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Genotype I African swine fever viruses emerged in domestic pigs in China and caused chronic infection

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Running title: Emergence of genotype I ASFVs in China.

Abstract

The Georgia-07-like genotype II African swine fever virus (ASFV) with high virulence has been prevalent in China since 2018. Here, we report that genotype I

ASFVs have now also emerged in China. Two non-hemadsorbing genotype I ASFVs, HeN/ZZ-P1/21 and SD/DY-I/21, were isolated from pig farms in Henan and Shandong province, respectively. Phylogenetic analysis of the whole genome sequences suggested that both isolates share high similarity with NH/P68 and OURT88/3, two genotype I ASFVs isolated in Portugal in the last century. Animal challenge testing revealed that SD/DY-I/21 shows low virulence and efficient transmissibility in pigs, and causes mild onset of infection and chronic disease. SD/DY-I/21 was found to cause necrotic skin lesions and joint swelling. The emergence of genotype I ASFVs will present more problems and challenges for the control and prevention of African swine fever in China.

Keywords: African swine fever virus, genotype I, virulence, pig, China

Introduction

African swine fever (ASF) is a highly contagious, hemorrhagic swine disease caused by the African swine fever virus (ASFV) [1, 2]. The disease continues to pose a major threat to the pig industry, food security, and rural development worldwide, and is listed as a notifiable disease by the World Organization for Animal Health (OIE).

The disease signs and mortality rates of ASF vary among viral strains and animal species [1]. Acute disease presents with high fever, depression, hemorrhages, cyanosis, and death within 15 days with near 100% mortality [3]. Subacute and chronic diseases are usually caused by less virulent strains with a longer course of disease and lower mortality. Chronic disease signs include intermittent fever, weight loss, chronic skin

ulcers, and arthritis [1].

ASF was first described in Kenya in 1921 [1] and spread throughout sub-Saharan Africa (www.ioe.int). ASFVs are classified into different genotypes based on the 3′-end sequences of the B646L gene, which encodes the major capsid protein p72 [3, 4]. To date, 24 different ASFV genotypes have been identified in Africa. Multiple virus strains and genotypes may circulate in any given region of Sub-Saharan Africa [3-5]. Genotype I virus was first found in Portugal outside Africa in 1957 and caused acute onset of infection [6]; it then appeared in Spain, France, Madeira, Italy, Cuba, Malta, Sardinia, Brazil, the Dominican Republic, and Haiti in the 1960s and 1970s [7-9]. Genotype I ASFVs have since been eradicated in all of these countries except Italy-Sardinia, where it has been endemic since 1978 [8].

In 2007, genotype II ASFV was first introduced into Georgia outside Africa and then spread to other Caucasian and European countries [6]. In 2018, Georgia-07-like genotype II ASFV emerged in China, and spread to 15 other Asian countries (www.oie.int) [10-12]. This virus causes very acute disease with near 100% mortality, and has been prevalent in China for almost 3 years (www.oie.int) [4, 10, 12-14]. A subsequent surveillance study found that lower virulent genotype II ASFVs emerged in China in 2020 due to natural mutations in the genomes of the highly virulent viruses [15]. These natural mutants showed lower virulence and high transmissibility, caused chronic and persistent infections in pigs, but were continuously shed via the oral and rectal routes at a low level, which causes more difficulties and challenges for the early diagnosis and control of ASF in China [15].

In the present study, we isolated genotype I ASFVs from pigs in two provinces in China, and sequenced the whole viral genomes of two isolates for phylogenetic analysis. Specific-pathogen-free (SPF) pigs were inoculated with one of the viruses at different doses to determine its pathogenicity. Disease signs, lesions, viremia, contact transmission, and viral loads in different organs and tissues were assessed.

Materials and Methods

Facility and ethics statements

All experiments including animal studies with live ASFVs were performed in the enhanced biosafety level 3 (P3+) facilities in the Harbin Veterinary Research Institute (HVRI) of the Chinese Academy of Agricultural Sciences (CAAS), which are approved for such use by the Ministry of Agriculture and Rural Affairs and the Animal Experimentation and Laboratory Animal Welfare Committee of HVRI.

Cell culture and virus isolation

Primary porcine alveolar macrophages (PAMs) were obtained from bronchoalveolar lavage of SPF pigs as previously described [16]. Peripheral blood mononuclear cells (PBMCs) were prepared from EDTA-treated swine blood by using a Pig PBMC Isolation Kit (TBD sciences, China).

Homogenized pig tissue samples (ASFV p72 gene positive by qPCR) were used to inoculate primary PAMs. At 3 days post-inoculation (p.i.), the ASFVs in the cell supernatants and PAMs were detected by using qPCR. The cell supernatants were collected and used to inoculate PAMs to propagate virus stocks. Each virus stock was examined to confirm the lack of bacterial contamination, classical swine fever virus (CSFV), porcine respiratory and reproductive syndrome virus (PRRSV), pseudorabies

virus (PRV), and porcine circovirus type 2 (PCV2). Virus stocks were aliquoted and stored at -80℃.

HAD assay

PBMCs were cultured in 96-well plates and infected with 10-fold diluted ASFVs [10]. Hemadsorption in the cultures was observed for at least 7 days by microscopy. The 50% HAD dose (HAD_{50}) was measured by using the Reed and Muench method [17].

Immunofluorescence assay

PAMs were seeded in 96-well plates and infected with different doses of ASFVs. Virus replication were confirmed by using an Immunofluorescence assay (IFA) with an ASFV-specific antibody, as described previously [15, 18]. The 50% tissue culture infective dose ($TCID_{50}$) was measured by using the Reed and Muench method [17].

Electron microscopy

PAMs were cultured in 6-well plates and infected with the indicated ASFVs at an MOI of 0.2. At 48 h p.i., ASFV-infected cells were collected for morphological observation under an electron microscopy, as described previously [10].

Quantitative PCR

ASFV genomic DNA from cell supernatants, whole peripheral blood samples, swabs, and tissue homogenates was extracted by using QIAamp® DNA Mini Kits (Qiagen, Germany). Quantitative PCR (qPCR) was carried out on an *ABI* QuantStudio5 (Q5) (ABI, USA) as previously described [19].

Viral gene sequencing and genetic analysis

Viral genes and genome segments were amplified by PCR and sequenced by using the first generation Sanger DNA sequencing method [20]. Multiple-sequence alignments and phylogenetic analyses of viral genes and genome segments were carried out by using the software DNAStar and MEGA X. Primer sequences for PCR

amplification and sequencing of genotype I ASFV genomes are available upon request. The genomes of the ASFVs were aligned by using E-INS-i of the program MAFFT v7 [21] and ambiguously aligned regions were excluded by using Gblocks-0.91 [22]. Phylogenetic analysis was hypothesized using maximum likelihood (ML), and ModelFinder was used to select the best-fit model according to the Bayesian information criterion (BIC) [23]. ML analysis was hypothesized using IQ-Tree [24] and the best model was TVM+F. Bootstrap branch support values (MLBS) were obtained with 1000 rapid bootstrap inferences and subsequently sought in a thorough ML search of the dataset.

Animal experiments

SPF Large White and Land race-crossed pigs without PRRSV, PCV2, PRV, or CSFV infection were obtained from the Laboratory Animal Center of HVRI. The 7-week-old SPF pigs were divided into groups of six pigs, and then intramuscularly inoculated with different doses of ASFV. Two additional naïve pigs were cohoused with the infected pigs from the first day of challenge to determine ASFV contact transmission. Each pig was monitored daily for body temperature changes and disease signs including anorexia, depression, fever, purple skin discoloration, staggering gait, diarrhea, and cough for 28 days p.i. or post-contact (p.c.). Oral and rectal swabs, and blood were collected for virus detection by using qPCR at different timepoints p.i. or p.c.. Necropsy was immediately performed if an animal died. Tissue samples including heart, liver, spleen, lung, kidney, tonsil, thymus, adrenal gland, marrow, synovial fluid, and lymph nodes (intestinal lymph node, inguinal lymph node, submaxillary lymph node, bronchial lymph node, gastrohepatic lymph node, and mediastinal lymph node) were collected during necropsy, and viral DNA was detected by using qPCR. Serum samples were collected and assessed for IgG against the ASFV

p72 protein by using a commercial ELISA kit (Harbin Weike Biotechnology Co., Ltd, China).

