Isolation and Characterization of a Moderately Virulent Classical Swine Fever Virus Emerging in China


Keywords:
classical swine fever virus; molecular characterization; antigenicity; pathogenicity; subgenotype 2.1d

Correspondence:
Y. Sun and H.-J. Qiu. State Key Laboratory of Veterinary Biotechnology, Harbin Veterinary Research Institute, Chinese Academy of Agricultural Sciences, Harbin, China.

Summary
Classical swine fever (CSF) is a devastating infectious disease of pigs caused by classical swine fever virus (CSFV). In China, CSF has been under control owing to extensive vaccination with the lapinized attenuated vaccine (C-strain) since 1950s, despite sporadic or endemic in many regions. However, recently, CSF outbreaks occurred in a large number of swine herds in China. Here, we isolated 15 CSFV strains from diverse C-strain-vaccinated pig farms in China and characterized the genetic variations and antigenicity of the new isolates. The new strains showed unique variations in the E2 protein and were clustered to the subgenotype 2.1d of CSFV recently emerging in China in the phylogenetic tree. Cross-neutralization test showed that the neutralizing titres of porcine anti-C-strain sera against the new isolates were substantially lower than those against both the highly virulent Shimen strain and the subgenotype 2.1b strains that were isolated in China in 2006 and 2009, respectively. In addition, experimental animal infection showed that the HLJZZ2014 strain-infected pigs displayed lower mortality and less severe clinical signs and pathological changes compared with the Shimen strain-infected pigs. The HLJZZ2014 strain was defined to be moderately virulent based on a previously established assessment system for CSFV virulence evaluation, and the virus shedding and the viral load in various tissues of the CSFV HLJZZ2014 strain-infected pigs were significantly lower than those of the Shimen strain-infected pigs. Taken together, the subgenotype 2.1d isolate of CSFV is a moderately virulent strain with molecular variations and antigenic alterations.

Introduction
Classical swine fever (CSF) is a World Organization for Animal Health (OIE)-listed, highly contagious disease of pigs, which has led to huge economic losses in the pig industry worldwide (Edwards et al., 2000; Leifer et al., 2013; Rossi et al., 2015). The disease is caused by classical swine fever virus (CSFV), a member of the Pestivirus genus within the Flaviviridae family. CSFV is genetically and serologically related to other pestiviruses, including bovine viral diarrhoea virus 1 (BVDV-1), BVDV-2 and border disease virus.

CSFV is a small enveloped virus with a single-stranded, positive-sense RNA genome of approximately 12.3 kb in length. The CSFV genome contains a single open reading frame encoding a polyprotein of 3898 amino acids that undergoes co- and post-translational processing by cellular and viral proteases, giving rise to four structural proteins C, E\textsuperscript{ns}, E1 and E2, and seven non-structural proteins N\textsuperscript{pro}, p7, NS2-3, NS4A, NS4B, NS5A and NS5B (Rümenapf et al., 1993; Meyers and Thiel, 1996; Tautz et al., 1997).

In China, CSF has been under control following massive vaccination of pigs with the lapinized attenuated vaccine (C-strain) since 1950s, although it is still sporadic or endemic in some regions (Luo et al., 2014). Several subgroups/subgenotypes of CSFV including 2.1, 2.2 and 1.1, and occasionally 2.3 have been identified in Mainland China, and
group 3 was found only in Taiwan from pig samples collected in 1994 (Tu et al., 2001; Tu, 2003; Deng et al., 2005; Chen et al., 2008; Shen et al., 2011). Subgroup 2.1, particularly clade 2.1b, has long been predominant in China (Tu et al., 2001; Chen et al., 2008, 2010; Luo et al., 2011). A new clade 2.1c has been identified in South China (Jiang et al., 2013).

Recently, CSF outbreaks occurred in a large number of C-strain-vaccinated swine herds in many regions of China and a new clade 2.1d has been reported (Zhang et al., 2015). Accordingly, it is necessary to comprehensively analyse the pathogenicity and molecular characteristics of the current CSFV isolates.

Here, we analysed the genetic diversity and antigenic alterations of the subgenotype 2.1d isolates of CSFV newly emerging in China and characterized the pathogenicity of a representative isolate in pigs.

