group to get modified clinical score (mCS), which was used to define the virulence of CSFV field isolates as follows: high virulence, mCS $\geq$ 16; moderate virulence, 6–16; low virulence, < 6.

Piglets who were dead or moribund, following euthanization in the latter case, were subjected to autopsy for pathological scoring as previously described by Floegel-Niesmann et al. (2009). Observations were made on skin, subcutis and serosae, tonsils, spleens, kidneys, lymph nodes, ileum and rectum, and lungs, and scored as 0–3 points according to the severity.

2.4. Sample test and analysis

Five ml blood samples were taken from each infected and control piglets for hematological, virological and serological examinations at time intervals up to 21 DPI. Leukocyte counts were determined using a Mindray BC-2800 Vet analyzer (Mindray Medical USA Corp, Mahwah, USA). The anti-clotted blood samples were also subjected to real time reverse transcription polymerase chain reaction (qRT-PCR) as previously described (Yang et al., 2015). CSFV E2 antibody and E[rns] protein in the sera were determined using the CSFV Antibody Test kit and the CSFV-Ag/Serum kit (IDEXX, Switzerland) according to the manufacturer's instructions. E[rns] antibody was determined with the *Pigtype* CSFV E[rns] ELISA Kit (QIAGEN, Germany). Neutralizing antibody titers were determined as previously described (Floegel-Niesmann et al., 2009) and expressed as ND_{50}.

3. Results

3.1. Clinical signs observed in CSFV-infected piglets

There was a 3 day incubation period before piglets infected with the CSFV field isolates developed clinical symptoms. Rectal temperatures were > 40°C at 3 or 4 DPI and remained between 39.7°C and 41.6°C during the entire observation period (Fig. 1A). The dynamics of rectal temperatures of animals infected with different field isolates were distinct with highest temperatures noted in the JL23-infected group (40.8–41.6°C between 5 and 21 DPI) (Fig. 1A). All piglets infected with
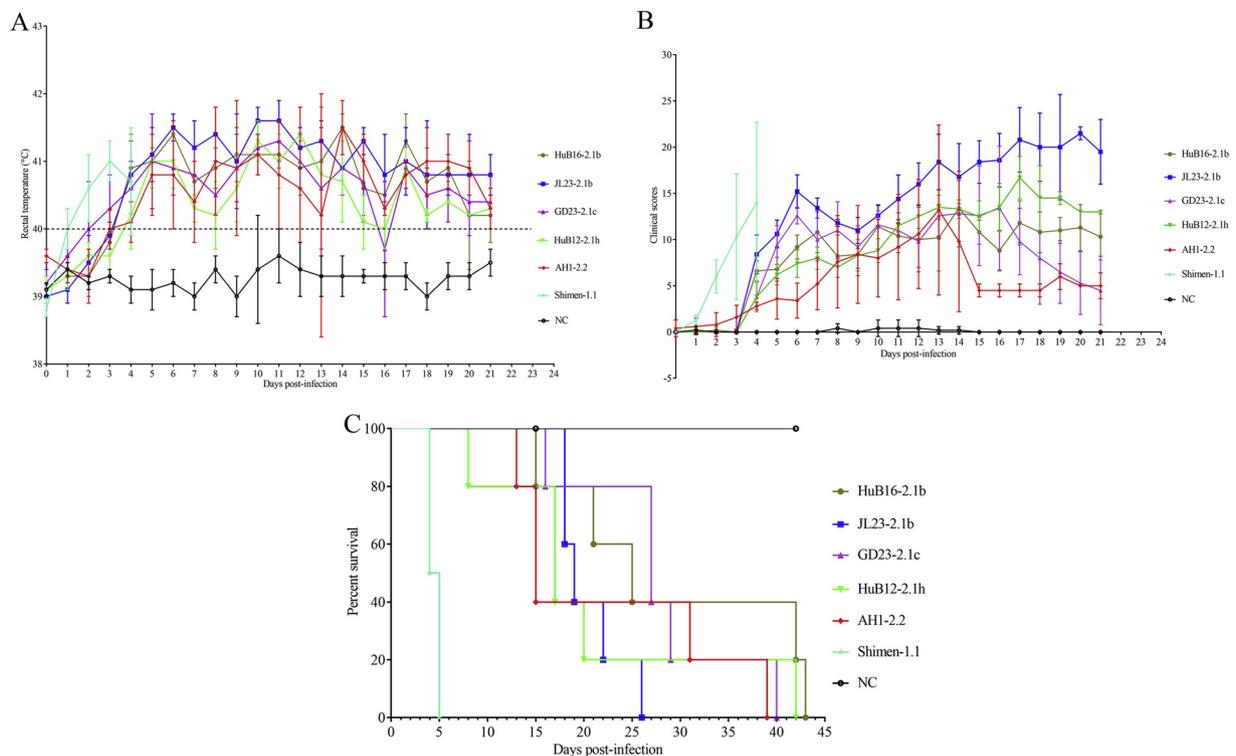
Fig. 1. Piglets infected with CSFV field isolates displayed high fever, typical clinical signs and all died from 8 to 43 DPI. A, rectal temperatures over 21 DPI. B, clinical scoring within 21 DPI (consisting of appetite, liveliness, breath, eyes, walking, body tension, body shape, defecation, skin and fever). C, mortality of animals in the 5 field isolate groups and 2 control groups. About 70% of infected piglets died between 14 and 28 DPI, and all died by 43 DPI.