Results

Isolation of genotype I ASFVs in China

In June 2021, a fattening pig weighing about 80 Kg showed paralytic symptoms in a farm in Shandong province, and was euthanized for autopsy. The lung sample was collected and delivered to the Chinese National African Swine Fever Para-reference Laboratory (CNASFPL) for ASFV detection. On another farm in Henan province, the fattening pigs developed chronic infection signs including weight loss, intermittent fever, skin ulcers, and arthritis; sporadic deaths were also observed. Samples from four dead pigs, including lymph nodes and spleens, were collected and delivered to the CNASFPL for ASFV detection. All samples were confirmed to be ASFV positive by qPCR targeting the viral p72 gene [19]. Further sequence analysis of the p72 genes indicated that the ASFVs in these samples belonged to genotype I.

Subsequently, two genotype I ASFVs were isolated from the samples from the Shandong and Henan province farms, and were named Pig/Shandong/DY-I/2021 (SD/DY-I/21) and Pig/Henan/ZZ-P1/2021 (HeN/ZZ-P1/21), respectively. Both isolates replicated well in PAMs with viral titers of $> 5 \times 10^7$ $TCID_{50}$/ml in cell culture supernatants, and showed non-hemadsorption activity (Figure 1). Electron microscopy (EM) observation revealed typical morphology of ASFV particles within the infected PAMs (Figure 1).

Genome analysis of the genotype I ASFV isolates SD/DY-I/21 and HeN/ZZ-P1/21

To characterize the phylogenic specificity of the genotype I ASFVs isolated in China, we sequenced the whole genomes of SD/DY-I/21 and HeN/ZZ-P1/21 by using a segmentation PCR strategy described previously [12]. The sequence data have been deposited in the GenBank and the accession numbers are MZ945537 and MZ945536. The phylogenetic trees were constructed by using the whole genome sequences of SD/DY-I/21 and HeN/ZZ-P1/21, as well as 70 reference strains in the GenBank database (Figure 2). SD/DY-I/21 and HeN/ZZ-P1/21 belong to the same clade with the genotype I Portuguese isolates NH/P68 (GenBank: KM262845) and OURT88/3 (GenBank: AM712240), and differ from the previous genotype II ASFV isolates in China (Figure 2). Sequence analysis showed 172, 025 bp and 158 ORFs for SD/DY-I/21, and 171, 235 bp and 157 ORFs for HeN/ZZ-P1/21.

Compared to the whole genomes of virulent L60 and Benin 97 strains, SD/DY-I/21 and HeN/ZZ-P1/21 as well as NH/P68 and OURT88/3 deleted 10 ORFs (MGF_110-11L, MGF_110-12L, MGF_360-6L, MGF_360-10L, MGF_360-11L, MGF_505-1R, MGF_360-12L, MGF_360-13L, MGF_360-14L, and MGF_505-2R), and had 4 truncated ORFs (MGF_360-9L, EP153R, EP402R, and MGF_110-2L) (Figure 3 A). Phylogenetic analysis of B646L, E183L, and B602L genes grouped L60, Benin 97, NH/P68, OURT88/3, SD/DY-I/21, and HeN/ZZ-P1/21 to other genotype I ASFV strains from Europe and West Africa (Supplementary Figure 1), consistent with the previous assumption that all genotype I ASFVs were originally derived from Africa [25]. The central variable region (CVR) profiles of the B602L gene of SD/DY-I/21 and HeN/ZZ-P1/21 showed no similarity with that of publicly available ASFVs including NH/P68 and OURT88/3 (Supplementary Table 1).

Compared to the whole genome of NH/P68 in the coding regions, SD/DY-I/21

differs by 18 single nucleotide changes in 13 open reading frames (ORFs), resulting in 12 amino acid (AA) changes (Figure 3B and Supplementary Table 2); HeN/ZZ-P1/21 differs by 18 single nucleotide mutations in 15 ORFs, resulting in 13 AA changes (Figure 3B and Supplementary Table 2); and OURT88/3 has 3 single nucleotide changes in 3 ORFs (Figure 3B). SD/DY-I/21 has 96 nucleotides deleted from sites 87541 to 87636 in its B602L ORF, a single C deletion at site 169370 in its MGF_360-17R ORF, a single T insertion at site 110751 in its CP204L ORF, and a single A insertion at site 163390 in its MGF_360-16R ORF (Figure 3C); HeN/ZZ-P1/21 has a 686-nucleotide deletion from sites 7105 to 7790 in MGF_110-4/5L, a 22-nucleotide fragment replacement from sites 8123 to 8144 in MGF_110-5L, a single C deletion at site 11607 in MGF_110-13/14L, 108 nucleotides deleted from sites 87456 to 87563 in its B602L ORF, single T and C deletions at sites 169200 and 169370, respectively, in MGF_360-17R, a single C insertion at site 70851 in its C257L ORF, a single T insertion at site 110751 in its CP204L ORF, and two A insertions at site 163390 in its MGF_360-16R ORF (Figure 3C).

Compared to the whole genome of NH/P68 in the non-coding regions, SD/DY-I/21 has 4 nucleotides deleted at 3 positions and 10 nucleotides inserted at 9 positions (Figure 3D); HeN/ZZ-P1/21 has one mutation, 31 nucleotides deleted at 9 positions, and 7 nucleotides inserted at 7 positions (Figure 3D); OURT88/3 has 6 nucleotide deletions at 4 positions, and 7 nucleotides inserted at 7 positions (Figure 3D).

Compared to NH/P68, the nucleotide deletions and insertions in the genome of SD/DY-I/21 resulted in changes in 4 ORFs: three ORFs (MGF_360-16R, CP204L, and B602L) had deletions ranging from 2 to 32 AAs in length, and one ORF (MGF_360-17R) had an insertion of 182 AAs in length (Supplementary Table 2); the nucleotide deletions, insertions, and replacement in the genome of HeN/ZZ-P1/21

resulted in changes in 9 ORFs: one ORF (MGF_110-4L) exists in NH/P68 but not in HeN/ZZ-P1/21, one ORF (MGF_110-5L) has 4 AA mutations, 5 ORFs (MGF_110-13L, MGF_110-14L, B602L, CP204L, and C257L) have deletions ranging from 3 to 150 AAs in length, and two ORFs (MGF_360-16R and MGF_360-17R) have insertions of 41 and 165 AAs, respectively (Supplementary Table 2).

It is surprising that the HeN/ZZ-P1/21 genome differs significantly from that of SD/DY-I/21: 32 nucleotide mutations in 23 ORFs, 700 nucleotide deletions in 4 ORFs, 3 nucleotide insertions in 3 ORFs, and a replacement of 22 nucleotides in MGF_110-5L (Figure 3 and Supplementary Table 2). These results indicate that the two genotype I ASFV isolates, SD/DY-I/21 and HeN/ZZ-P1/21, come from different sources.

The genotype I ASFV isolate SD/DY-I/21 causes chronic infection in pigs

To determine the virulence of genotype I ASFVs isolated in China, SD/DY-I/21 was tested in pigs. Groups of six SPF pigs were intramuscularly inoculated with 10^3 or 10^6 $TCID_{50}$ of SD/DY-I/21, respectively. All the challenged pigs showed intermittent fever of different degrees from Day 3 to 18 p.i. (Figure 4 and Table 1). In the 10^6 $TCID_{50}$-inoculated group, three pigs developed papules in the skin of neck, ear hind, abdomen, or even the whole body from Day 11 p.i. (Table 1 and Figure 5). All six pigs started to develop arthroncus from Days 13 and 17 p.i.; `two pigs started to limp on Days 14 and 25 p.i., respectively (Table 1 and Figure 5). Two pigs developed multiple focal cutaneous necrosis on Days 17 and 20 p.i., respectively (Table 1 and Figure 5). All of the pigs survived for the duration of the 28-day observation period (Table 1 and Figure 5).