**Materials and Methods**

**Clinical samples, cells and virus strains**

A total of 22 clinical samples (tonsils, lymph nodes, lung, spleen and kidney) were collected from CSF-suspected pigs on 15 pig farms of China (Hei Longjiang, Jilin and Beijing) during 2012–2015. Permissive PK-15 cells (ATCC CCL-33) were cultured in Dulbecco's modified Eagle's medium (DMEM) (Gibco, Shanghai, China) supplemented with 5% foetal bovine serum (Sigma-Aldrich, St. Louis, MO, USA), 100 µg/ml streptomycin and 100 IU/ml penicillin at 37°C and 5% CO2. The CSFV Shimen strain is a highly virulent strain isolated in China in 1945, which has been used as a reference challenge virus to evaluate the efficacy of CSF vaccines in China.

**Preparation of porcine anti-C-strain sera**

Eight 5-week-old healthy pigs from CSFV-free swine herds were each inoculated intramuscularly (i.m.) with one dose of C-strain vaccine from Harbin Weike Biotechnology Development Company, China (batch No. 2015015) and then given a booster 21 days later. The anti-C-strain sera were collected 21 and 28 days after the first immunization for cross-neutralization test against different CSFV strains (Table 2). All animal experiments were conducted according to the Guide for the Care and Use of Laboratory Animals of Harbin Veterinary Research Institute, Chinese Academy of Agricultural Sciences, China.

**Virus isolation and characterization**

The pooled tissue samples (tonsils, lung, lymph nodes, spleen and kidney) were homogenized and resuspended in 10 volumes of DMEM. For virus isolation, after three freeze–thaw cycles the tissue homogenate supernatant was passed through a 0.45-µm filter (Merck Millipore, Cork, Ireland) and inoculated into confluent monolayers of PK-15 cells (V/V, 1:10) and incubated at 37°C and 5% CO2 for 72 h. After 72 h post-inoculation (HPI), the cultures were harvested, and the supernatant was inoculated into new confluent monolayers of PK-15 cells after two freeze–thaw cycles. At each passage, the culture was verified by PCR, and at the same time, the cells were also seeded into a 96-well plate to determine the presence of the virus by indirect immunofluorescence assay using the polyclonal antibodies against CSFV E2 (working dilution: 1:200) according to the EU Diagnostic Manual for CSF Diagnosis (Anonymous, 2007).

RNA was extracted from the tissue homogenates with the QIAamp viral RNA kit (Qiagen, Hilden, Germany) according to the manufacturer’s manual. A partial NS5B gene fragment of CSFV was amplified by a reverse transcription polymerase chain reaction (RT-PCR) from the genomic RNA as described previously (Li et al., 2007). The PCR products were subjected to electrophoresis through a 2% agarose gel in TAE buffer (40 mM Tris-acetate, pH 8.0; 1 mM EDTA) and stained with ethidium bromide for visualization using a Gel Doc XR® System (Bio-Rad, CA, USA).

**Genome sequencing**

A pair of primers were designed based on all CSFV genome sequences available in GenBank to amplify the fragment covering the complete E2 gene of CSFV for direct sequencing without cloning, forward primer (E2-F) 5'-TTG AAG AGG YRG GAC AGG T-3' (Y=C or T; R=A or G) and reverse primer (E2-R) 5'-AGT ATC CAT TTT TTT AT-3'. Complete E2 gene fragment of CSFV was amplified from the genomic RNA of the CSFV PCR-positive samples by RT-PCR using a One-Step RT-PCR kit (Qiagen) according to the manufacturer’s instructions. The PCR profile consisted of an initial denaturation at 95°C for 5 min, followed by 35 amplification cycles (95°C for 30 s, 51°C for 1 min and 72°C for 1 min 40 s) and a final extension step at 72°C for 10 min using a PCR Thermal Cycler (TaKaRa, Otsu, Shiga, Japan). The nucleotide sequences of the PCR products were determined using an ABI Prism 3100 Genetic Analyzer (Applied Biosystems, Foster City, CA, USA).

**Phylogenetic analysis and sequence alignment**

The phylogenetic tree was constructed based on the deduced amino acids of the complete E2 gene of 15 strains isolated in the present study (Table 1) and 183 isolates available in GenBank, using the neighbour-joining (NJ) method. Multiple sequence alignments were generated based on the DNASTAR (Madison, WI, USA) and MEGA
software (Center for Evolutionary Functional Genomics, The Biodesign Institute, Tempe, AZ, USA).