the Shimen strain developed fever more rapidly and reached 40℃ at 1 DPI and 41℃ at 3 DPI. In contrast, all mock-infected pigs maintained normal body temperatures without significant fluctuations (39.0–39.6℃).

Clinical signs in infected piglets were depression, anorexia, conjunctivitis, constipation, cough, stiffness and bent back, and hesitant gait, which became more severe with disease progression (Fig. 1B). The severity of the clinical signs varied in piglets either within or between groups. JL23-infected animals generally displayed higher severity than the other 4 groups, gaining higher clinical scores as shown in Fig. 1B. Of note, watery diarrhea occurred following constipation in 4 of 5 JL23-infected piglets from 10 DPI, which was also observed in 1 of 5 HuB16- and 1 of 5 GD23-infected piglets. Skin hemorrhages were observed in the hips in 4 of 5 GD23-infected animals, or around the edge of the ears in 1 of 5 HuB12- and 1 of 5 AH1-infected piglets, or over the entire body in 1 of 5 JL23-infected piglets. The remaining piglets displayed only minor clinical signs prior to becoming moribund or dying, with clinical scores in groups GD23 and AH1 decreasing from about 15 DPI onward. In Shimen strain-infected animals the disease developed so rapidly that all 4 infected piglets died at 5 DPI without development of typical signs; consequently, the clinical scores of this group were not as high as those of field isolates. Mock-infected piglets did not show any clinical signs.

Although all field isolate-infected piglets died by 43 DPI, case fatalities varied within the experimental groups during the 21-day observation period. As shown in Fig. 1C, compared with the Shimen strain group, the times of death of the field virus-infected animals were significantly delayed with the earliest observed at 8 DPI in the HuB12-infected group, in which 4/5 piglets were dead by 21 DPI, while in the other four groups only 1–3 infected piglets in each group had died. According to the mean clinical scores and fatalities, therefore, strains JL23, HuB12 and AH1 were defined as highly virulent, with HuB16 and GD23 as moderately virulent (Table 1).

Table 1
Classification of virulence of the analyzed CSFV field isolates in this study.

Strain	Mean clinical score (21 DPI)	Case fatality (21 DPI)	Modified clinical score	Virulent
HuB16-2.1b	13.8	1 (40%)	14.8	moderately
JL23-2.1b	23.2	2 (60%)	25.2	highly
GD23-2.1c	14.2	1 (20%)	15.2	moderately
HuB12-2.1 h	15.0	2 (80%)	17.0	highly
AH1-2.2	16.2	2 (60%)	18.2	highly
Shimen	13.8	3 (100%)	16.8	highly
NC	–	–	–	–

Table 2
Mean pathological scores of piglets infected by CSFV subgenotype 2.1 and 2.2 isolates in each group.

Parameter	HuB16-2.1b	JL23-2.1b	GD23-2.1c	HuB12-2.1 h	AH1-2.2
Skin	0.3	1.4	1.3	1.3	0.8
Subcutis and serosae	0.3	0.6	1.3	0	0.5
Tonsil	1.7	1.2	1	1.3	0.3
Spleen	1.3	1.2	0.8	1	0.5
Kidney	0.3	2	2	0.8	2
Lymph nodes	2	2	2.8	2.5	2
Ileum and rectum	1.3	2.2	0.8	2.8	0
Lung	3	3	2.5	2	2

3.2. Pathological lesions in CSFV-infected piglets

Of all pathological parameters assessed respiratory lesions, particularly pulmonary edema and hemorrhage or cellulosic exudation, were the mostly frequently observed (Table 2). Swelling and hemorrhage of lymph nodes were also commonly found. Two piglets in each infected group displayed hyperemia and necrosis of their tonsils.