In the 10^3 $TCID_{50}$-inoculated group, three pigs developed sporadic papules in the skin from Day 13 p.i., and five pigs started to develop arthroncus from Day 14 p.i. (Table 1 and Figure 5). One pig developed disease and died on Day 16 p.i. (Table

1). Nostril bleeding before death, and hyperemia and swelling of the spleen, liver, and lymph nodes were observed on autopsy. The remaining five pigs survived for the duration of the 28-day observation period (Table 1). These results demonstrate that the genotype I ASFV SD/DY-I/21 is pathogenic and mainly causes chronic disease signs in pigs.

Replication and shedding of SD/DY-I/21 in pigs

To evaluate viral shedding and viremia, oral and rectal swabs and blood samples from the pigs were collected to detect ASFV genome DNA by using qPCR every other day p.i.. For the pigs inoculated with 10^6 $TCID_{50}$ of SD/DY-I/21, viral DNA was detectable in the oral swabs from Day 5 p.i., in the rectal swabs from Day 7 p.i., and in the blood from Day 7 p.i. (Figure 6). For the pigs inoculated with 10^3 $TCID_{50}$ of virus, viral DNA was detectable in the oral swabs from Day 9 p.i., in the rectal swabs from Day 11 p.i., and in the blood from Day 7 p.i.. Viral DNA was detected in the oral and rectal swabs for more than 28 days after infection in all inoculated pigs (Figure 6). In general, virus shedding was detected earlier and at a higher level in the oral swabs than in the rectal swabs (Figure 6).

Tissue samples were collected from the one necropsied pig on the day of its death and from all of the surviving pigs, which were euthanized on Day 28 p.i., for viral genome DNA detection by qPCR. The viral loads in the tissues from the dead pig inoculated with the 10^3 $TCID_{50}$ dose were much higher than those of the surviving pigs (Figure 7A). Viral DNA was detected in the tissues of all of the surviving pigs, especially in the spleens, lungs, adrenal gland, marrow, and certain lymph nodes **(**Figure 7A).

Transmission of genotype I ASFV SD/DY-I/21 in pigs

Two naive pigs were cohoused with pigs inoculated with 10^3 $TCID_{50}$ or 10^6

$TCID_{50}$ of SD/DY-I/21 from the first day of infection for contact transmission test, respectively. One contact pig in the 10^6 $TCID_{50}$ group started to have a fever from Day 26 p.c. (Figure 4A and Table 1) and developed arthroncus from Day 17 p.c. (Table 1). Viral DNA was detectable in oral swabs from Days 5 and 7 p.c., respectively, in the rectal swabs from Days 9 and 13 p.c., respectively, and in the blood from Days 15 and 23 p.c., respectively (Figure 6). Both contact pigs survived for the duration of the 28-day observation period, and were euthanized on Day 28 p.c.. Tissue samples were collected for viral DNA detection by qPCR. Low viral DNA loads were detected in different tissues of both contact pigs (Figure 7A).

Both contact pigs in the 10^3 $TCID_{50}$ group showed varying degrees of intermittent fever during the 28-day observation period (Figure 4B). One contact pig developed phyma on its ridge on Day 25 p.c. (Table 1). Viral DNA was detectable in the oral swabs from Days 9 and 11 p.c., respectively, in the rectal swabs from Days 13 and 15 p.c., respectively, and in the blood from Days 23 and 25 p.c., respectively (Figure 6). Both pigs survived for the duration of the 28-day observation period, and were euthanized on Day 28 p.c., when tissues were collected for viral DNA detection by qPCR. High viral DNA loads were detected in the lungs of both contact pigs, and in the tonsil of one contact pig (Figure 7A). Low viral DNA loads were detected in other tissues including heart, thymus, adrenal gland, marrow, and certain lymph nodes (Figure 7A).

ASFV-specific antibody responses in inoculated and contact pigs

To evaluate the antibody response after SD/DY-I/21 infection, sera from inoculated and contact pigs were collected every other day p.i. or p.c. to detect IgG against ASFV p72 protein by using an ELISA. In the 10^6 $TCID_{50}$-inoculated group, antibody was detected in two pigs on Day 7 p.i., and in all six pigs from Day 9 p.i. (Figure 7B).

In the 10^3 $TCID_{50}$-inoculated group, antibody was detected in five pigs on Day 9 p.i., and in all six pigs from Day 11 p.i. (Figure 7B). The antibody levels in pigs inoculated with virus gradually increased until Day 25 p.i. (Figure 7B). Two contact pigs in the 10^6 $TCID_{50}$ group seroconverted on Days 21 and 27 p.i., respectively, and two contact pigs in the 10^3 $TCID_{50}$ group seroconverted on Days 21 and 25 p.i., respectively (Figure 7B). These data further demonstrate that SD/DY-I/21 is highly transmissible in pigs.

Discussion

In this study, we isolated and characterized what we believe are the first genotype I ASFV strains in China. Two genotype I isolates, SD/DY-I/21 and HeN/ZZ-P1/21, were isolated from domestic pig farms in two provinces. Phylogenetic analysis based on the whole genome sequences suggested that both viruses are highly similar to NH/P68 and OURT88/3, two genotype I Portuguese early isolates. NH/P68 was isolated from a domestic pig with chronic clinical signs in Portugal in 1968 [26-28], and OURT88/3 was isolated from soft ticks (*Ornithodoros erraticus*) on a Portuguese pig farm in 1988 [29]. However, HeN/ZZ-P1/21 and SD/DY-I/21 showed substantial genetic differences from NH/P68 and OURT88/3 in terms of nucleotide mutations, deletions, insertions, and short-fragment replacement across the whole genome. Of particular interest, whole genome sequence analysis clearly showed differences between HeN/ZZ-P1/21 and SD/DY-I/21. These results provide important information to help trace the source of these viruses through field surveillance and imply that two separate events may have led to the emergence of these genotype I ASFVs in China.

The genotype I Portuguese ASFV isolate was attenuated by serial passage in

bone marrow cell cultures and then tested in the field in Portugal in the 1960s [30, 31]. As chronic ASF developed post-vaccination, the field trial for this vaccine candidate was soon stopped [32]. After that, NH/P68 and OURT88/3 were isolated in the field [25, 26, 29], even though there is still no definitive proof for their origins. Through 30 years of efforts, Portugal and Spain successfully eradicated ASF in the 1990s (www.oie.int)[31]. NH/P68, OURT88/3, and similar viruses have not been detected in the field since the 1990s [2, 31]. Lower virulent genotype II ASFVs can be evolved from their parental field isolates [15, 33, 34]. Genotype I ASFVs have been continuously circulating in Africa since 1921, and there is no doubt that attenuated genotype I viruses emerge in nature. However, the genomes and phenotypes of these isolates in Africa has been little characterized. It is one of possibilities that the attenuated genotype I isolates including NH/P68 and OURT/88 may be introduced from Africa. Therefore, how these genotype I viruses invaded China needs further investigation.

Animal challenge tests revealed that the genotype I ASFV isolated in China shows similar low pathogenicity in pigs to that of NH/P68 and OURT88/3. The SD/DY-I/21 isolate showed moderate virulence and efficient transmissibility in pigs, and caused chronic disease and even death in one pig. Except for one contact pig, all of the pigs in our study developed fever. Moreover, almost all of the inoculated and contact pigs developed joint swelling. It has been reported that NH/P68 and OURT88/3 have low pathogenicity and cause chronic and persistent infection in domestic pigs [26, 29]. NH/P68 infection caused 47% of pigs to experience clinical disease including necrotic skin areas and joint swelling, 79% of pigs had high fever, and 84% of pigs had viremia [26]. Less severe post-vaccination reactions including fever and joint swelling were apparent with OURT88/3 [29]. Mutations in the

EP402R gene that encodes CD2v may cause the virus to lose its hemadsorbing activity and could partially attenuate its virulence in domestic pigs and wild boar [15, 33-35]. MGF505/360 regions including MGF_505-1R, -2R, -3R, and MGF_360-12L, -13L, -14R genes are involved in virus replication in tick cells [36], the inhibition of interferon (IFN) production [37], and virus virulence in pigs [38]. Our genotype I isolates, HeN/ZZ-P1/21 and SD/DY-I/21, both had the truncated CD2v protein with non-hemadsorbing phenotype, and deleted MGF505/360 regions as same as NH/P68 and OURT/88, but different from virulent L60 and Benin 97 [25, 39]. The change of these genes may partially explain the low virulence phenotype of these viruses.