Cross-neutralization test

Antigenic differences between the new isolates and the classical Shimen strain were assessed by cross-neutralization of 100 TCID<sub>50</sub> CSFV Shimen strain or four randomly selected new subgenotype 2.1d isolates, including HLJZZ2014, HLJWC2014-2, BJSN2013 and HLJYC2014, against three porcine anti-C-strain sera. Subsequently, HLJZZ2014 and two subgenotype 2.1b strains [HuN06 (GenBank accession no. KX759643) and SX(09) (KX759642)] that were isolated in China in 2006 and 2009, respectively, were further evaluated against the porcine anti-C-strain sera (Table 2). The CSFV-specific neutralizing antibody titres were expressed as the reciprocal of the highest serum dilution that inhibited the infection of PK-15 cells in 50% of the culture wells according to the EU Diagnostic Manual for CSF Diagnosis (Anonymous, 2007). The antibody blocking rates of the sera ranged from 40.36 to 65.37%, as determined by an IDEXX HerdCheck CSFV Ab ELISA (IDEXX Laboratories, Schiphol-Rijk, the Netherlands).

One-step growth curve

The virus was propagated in PK-15 cells and the viral titres of the culture were determined by the method of Reed and Muench (1938). The confluent monolayers of PK-15 cells in a 24-well cell culture plate (Corning, USA) were inoculated with the CSFV Shimen or HLJZZ2014 strain at a multiplicity of infection (MOI) of 5 and incubated on ice for 1 h. Thereafter, the inoculum was replaced with pre-warmed fresh medium and cells were further incubated for 1 h at 37°C. Extracellular virus was inactivated by low pH treatment (Mettenleiter, 1989) and the cell culture was harvested at 0, 12, 24, 36, 48, 60, 72 and 84 HPI. After two freeze–thaw cycles, the cellular debris was removed by centrifugation and the supernatant was titrated on PK-15 cells. Average values and standard deviations of three independent experiments were calculated.

Experimental infection of pigs with the HLJZZ2014 strain

To determine the level of virulence of the emerging subgenotype 2.1d CSFV strain studied in this work, we utilized the previously established clinical scoring system

<table>
<thead>
<tr>
<th>No.</th>
<th>Isolate</th>
<th>GenBank accession No.</th>
<th>Year</th>
<th>Area</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>HLJWC2014-1</td>
<td>KU375249</td>
<td>2014</td>
<td>Heilongjiang</td>
</tr>
<tr>
<td>2</td>
<td>HLJWC2014-2</td>
<td>KU375250</td>
<td>2014</td>
<td>Heilongjiang</td>
</tr>
<tr>
<td>3</td>
<td>JLHN2014</td>
<td>KU375251</td>
<td>2014</td>
<td>Jilin</td>
</tr>
<tr>
<td>4</td>
<td>JLHD2012</td>
<td>KU375252</td>
<td>2012</td>
<td>Jilin</td>
</tr>
<tr>
<td>5</td>
<td>BJSN2013</td>
<td>KU375253</td>
<td>2013</td>
<td>Beijing</td>
</tr>
<tr>
<td>6</td>
<td>JLCL2015</td>
<td>KU375254</td>
<td>2015</td>
<td>Jilin</td>
</tr>
<tr>
<td>7</td>
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<td>KU375255</td>
<td>2015</td>
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<tr>
<td>8</td>
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<td>KU375256</td>
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</tr>
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<td>KU375257</td>
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<td>Heilongjiang</td>
</tr>
<tr>
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<td>KU375262</td>
<td>2014</td>
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</tr>
<tr>
<td>15</td>
<td>HLJBY2015</td>
<td>KU375263</td>
<td>2015</td>
<td>Heilongjiang</td>
</tr>
</tbody>
</table>

Table 2. Neutralizing titres of different swine anti-C-strain sera against various CSFV strains