Multiple kidney petechiae, the typical pathological lesions of CSF, were observed only in a few animals infected with JL23 (2/5), GD23 (1/5), and AH1 (1/5). In addition, pinpoint hemorrhages in the kidneys were also not observed in most infected animals. However, large blood clots were found in the stomach of AH1 (1/5) and HuB12 (4/5)-infected animals, and blood was apparent in the stool of HuB12 (2/5) and GD23-infected piglets (1/5). Multiple petechiae were also observed in the serosa of the stomachs of animals infected with HuB16 (2/5), JL23 (2/5) and GD23 (1/5). Button-shaped ulcers were found in the large intestine of one piglet of the JL23 group, while spleen infarctions were not found in any.

3.3. Leukopenia, viremia and E^{rns} levels in CSFV-infected piglets

Leukopenia was observed in all infected piglets from 3 DPI to the end of the study, and different groups showed similar dynamic changes of leukocyte depletion. At 3 DPI, leukocyte counts decreased from 13.0 to 16.9×10^9/l to 7.84–15.3×10^9/l (2.1–50.3% depletion) in the five experimental groups, with further depletion at 7 DPI (29.2–55.6%, 6.74–11.00×10^9/l) and 21 DPI (28.6–72.9%, 4.57–9.3×10^9/l). Shimen strain-infected piglets did not develop significant leukocyte depletion (a decrease of only 8.4% at 3 DPI, before death within 5 DPI). Leukocyte counts of mock-infected piglets remained within the healthy range.

Corresponding to the leukopenia, viremia detected by qRT-PCR was observed in all CSFV-infected animals and lasted for the entire observation period, although the onset time differed between the groups. At 3 DPI, viral loads in blood were detected in all piglets of the HuB12 and AH1 groups and in 3/4 piglets of the Shimen control ($10^{3.35-7.03}$ genome copies/ml), but only in 1/5, 1/5 and 3/5 piglets of the HuB16, JL23 and GD23 groups respectively ($10^{1.83-4.30}$ genome copies/ml) (Fig. 2A). At 5 DPI, there was a rapid elevation of viremia in all infected piglets. Viremia following HuB12 and AH1 infection reached a peak at 10 DPI, earlier than in the other 3 groups (Fig. 2A). It is worth noting that the Shimen strain group had much higher viral load at 3 and 5 DPI than all the field isolates (Fig. 2A), indicating that the Shimen strain replicated more rapidly *in vivo,* resulting in peracute infection and 100% death within 5 days.

The appearance of E^{rns} protein in the blood of most infected piglets correlated with the onset of viremia, being detected in all 5 field isolate groups as early as the onset of viremia at 3 DPI although only in a few piglets and at levels lower than the positive threshold (Fig. 2B). At 5 DPI E^{rns} was present in all animals of the HuB16, JL23, HuB12 and GD23 groups. E^{rns} protein appeared earlier in the Shimen group and later in the AH1 group. Levels of E^{rns} in the blood of all infected pigs were maintained over the entire 21-day observation.

3.4. Development of CSFV E2 and E^{rns} antibodies in infected animals

As determined by E2-based antibody ELISA (IDEXX, Switzerland), the antibody response against E2 was negative in all CSFV-infected piglets (Fig. 3), which was confirmed by neutralization tests showing NT titers < 5 ND_{50} at 14 DPI. In contrast, E^{rns} antibody was detected as early as 10 DPI (Fig. 4), about a week following the appearance of E^{rns} protein and viremia, but it was transient with positive rates of only 4–56% between 10–21 DPI (Table 3). In detail, E^{rns} antibody was detected at 10 DPI in 1 piglet of the JL23 group, then in 5 piglets of 4 groups (HuB16, JL23, GD23 and HuB12) at 14 DPI, and in 10 at 17 DPI. None of the piglets in the AH1-infected group developed measurable E^{rns} antibody (Fig. 4).

4. Discussion

Nation-wide compulsory vaccination strategy with C-strain plays a critical role in prevention and control of CSF in the pig industry of China, and vaccination coverage > 90% has resulted in effective control of the disease with large scale outbreaks only rarely occurring (Sun et al., 2001; Chen et al., 2008; Ji et al., 2015). However, vaccination alone has not eliminated the disease and sporadic outbreaks still occur in some vaccinated pig farms, where sows with persistent CSFV infection are considered the main viral transmission source to suckling and weaned piglets. Previous studies showed that 11.12% of tonsils from 21,014 sows collected from 29 pig farms in 13 Chinese provinces were CSFV positive, with all 29 farms having sows with positive rates of 4.4–30.1% (Qiu, 2004). Further study showed that C-strain vaccination was unable to prevent the mortality of congenital infected piglets because of induction of insufficient protection immunity (Ning et al., 2004), although this modified live vaccine can provide complete protective immunity against various CSFV genotypes (Huang et al., 2014). Currently CSF cases in the vaccinated swine herds were frequently reported (Hu et al., 2016), and the five CSFV field isolates evaluated in the present study were also collected from weaned piglets in C-strain vaccinated swine farms, where CSFV carries sows were one of the main reasons of vaccination failure.