Since 2018, Georgia-07-like genotype II ASFVs with high virulence have been prevalent in China. The naturally mutated genotype II low virulent strains were found in the field in 2020 [15]. Now, NH/P68- and OURT88/3-like genotype I epidemic strains with lower virulence than attenuated genotype II viruses have emerged in the field. These low virulent ASFVs have longer incubation periods, efficient transmissibility, and cause mild onset of infection and chronic disease. Infected pigs continuously shed viruses and develop low-level viraemia, which makes early diagnosis more difficult than attenuated genotype II viruses in the field. Meanwhile, new reassortants with unknown virulence among genotype II virulent and attenuated viruses, and genotype I viruses may subsequently emerge in the field. Therefore, the emergence of the genotype I ASFVs will cause more problems and pose bigger challenges for ASF eradication in China. The newly emerging genotype I ASFVs may cause severe and continuous economic losses to the pig industry, once they spread in swine herds or infect breeding sows and boars. Nationwide surveillance of genotype I ASFVs is therefore urgently needed to minimize the losses caused by their infection in China.

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Author Contributions

The manuscript was written by D.Z. and Z.B; experiments and data analysis were performed by E.S., L.H., X.Z., J.Z., D.S., Z.Z., Z.W., H.H., W.W., H.H., W.W., F.L., R.L., J.S., Z.T., W.X., Y.G., X.H., and Y.Z.; and the study was designed by D.Z. and Z.B.

Conflict of interest

The authors declare that they have no conflicts of interest. The funding source was not involved in the current analysis or in the preparation of this report. The corresponding author had full access to all the data and accepts the final responsibility for the decision to submit this manuscript for publication.

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Figure 1 Characterization of genotype I ASFV isolates *in vitro*. PAMs were infected with the genotype I isolates SD/DY-I/21 and HeN/ZZ-P1/21, and the genotype II

strain HLJ/18 as a control at an MOI of 0.1. The cells were fixed and analyzed by using an immunofluorescence assay (IFA) at 24 h p.i. The hemadsorption (HAD) assay was performed with the indicated viruses in PBMCs. PAMs were infected with ASFVs in 6-well plates (MOI=0.2), and the cell pellets were harvested for morphological assessment by using an electron microscope (EM).

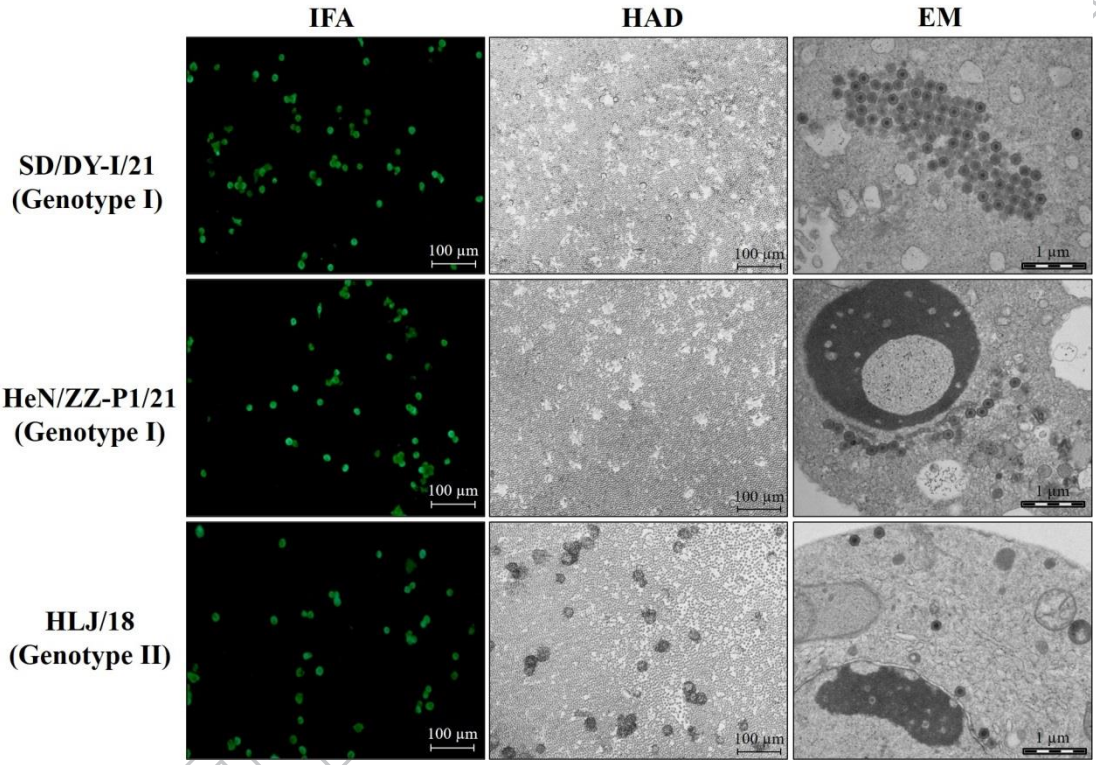


Figure 2 Phylogenetic tree based on the full genome sequences derived from SD/DY-I/21, HeN/ZZ-P1/21, and 70 reference strains from the GenBank database (accession numbers are reported in brackets). Gi, all strains are genotype I ASFVs. Gii, all strains are genotype II ASFVs. The red boldface type indicates the isolates in this study.

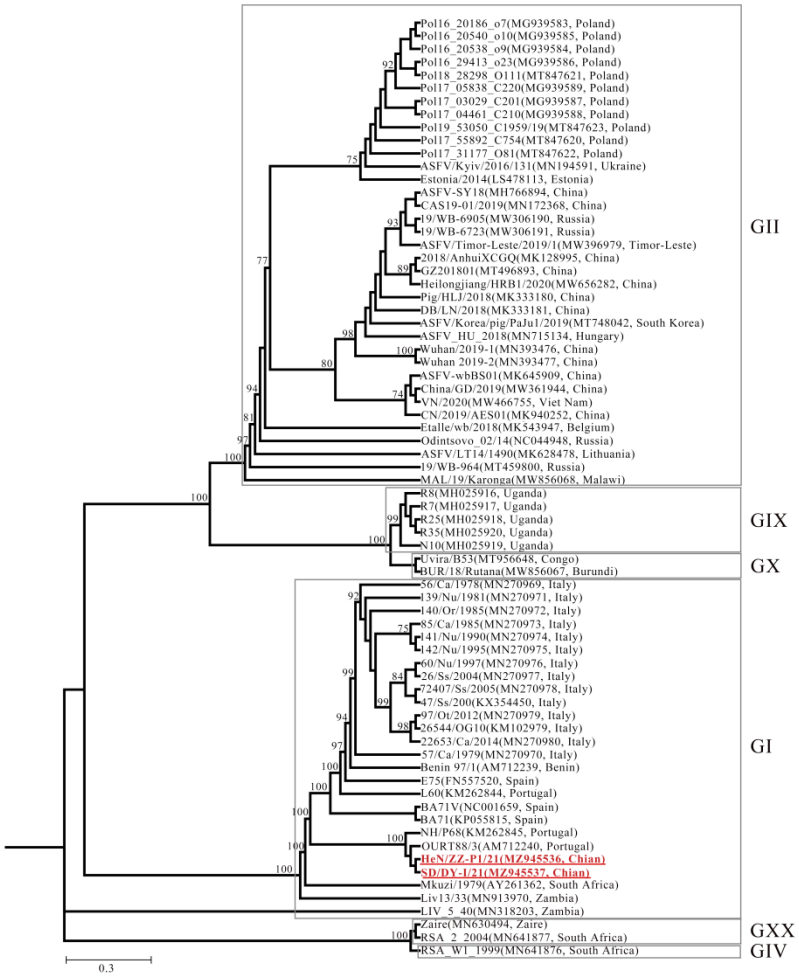


Figure 3 ORFs, nucleotide mutations, deletions, insertions, and replacement in the genomes of SD/DY-I/21 and HeN/ZZ-P1/21. Analysis of the deletion, shortening and lengthening of all ORFs of SD/DY-I/21 and HeN/ZZ-P1/21 compared with virulent isolates L60 and Benin 97, and attenuated isolates NH/P68 and OURT88 (A). The whole genome sequences of SD/DY-I/21 and HeN/ZZ-P1/21 were respectively compared with those of low virulence isolates NH/P68 and OURT88/3 for nucleotide mutations in ORFs (B), nucleotide deletions, insertions, and replacement in ORFs (C); and nucleotide mutations, deletions, and insertions in the noncoding regions (D). The names of the ORFs are shown on the bottom of each panel.