<table>
<thead>
<tr>
<th>Antisera ID</th>
<th>Pig No.</th>
<th>Pre-booster vaccination</th>
<th>Neutralizing titres against CSFV isolates</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td>HLJZZ2014 (2.1d)</td>
</tr>
<tr>
<td>#1</td>
<td>1</td>
<td>✓</td>
<td>80</td>
</tr>
<tr>
<td>#2</td>
<td>2</td>
<td>✓</td>
<td>20</td>
</tr>
<tr>
<td>#3</td>
<td>3</td>
<td>✓</td>
<td>&lt;5</td>
</tr>
<tr>
<td>#4</td>
<td>4</td>
<td>✓</td>
<td>&lt;5</td>
</tr>
<tr>
<td>#5</td>
<td>5</td>
<td>✓</td>
<td>10</td>
</tr>
<tr>
<td>#6</td>
<td>6</td>
<td>✓</td>
<td>7.5</td>
</tr>
<tr>
<td>#7</td>
<td>7</td>
<td>✓</td>
<td>7.5</td>
</tr>
<tr>
<td>#8</td>
<td>8</td>
<td>✓</td>
<td>&lt;5</td>
</tr>
<tr>
<td>#9</td>
<td>9</td>
<td>✓</td>
<td>7.5</td>
</tr>
<tr>
<td>#10</td>
<td>10</td>
<td>✓</td>
<td>20</td>
</tr>
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<td>11</td>
<td>✓</td>
<td>30</td>
</tr>
<tr>
<td>#12</td>
<td>12</td>
<td>✓</td>
<td>20</td>
</tr>
</tbody>
</table>

/ not detected.

The CSFV strains tested included four randomly selected new isolates (subgenotype 2.1d), two earlier isolates (subgenotype 2.1b) and the Shimen strain (subgenotype 1.1) isolated in China.
(Mittelholzer et al., 2000) to define the virulence of CSFV strains under identical experimental conditions. One of the emerging subgenotype 2.1d strain (HLJZZ2014 strain) and highly virulent Shimen strain were used to infect pigs.

Fifteen 5-week-old healthy pigs from CSFV-free swine herds were randomly divided into three groups of five pigs each. Pigs in Groups 1 and 2 were each inoculated i.m. with 10^5 TCID_{50} CSFV Shimen or new isolate HLJZZ2014 strain, and Group 3 injected with DMEM served as uninfected control. Each group was housed in an individual room. Following inoculation, clinical signs and rectal temperature were monitored daily throughout the experiment according to a previously established system (Mittelholzer et al., 2000). Rectal swabs were collected daily post-challenge and subjected to virus titration as described above. The serum samples of the pigs were collected at different time points and detected for the presence of E2-specific antibodies by using the IDEXX HerdCheck CSFV Ab ELISA, according to the manufacturer’s instructions. The anti-coagulation blood samples were collected at 0, 3, 6, 9, 12 and 15 days post-challenge (DPC) and subjected to detection of the CSFV RNA by a real-time RT-PCR as described previously (Zhao et al., 2008). Various organ samples (lymph nodes, spleen, kidney, tonsils, heart, liver, lung and bladder) were collected from the pigs died during the experiment and from the surviving pigs euthanatized at 21 DPC and tested for the presence of CSFV using the E2- and NS5B-specific PCRs as mentioned above. In addition, the quantity of viral genomic copies in individual organs was determined by the real-time RT-PCR as described above. The collected organs were also subjected to pathological examinations as described previously (Sun et al., 2010).

Statistical analysis
Data were analysed using the SPSS 14.0 software (SPSS Inc., Chicago, IL, USA). Nonparametric one-way analysis of variance (ANOVA) was used to compare the data. A P-value of less than 0.05 was considered significant.

Results

Phylogeny of the new CSFV isolates
In this study, tissue samples of 22 CSF-suspected pigs from 15 different pig farms in Heilongjiang, Jilin and Beijing were tested positive for CSFV by RT-PCR, and 15 CSFV strains were isolated in PK-15 cells (Table 1).

As shown in Fig. 1, out of the 15 new isolates, 14 isolates (HLJZZ2014, HLJWC2014-1, HLJWC2014-2, JLHN2014, JLHD2012, BJSN2013, JLC2015, HLJQH2015, HLJYC2014, HLJAC2014, JLFY2014-1, HLJBY2015, JLFY2014-2 and HLJB2014) were clustered into the new subgenotype 2.1d together with some recent isolates, and one isolate (HLJHRB2014) belonged to subgenotype 2.1b.

Genetic variations of the new CSFV isolates
Alignment of the E2 protein sequences of different CSFV strains showed that the new isolates contain unique variations at positions 31 (K to R), 34 (N to S), 182 (L to W), 205 (R to K), 303 (R to K) and 331 (V to A) (Fig. 2).

Replication kinetics of the CSFV HLJZZ2014 strain
The sequence alignment showed that the 14 isolates of subgenotype 2.1d have nearly identical E2 sequences, and these isolates showed similar replication curves in PK-15 cells. Therefore, we randomly chose one of the 14 isolates, HLJZZ2014, for further study.