Since CSF has been enzootic in China for several decades, the genetic diversity and evolutionary dynamics of field CSFVs have been altered over time. In the 1990s, CSF subgenotypes 2.1 and 2.2 were almost equally predominant (Sun et al., 2001); however, subgenotype 2.1 has become the most dominant since 2000, causing > 87% CSF cases. Moreover, subgenotype 2.1 viruses have likely evolved more rapidly than other subgenotypes and have generated ten sub-sub-genotypes (2.1a-j) so far, of which subgenotypes 2.1b, c and h predominate in China (Chen et al., 2008; Jiang et al., 2013; Gong et al., 2016a). Although CSFV diversity and evolution in China have been well documented, virulence of CSFV field isolates over the last few decades has not been systematically characterized. The results of the present
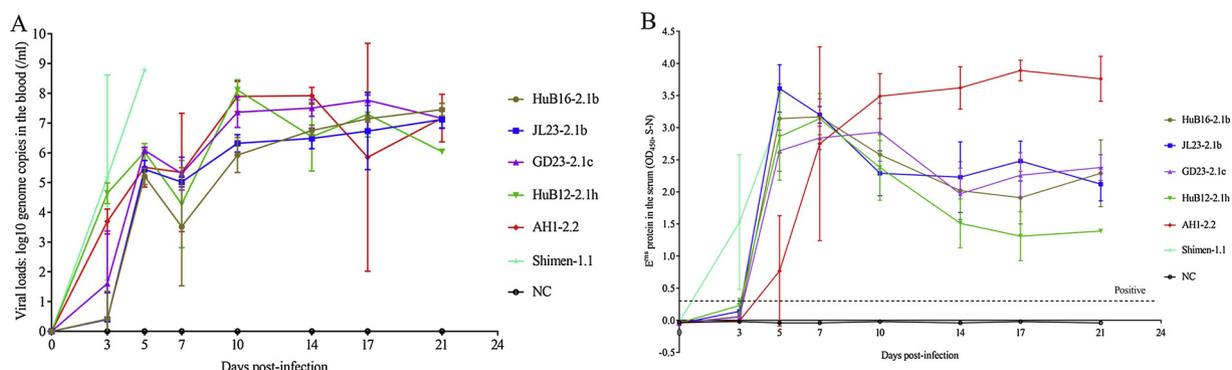

Fig. 2. Viral RNA loads (A) and E^{rns} protein levels (B) in the blood of all infected and control animals within 21 DPI. The positive threshold of E^{rns} protein levels is $OD_{450} \geq 0.3$ (S-N).
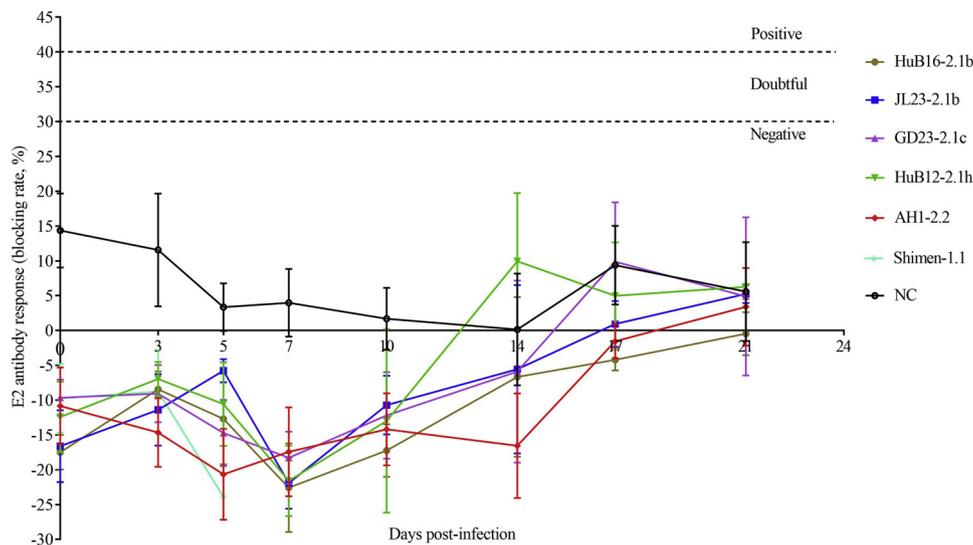
Fig. 3. E2 antibody response of all infected and control animals within 21 DPI. A blocking rate of at least 40% is associated with protection.
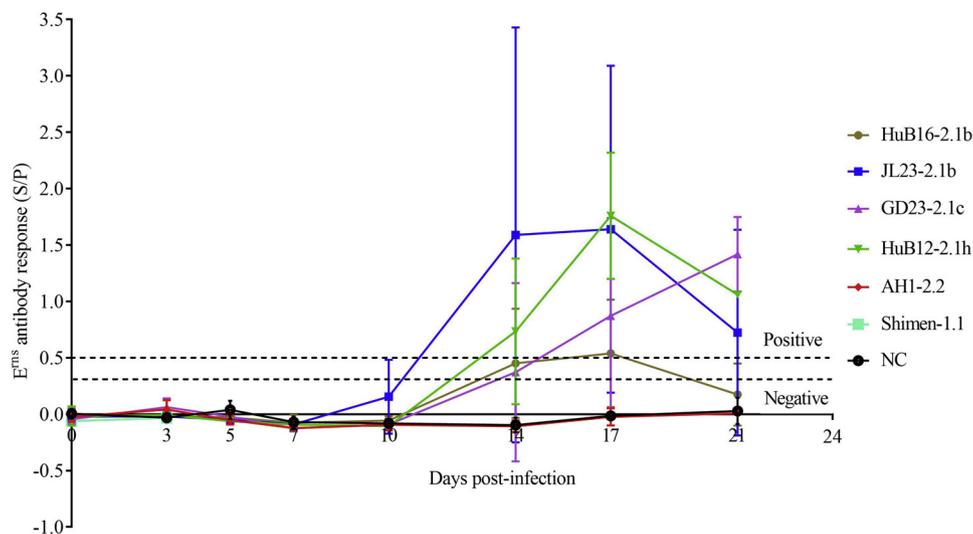