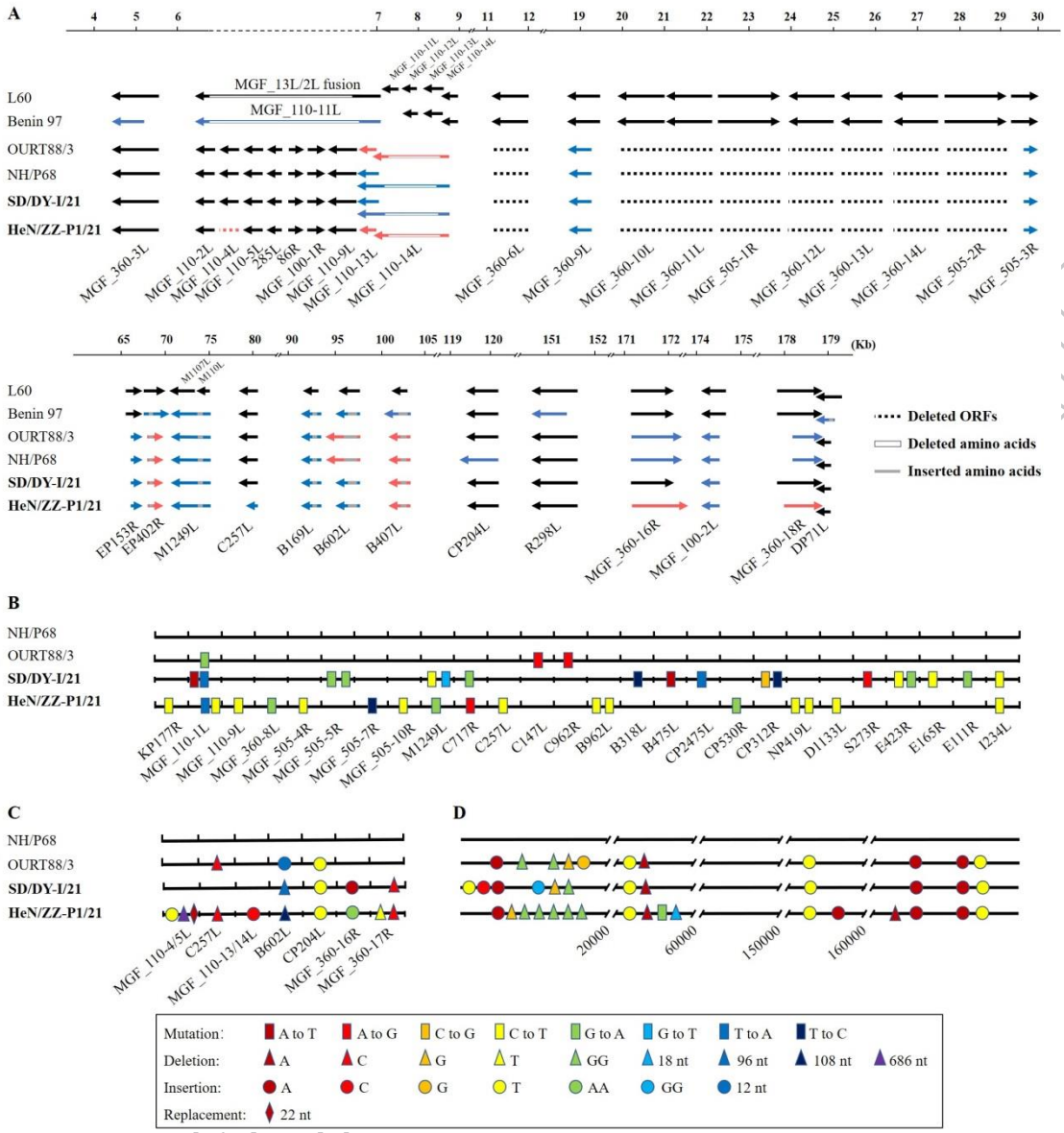


Figure 4 Rectal temperature changes in pigs infected with SD/DY-I/21 at a dose of 10^6 $TCID_{50}$ (A) or 10^3 $TCID_{50}$ (B). Pig 1-Pig 6, 6 pigs inoculated with SD/DY-I/21, Contact 1-Contact 4, 4 non-inoculated pigs cohoused to test for contact transmission. The dashed black lines in these panels indicate the threshold of normal rectal temperature.

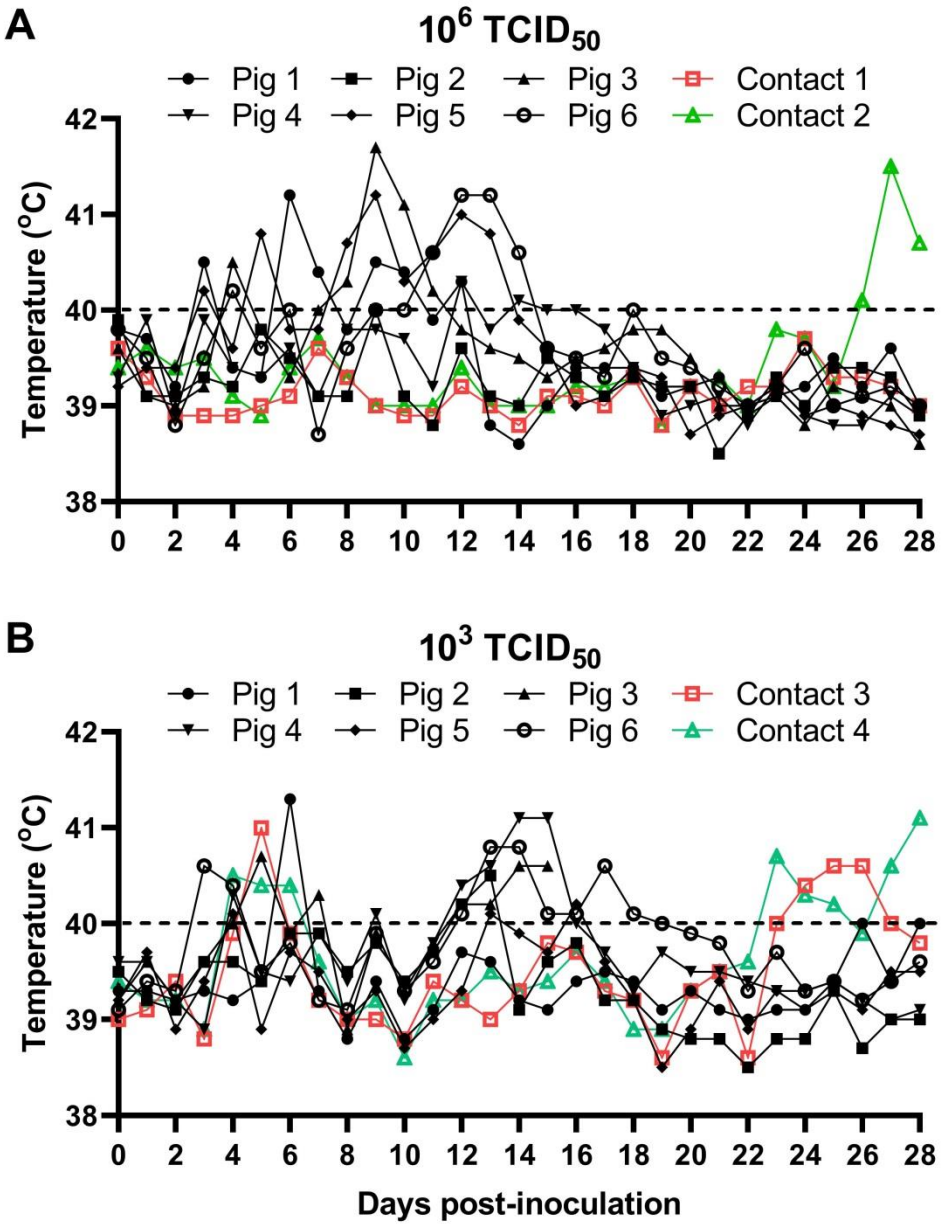


Figure 5 Disease signs in pigs infected with the genotype I isolate SD/DY-I/21. Disease signs include papules (A and B), cutaneous necrosis (B and C), and arthroncus of hind legs (B and D) in surviving pigs.