To determine the replication characteristics of the CSFV subgenotype 2.1d strains, one of the new isolates, HLJZZ2014, was selected to test its growth kinetics. As shown in Fig. 3, HLJZZ2014 exhibited significantly lower replication level at 36, 48, 60, 72 and 84 HPI (P < 0.05) compared with the Shimen strain.

Antigenicity of the new CSFV isolates
Cross-neutralization test showed that the neutralizing titres of the swine anti-C-strain sera against the randomly selected four new isolates of subgenotype 2.1d (HLJZZ2014, HLJWC2014-2, BJSN2013 and HLJYC2014) were much lower than those against both the Shimen strain (subgenotype 1.1) (Table 2). Interestingly, the further test showed that the neutralizing titres of the porcine anti-C-strain sera against the HLJZZ2014 strain were significantly lower (4- to 32-fold) than those against both the Shimen strain and the subgenotype 2.1b HuN06 and SX(09) strains that were isolated in China in 2006 and 2009, respectively (Table 2).

Pathogenicity of the CSFV HLJZZ2014 strain
Based on the clinical score (CS) list as described previously (Mittelholzer et al., 2000), each pig was judged daily after experimental infection. As shown in Fig. 4a, the CS of the HLJZZ2014 strain-infected pigs was determined to be 9–11, compared with those of the highly virulent Shimen strain-infected pigs (CS was 24), indicating that the HLJZZ2014 strain was moderately virulent (5 < CS ≤ 15).

Following challenge, the HLJZZ2014 strain-infected pigs exhibited fever from 7 DPC, which was 5 days delayed compared with that of the Shimen strain-infected pigs (fever from 2 DPC) (Fig. 4b). In the HLJZZ2014 strain
Fig. 1. Phylogenetic analysis of complete amino acid sequences of E2 of CSFV isolates. The phylogenetic tree was constructed based on 15 strains isolated in the present study (indicated as diamonds or square) and 183 isolates available in GenBank, using the neighbour-joining (NJ) method.
group, two out of five pigs displayed milder clinical signs than those of the Shimen strain-infected group, and died at 18 and 19 DPC, respectively. In the Shimen group, all the pigs displayed typical CSF symptoms (anorexia, depression, chill, prostration, incoordination and constipation followed by diarrhoea, locomotor ataxia and posterior paresis) with high fever from 2 DPC and all the pigs in this group died from 12 to 14 DPC (Table 3).

Viral RNA was detected in all CSFV HLJZZ2014 strain-infected pigs, with a viral RNA load of about $10^2$ to $10^4$ copies/μl from 9 DPC, and viral RNA greater than $10^3$ copies/μl was
detected in the CSFV Shimen strain group from 3 DPC, up to 10^6 copies/μl (data not shown).

Tissue distribution of CSFV genomic RNA and virus shedding in the HLJZZ2014-infected pigs

The HLJZZ2014 strain-infected pigs had a significantly lower viral load than the Shimen strain-infected pigs in various tissue samples, including heart, liver, spleen, lung, tonsil, bladder and inguinal and submaxillary lymph nodes (Fig. 6).

Moreover, virus titration of rectal swabs of infected pigs showed that the virus shedding in the HLJZZ2014 strain-infected pigs was significantly delayed (from 8 DPC) and much lower than that of the Shimen strain-infected pigs (from 4 DPC) (Fig. 7).

Discussion

CSF has been well controlled by compulsory vaccination with the lapinized attenuated vaccine (C-strain) in China for decades, and no large-scale outbreaks have been reported. However, recently CSF outbreaks occurred in a large number of C-strain-vaccinated farms in many regions of China and caused a great impact on the swine production. Recent reports have shown that a new clade 2.1c (Jiang et al., 2013) and a new clade 2.1d (Zhang et al., 2015) have been identified in some regions of China, indicating the genetic variations of CSFV contribute to the current outbreaks. Therefore, we attempted to analyse the pathogenicity and molecular characteristics of the current CSFV isolates.