Fig.4. E^{rns} antibody response of all infected and control pigs within 21 DPI. The positive threshold of Erns antibody levels is 0.5 (S/P).

Table 3
E^{rns} antibody response in piglets after infection with CSFV field isolates[a].

Group	0 DPI	3 DPI	5 DPI	7 DPI	10 DPI	14 DPI	17 DPI	21 DPI
HuB16-2.1b	0/5	0/5	0/5	0/5	0/5	2/5	2/4[b]	0/3[b]
JL23-2.1b	0/5	0/5	0/5	0/5	1/5	3/5	3/5	1/2[b]
GD23-2.1c	0/5	0/5	0/5	0/5	0/5	1/5	2/4[b]	4/4[b]
HuB12-2.1 h	0/5	0/5	0/5	0/5	0/4[b]	1/4[b]	3/3[b]	1/1[b]
AH1-2.2	0/5	0/5	0/5	0/5	0/5	0/4[b]	0/2[b]	0/2[b]
Shimen-1.1	0/4	0/4	0/1[b]	–	–	–	–	–
Positive rate	0	0	0	0	4%	30%	56%	50%

[a] Criteria of positive E^{rns} antibody is S/P ≥ 0.5 (OD$_{450}$) based on the kit introduction, and the S/P values of the positive samples were.0.56–3.56.
[b] Deaths were excluded.

study of 5 field isolates have contributed to our understanding about the virulence and pathogenicity of current CSFVs in China. As shown here, infected pigs developed typical signs and pathological lesions at the herd level, but significant individual variation existed in infected pigs. Only a few typical signs and pathological lesions were observed, with few consistently seen on infected animals, thereby presenting difficulties in making a clinical diagnosis. This explains why only atypical CSF is commonly observed in field studies. Diagnosis according to

clinical signs and pathological symptoms may therefore result in a faulty diagnosis, leaving laboratory diagnosis necessary for confirmation of the cases. Since a 21-day observation period was used in previous studies for CSFV challenge experiments (Floegel-Niesmann et al., 2009; Luo et al., 2017 ; Lim et al., 2017), this regimen was also used in our study, with the result that the 5 field isolates were characterized as 3 highly and 2 moderately virulent strains. However, to rule out the possibility that any diseased pig might recover or form a persistent infection the observation time was extended. Since all infected animals were dead by 43 DPI, it is clear that these outcomes did not occur.

It has been reported that genetic typing of CSFV does not correlate with antigenic typing (Kosmidou et al., 1995; Nishimori et al., 1996; Zhu et al., 2009), and we found in the present study that genetically close CSFV field isolates showed distinct differences in virulence and clinical manifestations. The moderately virulent HuB16 and highly virulent JL23 grouped closely together in subgenotype 2.1b sharing 99.1% nt and 100% aa full E2 gene identity respectively, but caused different clinical symptoms in infected pigs. In previous studies, subgenotype 2.1b isolates HLJZZ2014 and HLJ1 shared 99.0–99.4% nt and 99.2–99.7% aa full E2 gene identities respectively with our isolates HuB16 and JL23, but were defined as moderately virulent (Luo et al., 2017; Zhang et al., 2018). The clinical scores of HuB16-infected piglets

(13.8) were comparable to that of HLJ1-infected pigs (13), but higher than that of HLJZZ2014-infected pigs (6). In contrast, JL23 infection caused more severe clinical signs and obtained the highest score (23.2). Onset of fever in piglets infected with HuB16, JL23 or HLJ1 (4 DPI) was also earlier than with HLJZZ2014 (7 DPI) although mortalities due to these four isolates by 21 DPI were similar (HLJZZ2014, 2/5; HLJ1, 2/3; HuB16, 2/5; JL23, 3/5).