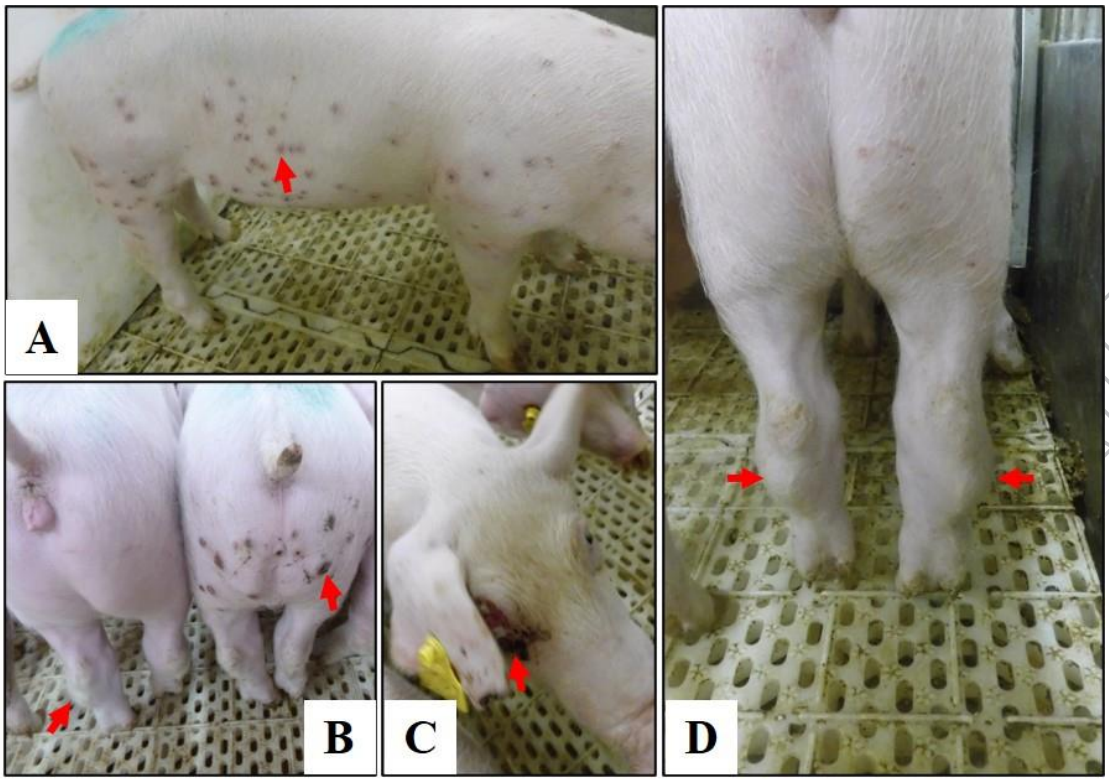


Figure 6 Detection of virus shedding and viremia in infected and contact pigs by use of qPCR. Oral and rectal swab samples, as well as blood, were collected from pigs infected with SD/DY-I/21 and contact pigs at the indicated days post-infection. Viral DNA was extracted and detected by using qPCR. The data on the contact pigs cohoused with the 10^6 $TCID_{50}$-inoculated pigs and 10^3 $TCID_{50}$-inoculated pigs are labeled in red and blue, respectively. The different shaped black dots represent individual pigs.

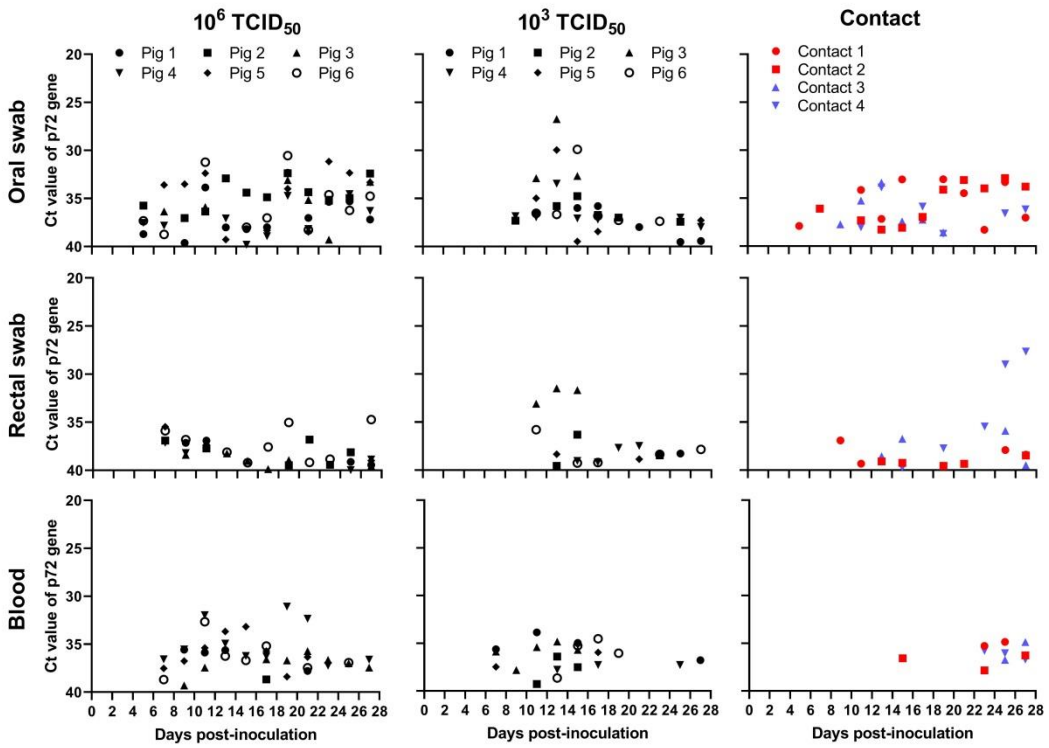
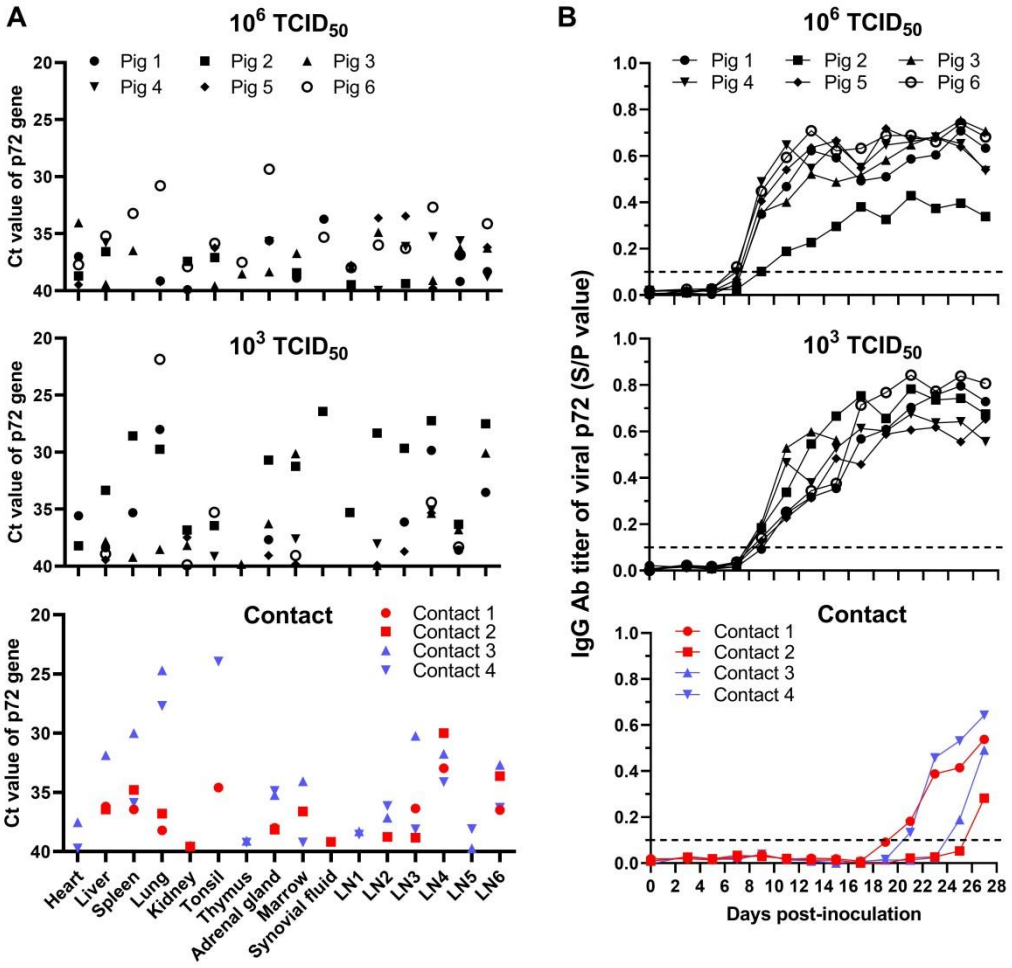