In this study, 15 new strains of CSFV were isolated in various C-strain-vaccinated pig farms in some regions of China. Firstly, we chose the complete E2 sequence of these strains to characterize the genetic diversity, considering that the full-length E2 sequence provides better resolution for genetic analysis (Postel et al., 2012). Fourteen out of the 15 new isolates were clustered to the new subgenotype 2.1d, together with some recent CSFV isolates in China in the phylogenetic tree, which is consistent with a previous report (Zhang et al., 2015) that most isolates in China were identified to be subgenotype 2.1d rather than 2.1b. This implies that the subgenotype 2.1d is becoming predominant over 2.1b in China (Tu et al., 2001; Chen et al., 2008, 2010; Luo et al., 2011).
Alignment of the E2 protein sequences of different CSFV strains showed that the new isolates contain unique variations at some positions. As an immunodominant protein, E2 can induce protective neutralizing antibodies (NAbs) in pigs (Weiland et al., 1999). Two independent antigenic units, B/C (aa 690-779 of the polyprotein or aa 1-90 of E2) and A/D (aa 770-879 of the polyprotein or aa 81-190 of E2), have been identified in the N-terminal half of E2 (van Rijn et al., 1994; Chang et al., 2010). It has been reported that single mutations on the B/C domain of E2 could lead to variations in viral neutralization (Chen et al., 2010).

To determine whether the variations potentially shape the antigenic alterations, cross-neutralization test was carried out and the results showed that the neutralizing titres of swine anti-C-strain sera against the new isolates were significantly lower than those against both the classical highly virulent Shimen strain (the first CSFV strain isolated in China in 1945) and the subgenotype 2.1b HuN06 and SX (09) strains that were isolated in China in 2006 and 2009, respectively. The C-strain vaccine was developed in China in 1956, which was attenuated from a highly virulent Shimen strain (the first CSFV strain isolated in China in 1945) and the subgenotype 2.1b HuN06 and SX (09) strains that were isolated in China in 2006 and 2009, respectively. The C-strain vaccine was developed in China in 1956, which was attenuated from a highly virulent Shimen strain after at least 480 passages in rabbits (Luo et al., 2014). Our findings indicate that the new 2.1d isolates represent an increased risk of vaccine failure compared with previous other subgenotype 2.1 strains, although the C-strain vaccine had been proved to provide complete protection against CSFV of different genotypes (Research Group of CSF Vaccine, 1979; Qiu et al., 2006). The genetic variations and antigenic alterations of the emerging genotype 2.1d strains partially explain why the outbreaks occurred in traditional C-strain-vaccinated farms. Whether the mutations in the E2 protein of the emerging 2.1d subgenotype isolates.
of CSFV, particularly B/C domain, are associated with viral escape from NAbs needs further study.

Next, we characterized the pathogenicity of the new isolates. To determine the virulence of the emerging subgenotype 2.1d CSFV, we utilized the previously established system for CSFV virulence assessment (Mittelholzer et al., 2000). The HLJZZ2014 strain was defined to be moderately virulent (5 ≤ CS ≤ 15), in contrast to that of the highly virulent Shimen strain (CS > 24). The HLJZZ2014 strain displayed delayed fever from 7 DPC and lower mortality compared with the virulent Shimen strain. Furthermore, the rectal virus shedding of the HLJZZ2014 strain-infected pigs was significantly delayed and much lower than that of the Shimen strain-infected ones. Significantly lower viral load in various tissues of the CSFV HLJZZ2014 strain-infected pigs was correlated with moderate disease presentations compared with the highly virulent Shimen strain-infected pigs. All these findings support that the HLJZZ2014 strain is a moderately virulent CSFV. The moderate clinical presentations of the disease caused by the subgenotype 2.1d strain in this study were consistent with atypical clinical signs of the disease observed in pig farms in China during the last years (Luo et al., 2014). A practical consequence of such a mild and chronic presentation of the disease is delayed or wrong diagnosis due to non-specific clinical signs of CSF (Pérez et al., 2012). In addition, sequence variations might lead to false negative or low sensitivity of the currently used diagnostic tests for CSF due to the mismatches of currently available primers with new isolates.

In summary, the CSFV subgenotype 2.1d isolate is a moderately virulent strain with unique molecular variations and antigenic alterations, which highlights the fact that the existing vaccines appear to be less effective than they would be desirable against the emerging CSFV genotype that seems to overwhelm earlier genotypes to be predominant ones. The study provides evidence of a real need to develop new vaccines for the new strains.

Acknowledgements

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References


EU and OIE Reference Laboratory for Classical Swine Fever Virus. (accessed November 12, 2014).