Viremia is an important characteristic of CSFV infection (Blome et al., 2017). In addition, glycoprotein E^{rns} of CSFV is a virulence determinant associated with pathogenicity. This protein is secreted into the serum of infected animals and the culture supernatant of infected cells (Rümenapf et al., 1993), and induces apoptosis in lymphocytes resulting in leukopenia and immunosuppression (Bruschke et al., 1997). Currently there is a poor understanding of the correlation of parameters such as the dynamics of viremia and E^{rns} expression, and even of E2 and E^{rns} antibody levels in blood, with CSFV virulence and pathogenicity in pigs. In our study five field isolates produced considerable levels of viremia and E^{rns} protein in blood lasting for the entire 21 days of observation (Fig. 2), but the standard deviation among piglets in each group of these two parameters was high. Moreover, the viremia and E^{rns} protein in the blood of Shimen-infected piglets developed earlier and to higher levels than in the field isolate groups. Piglets in the Shimen group developed fever and clinical signs as early as 1 DPI without a significant incubation period, resulting in peracute death within 5 DPI (Fig. 1), which made it impossible to evaluate the viremia, E^{rns} protein and antiviral antibodies at 5 DPI and later. In comparison, the 5 field isolate groups had 3 days incubation before developing fever and clinical signs, concurrent with the appearance of viremia and E^{rns} protein in their blood which rapidly increased over the next days along with the disease progression. These results indicate that the onset of fever and clinical manifestations, or the virulence and pathogenicity of CSFV, were correlated with viremia and E^{rns} levels in the blood.

Shimen strain is a well-known Chinese challenge strain, and usually used as blood stock. This strain was obtained through *in vivo* adaption to susceptible pigs for > 100 passages, which process greatly increased its virulence and infectivity for pigs and made it one of the most virulent CSFVs in existence (unpublished results from Prof. Hongwu Lang in China Institute of Veterinary Drug Control). In the present study the Shimen strain showed extreme virulence, with a very rapid onset of high fever and clinical signs compared with field isolates, all four Shimen-infected pigs showing fever and clinical signs from 1 DPI, followed by a rapid increase and higher levels of viral load and E^{rns} expression in blood. In addition, all 4 Shimen-infected pigs died within 5 DPI without complete development of clinical signs and pathological lesions (Figs. 1 and 2). While the parameters associated with its high virulence remain largely unknown, the rapid increase and high levels of viremia and E^{rns} protein in the blood may be important factors.

Glycoprotein E2 is a primary neutralizing antigen of CSFV, which can be used as subunit vaccine to elicit strong neutralizing antibody-mediated immunity against viral infection (Risatti et al., 2005; Ji et al., 2015; Blome et al., 2017). In addition, glycoprotein E^{rns} is a minor neutralizing antigen but also able to induce protective immunity (Ji et al., 2015). Levels of neutralizing antibodies, particularly anti-E2, determine the outcome of CSFV infection, and pigs recovering from CSFV infection are found to have mounted effective levels of E2-specific antibodies by 10–14 DPI (Blome et al., 2017). In a study comparing the virulence of six field CSFV isolates Floegel-Niesmann et al. (2009) showed that all infected pigs became ill due to inadequate development of anti-CSF antibody, None of the pigs infected by either of two highly virulent isolates produced a detectable antibody titer, and in pigs infected with any of the four other isolates (one highly and 3 moderately virulent) only one in each group had detectable anti-CSF antibody at 14 DPI. A similar result was obtained in our study: piglets infected by any of the 5 isolates produced very low anti-E2 antibody levels, much lower than the 40% blocking rate considered the threshold for protection (Fig. 3). In addition, serum NT titers of all infected piglets at 14 DPI

were also very low (< 5), and E^{rns} antibody levels were transient, with positive rates at varying time points and ranging from 4.2 to 55.6%. Such limited immune responses likely explain why the infected piglets developed severe disease with none recovering from the experimental infection.