Figure 7 Detection of virus load in tissues and serum conversion in SD/DY-I/21-infected pigs and contact pigs. (A) The indicated tissue samples were collected from the dead pig and surviving pigs that were euthanized on Day 28 post-inoculation or post-contact to detect viral DNA by using qPCR. LN1, intestinal lymph node; LN2, inguinal lymph node; LN3, submaxillary lymph node; LN4, bronchial lymph node; LN5, gastrohepatic lymph node; and LN6, mediastinal lymph node. (B) ASFV-specific antibody in sera from infected and contacted pigs was detected at the indicated times post-infection or -contact by using a commercial ELISA kit coated with viral p72 protein. The data on the contact pigs cohoused with the 10^6 $TCID_{50}$- and 10^3 $TCID_{50}$-inoculated pigs are labeled in red and blue, respectively. The different shaped black dots represent individual pigs.



Supplementary Figure 1 Phylogenetic trees based on selected ORFs derived from SD/DY-I/21 and HeN/ZZ-P1/21. Phylogenetic trees based on the partial B646L (A), E138L (B) and B602L (C) genes, depicting the genetic relationships of China isolates with representatives of the known ASFV genotype I isolates from different countries. GI, all strains are genotype I ASFVs; GII, all strains are genotype II ASFVs, and etc. Bootstrap values lower than 60 were not displayed in the trees. The red boldface type indicates the isolates in this study.

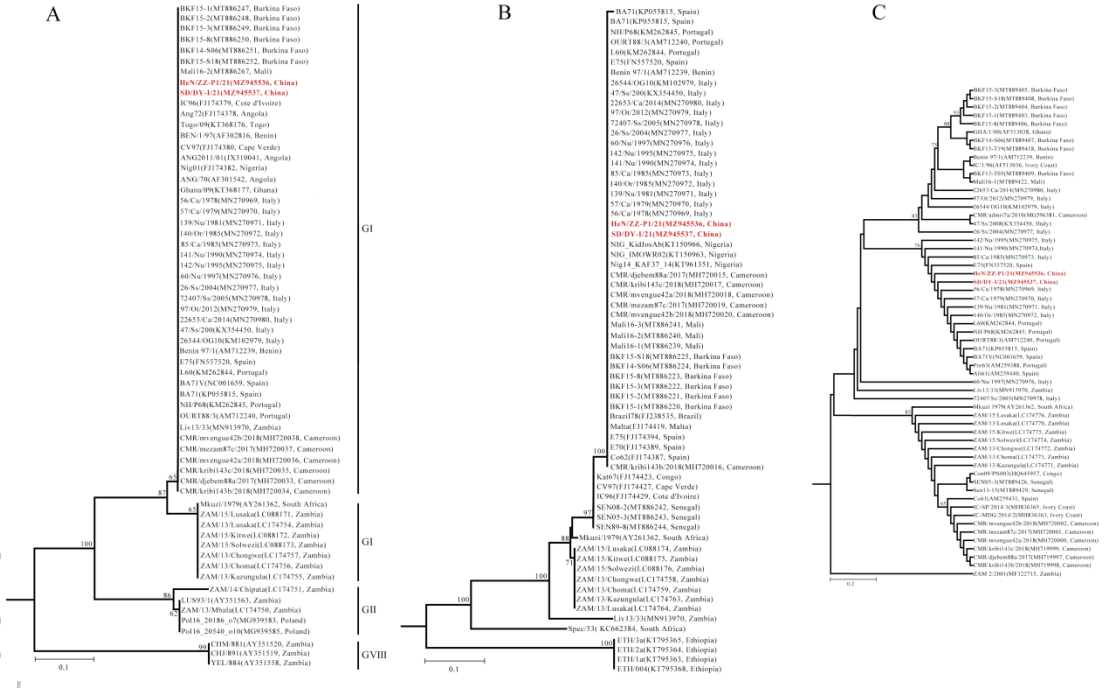
Table 1. Disease signs in pigs inoculated with different doses of the genotype I ASFV SD/DY-I/21.

Group	Treatment	Pig	Earliest appearance of disease signs (Day post-inoculation, dpi)

		No.	Fever (>40 ^{o}C)	Papule	Arthroncus	Limp	Phyma	Cutaneous necrosis	Death
10^6 $TCID_{50}$	Infection	1	3	13	14	/[a]	/	/	NA[b]
		2	9	/	14	/	/	/	NA
		3	4	/	13	25	/	17	NA
		4	12	/	14	14	/	/	NA
		5	3	12	17	/	/	/	NA
		6	4	11	17	/	/	20	NA
	Contact	1	/	/	/	/	/	/	NA
		2	26	/	17	/	/	/	NA
10^3 $TCID_{50}$	Infection	1	6	13	14	/	/	/	NA
		2	12	13	14	/	/	/	NA
		3	4	13	14	/	/	/	16
		4	4	/	14	/	/	/	NA
		5	4	/	/	/	/	/	NA
		6	3	/	14	/	/	/	NA
	Contact	3	5	/	/	/	25	/	NA
		4	4	/	/	/	/	/	NA

a, no signs of disease.

b, the pig survived the infection.



Supplementary Table 1: CVR profiles within the B602L of genotype I Chinese isolates and viruses selected from previous reports.

Virus designation	Country of origin	Year of sampling	CVR profile	No. repeats	Accession number
ZAM 2/2001	Zambia	2001	BNAFNBTDBNAG	12	MF322715
Mkuzi 1979	South	1979	BVWAFNBNAAAF	12	AY2613

	Africa				62
85/Ca/1985	Italy	1985	ABNAAADBNAFA	12	MN270973
141/Nu/1990	Italy	1990	ABNAAADBNAFA	12	MN270974
142/Nu/1995	Italy	1995	ABNAAADBNAFA	12	MN270975
60/Nu/1997	Italy	1997	ABNAAADBNAFA	12	MN270976
26/Ss/2004	Italy	2004	ABNAAADBNAFA	12	MN270977
72407/Ss/2005	Italy	2005	ABNAAADBNAFA	12	MN270978
47/Ss/2008	Italy	2008	ABNAAADBNAFA	12	KX354450
26544/OG10	Italy	2010	ABNAAADBNAFA	12	KM102979
97/Ot/2012	Italy	2012	ABNAAADBNAFA	12	MN270979
22653/Ca/2014	Italy	2014	ABNAAADBNAFA	12	MN270980
Sen13-15	Senegal	2013	ABNABTDBNAAAA	13	MT889429
BKF15-8	Burkina Faso	2015	ABNAAAAACBNAFA	14	MT889406
Con09/PN003	Republic of the Congo	2009	AABNABTDBNAAAA	14	HQ645957
SEN05-3	Senegal	2005	AABNABTDBNAAAA	14	MT889426
Liv13/33 (OmLF2)	Zambia	2017	BNADBNAFTBTDBNAF	16	MN913970
CMR/admri7a/2010	Cameroon	2010	ABNAAAACBNABTDBNAFA	19	MG596381
IC-MDG/2014/2	Ivory Coast	2014	ABNAAAACBNABTDBNAFA	19	MH836363
IC-SP/2014/3	Ivory Coast	2014	ABNAAAAACBNABTDBNAFA	20	MH836365
BKF14-S06	Burkina Faso	2014	ABNAAAAACBNAAAAACBNAAA	22	MT889407
BKF15-T19	Burkina Faso	2015	ABNAAAAAAACBNAAAAACBNAFA	24	MT889418
56/Ca/1978	Italy	1978	ABNAAAACBNABNABNABTDBNAFA	25	MN270969
57/Ca/1979	Italy	1979	ABNAAAACBNABNABNABTDBNAFA	25	MN270970
139/Nu/1981	Italy	1981	ABNAAAACBNABNABNABTDBNAFA	25	MN270971
140/Or/1985	Italy	1985	ABNAAAACBNABNABNABTDBNAFA	25	MN270972
L60	Portugal	1960	ABNAAAACBNABNABNABTDBNAFA	25	KM262844
Ali61	Spain	1961	ABNAAAACBNABNABNABTDBNAFA	25	AM259440
E75	Spain	1975	ABNAAAAACBNABNABNABTDBNAFA	26	FN557520
Mali16-1	Mali	2016	ABNAAAAAACBNAAAAAAAACBNAFA	26	MT889422
Co61	Spain	1961	AAABNAAAAAAAACBNABNABNABTDFA	28	AM259431
Por63	Portugal	1963	ABNAAAAAAACBNABNABNABTDBNAFA	28	AM259388
BA71V	Spain	1975	ABNAAAAAAACBNABNABNABTDBNAFA	28	NC001659
BA71	Spain	1971	ABNAAAACBNABNABNABNABTDBNAFA	28	KP055815
BKF13-T05	Burkina Faso	2013	ABNAAAAACBNAAAAAAAAAAAACBNAFA	29	MT889409
GHA/1/00	Ghana	2000	ABNAAAAACBNAAAACBNAAAACBNAAAAACBNAFA	36	AF5130

					38
Benin 97/1	Benin	1997	ABNAAAACBNAAAAACBNAAAAACBNAAAACBNAFA	36	AM712239
IC/1/96	Ivory Coast	1996	ABNAAAACBNAAAAACBNAAAAACBNAAAACBNAFA	36	AF513036
HeN/ZZ-P1/21	**China**	**2021**	**ABNAAAAAAAAAAAAAAACBNABNABNABTDBNAFA**	**36**	**MZ945536**
SD/DY-I/21	**China**	**2021**	**ABNAAAACCBNABNAAAAACBNABNABNABTDBNAFA**	**37**	**MZ945537**
NH/P68	Portugal	1968	ABNAAAAAAAAAAAAACCBNABNAAAAACBNABNABNABTDBNAFA	45	KM628245
OURT88/3	Portugal	2004	ABNAAAAAAAAAAAAAACCBNABNAAAAACBNABNABNABTDBNAFA	46	AM712240

Notes: CVR codes as previously described: CAST/CVST/CTST = A, CADT/CVDT/CTDT = B, GAST/GANT = C, CASM = D, CANT = F, CTNT=G, NVDT/NVDI/NVGT = N, SAST = S, NVNT = T, NAST/NADT/NANT = V, and SADT= W. The lighter color indicates the different CVR profile of the isolates.

Supplementary Table 2 The changes of nucleotide, amino acid, and ORFs of genotype I ASFVs compared with that of NH/P68 virus.

The location of nucleotide change in viral genome[a]		The change of nucleotide, and amino acid of ORF at the indicated site		
Position in viral genome	ORF/Region name	SD/DY-I/21	HeN/ZZ-P1/21	OURT88/3
3103	KP177R	—[b]	C-T, P35L	—
5646/7	Noncoding region	T insertion	—	—
5795	MGF_ 110-1L	A-T, I240K	—	—
5991	MGF_ 110-1L	T-A, I175F	T-A, I175F	—
6353	MGF_ 110-1L	—	C-T, G54D	—
6411	MGF_ 110-1L	—	—	G-A, L35F
7104/5	MGF_ 110-4/5L	—	T insertion, E120K	—
7105-7790	MGF_ 110-4/5L	—	686 nt deletion, MGF_110-4L deleted	—
8123 /44	MGF_ 110-5L	—	22 nt replacement, 3 AA mutations at its N terminal	—
8853/4	Noncoding region	C insertion	—	—
10529	MGF_110-9L	—	C-T*	—
11607	MGF_110-13/14L	—	C deletion, initiation codon shift, 160 AAs of MGF_110-13L; stop codon shift, 126 AAs of MGF_110-14L	C deletion, initiation codon shift, 160 AAs of MGF_110-13L; stop codon shift, 126 AAs of MGF_110-14L
11993/4	Noncoding region	A insertion	A insertion	A insertion
13577	Noncoding region	—	G deletion	—
13767/68	Noncoding	—	GG deletion	GG deletion

	region			
15394/5	Noncoding region	GG insertion	GG deletion	—
15621/2	Noncoding region	G deletion	GG deletion	GG deletion
15749/50	Noncoding region	GG deletion	GG deletion	G deletion
15943/4	Noncoding region	—	GG deletion	G insertion
18707	MGF_360-8L	—	G-A*	—
21296	MGF 505-4R	—	C-T, H34Y	—
22889	MGF_505-5R	G-A*	—	—
22932	MGF_505-5R	G-A, E64K	—	—
25808	MGF_505-7R	—	T-C, I458T	—
28018/9	Noncoding region	T insertion	T insertion	T insertion
31037	MGF_505-10R	—	C-T, T387I	—
31583	Noncoding region	A deletion	A deletion	A deletion
58306	Noncoding region	—	G-A	—
59790-59807	Noncoding region	—	18 nt deletion	—
61454	M1249L	C-T, E1167K	—	—
62169	M1249L	—	G-A*	—
64797	M1249L	G-T, F52L	—	—
68100	C717R	G-A, V188I	—	—
68130	C717R	—	A-G, M198V	—
70851/2	C257L	—	C insertion, stop codon shfit, 115 AAs	—
70985	C257L	—	C-T, A62T	—
73891	C147L	—	—	A-G, F74S
76282	C962R	—	—	A-G, H581R
77745	B962L	—	C-T, G904D	—
79169	B962L	—	C-T*	—
80920	B318L	T-C*	—	—
84763	B475L	A-T, F71L	—	—
87456/563	B602L	—	108 nt deletion, 634 AAs	—
87541/636	B602L	96 nt deletion, 638 AAs	—	—
87683/4	B602L	—	—	12 nt insertion, 674 AAs
110177	CP2475L	T-A, I154L	—	—
110751/2	CP204L	T insertion, 194 AAs	T insertion, 194 AAs	T insertion, 194 AAs
112741	CP530R	—	G-A, G463E	—
113808	CP312R	C-G*	—	—
113882	CP312R	T-C, L222S	—	—

120184	NP419L	—	C-T, C166Y	—
120316	NP419L	—	C-T, G122E	—
127759	D1133L	—	C-T, V636M	—
132817	S273R	A-G, T74A	—	—
148762	E423R	C-T*	—	—
149670	E423R	G-A, S315N	—	—
152776	E165R	C-T, P146S	—	—
155245	E111R	G-A*	—	—
155316/7	Noncoding region	T insertion	T insertion	T insertion
157227	I243L	—	C-T*	—
157539	I243L	C-T*	—	—
159449/50	Noncoding region	—	A insertion	—
161525	Noncoding region	—	A deletion	—
163390/1	MGF_360-16R	A insertion, 309 AAs	AA insertion, 352 AAs	—
165269/70	Noncoding region	A insertion	A insertion	A insertion
169200	MGF_360-17R	—	T deletion, initiation codon shift, 237 AAs	—
169370	MGF_360-17R	C deletion, stop codon shift, 254 AAs	C deletion	—
170513/4	Noncoding region	A insertion	A insertion	A insertion
170515/6	Noncoding region	T insertion	T insertion	T insertion

Note: a the site compared with the viral genome of the NH/P68 virus; b means no difference compared with that of NH/P68 virus; * Silent mutation.