A caveat is necessary for interpretation of the results of the E^{rns}-specific antibody ELISA, which was developed as a DIVA (differentiation of infected from vaccinated animals) assay to accompany application of genetically engineered DIVA vaccines (Floegel-Niesmann, 2001, 2003; Aebischer et al., 2013; Meyer et al., 2017). Using this ELISA, Meyer et al. (2017) reported that 52% (24/46) of serum samples collected between 10 and 14 DPI from 24 pigs infected with 13 field isolates belonging to various CSF genotypes were positive for E^{rns} antibody, and all became positive after 14 DPI. However, using the same kit, we found lower positive E^{rns} antibody rates (4–56%, 10–21 DPI; Table 3) in serum samples from pigs infected with CSFV field isolates. Titers were negative in all piglets infected with the highly virulent AH1 isolate while viremia and E^{rns} protein in blood were present in all during the entire observation period. These observations have the following implications: (1) the current E^{rns}-based antibody ELISA may not be able to identify all infected animals, particularly those suffering the disease; (2) the E^{rns}-based companion assay requires more extensive evaluation and validations using different panels of serum samples collected from pigs infected by various CSFV subgenotypes; and (3) the dynamics of E^{rns}-specific antibody response in pigs infected by the various CSFV subgenotypes require a further investigation.

In conclusion, four current and one earlier CSFV field isolates in China were classified in the present study as either highly or moderately virulent strains. E2 and E^{rns} antibody responses during the disease course were generally inadequate for protection, or even absent in some infected animals. Viremia and the E^{rns} protein were present and long-lasting in the blood of all infected animals, which could characterize the progression of CSF. Nevertheless, there were considerable differences in the clinical, virological and serological responses of the animals to infection, depending on the individual infecting strains but also on different animals within the same group. While the present study has improved our understanding of the virulence and pathogenicity of predominant CSFV field isolates, an explanation for the current situation still remains elusive and understanding the roles of host factors in the atypical disease course should be emphasized in future study.

Acknowledgements

References

Aebischer, A., Müller, M., Hofmann, M.A., 2013. Two newly developed E^{rns}-based ELISAs allow the differentiation of classical swine fever virus-infected from marker-vaccinated animals and the discrimination of pestivirus antibodies. Vet. Microbiol. 161, 274–285.

Blome, S., Staubach, C., Hende, J., Carlson, J., Beer, M., 2017. Classical swine fever-an updated review. Viruses 9 (4), E86.

Bruschke, C.J., Hulst, M.M., Moormann, R.J., van Rijn, P.A., van Oirschot, J.T., 1997. Glycoprotein E^{rns} of pestiviruses induces apoptosis in lymphocytes of several species. J. Virol. 71, 6692–6696.

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.

Enkhbold, B., Shatar, M., Wakamori, S., Tamura, T., Hiono, T., Matsuno, K., Okamatsu, M., Umemura, T., Damdinjav, B., Sakoda, Y., 2017. Genetic and virulence characterization of classical swine fever viruses isolated in Mongolia from 2007 to 2015. Virus Genes 53 (3), 418–425.

Floegel-Niesmann, G., 2001. Classical swine fever (CSF) marker vaccine. Trial III. Evaluation of discriminatory ELISAs. Vet. Microbiol. 83, 121–136.

Floegel-Niesmann, G., 2003. Marker vaccines and companion diagnostic tests for classical swine fever. Dev. Biol. (Basel) 114, 185–191.

Floegel-Niesmann, G., Bunzenthal, C., Fischer, S., Moenning, V., 2003. Virulence of recent

and former classical swine fever virus isolates evaluated by their clinical and pathological signs. J. Vet. Med. B Infect. Dis. Vet. Public Health 50, 214–220.

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., 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., 2016b. *In vitro* adaptation and genome analysis of a sub-subgenotype 2.1c isolate of classical swine fever virus. Virus Genes 52, 651–659.

Hu, D.F., Lv, L., Gu, J.Y., Chen, T.Y., Xiao, Y.H., Liu, S.D., 2016. Genetic diversity and positive selection analysis of classical swine fever virus envelope protein gene E2 in east China under C-Strain vaccination. Front. Microbiol. 7, 85.

Huang, Y.L., Deng, M.C., Wang, F.I., Huang, C.C., Chang, C.Y., 2014. The challenges of classical swine fever control: modified live and E2 subunit vaccines. Virus Res. 2014 (179), 1–11.

Ji, W., Guo, Z., Ding, N.Z., He, C.Q., 2015. Studying classical swine fever virus: making the best of a bad virus. Virus Res. 197, 35–47.

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., 2013. Phylogenetic analysis using E2 gene of classical swine fever virus reveals a new subgenotype in China. Infect. Genet. Evol. 17, 231–238.

Kosmidou, A., Ahl, R., Thiel, H.J., Weiland, E., 1995. Differentiation of classical swine fever virus (CSFV) strains using monoclonal antibodies against structural glycoproteins. Vet. Microbiol. 47, 111–118.

Lim, S.I., Kim, Y.K., Lim, J.A., Han, S.H., Hyun, H.S., Kim, K.S., Hyun, B.H., Kim, J.J., Cho, I.S., Song, J.Y., Choi, S.H., Kim, S.H., An, D.J., 2017. Antigenic characterization of classical swine fever virus YC11WB isolates from wild boar. J. Vet. Sci. 18 (2), 201–207.

Luo, Y.Z., Li, S., Sun, Y., Qiu, H.J., 2014. Classical swine fever in China: a minireview. Vet. Microbiol. 172 (1-2), 1–6.

Luo, Y.Z., Ji, S., Liu, Y., Lei, J.L., Xia, S.L., Wang, Y., Du, M.L., Shao, L., Meng, X.Y., Zhou, M., Sun, Y., Qiu, H.J., 2017. Isolation and characterization of a moderately virulent classical swine fever virus emerging in China. Transbound. Emerg. Dis. 64 (6), 1848–1857.

Meyer, D., Fritsche, S., Luo, Y., Engemann, C., Blome, S., Beyerbach, M., Chang, C.Y., Qiu, H.J., Becker, P., Postel, A., 2017. The double-antigen ELISA concept for early detection of E^rms^-specific classical swine fever virus antibodies and application as an accompanying test for differentiation of infected from marker vaccinated animals. Transbound. Emerg. Dis. 64 (6), 2013–2022.

Mittelholzer, C., Moser, C., Tratschin, J., Hofmann, M.A., 2000. Analysis of classical swine fever virus replicationkinetics allows differentiation of highly virulent from avirulent strains. Vet. Microbiol. 74, 293–308.

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.

Ning, Y.B., Wang, Q., Qiu, H.S., Zhang, G.C., Song, L., Zhao, Y., Wang, Z.S., Shen, Q.C., 2004. Influence of persistent HCV infection on sows reproduction ability and piglets HCLV immune efficacy (in Chinese). Acta Veterinariaet Zootechnica Sinca 35, 449–453.

Nishimori, T., Yamada, S., Shimizu, M., 1996. Production of monoclonal antibodies against classical swine fever and their use for antigenic characterization of Japanese isolates. J. Vet. Med. Sci. 58, 707–710.

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.

Qiu, H.S., 2004. Clinical healthy sows with persistent infection are the origin of CSF circulation in vaccinated swine farms (in Chinese). Swine Prod. 3, 29–30.

Risatti, G.R., Borca, M.V., Kutish, G.F., Lu, Z., Holinka, L.G., French, R.A., 2005. The E2 glycoprotein of classical swine fever virus is a virulence determinant in swine. J.Virol. 79, 3787–3796.

Rümenapf, T., Stark, R., Meyers, G., Thiel, H.J., 1993. Structural proteins of hog cholera virus expressed by vaccinia virus: further characterization and induction of protective immunity. J. Virol. 67, 589–597.

Simmonds, P., Becher, B., Bukh, J., Gould, E.A., Meyers, G., Monath, T., Muerhoff, S., Pletnev, A., Rico-Hesse, R., Smith, D.B., Stapleton, J.T., ICTV Report Consortium, 2017. ICTV virus taxonomy profile: *flaviviridae*. J. Gen. Virol. 98, 2–3.

Sun, J.M., Wei, L., Lu, Z.J., Mi, S.J., Bao, F., Guo, H.C., Tu, C., Zhu, Y., Gong, W.J., 2018. Retrospective study of porcine circovirus 3 infection in China. Transbound. Emerg. Dis . 65, 607–613.

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

Wang, Q., Tu, C., 2015. Classical Swine Fever (in Chinese). China Agriculture Press, Beijing, pp. 13–15.

Yang, Z., Shi, Z.X., Guo, H.C., Qu, H., Zhang, Y., Tu, C., 2015. Annexin 2 is a host protein binding to classical swine fever virus E2 glycoprotein and promoting viral growth in PK-15 cells. Virus Res. 201, 16–23.

Zhang, H.L., Leng, C.L., Tian, Z.J., Liu, C.X., Chen, J.Z., Bai, Y., Li, Z., Xiang, L.R., Zhai, H.Y., Wang, Q., Peng, J.M., An, T.Q., Kan, Y.C., Yao, L.G., Yang, X.F., Cai, X.H., Tong, G.Z., 2018. Complete genomic characteristics and pathogenic analysis of the newly emerged classical swine fever virus in China. BMC Vet. Res. 14, 204.

Zhu, Y., Shi, Z.X., Drew, T.W., Wang, Q., Qiu, H.J., Guo, H.C., Tu, C., 2009. Antigenic differentiation of classical swine fever viruses in China by monoclonal antibodies. Virus Res. 142, 169–174.